PRESENTATION OF FOREIGN ANTIGENIC DETERMINANTS AT THE CELL SURFACE OF ENTERIC BACTERIA USING THE TRAT PROTEIN.

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ABSTRACT

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PRESENTATION OF FOREIGN ANTIGENIC DETERMINANTS AT THE CELL SURFACE OF ENTERIC BACTERIA USING THE TRAT PROTEIN.

by Jacqueline Laura Harrison.

The TraT protein is an oligomeric, highly cell-surface-exposed lipoprotein, specified by F-like plasmids. It confers resistance to the bactericidal effects of serum and blocks the conjugal transfer of plasmids to cells bearing identical or closely-related plasmids, a process known as surface exclusion. This thesis describes studies on the structure and function of TraT and on its use for presentation of foreign antigenic determinants at the bacterial cell surface. The protein specified by the antibiotic resistance plasmid R6-5 was purified to homogeneity by a new procedure. Circular dichroism spectroscopy indicated that the protein contained significant α-helical structure (22%), in contrast to other previously-studied outer membrane proteins. The purified protein had a protective effect on bacterial cells incubated in serum, suggesting that it does not have to be located on the cell surface to mediate serum resistance. Additionally, when added to mating mixtures, the purified TraT inhibited the conjugal transfer of plasmids belonging to surface exclusion group IV, but had no significant effect on the transfer of plasmids belonging to other groups, confirming that TraT is specific in its surface exclusion effect. Since the mature form of the R6-5 protein differs from that of F (which belongs to surface exclusion group I) by a single amino acid (Ala_{120} rather than Gly_{120}), the result indicates that this residue is a critical determinant of surface exclusion specificity. A model for surface exclusion specificity is presented. The TraT protein structure and function was further probed, around this specificity region, by genetic insertion of a foreign antigenic determinant (the C3 epitope of poliovirus) at residue 125 of the protein. The hybrid protein was transported to the outer membrane and assembled into trypsin-resistant oligomers, characteristic of the wild-type protein. However, the C3 epitope inserted in this region was not recognised by monoclonal antibodies added to whole cells and disrupted the permeability barrier effect of the outer membrane. In parallel with these studies, plasmids specifying hybrid TraT proteins with the C3 epitope of poliovirus inserted at positions 61 or 180 were introduced into an *aroA*$^-$ attenuated strain of *Salmonella typhimurium*. The plasmid-bearing derivatives which efficiently expressed the C3 epitope without detectably altering any of the important characteristics of the vaccine strain, were used for oral immunisation of BALB/c mice. The bacteria invaded the livers of the mice and were detectable until day 14 after inoculation. However, no immune response to C3 was elicited, probably due to the instability of the plasmids following inoculation. To circumvent these problems and to improve expression of the hybrid TraT proteins *in vivo*, a modified inducible expression system with improved stability was developed. The hybrid *traT/C3* genes were placed under the control of the *groEL* promoter (which is strongly induced when *S. typhimurium* enters macrophages) and cloned into a vector that allows homologous recombination of the promoter-gene cassette into the *aroC* chromosomal locus. The TraT/C3 hybrid proteins accounted for more than 4% of the total cellular protein in the *Salmonella* vaccine strain.

In memory of my Grandma, Winifred Ethel Harrison.

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PUBLICATIONS.

1) TAYLOR, I.M., HARRISON, J.L., TIMMIS, K.N., AND O'CONNOR, C.D. (1990) The TraT lipoprotein as a vehicle for the transport of foreign antigenic determinants to the cell surface of *Escherichia coli* K-12: structure-function relationships in the TraT protein. Molec. Micro. **4**: 1259-1268.

2) HARRISON, J.L., TAYLOR, I.M., and O'CONNOR, C.D. (1990) Presentation of foreign antigenic determinants at the bacterial ell surface using the TraT lipoprotein. Res. Microbiol. **141**: 1009-1012.

3) HARRISON, J.L., TAYLOR, I.M., PLATT, K., and O'CONNOR, C.D. (1992) Surface exclusion specificity of the TraT lipoprotein is determined by single alterations in a five amino acid region of the protein. Molec. micro. - submitted.

AMINO ACIDS.

Amino Acid.	Three-letter Abbreviation.	One-letter Symbol.
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ABBREVIATIONS.

A	adenine
α-[^{35}S]-dATP	dATP radiolabelled at the α-phosphate with sulphur-35
A_{600}	absorbance at 600 nm (or similarly at 260 or 280 nm)
Ac	acetate
Ap	ampicillin
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
BCG	Bacille Calmette-Guérin
BSA	bovine serum albumin fraction V
C	cytosine
CD	circular dichroism
CFU	colony forming units
Cm	chloramphenicol
Conc^{n}	concentration
DEPC	diethyl pyrocarbonate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DHB	2,3-dihydroxybenzoate
dITP	2'-deoxyinosine 5'-triphosphate
DMSO	dimethyl sulphoxide
DN'ase	deoxyribonuclase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fd	fusidic acid
FPLC	fast protein liquid chromatography
FSB	final sample buffer

G	guanine
g	gravity (9.81 m/s^2)
gal	galactose
HCC	hexamine cobalt chloride
HEPES	N-2-hydroxyethyl piperazine-N-ethanesulphonic acid
HRP	horse radish peroxidase
IgG	immunoglobulin
^{125}I	iodine (radioactive 125 isotope)
IPTG	isopropyl-ß-D-thiogalactoside
k	thousand
kb	kilobase
kDa	kilo Daltons
Km	kanamycin
*lac*P	*lac* Promoter
λP_L	leftward promoter from bacteriophage lambda
LD_{50}	50% lethal dose
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MOPS	3-(N-morpholino) propane-sulphonic acid
Nal	naladixic acid
OMP	outer membrane protein
PABA	para-aminobenzoic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PTH	phenylthiohydantoin
^{R}	resistance
RES	reticulo endothelial system
Rif	rifampicin
RN'ase	ribonuclease
RNA	ribonucleic acid

rpm	revolutions per minute
r.t.	room temperature
SDS	sodium dodecyl sulphate
SET	sucrose, EDTA, tris buffer
Sfx	surface exclusion
Sfx_I	surface exclusion group I (similarly with groups II, III, IV and V)
Spc	spectinomycin
T	thymine
Tc	tetracycline
TE	tris, EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TraT/C3	TraT protein with the C3 epitope inserted in one of its sites
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
TS	tris, saline buffer
TWEEN 20	polyoxyethylenesorbitan monolaurate
UDP	uridine 5'-pyrophosphate
wt	wild-type
X-gal	5-bromo-4-chloro-3-indoyl-ß-phosphate

CONTENTS.

TABLES.

FIGURES.

CHAPTER 1:

GENERAL INTRODUCTION.

1. GENERAL INTRODUCTION.

Proteins exposed on the cell surface of bacteria have numerous diverse and specific interactions with constituents of the surrounding environment. This thesis describes studies on one such protein, TraT, aimed at characterising its structure and functions in more detail and, additionally, with the goal of determining its suitability for the transport and presentation of foreign antigenic determinants (epitopes) to the immune system. Before such work is described, however, it is necessary to summarise what is known in general about the structure of the cell envelope of Gram-negative bacteria (Section 1.1.); the TraT protein in particular (Section 1.2.) and the use of outer membrane proteins for epitope presentation (Section 1.3.).

1.1. THE CELL ENVELOPE OF GRAM-NEGATIVE BACTERIA.

Examination of high-resolution electron micrographs of sections through Gram-negative and Gram-positive bacteria reveals gross morphological differences, fundamentally in the composition of their cell envelopes. Gram-positive bacteria are encased within a thick peptidoglycan layer (typically 20-50 nm) and in some species an extracellular capsule. The cell envelope of Gram-negative bacteria is more complex with the peptidoglycan layer being less substantial (typically 2 nm) and the presence of a second membrane, termed the outer membrane, exterior to the peptidoglycan. Like their Gram-positive counterparts, capsules can also be present in some species.

The outer membrane of Gram-negative bacteria is a vital part of the cell for three main reasons. Firstly, it forms an important physical and functional barrier that protects the cell from powerful surfactants, digestive enzymes and fluctuating osmotic conditions in the immediate environment. For example, many Gram-negative pathogens are intrinsically resistant to hydrophobic antibiotics that are highly effective against Gram-positive bacteria. Secondly, it allows the selective movement of molecules into and out of the cell. Gram-negative bacteria are generally freely

permeable to ions and many small polar molecules less than about 600 Da in size, but require specific uptake pathways for larger molecules. Finally, the membrane has an important role in the biochemically significant cell surface events which are vital for proper cell functioning and survival. For example, it is intrinsically involved in cell-cell interactions during conjugation and largely determines the immunogenicity of the cell within a host.

1.1.1. Layers that make up the Cell Envelope.

The cell envelope of Gram-negative bacteria (see Figure 1.1) can be divided structurally into four distinct layers: a cytoplasmic (or inner) membrane, a peptidoglycan layer and a periplasmic space (also called the periplasm) bounded by the outer membrane (Inouye, 1979; 1987; Hammond *et al.*, 1983; Lugtenberg & Van Alphen, 1983). Each of these structures are considered briefly below with reference mainly to enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium.*

1.1.1.1. The Cytoplasmic Membrane.

The cytoplasmic membrane is the major permeability barrier of the cell although it is freely permeable to hydrophobic compounds. It contains phospholipids and proteins in about equal amounts, the proteins allowing certain nutrients and other chemicals to pass into and out of the cell, while selectively preventing others from doing so. It contains the enzyme systems of the electron transport chain and oxidative phosphorylation, systems for the active transport of solutes and excretion of waste products and, additionally, the synthetic apparatus necessary for the production of exterior layers.

1.1.1.2. The Peptidoglycan Layer.

This is a wall-like complex of peptides and oligosaccharides which are highly cross-linked giving the cell its shape and rigidity. The layer is covalently linked by Murein Lipoprotein molecules to the outer membrane. Gram-negative bacteria have

a single supermolecular layer of peptidoglycan, unlike Gram-positive bacteria whose peptidoglycan is multilayered.

1.1.1.3. The Periplasmic Space.

This is the compartment between the peptidoglycan and outer membrane and is occupied mainly by proteins. The periplasmic space accounts for approximately 4% of the total cell protein and 20-40% of the total cell volume. The periplasm is distinguished by a distinct ionic composition and the possession of a unique series of proteins. The restrictive properties of the outer membrane ensure that periplasmic components can not easily leak into the environment. Certain of the proteins have catalytic functions, converting a variety of compounds into a form amenable to translocation by specific carriers in the cytoplasmic membrane. A second group of periplasmic enzymes catalyse the destruction of certain antibacterial agents able to permeate the outer membrane. The periplasm also contains a series of nutrient-binding proteins which are essential components of certain active transport systems. Therefore the periplasm plays an important role in cell physiology.

1.1.1.4. The Outer Membrane.

As described above, this layer forms the interface between the cell and its immediate environment and therefore accounts for the characteristic properties of the cell surface of Gram-negative bacteria. The layer thus protects the cell from the harsh external environment, being especially important in Gram-negative enteric organisms (Nikaido & Nakae, 1979) where the outer membrane has to protect the cell from degradation by the host's proteolytic enzymes, the unfavourable pH of the host's gut, the emulsifying action of the host's bile salts and also a number of natural and synthetic antibiotics (Nikaido, 1979).

The outer membrane effectively regulates the passage of solutes through the cell envelope into the cytoplasmic membrane (Nikaido & Vaara, 1985), allowing the passage of small hydrophilic molecules through the membrane into the periplasmic

space (Nikaido & Nakae, 1979). In contrast to other biological membranes the outer membrane selectively excludes hydrophobic compounds (Sukupolvi & Vaara, 1989). Another important function of the outer membrane is to endow the bacterial surface with strong hydrophilicity, which is important for avoidance of specific immune attack, such as phagocytosis by macrophages or the bactericidal effects of serum complement. These three main functions of the outer membrane are a direct consequence of its composition.

1.1.2. Components of the Outer Membrane.

The composition of the outer membrane of Gram-negative bacteria has been summarised by Lugtenberg & Van Alphen, (1983); Nikaido & Nakae, (1979) and Nikaido & Vaara, (1985; 1987). Its principal components are:- phospholipids, (accounting for 50% of the total cellular phospholipids); lipopolysaccharide, LPS (accounting for 90% of the total cellular LPS) and a limited number of characteristic proteins (which account for 9-12% of the total cellular proteins and 60% of the cellular membrane proteins).

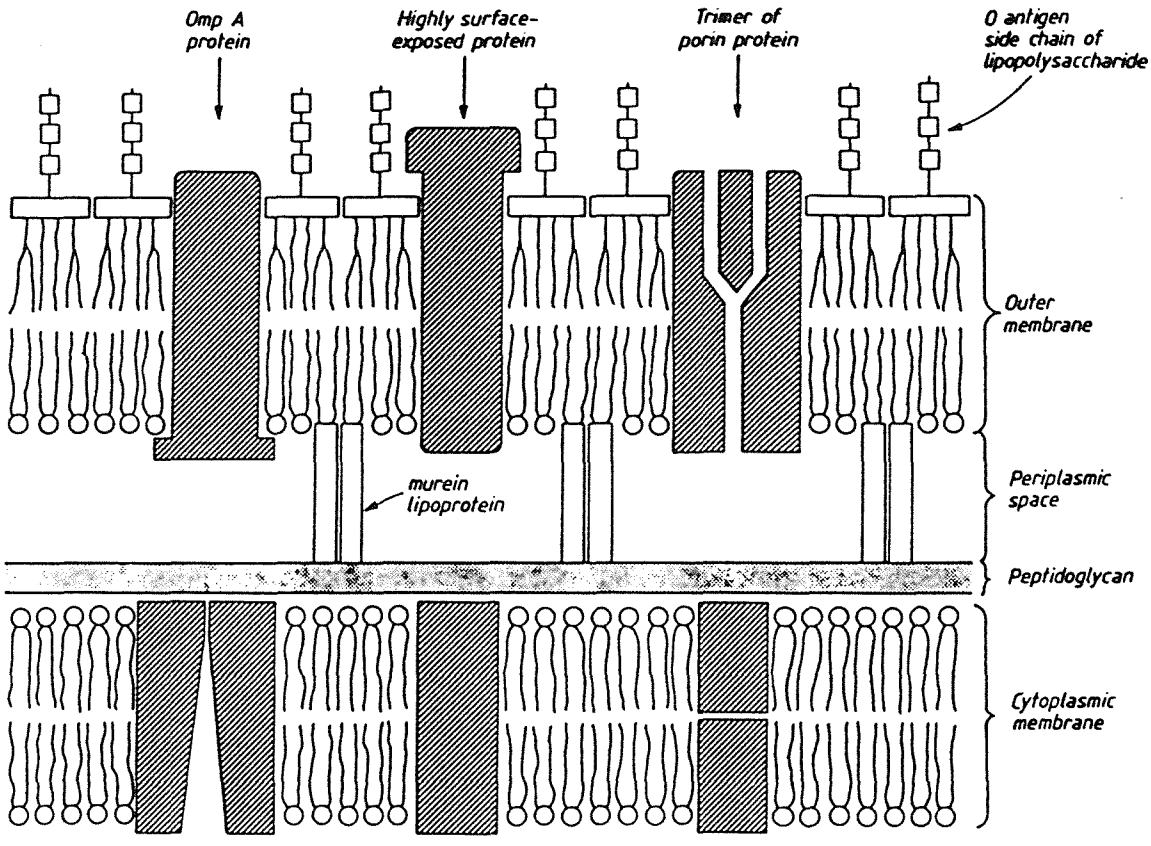
1.1.2.1. Phospholipids.

The phospholipid content of the outer membrane is less than that of the cytosolic membrane. Indeed, the outer membrane seems to only contain 50% of the amount of phospholipid required to form a bilayer, with most of the phospholipid being in the inner leaflet of the outer membrane, forming a monolayer. This has been shown using covalent labelling with cyanogen bromide-activated dextran, which can not diffuse across the outer membrane (Nikaido & Nakae, 1979). The outer membrane bilayer is therefore made complete by LPS forming the outer leaflet of the outer membrane (see Figure 1.1). This has been established in labelling studies with LPS-specific, ferritin-labelled antibody as well as degradation experiments with galactose oxidase (Nikaido & Nakae, 1979).

FIGURE 1.1.

Schematic Representation of a Section through the Cell Envelope of *E. coli* or *S. typhimurium.*

(adapted from Sukupolvi & O'Connor, 1990)



In this diagram, enzymes present in the periplasmic space have been omitted for clarity and the O-antigen side chain (not present in *E. coli* K-12) has been shortened. Additionally, only two of the three pores thought to be present in each porin trimer are visible. Other strain specific cell surface components such as pili, fimbriae and capsules are not shown.

1.1.2.2. Lipopolysaccharide (LPS).

LPS is a large (10 kDa) amphipathic molecule composed of a proximal hydrophobic portion, termed lipid A, and a distal hydrophilic polysaccharide portion (see Figure 1.2).

The hydrophobic lipid A portion, which is well conserved in Gram-negative bacteria and *E. coli*, contains six saturated fatty acid chains that are linked to two glucosamine residues. These fatty acyl chains constitute about half of the outer leaflet of the outer membrane. However, they are absent from the inner leaflet which instead contains phospholipids.

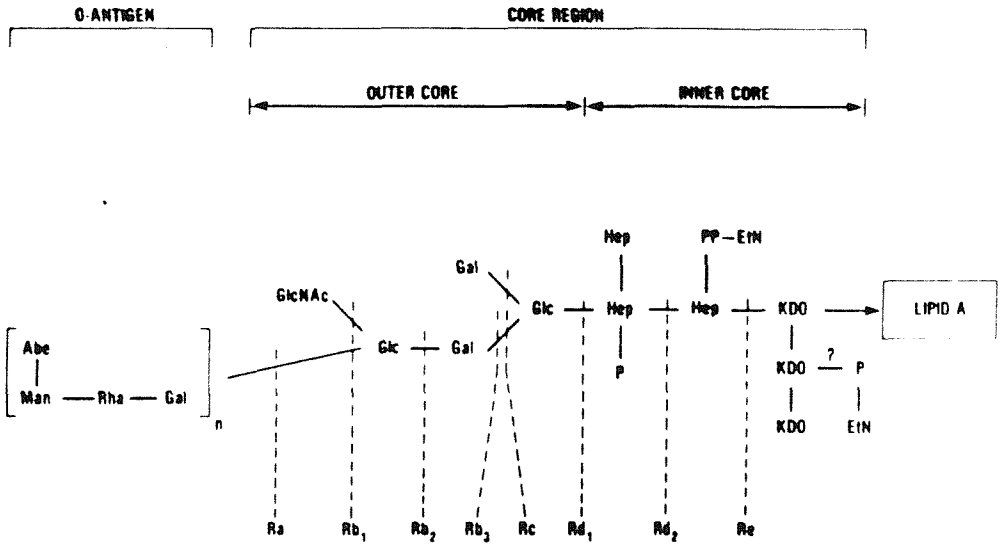
The hydrophilic portion is usually considered to exist as two parts. Firstly, there is the proximal portion of the polysaccharide which is called the core region. This consists of about 10 sugar units linked to lipid A by the sugar acid, KDO (2-keto-3-deoxyoctulosonic acid). The core can be divided into two subdomains, the inner and the outer core regions. The inner core sugars of *E. coli* and *S. typhimurium* include certain unique species that are characteristic of LPS, such as KDO and Hep (L-glycero-D-manno-heptose), whereas the outer core consists of hexoses, primarily glucose, galactose and GlcNac (N-acetyl-D-glucosamine). Most Gram-negative bacteria appear to contain two KDO and two Hep residues per LPS molecule.

Covalently attached to the core in smooth strains is a carbohydrate polymer termed the O-antigen. The O-antigen consists of 3 to 6 sugar residues repeated 20 to 50 times. In contrast to the lipid A and the core region, the polysaccharides of the O-antigen, where present, exhibit gross differences in composition and structure even within a single species (or genera, such as *Escherichia* or *Salmonella*). For this reason, the structure of this portion has been used in the fine serological typing of the strains, especially in *Enterobacteriaceae*. In smooth stains, the LPS occupies about 41% of the surface of the outer membrane (Nikaido & Nakae, 1979).

FIGURE 1.2.

Schematic Representation of the Three Constituent Domains of *S. typhimurium* Lipopolysaccharide.

(adapted from Rick, 1987)



The lipid A, core and O-antigen domains are shown. The O-antigens usually consist of 20-50 repeating units of four or five distinct sugars. *E. coli* K-12 does not contain the O-antigen domain (Rietschel, 1984). The LPS structures and corresponding chemotypes of mutants defective at various stages of core biosynthesis are indicated by the broken lines and associated letter designations (i.e. Ra, Rb_1, etc., respectively).

Abbreviations:

Abe	abequose;	Man	D-manose;
Rha	L-rhamnose;	Gal	D-galactose;
GlcNAc	N-acetyl-D-glucosamine;	Glc	D-glucose;
EtN	ethanolamine;	P	phosphate.
Hep	L-glycero-D-mannoheptose;		
KDO	2-keto-3-deoxyoctulosonic acid;		

Several types of mutants exist that produce incomplete LPS molecules. The properties of these mutants help to define the biological functions performed by various parts of the LPS. Semi-rough mutants (that cannot polymerise the O-antigen polysaccharide monomers) and rough mutants (which cannot synthesise or attach the O-side chains) show a loss of virulence, suggesting that this portion of LPS is important in host-parasite interaction. Mutants with defects in the outer core sugars are common. These, however, have no obvious phenotypes except for bacteriophage resistance (Nikaido & Vaara, 1987), but less obvious changes in outer membrane function cannot be excluded. Mutants that have lost the outer core and part of the inner core sugars (deep-rough mutants) are exceptionally sensitive to a wide range of compounds, including dyes, long-chain fatty acids, antibiotics (such as novobiocin and rifampicin), bile salts, other detergents and mutagens (Nikaido, 1976). This sensitivity is thought, in part, to be because the absence of critical polysaccharides apparently blocks the incorporation of many proteins into the outer membrane, producing a compensatory increase in its phospholipid content. The head groups of much of the outer membrane phospholipid then become exposed and hence allow the rapid penetration of hydrophobic molecules, just as model bilayers do. Thus, normal LPS must be important in maintaining the permeability barrier properties of the outer membrane. Heptose-deficient LPS strains of *E. coli* and *S. typhimurium* are very poor recipients in conjugation experiments (Havekes *et al.*, 1976; Sanderson *et al.*, 1981). This suggests that LPS also plays an important role in conjugation.

LPS is a known endotoxin, the toxic properties of LPS residing in the hydrophobic lipid A moiety, and accounts for the toxicity of the Gram-negative bacterial cell envelope when present in mammalian blood. LPS, containing mainly anionic groups, confers an overall negative charge and hydrophilicity on the cell surface. It is this that helps the cell to avoid phagocytosis by macrophages and the bactericidal effects of serum complement.

In this study the *E. coli* K-12 strains used were rough and hence do not have long chain LPS on their outer membrane. By contrast, the *aroA*$^-$ *S. typhimurium* LT2 strains used were smooth. The *S. typhimurium galE*$^-$ (galactose epimerase-deficient) strains

used are rough when grown on minimal solid media since they cannot synthesise their own galactose-containing O-antigen. However, when exogenous galactose is supplied in the medium they are able to synthesise a complete LPS O-antigen.

1.1.2.3. Proteins.

The outer membrane proteins constitute 9-12% of the total cellular proteins (Lugtenberg & Van Alphen, 1983). Unlike the cytoplasmic (inner) membrane, the outer membrane contains just a small number of proteins, but in very large quantities presumably reflecting the more specialised nature of the membrane. The outer membrane of wild-type *E. coli* strains contains at least three main proteins:- Murein (also called Braun's) Lipoprotein; OmpA and the porins. However, in addition to these a number of minor proteins and proteins that are only present under certain conditions exist.

As might be expected from their position at the cell surface, outer membrane proteins have unusual properties, such as resistance to proteases and to solubilisation in denaturants such as detergents and bile salts. Some of the proteins are found as heat-resistant oligomers and seem to be very tightly packed. The loss of a protein from the membrane due to mutation of its gene generally leads to a compensatory increase in the amount of one or more of the other outer membrane proteins (Henning *et al.*, 1979b). This may be a reflection of competition for common sites where these proteins are inserted into the outer membrane.

Murein Lipoprotein in *Escherichia coli* is the most abundant protein, in terms of molecules, in the cell (each cell containing around $7x10^5$ copies) - (Braun, 1975). It has a molecular weight of 7.2 kDa and its function appears to be to bind the outer membrane covalently to the peptidoglycan layer (see Figure 1.1), thereby maintaining the integrity of the outer membrane. It can covalently link the underlying peptidoglycan layer via the ε-amino group of its C-terminal lysine residue (Braun, 1975). Hence it is thought that the main function of the lipoprotein is structural in that it stabilises the outer membrane-peptidoglycan complex. A lipoprotein similar to

Braun's Lipoprotein is also present in *Salmonella* strains.

OmpA is another protein that exists in large quantities in the outer membrane of *E. coli*, with a copy number of around 10^5 per cell (Chen *et al.*, 1980). It has a subunit molecular weight of about 30 kDa and has a high ß-sheet structural content. It appears to be transmembranous and is partly exposed to the cell surface (see Figure 1.1). Unlike *E. coli* porins there is little evidence that OmpA exists as an oligomer or forms a stoichiometric complex with other proteins. The exact role of the OmpA protein in the physiology of the outer membrane is not yet fully understood. However, mutants lacking both OmpA and Murein Lipoprotein produce unstable outer membranes indicating that the OmpA protein is implicated in the maintenance of the integrity of the outer membrane and cell shape (Sonntag *et al.*, 1978). OmpA is also known to act as a phage receptor, binding OmpA-specific bacteriophages, such as Ox2 and K3 (Morona *et al.*, 1985). OmpA is also important for F-dependent conjugation as it is required in the *recipient* cell for efficient conjugation in liquid media (Achtman *et al.*, 1978d). Ried & Henning, (1987) showed that a single amino acid substitution of Gly to Asp at position 154 of the OmpA protein caused the cells to be Con$^-$. This region of OmpA has also been implicated in binding the OmpA-specific bacteriophage, Ox2, and hence may be at the cell surface (Morona *et al.*, 1985). OmpA may play a role in the uptake of amino acids (Manning *et al.*, 1977) and is also required for the actions of colicins K and L (Lugtenberg & Van Alphen, 1983).

The remaining major outer membrane proteins are the porin proteins. The outer membrane of *E. coli* contains, under standard growth conditions, just two porin proteins, OmpC and OmpF, which act as channels. They allow the passive movement of small hydrophilic molecules through the outer membrane into the cell and the efflux of waste products out of the cell. The porins are some of the most abundant proteins present in *E. coli* in terms of mass, accounting for up to 2% of the total cellular proteins (Nikaido & Vaara, 1987). Although the total amount of porin present in the outer membrane is fairly constant, the relative abundance of each type of porin present is efficiently regulated in response to environmental changes, such as osmolarity and temperature (Forst *et al.*, 1989). In this way the bacteria alter the

permeability of the outer membrane, since each protein has a slightly different pore size and distribution of charged and polar residues within the water-filled pore. The OmpF and OmpC pores of *E. coli* have diameters of 1.16 and 1.08 nm, respectively (Nikaido & Vaara, 1987). Thus the OmpC porin has a slightly lower cut off point for small hydrophilic molecules. Nikaido & Vaara, (1985) have shown, using related oligosaccharides of defined molecular mass, that OmpF allows hydrophilic molecules of up to 600 Da to traverse the membrane. The OmpF and OmpC porins of *E. coli* exist as trimers, with subunit molecular masses of 38.3 and 37 kDa, respectively, with very little of their structure being extramembranous, even though they are transmembranous. In contrast to other integral membrane proteins, the porin proteins have a strikingly high content of ß-structure but virtually no α-helical segments or any long stretches of hydrophobicity (Rosenbusch, 1974). Recently, the crystal structure of OmpF porin has been obtained at 2.7 Å resolution (J. Rosenbusch, unpublished results). Each subunit consists of a 16-strand antiparallel ß-barrel analogous to that of the previously characterised *Rhodobacter capsulatus* porin (Weiss *et al.*, 1991).

There are other proteins, apart from these three classes of major proteins, that are present in the outer membrane but not at such a frequency. However, these minor proteins, may become major proteins under certain growth conditions where they are fully induced. For example, when *E. coli* cells are grown in the presence of maltose the LamB protein is seen in the outer membrane. Its function is to transport maltose and maltodextrins through the membrane; it also acts as a receptor for phage lambda. The LamB protein is seen to have the largest pore size (1.3 nm) of the *E. coli* pore proteins (Hammond *et al.*, 1983) and hence its principal function is to transport maltodextrins which are too large to use the general porin route. It has a subunit molecular weight of 47 kDa and resembles the other porins in its affinity towards the peptidoglycan layer and its tendency to form stable oligomers in SDS. However, it has limited chemical or immunological similarity with other major outer membrane proteins (Overbeeke *et al.*, 1980). The *lamB* gene sequence (Clement & Hofnung, 1981) predicts the LamB protein to contain many negatively charged amino acids and a high content of ß-structure. Charbit *et al.*, (1988c) have proposed a detailed model for the topology of the protein.

Similarly, if *E. coli* are grown under conditions of phosphate limitation then the porin PhoE is induced. The monomeric form of this porin protein is 36.7 kDa in size (Rosenbusch, 1974) and specifies a pore size the same as OmpF, 1.16 nm (Nikaido & Vaara, 1987). This porin, too, shares properties with the OmpF and OmpC proteins, being trimeric in its undenatured form, rich in ß-sheet structure (Rosenbusch, 1974) and its gene having very strong sequence homology to the other porins (Mizuno *et al.*, 1983; Overbeeke *et al.*, 1983). PhoE, however, forms pores which are more efficient at allowing transfer of phosphate-containing compounds or negatively-charged compounds through the outer membrane, unlike OmpC and OmpF which form cation selective pores. It appears to be synthesised in order to scavenge the last traces of phosphate or phosphate-containing components from the medium. The PhoE protein also serves as a receptor for phage TC45 (Agterberg *et al.*, 1987).

Some outer membrane proteins are specified by extrachromosomal elements, such as plasmids or phage, and hence are only present under certain circumstances. One such protein is the TraT lipoprotein, a highly cell-surface-exposed component specified by plasmids of the IncF incompatibility group. Since this protein is employed in the present study, its properties are described below.

1.2. THE TRAT PROTEIN.

The TraT protein is an outer membrane lipoprotein specified by F-like plasmids of Gram-negative bacteria. It is so named because it is determined by the *traT* gene which is generally part of the *tra* (transfer) operon of such plasmids. The gene products of the *tra* operon are involved in the conjugal transfer of DNA, via cell-cell contacts.

1.2.1. Expression of the TraT Protein.

The best-studied *tra* operon is that of the F (fertility) factor, a covalently closed, circular, double stranded DNA molecule of around 100 kb in length (reviewed by

Ippen-Ihler & Minkley, 1986). The *tra* operon is comprised of approximately one third (a 35 kb segment) of the total F plasmid DNA and contains around two dozen genes. These genes have been shown by DNA hybridisation studies to be highly similar to those of other F-like plasmids (such as the Col factors: ColV2 and ColVB*trp*; and the R factors: R1-19 and R100-1). However, the structure of the *tra* operon of the F plasmid is the only one known in detail and hence, it is this operon which is reviewed here.

The map positions of the *tra* cistrons on the F factor were defined by genetic analysis of cloned *EcoRI* fragments of the *tra* operon of F DNA (Achtman *et al.*, 1978b). The function of the separate genes were determined by isolation and complementation analysis of nonsense, missense and frame shift mutations of the operon. Analysis of lambda-phage insertions into the *tra* region also helped identify the genes responsible for conjugation (reviewed by Ippen-Ihler & Minkley, 1986; Willetts & Skurray, 1980). On analysis of mutations of the *tra* operon it was found that the *tra* gene products fell into four main categories according to their function:-

Firstly, the *traS* and *traT* genes code for proteins that bring about the phenomenon of surface exclusion. This is the process whereby the unfavourable self-mating of two *donor* cells is prevented (for more details see section 1.2.6.1.).

The majority of the genes in the operon code for products involved in the assembly of F-pili. There are on average between 0.5-1.5 of these 1-2 μm long proteinaceous appendages projecting from the cell surface of each *donor* cell and each is about 8 nm in diameter with a central hole of 2 nm (Ippen-Ihler & Minkley, 1986).

Pili are essential for the formation of *donor-recipient* cell contacts although their precise role in DNA transfer is still controversial. It is thought that the pili exist transiently in the inner membrane with F-pilin subunits being continuously recycled between assembled and disassembled states (Silverman, 1986). Hence, the pili may depolymerise to pull the two cells into close contact so that DNA can be transferred between them. It is presently unclear if DNA is actually transferred through the pilus

itself. Until recently it was thought that DNA transfer occurred through a special DNA channel or pore between *donor* and *recipient* cells and not through the pilus. This was inferred from mating experiments with low levels of SDS (0.01%) which remove F-pili from *donor* cells and prevent their regeneration from the F pilin pools in the inner membrane (Sowa *et al.*, 1983). As SDS-induced loss of pilation has only a small effect on transfer frequency if aggregates are allowed to form prior to SDS exposure (Achtman *et al.*, 1978b) it is possible that the pilus is not the conduit for DNA. Recently, however, Harrington & Rogerson, (1990) reported that when *donor* and *recipient* cells were separated by use of polycarbonate filters that were about 6 μm thick (i.e. 2 to 3 times the length of an *E. coli* cell) and that had pores of submicrometer diameter (about 0.1 to 0.01 times the diameter of an *E. coli* cell), DNA transfer still occurred. This implies that DNA transfer may indeed be via the pilus after all. Although further work is required to resolve the question, it is clear that the pilus is essential under normal conditions of DNA transfer, whatever its role.

The *traN* and *traG* gene products are responsible for stabilisation of *donor-recipient* surface associations (Manning *et al.*, 1981), although again, their mode of action has not been characterised. The *traG* gene is the only gene in the operon whose protein product is assigned two functions (F-pilin biosynthesis and stabilisation of *donor-recipient* associations).

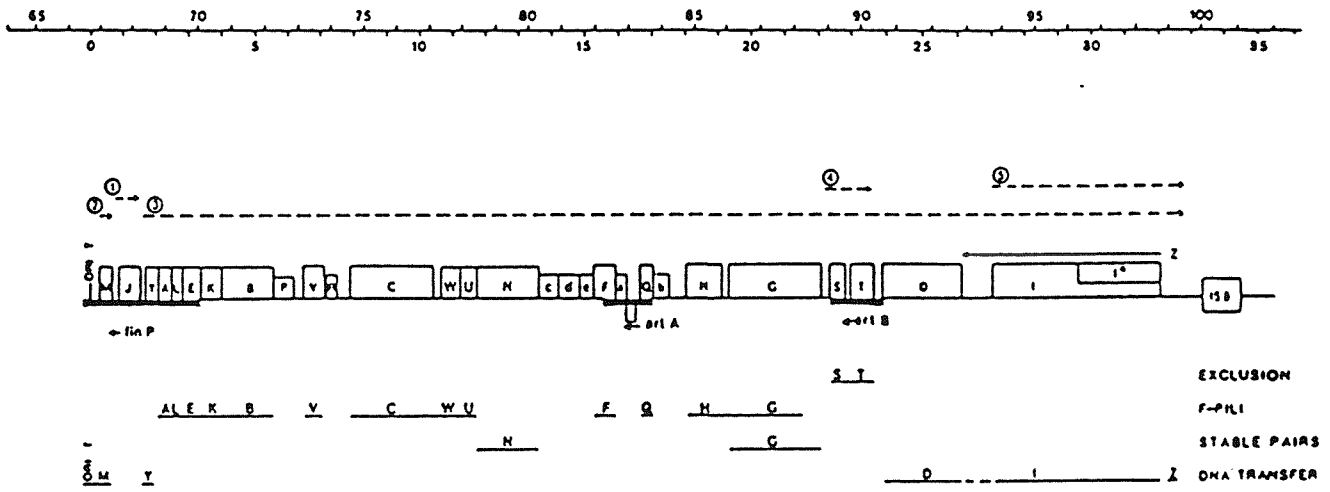
The remaining genes specify proteins involved in DNA transfer (Ippen-Ihler & Minkley, 1986). One strand of the F plasmid is nicked specifically at *oriT* (involving the TraY protein) allowing the DNA to unwind. This single strand of DNA is displaced (by TraD) and transported to the *recipient* cell where it is recircularised and converted into a duplex by complementary strand synthesis. The *donor* strand is then replaced by a modified rolling circle type of replication (involving TraM & I).

The physical, genetic and functional mapping of these genes in the *tra* region of the F-plasmid is summarised in Figure 1.3.

FIGURE 1.3.

Physical, Genetic and Functional Map of the *tra* Operon of the F Conjugative Region.

(adapted from Ippen-Ihler & Minkley, 1986)



Top line: Represents the kilobase coordinates for F (66.7-100) and the *tra* region (0-33.3).

Centre line: The boxes indicate the placement and size of *tra* (capital letters) and *trb* (small letters) genes. A small box height represents a gene with an uncharacterised function. ORI T is the origin of transfer. The long arrow represents the uncertainty in *traZ* placement. The small arrows represent the positions and directions of additional *tra* upstream promoters, *finP, artA* and *artB*. Dashed arrows represent *tra* transcripts.

Bottom line: Represents the functional assignment of the non-regulatory *tra* region products.

In addition to the plasmid-encoded *tra* genes certain chromosomal genes of *E. coli* K-12 are required for *donor* activity (Silverman, 1986). However, these gene products are not totally devoted to DNA transfer and have other functions in the cell.

The only gene in the operon whose product does not fit into the above functional categories is the *traJ* gene. The product of *traJ* gene is a transcriptional activator of the *tra* operon that promotes the transcription of all the *tra* genes from promoter sites that precede *traM* and *traY*. Evidence for this role comes from *traJ* mutants which are pleiotropic, being defective in transfer, pilus formation and surface exclusion (Achtman *et al.*, 1971; 1972; Willetts & Achtman, 1972; Achtman, 1973; Finnegan & Willetts, 1973) and also from hybridisation studies (Willetts, 1977). TraJ is regulated through the *finP* (fertility inhibition) product which is a RNA with a 6-7 nucleotide 'loop' formed between a 12-base inverse repeat 'stem'. It is this 'loop' part of the *finP* RNA that interacts with a complementary 105-base non-coding leader region of *traJ*. Most IncF plasmids carry two fertility inhibition genes, *finO* and *finP*, the products of which act in concert to prevent transcription of *traJ*. F itself lacks *finO*, but carries both the *finP* gene and the site at which FinOP regulation occurs, *fisO* (Mullineaux & Willetts, 1985). Hence, if a compatible, co-resident F-like plasmid produces the *finO* product it then acts with the *finP* product to repress the expression of the *traJ* gene and hence prevent expression of all the other *tra* genes (Finnegan & Willetts, 1971).

Recent work has uncovered several internal promoters, suggesting that the control of expression of the *tra* genes may be more complex than originally thought. For example, the *traT* gene has a relatively weak *traJ*-independent promoter, the start and termination point of which has been precisely mapped by S1 nuclease protection studies to within the *traS* gene (Ham *et al.*, 1989). RNA stability experiments in the same study also indicated that the mRNAs for both *traS* and *traT* are unusually stable. Therefore, it seems that the *traJ*-independent expression of the F TraT protein is due partly to a *traJ*-independent promoter and partly to stable mRNA.

Another interesting factor that affects the expression of the F TraT protein is, like

other products of the *tra* operon, temperature. Essentially no protein is detectable at 25°C, but maximal levels are synthesised at 42°C (Sowa *et al.*, 1983).

1.2.2. Physico-chemical Properties of the TraT Protein.

The TraT protein of F is a basic protein with a pI of around 9 (Manning *et al.*, 1980). The mature form is 223 amino acids long with a subunit molecular weight of 23,709 Da (Jalajakumari *et al.*, 1987). The mature protein is present at very high concentrations, 20,000 to 30,000 copies per cell (Achtman *et al.*, 1977; Minkley & Ippen-Ihler, 1977), in cells bearing F-like plasmids. This corresponds to about 0.4% of the total cellular protein. The TraT protein is thought to exist in the outer membrane in a multimeric form (Manning *et al.*, 1980; Minkley & Willetts, 1984). Manning *et al.*, (1980) showed that the TraT protein favours oligomer formation because before boiling the protein in the presence of SDS less than 35% was in the monomeric from. Electron micrographs of the purified protein suggest it is an oligomeric doughnut-shaped molecule (Minkley, unpublished results - cited Ippen-Ihler & Minkley, 1986). The outer diameter of the doughnut being approximately 18.5 nm and the diameter of the central region being approximately 8 nm. This central diameter is seen to be the appropriate size for binding the F-pilus.

The native TraT protein shares a number of properties in common with other outer membrane proteins, in particular the porins. For example, not only is it oligomeric, transmembranous and associated with the peptidoglycan of the cell envelope, but it is also highly heat stable up to temperatures of 70 to 80°C, is very strongly resistant to digestion by broad spectrum proteases (e.g. trypsin or pronase) and is rendered insoluble in a variety of detergents (e.g. TraT is only partially soluble in SDS at temperatures below 70°C) (Manning & Achtman, 1979; Manning *et al.*, 1980). These properties might be expected because *E. coli* normally inhabits the gut, where it is exposed to a number of proteolytic and chaotropic agents. They also suggest that the native protein is very tightly packed, preventing the proteases and denaturants from accessing the required sites in the molecule. Despite these similarities, however, the TraT and porin proteins have virtually no similarity in their amino acid sequences.

FIGURE 1.4.

The R100 *traT* Gene and Amino Acid Sequence.

(Ogata *et al.*, 1982)

The numbers above the sequence represent the nucleotide bases of the gene and the numbers below the sequence represent the amino acid residues in the mature protein. The unique restriction sites, within the *traT* gene, that have been employed for the insertion of the C3 epitope (Taylor *et al.*, 1990), are shown. The seven tyrosine residues within the protein, at least some of which are labelled with ^{125}I by lactoperoxidase, are boxed.

FIGURE 1.4.

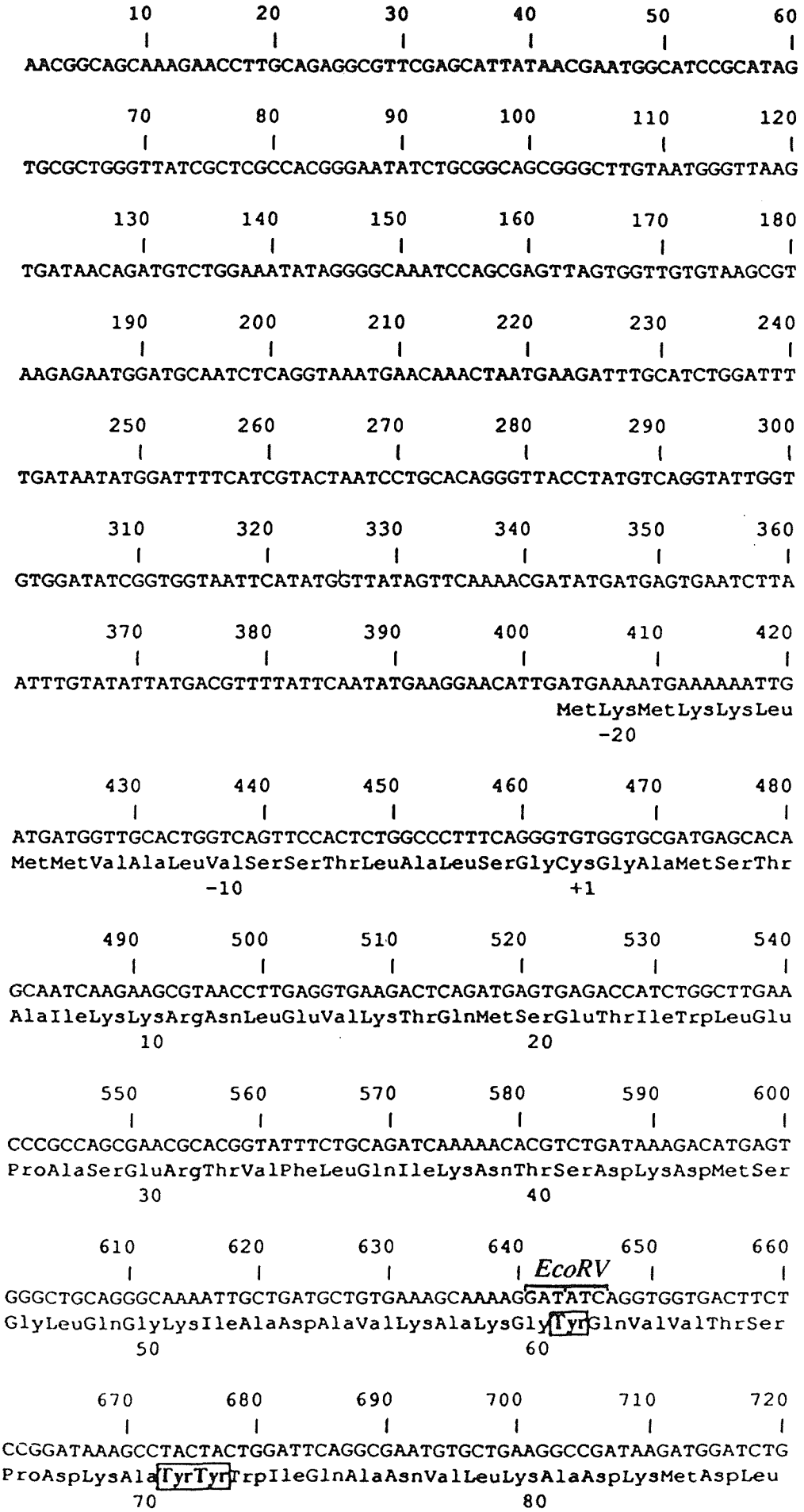

10 20 30 40 50 60
AACGGCAGCAAAGAACCTTGCAGAGGCGTTCGAGCATTATAACGAATGGCATCCGCATAG
70 80 90 100 110 120
TGCGCTGGGTTATCGCTCGCCACGGGAATATCTGCGGCAGCGGGCTTGTAATGGGTTAAG
130 140 150 160 170 180
TGATAACAGATGTCTGGAAATATAGGGGCAAATCCAGCGAGTTAGTGGTTGTGTAAGCGT
190 200 210 220 230 240
AAGAGAATGGATGCAATCTCAGGTAAATGAACAAACTAATGAAGATTTGCATCTGGATTT
250 260 270 280 290 300
TGATAATATGGATTTTCATCGTACTAATCCTGCACAGGGTTACCTATGTCAGGTATTGGT
310 320 330 340 350 360
GTGGATATCGGTGGTAATTCATATGGTTATAGTTCAAAACGATATGATGAGTGAATCTTA
370 380 390 400 410 420
ATTTGTATATTATGACGTTTTATTCAATATGAAGGAACATTGATGAAAATGAAAAAATTG
MetLysMetLysLysLeu
-20
430 440 450 460 470 480
ATGATGGTTGCACTGGTCAGTTCCACTCTGGCCCTTTCAGGGTGTGGTGCGATGAGCACA
MetMetValAlaLeuValSerSerThrLeuAlaLeuSerGlyCysGlyAlaMetSerThr
-10 +1
490 500 510 520 530 540
GCAATCAAGAAGCGTAACCTTGAGGTGAAGACTCAGATGAGTGAGACCATCTGGCTTGAA
AlaIleLysLysArgAsnLeuGluValLysThrGlnMetSerGluThrIleTrpLeuGlu
10 20
550 560 570 580 590 600
CCCGCCAGCGAACGCACGGTATTTCTGCAGATCAAAAACACGTCTGATAAAGACATGAGT
ProAlaSerGluArgThrValPheLeuGlnIleLysAsnThrSerAspLysAspMetSer
30 40
610 620 630 640 650 660
EcoRV
GGGCTGCAGGGCAAAATTGCTGATGCTGTGAAAGCAAAAGGATATCAGGTGGTGACTTCT
GlyLeuGlnGlyLysIleAlaAspAlaValLysAlaLysGlyTyrGlnValValThrSer
50 60
670 680 690 700 710 720
CCGGATAAAGCCTACTACTGGATTCAGGCGAATGTGCTGAAGGCCGATAAGATGGATCTG
ProAspLysAlaTyrTyrTrpIleGlnAlaAsnValLeuLysAlaAspLysMetAspLeu
70 80

FIGURE 1.4. (Cont[d].)

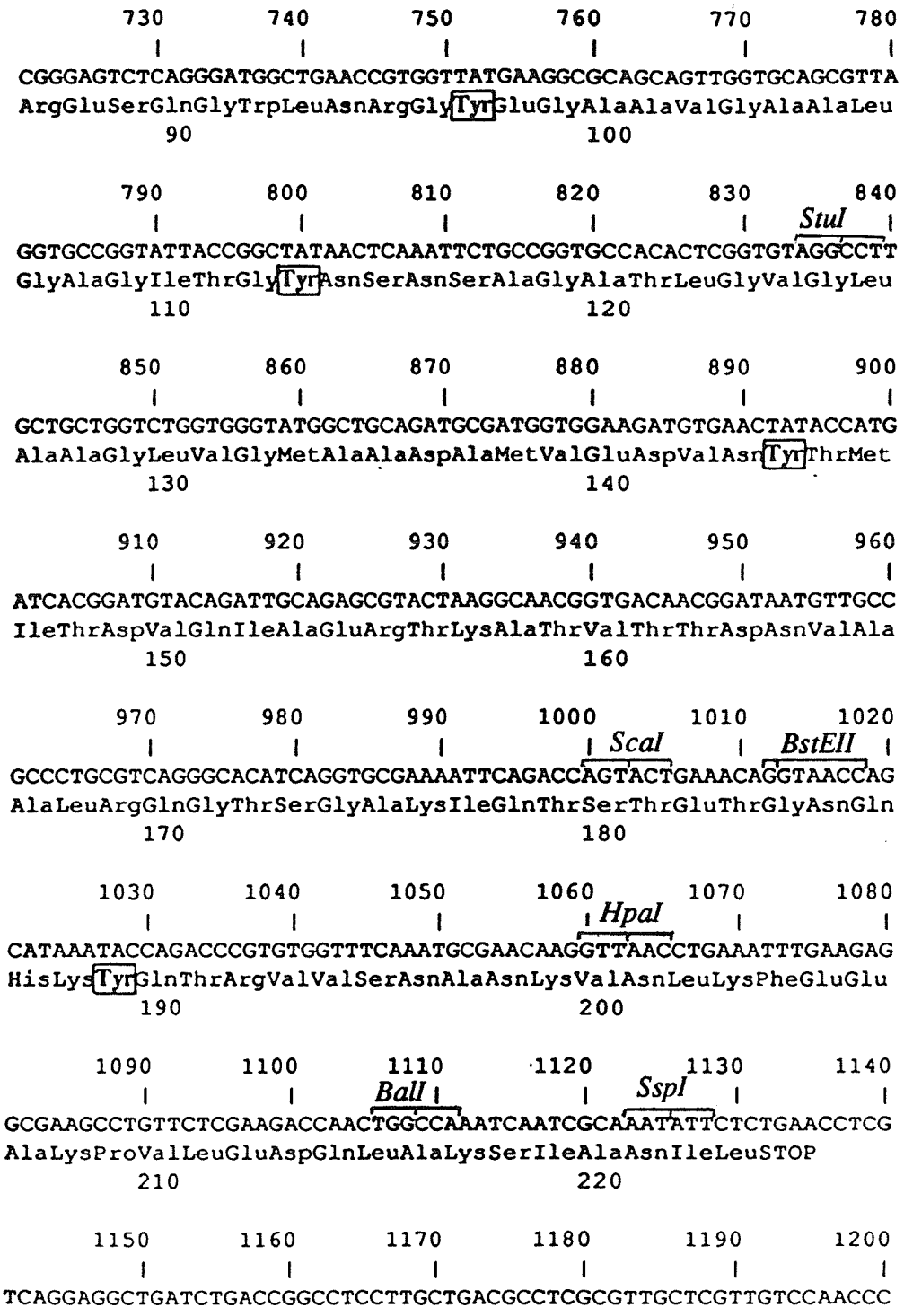
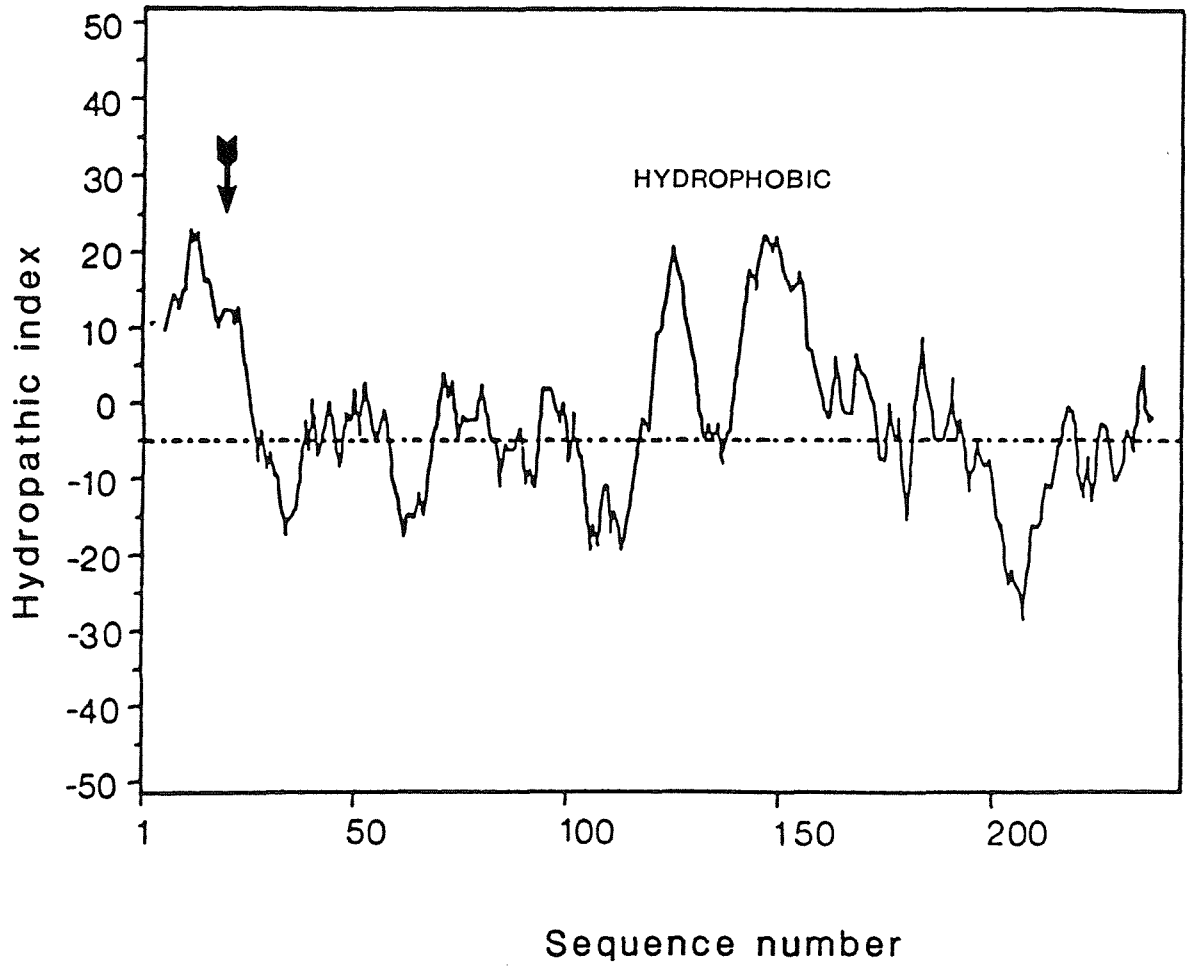


FIGURE 1.5.

Hydropathy Plot of the R6-5 TraT Protein.

(Kyte & Doolittle, 1982)

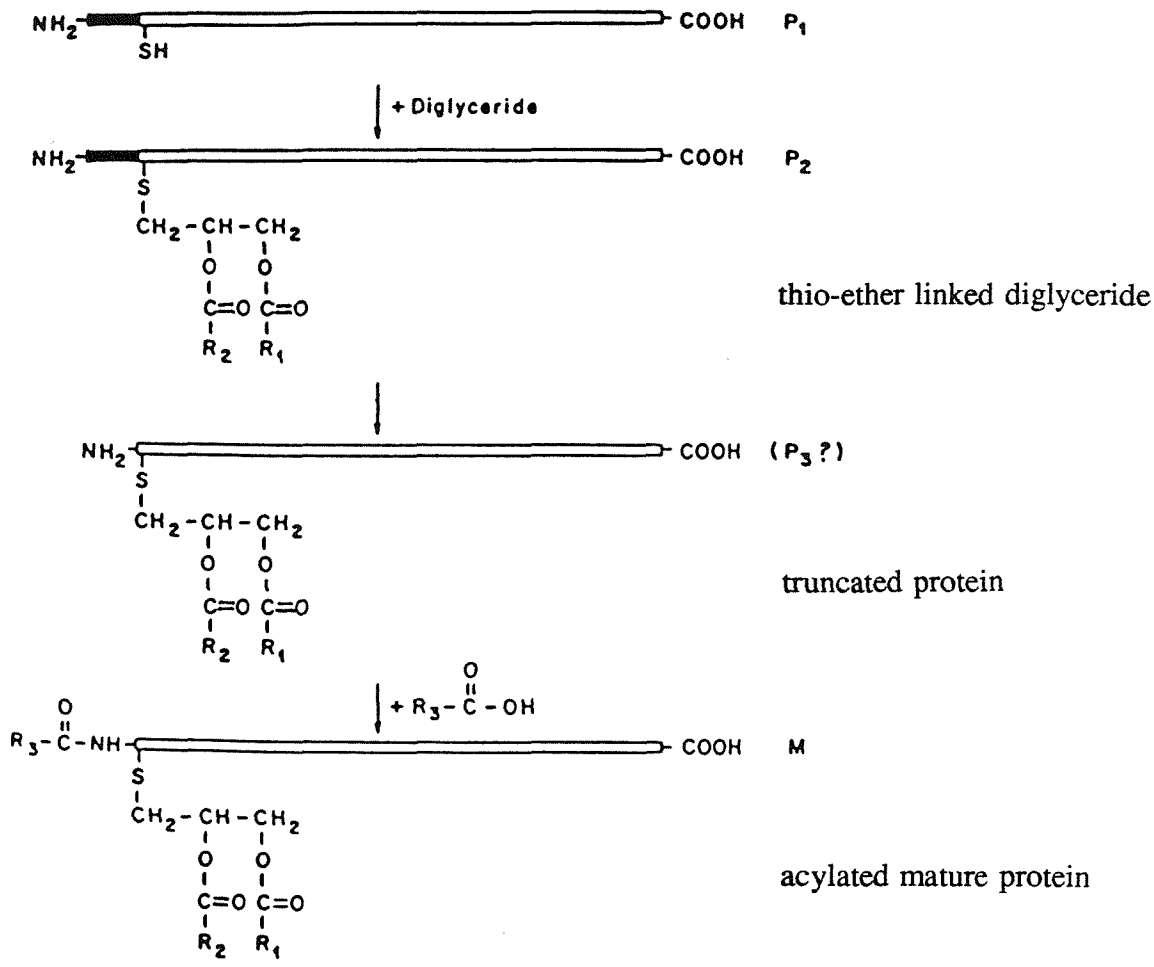


The hydropathy index, averaged over 11 residues, was plotted according to Kyte & Doolittle, (1982). The x-axis represents the number of residues of the protein and the y-axis represents the hydropathic index. A positive hydropathic index indicates a hydrophobic region of the protein. The two main hydrophobic regions of the TraT protein (residues 119-130 and 138-155 of the precursor protein) are shown. The arrow shows the first residue of the mature protein. Note that the amino terminus will also be hydrophobic due to the attachment of lipids (see text).

FIGURE 1.6.

Schematic Model for the Processing and Modification of the TraT Signal Sequence in *E. coli.*

(adapted from Perumal & Minkley, 1984)



The precursor forms are referred to as P_1, P_2 and P_3, whereas M refers to the mature form of the protein. R_1, R_2 and R_3 represent hydrocarbon chains of fatty acids.

The unmodified precursor, P_1, is covalently modified at the N-terminal proximal cysteine to form P_2. Signal sequence cleavage thus occurs between Gly-Cys_1 to give an, as yet unidentified, hypothetical intermediate P_3. The addition of fatty acid via an amide link to P_3 produces the mature TraT protein.

Their hydropathy plots and predicted amino acid secondary structures also show major differences (Sukupolvi & O'Connor, 1990). For example, OmpF, OmpC, OmpA and LamB have less than 15% α-helical content (Vogel & Jähnig, 1986), whereas that of the TraT protein is predicted to contain more. Also, the porins contain only small stretches of hydrophobic residues (less than 7 amino acids), whereas TraT has two larger stretches of non-charged amino acids at residues 99-110 and 118-135 of the mature protein (see Figures 1.4 and 1.5).

TraT, being an outer membrane lipoprotein (Achtman *et al.*, 1977; Achtman *et al.*, 1979; Manning *et al.*, 1980) is synthesised as a precursor that has a molecular weight of 25,932 Da and whose first twenty amino acids have characteristic features of a signal sequence (Ogata *et al.*, 1982; Minkley, 1984). The processing and modification of the precursor TraT protein is shown in Figure 1.6 (Perumal & Minkley, 1984). As the protein is transported to the outer membrane of the cell two fatty acids are attached to glycerol via ester linkages. This diglyceride is then attached via a thio-ether link to the single cysteine residue (amino acid 21 - see Figure 1.4) at the N-terminal end of the protein. Following cleavage of the first 20 amino acids (the signal peptide) by signal peptidase II, a further fatty acyl group is added to the cys residue (which forms the first amino acid of the mature protein) via an amide bond.

1.2.3. The TraT Protein Sequences of Different Plasmids of the IncF Group.

The *traT* genes of several of the IncF group plasmids (F, R100-1, R6-5, ColB, F_o*lac* of *Salmonella typhi*, pSLT of *Salmonella typhimurium* and pYV of *Yersinia enterocolitica*) have been cloned, sequenced and their corresponding TraT protein sequences deduced (Jalajakumari *et al.*, 1987; Ogata *et al.*, 1982; O'Connor & Timmis - unpublished; Harrison *et al.*, 1992 - submitted; Finlay & Paranchych, 1986; Sukupolvi *et al.*, 1990 and China *et al.*, 1990, respectively).

The *traT* gene sequences are highly conserved between these plasmids, unlike the sequences for the *traS* genes. At the amino acid level, the sequence of *Y. enterocolitica* TraT has 88% identity with that of F_o*lac* and 82% identity with that

of F and R100. The *S. typhimurium* pSLT TraT protein is more similar to R100 than to the pYV or F_o*lac* TraT.

The primary sequences of the corresponding proteins (see Figure 1.7) show both well conserved and relatively variable regions. The amino- and carboxyl- ends are highly conserved between the different TraT proteins, indicating that they are probably structurally or functionally important. The variable regions of the protein, however, are less likely to be structurally essential. In the outer membrane proteins PhoE, OmpC and OmpF the major differences in their primary structures are in the hydrophilic regions. Van der Ley *et al.*, (1985), showed, using monoclonal antibodies directed against cell-surface-exposed epitopes of these porins, that the most pronounced differences correlate with regions of the proteins that are exposed to the cell surface. This too, by analogy to these outer membrane proteins, may be true for the differences between the TraT proteins. However, at present there is essentially no experimental data on this point.

The TraT proteins are seen to have three variable regions (between residues 44-71, 88-102 and 157-161). There is another notable region (amino acids 116-120) within the TraT proteins which shows a small amount of variation between proteins, but not as much as the other variable regions of the protein. This region of the protein is of interest for two reasons:

Firstly, it may be important for the surface specificity of the protein (Willetts & Maule, 1974 & 1986). It is seen that the differences in the primary structure between the mature TraT proteins of F, R100, ColB and R6-5 are in this region. For example, Sukupolvi & O'Connor, (1990) noted that the only difference between the ColB and R6-5 TraT proteins is a single amino acid alteration (Ser to Asn at amino acid 116 of the mature protein). Similarly, the only amino acid difference between the TraT coded by the F plasmid and that coded by the R100 plasmid is a Gly at amino acid 120 instead of an Ala (see Table 1.1). Thus, assuming that TraT has surface exclusion specificity during conjugation of these F-like plasmids, the effect may be attributable to the differences in their TraT sequences around residues 116-120.

TABLE 1.1.

Amino Acid Differences in the Specificity Region of the F-like Plasmids.

Plasmid Specifying TraT Protein.	Surface Exclusion Group. [a]	Relevant Portion of Sequence (residues 116-120). [b]
F	I	...AsnSerAlaGlyGly...
ColB2-K98	II	...Ser---------Ala---...
R6-5	IV	...Asn---------Ala---...
R100-1	IV	...Asn---------Ala---...

[a] Based on the classification of Willetts & Maule, (1986), see Table 1.3.

[b] TraT sequences are taken from Jalajakumari *et al.*, (1987); Harrison *et al.*, (1992); O'Connor & Timmis - unpublished; Ogata *et al.*, (1982), respectively.

FIGURE 1.7.

Comparison of the TraT Protein Amino Acid Sequences of the F-like Plasmids.

(adapted from Sukupolvi & O'Connor, 1990)

This figure compares the TraT amino acid sequences (deduced form the *traT* gene sequences) of:

F (Jalajakumari *et al.*, 1987);

R100-1 (Ogata *et al.*, 1982);

ColB (Harrison *et al.*, 1992);

F_o*lac* (Finlay & Paranchych, 1986);

pSLT (Sukupolvi *et al.*, 1990) and

pYV (China *et al.*, 1990).

The *traT* gene sequence encoded by the plasmid R6-5 has been determined (O'Connor & Timmis - unpublished), but is not shown here as its amino acid sequence is identical to that of R100.

A, B and C represent the regions that show variation between the plasmids. Residues 116-120 is the semi-variable region that has been implicated in surface exclusion specificity between the different F-like plasmids (see text).

+1 represents the Cys residue that marks the first residue of the mature protein.

FIGURE 1.7.

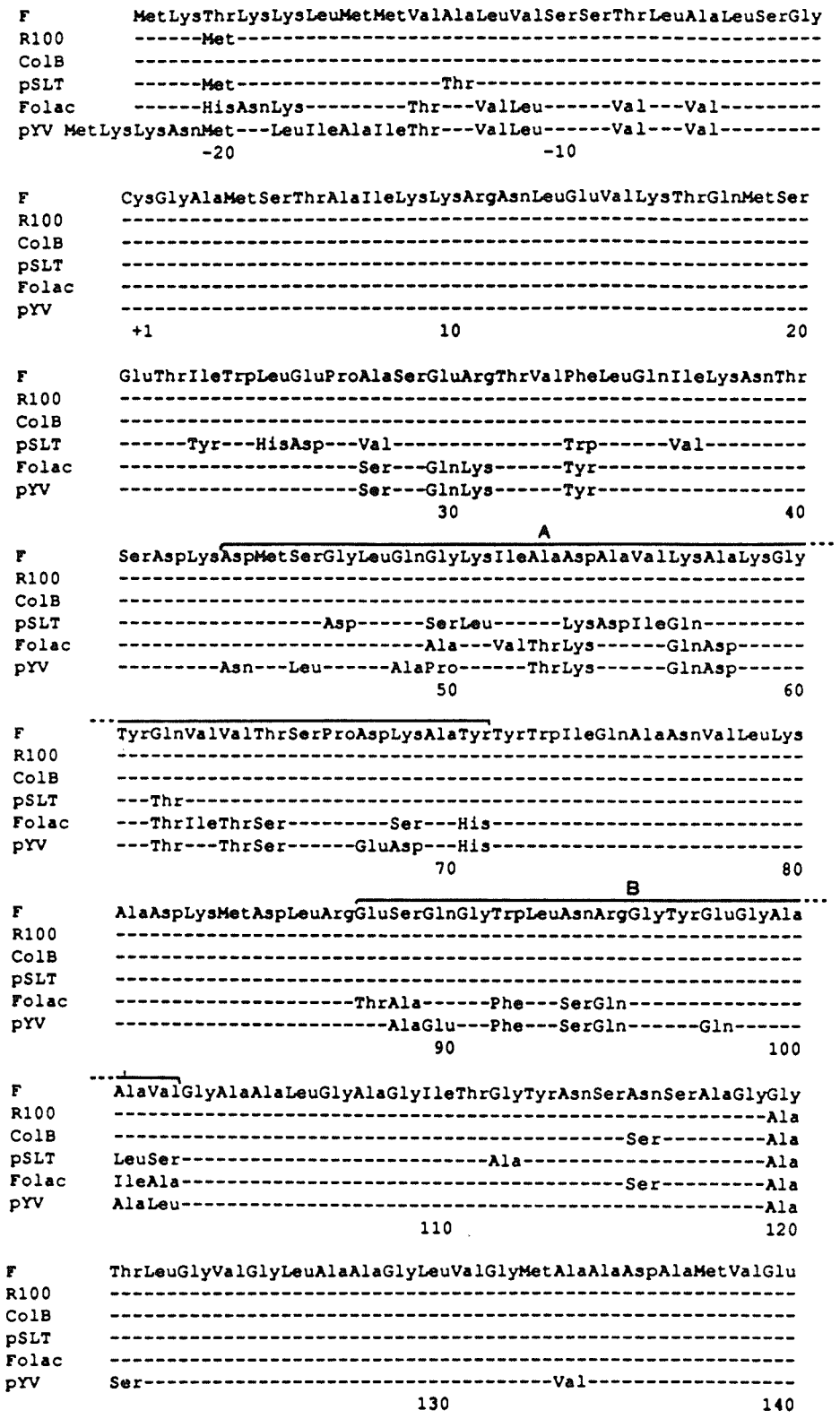
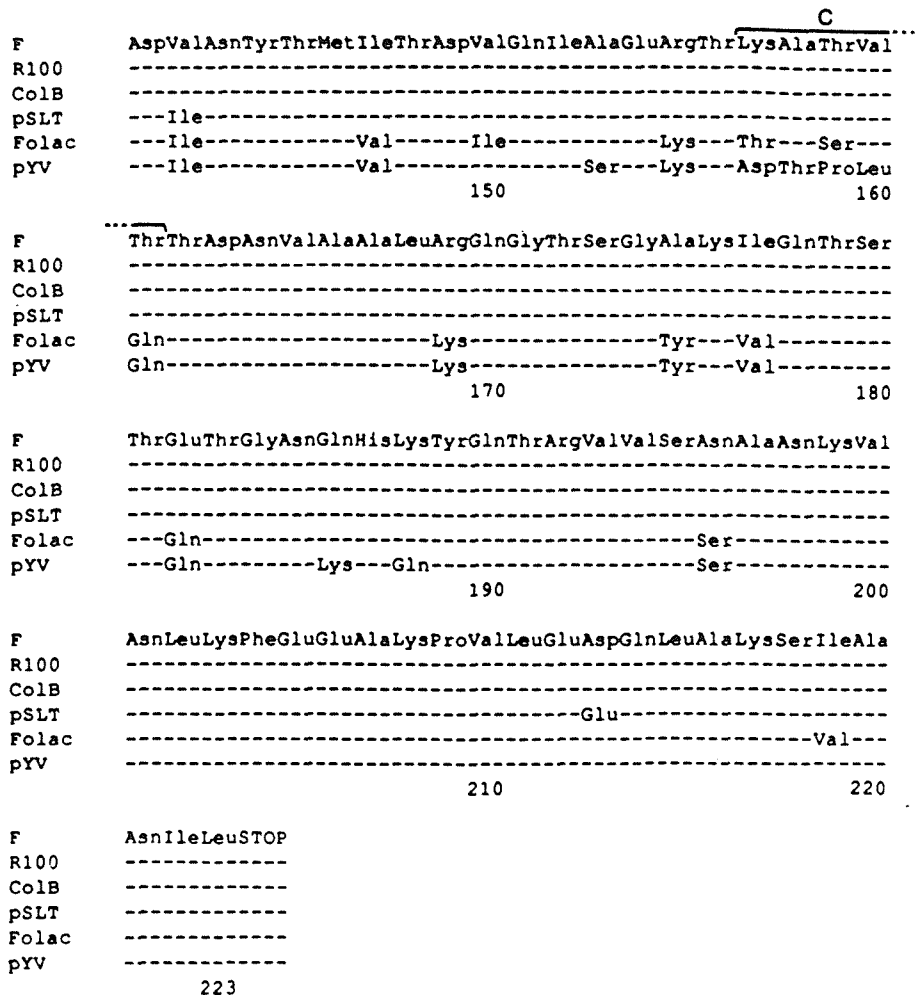

F MetLysThrLysLysLeuMetMetValAlaLeuValSerSerThrLeuAlaLeuSerGly
R100 ------Met----------
ColB ----------
pSLT ------Met----------Thr----------
Folac ------HisAsnLys---------Thr---ValLeu------Val---Val---------
pYV MetLysLysAsnMet---LeuIleAlaIleThr---ValLeu------Val---Val---------
-20 -10
F CysGlyAlaMetSerThrAlaIleLysLysArgAsnLeuGluValLysThrGlnMetSer
R100 ----------
ColB ----------
pSLT ----------
Folac ----------
pYV ----------
+1 10 20
F GluThrIleTrpLeuGluProAlaSerGluArgThrValPheLeuGlnIleLysAsnThr
R100 ----------
ColB ----------
pSLT ------Tyr---HisAsp---Val----------Trp------Val---------
Folac ----------Ser---GlnLys------Tyr----------
pYV ----------Ser---GlnLys------Tyr----------
30 40
A
F SerAspLysAspMetSerGlyLeuGlnGlyLysIleAlaAspAlaValLysAlaLysGly
R100 ----------
ColB ----------
pSLT ----------Asp------SerLeu------LysAspIleGln---------
Folac ----------Ala---ValThrLys------GlnAsp------
pYV ---------Asn---Leu------AlaPro------ThrLys------GlnAsp------
50 60
F TyrGlnValValThrSerProAspLysAlaTyrTyrTrpIleGlnAlaAsnValLeuLys
R100 ----------
ColB ----------
pSLT ---Thr----------
Folac ---ThrIleThrSer---------Ser---His----------
pYV ---Thr---ThrSer------GluAsp---His----------
70 80
B
F AlaAspLysMetAspLeuArgGluSerGlnGlyTrpLeuAsnArgGlyTyrGluGlyAla
R100 ----------
ColB ----------
pSLT ----------
Folac ----------ThrAla------Phe---SerGln----------
pYV ----------AlaGlu---Phe---SerGln------Gln------
90 100
F AlaValGlyAlaAlaLeuGlyAlaGlyIleThrGlyTyrAsnSerAsnSerAlaGlyGly
R100 ----------Ala
ColB ----------Ser---------Ala
pSLT LeuSer----------Ala----------Ala
Folac IleAla----------Ser---------Ala
pYV AlaLeu----------Ala
110 120
F ThrLeuGlyValGlyLeuAlaAlaGlyLeuValGlyMetAlaAlaAspAlaMetValGlu
R100 ----------
ColB ----------
pSLT ----------
Folac ----------
pYV Ser----------Val----------
130 140

FIGURE 1.7. (cont[d].)



The second reason for interest is that insertion of charged residues into this region of TraT results in increased outer membrane permeability to hydrophobic agents (Sukupolvi & O'Connor, 1990; Sukupolvi *et al.*, 1990). This implies that the region is important for the correct insertion of the protein into the membrane or for interaction of the protein with the surrounding environment. Similarly, it has been found that the permeability mutation, termed SS-A (Sukupolvi *et al.*, 1984), in the pSLT *traT* gene in *S. typhimurium* (Sukupolvi *et al.*, 1986) results in a Gly to Arg alteration at position 132, again within the prominently hydrophobic region of the protein (Sukupolvi *et al.*, 1990).

1.2.4. Mutants of the TraT Protein.

1.2.4.1. Mutants that Over-Produce the TraT Protein.

Manning *et al.*, (1982) isolated a series of plasmid mutant derivatives that over-produce the R6-5 TraT protein. Some of the mutants directed the synthesis of 10-fold more of the TraT protein (200,000 copies/cell) than did the parental plasmid. The proteins specified by all the mutant plasmids, except one, were correctly inserted into the outer membrane and exposed on the cell surface. Since the TraT protein is a non-essential component of the outer membrane, and its gain or loss from the cell is not known to be accompanied by any major changes in cell physiology, *traT* gene regulatory mutations might provoke less dramatic perturbations in membrane function than analogous mutations affecting the synthesis of essential outer membrane proteins. However, it was found that the strains that produced greater than normal amounts of the TraT protein grew slower and began to lyse in what was 'mid-log' phase for the bacteria that did not over-produce the TraT protein. Hence, over-production of TraT appears to be deleterious to bacterial growth.

1.2.4.2. Mutants that Increase Outer Membrane Permeability.

It has been found that certain mutated TraT proteins can lead to an increase in outer membrane permeability to hydrophobic compounds (Sukupolvi & O'Connor, 1987;

Rhen *et al.*, 1988). This was first shown by producing insertion mutations in the R6-5 *traT* gene (Sukupolvi & O'Connor, 1987). The constructs that altered outer membrane permeability had insertions in the same region of the protein, around the amino acid Gly_{125} which is located in a large hydrophobic and therefore possibly membrane spanning region of the protein (see Figure 1.5). Since the insertion mutations resulted in the introduction of a negatively charged amino acid into the hydrophobic region, the simplest explanation for the permeability phenotype associated with these specific insertions is that the alterations disturb the insertion of the protein in the membrane, or if the protein is inserted, they alter its interaction with the surrounding membrane components. The insertions also reduced the ability of the protein to confer serum resistance on the bacterium (Sukupolvi *et al.*, 1987).

Subsequently, oligonucleotide-mediated site-directed mutagenesis of Leu_{126} of the R6-5 TraT protein was carried out to determine whether the size, shape or charge of the inserted amino acid led to increased membrane permeability (Sukupolvi *et al.*, 1990). No effect on membrane permeability was found when the inserted amino acids were non-polar; however the insertion of either positively-charged (Arg or His) or negatively-charged (Asp or Glu) residues made the bacteria highly sensitive to hydrophobic antibiotics. The same sensitising effect was observed when proline was inserted which was probably due to the helix-disrupting effect of this residue.

As mentioned above, an alteration in the *S. typhimurium* pSLT TraT protein, which results in outer membrane permeability and which was produced by classical mutagenesis procedures, also maps to the hydrophobic region of the protein (Arg instead of Gly at 132). This further underlines the importance of this region in affecting outer membrane permeability. The wild-type *traT* genes of the F and R6-5 plasmids can suppress the phenotype caused by this mutant if placed in *trans* (Sukupolvi *et al.*, 1986). This suggests that the wild-type TraT protein in the cell may be able to bind the mutant subunit/s to give hetero-multimers that can probably adopt a conformation that resembles that of the normal protein thereby restoring the normal integrity and barrier properties of the outer membrane.

1.2.5. Surface Exposure of the TraT Protein.

Conjugation is a contact phenomenon and the TraT protein inhibits this process by preventing the formation of stable mating aggregates (see 1.2.6.1.). Thus, it would be expected that at least part of the protein is on the cell surface. Manning *et al.*, (1980) investigated this in two ways. Firstly, they coupled cyanogen bromide-activated dextran to the whole cells and thus labelled the surface-exposed proteins. They then fractionated the cell proteins, before and after coupling, on SDS polyacrylamide gels. The coupling of dextran onto the exposed protein prevented them from entering the gel. They found that 30 to 40% less TraT protein migrated on the gel suggesting that the TraT protein is surface-exposed.

In a separate series of experiments whole *traT*$^+$ cells were iodinated with ^{125}Iodine using lactoperoxidase and conditions where only the exposed proteins were labelled. The TraT protein was seen to be a minor band relative to all the other cell proteins in Coomassie Blue-stained SDS-polyacrylamide gels. However, autoradiography of the gel showed the TraT protein was the most strongly labelled of all the bands, implying that it was the major exposed protein in the cell. As the lactoperoxidase causes the radio-labelled iodine to selectively label tyrosine residues it was possible that the TraT protein had an unusually high proportion of surface-exposed tyrosines leading to an over-estimate of the degree of surface exposure. However, this possibility can now be discounted as, on sequencing of TraT and several other major outer membrane proteins, it was found that the former protein contains at least half the number, if not a third, of the tyrosine residues found in the other proteins (see Table 1.2).

Additional evidence for the surface exposure of TraT comes from a mutant which over-produces an altered TraT protein that is unable to be transported to the outer surface of the outer membrane. Consequently, the mutant did not mediate the expected levels of surface exclusion and serum resistance (Manning *et al.*, 1982).

TABLE 1.2.

Comparison of the Number of Tyrosine Residues in Five Major Outer Membrane Proteins.

Outer Membrane Protein.	Number of Tyrosine Residues in Mature Form.	Reference.
LamB	22	Clement & Hofnung, 1981
OmpA	17	Chen *et al.*, 1980
OmpF	29	Inokuchi *et al.*, 1982
PhoE	22	Overbeeke *et al.*, 1983
OmpC	29	Mizuno *et al.*, 1983
TraT (F)	**7**	Jalajakumari *et al.*, 1987

1.2.6. Functions of the TraT Protein.

1.2.6.1. Surface Exclusion (Sfx).

Surface exclusion is the phenomenon whereby cells carrying a sex factor are reduced in their ability to act as a *recipient* with other *donor* cells carrying the same or a closely related sex factor (Lederberg *et al.*, 1952; Willetts & Maule, 1974). Thus, a culture of cells carrying an F factor will not transfer DNA from one cell to another with any reasonable efficiency. Cells carrying the F factor typically have a 100- to 300- fold reduction in their ability to act as *recipients*, relative to an F^- cell (Achtman *et al.*, 1977; Willetts & Maule, 1974). It thus prevents the non-productive and highly energy-intensive mating of two cells harbouring the same conjugative plasmids.

Two *tra* gene products are independently responsible for this phenomenon of surface exclusion (Achtman *et al.*, 1977). These are the gene products TraT and TraS. The TraT protein is found to separately reduce *recipient* ability 15- to 20-fold; however its combined effect with TraS is cooperative and together gives a 400-fold reduction in DNA transfer (Achtman *et al.*, 1977).

For some unknown reason F^- phenocopies of *donor* cells are produced by growth of *donor* cells into late stationary phase (Lederberg *et al.*, 1952). These cells were found to have a *recipient* ability equivalent to an F^- cell (Willetts & Maule, 1974). Since these cells were found to be non-pilated it might have been expected that the cells had stopped synthesising the *traY-Z* operon products, including the TraS and TraT proteins. However, the *traT* product is still found in these F^- phenocopies and its concentration is unaffected (Achtman *et al.*, 1977). Thus, it may be that the F^--phenocopy effect is due solely to decreased amounts of the *traS* gene product.

The TraS protein, unlike the TraT protein, is an inner membrane protein and is smaller (16,861 Da) than the TraT protein (Jalajakumari *et al.*, 1987). It has no signal sequence and it is extremely hydrophobic. The protein causes surface exclusion by preventing the DNA transfer that normally follows mating pair formation (Achtman

et al., 1977; Manning & Achtman, 1979). Since *donor* conjugal DNA transfer does not occur in a population of F^+ cells TraS may, in accordance with its inner membrane location, block the triggering of conjugal DNA metabolism (Manning & Achtman, 1979). Hence, its mechanism of action, although synergistic with that of the TraT protein, is totally unrelated.

Achtman *et al.*, (1977) showed, using *traS*$^-$ and *traT*$^-$ mutants, that surface exclusion is mainly attributed to the TraS protein: the *traS*$^-$ *traT*$^+$ cells were only 16 to 26 times worse *recipients* than the control *recipient* cells whereas the *traS*$^+$ *traT*$^-$ cells were 90 to 215 times worse. This means that cells not carrying the TraT protein, but still expressing the TraS protein were far better at preventing conjugation than the *traS*$^-$ *traT*$^+$ cells.

It has been found that the extent of surface exclusion between cells containing plasmids of different surface exclusion groups varies greatly. Five surface exclusion groups (Sfx_I to Sfx_V) have now been identified amongst the F-like plasmids of E.coli (Willetts & Maule, 1974). Examples of these are shown in Table 1.3. Plasmids belonging to a specific surface exclusion group can transfer at essentially normal frequencies to cells carrying a plasmid of a different surface exclusion group. Therefore, an R100 plasmid (Sfx_{IV}) can be transferred with essentially no surface exclusion to a *recipient* carrying the F*his* plasmid (Sfx_I), whereas transfer is inhibited more than 300-fold when the R100 plasmid is crossed with a *recipient* cell carrying a plasmid from the same surface exclusion group, such as R6-5 (Willetts & Maule, 1974; 1986). This strongly suggests that both the TraT and TraS proteins are specific for each group of plasmids.

The specificity of these five groups might be due to one or a combination of the following. Firstly, it was found, following the cloning and sequencing of the *traT* genes of different F-like plasmids (Ogata *et al.*, 1982; Finlay & Paranchych, 1986; Jalajakumari *et al.*, 1987; Harrison *et al.*, 1992 - submitted), that their amino acid sequences vary very slightly (see 1.2.3.), although the proteins were identical in size. It was noted that the mature forms of TraT proteins specified by the F (Sfx_I),

ColB-K98 (Sfx_{II}) and R100 (Sfx_{IV}) plasmids differ by a single amino acid substitution in the same region (116-120) of the protein (see Table 1.1 and also Figure 1.7). This suggests that amino acids 116 to 120 determine the specificity of the TraT protein in surface exclusion. This was demonstrated by genetically substituting the relevant region (residues 116 to 120) of the ColB-K98 *traT* gene (Sfx_{II}) into the R6-5 *traT* gene (Sfx_{IV}) and showed that the surface exclusion specificity of the mutated R6-5 plasmid had switched surface exclusion specificity from Sfx_{IV} to Sfx_{II} (Harrison *et al.*, 1992 - submitted). Although this region of the TraT protein (residues 116 to 120) lies between two large hydrophobic regions, it is in fact hydrophilic itself, suggesting that it may be surface-exposed and hence able to interact with external agents.

Further endorsing this is the differences in the serological character of pili of the different F-like plasmids (Willetts & Maule, 1986). Sequence analysis of the pilin proteins of F-like plasmids shows that they are generally highly conserved (Frost *et al.*, 1985). However, the amino terminal regions show some variation between plasmids. The F-like plasmids have been classified into groups according to their pilus type (Forst *et al.*, 1985; Willetts & Maule, 1986). It can be seen that the pilus type correlates with the surface exclusion system (see Table 1.4). For example, F-like pili interact with Sfx_{I}, ColB-like pili interact with SfX_{II}, R1-19 pili interact with Sfx_{III} and R100-1-like pili interact with Sfx_{IV}. It is interesting to note that the plasmids are not all in the same surface exclusion and pilin group. For example, ColV2 and ColVB*trp* (F-like, or group I pili) recognise Sfx_{II} and Sfx_{III} systems, respectively, and are exceptions to the above rule.

The TraT protein, being an outer membrane protein, was found to bring about surface exclusion by preventing the formation of stable mating aggregates between two cells expressing the TraT protein (Achtman *et al.*, 1977). It has been proposed that the TraT protein achieves this by binding to the tips of the pili on the *donor* cell and thus prevents the pili from binding to their receptors which are thought to be present on the *recipient* cell surface (Willetts & Maule, 1986). If there is no pilus-receptor interaction then pilus retraction does not occur and the cells are not brought into close contact thereby preventing conjugation (Achtman *et al.*, 1978c, Silverman,

1986). This model explains the finding (Willetts & Maule, 1974) that when the *donor* cell contains two plasmids of different surface exclusion groups (and hence expresses mixed pili) and the *recipient* contains just one plasmid the same group as one of the plasmids in the *donor*, conjugation still takes place. Surface exclusion does not occur in this case as the second plasmid in the *donor*, which is of a different group to that in the *recipient* can still be transferred by conjugation between the two cells. This is because the pili specified by the second plasmid in the *donor* cannot interact with the TraT protein in the *recipient* because they are specified by plasmids of different surface exclusion groups. Hence, the pilus is free to bind the receptor in the *recipient*, the two cells can form stable mating aggregates and surface exclusion is abolished. Although the available experimental evidence is in agreement with the model, its validity would be greatly strengthened by a direct demonstration of pilus:TraT interactions.

The true receptor for the pilus, that is present in *recipient* cells, remains elusive. In the case of the F plasmid, OmpA may be a possible candidate for the pilus receptor as it is required on the *recipient* cell surface for efficient F-mediated conjugation in liquid media (Achtman *et al.*, 1978c).

However, OmpA is unlikely to act as a receptor for the pili of other F-like plasmids. For example, Manning & Reeves, (1975) showed that plasmids such as R100-1 transfer at normal frequencies to *ompA* mutant strains that prevent the transfer of F DNA. This result confirms the differences in specificity of the *recipient* ability of F and R100-1. Riede & Eschbach, (1986) have shown, using bacteriophage host range mutants, that single amino acid alterations in the adhesin proteins of the phages allowed completely different outer membrane proteins to act as phage receptors. Moreover, LPS also plays an important role in pilus receptor functions as *E. coli* strains lacking heptose in their LPS were conjugation-deficient (Havekes *et al.*, 1976). Thus, it seems that not just one component of the outer membrane may act as the pilus receptor and that the particular receptor used depends on the pilus type.

TABLE: 1.3.

Examples of the Plasmids Found in Each Surface Exclusion Group:

(Willetts & Maule, 1986).

Sfx Group.	Plasmid/s.
Sfx_I	F; F8; F*his*; F*lac*
Sfx_{II}	ColB-K98; ColB::Tn5 Seq1; ColB2
Sfx_{III}	ColVB*trp*; R1-19; R1
Sfx_{IV}	R100-1; R6-5
Sfx_V	F_o*lac*

TABLE: 1.4.

Examples of the Plasmids Found in Each Pilus Group:

(Frost *et al.*, 1985).

Pilus Group.	Pilus type.	Plasmid/s.
I	F-like	F; ColV2; ColVB*trp*
II	ColB-like	ColB-K98; ColB2
III	R1-19	R1
IV	R100-1-like	R100-1; R6-5
V	F_o*lac*	F_o*lac*

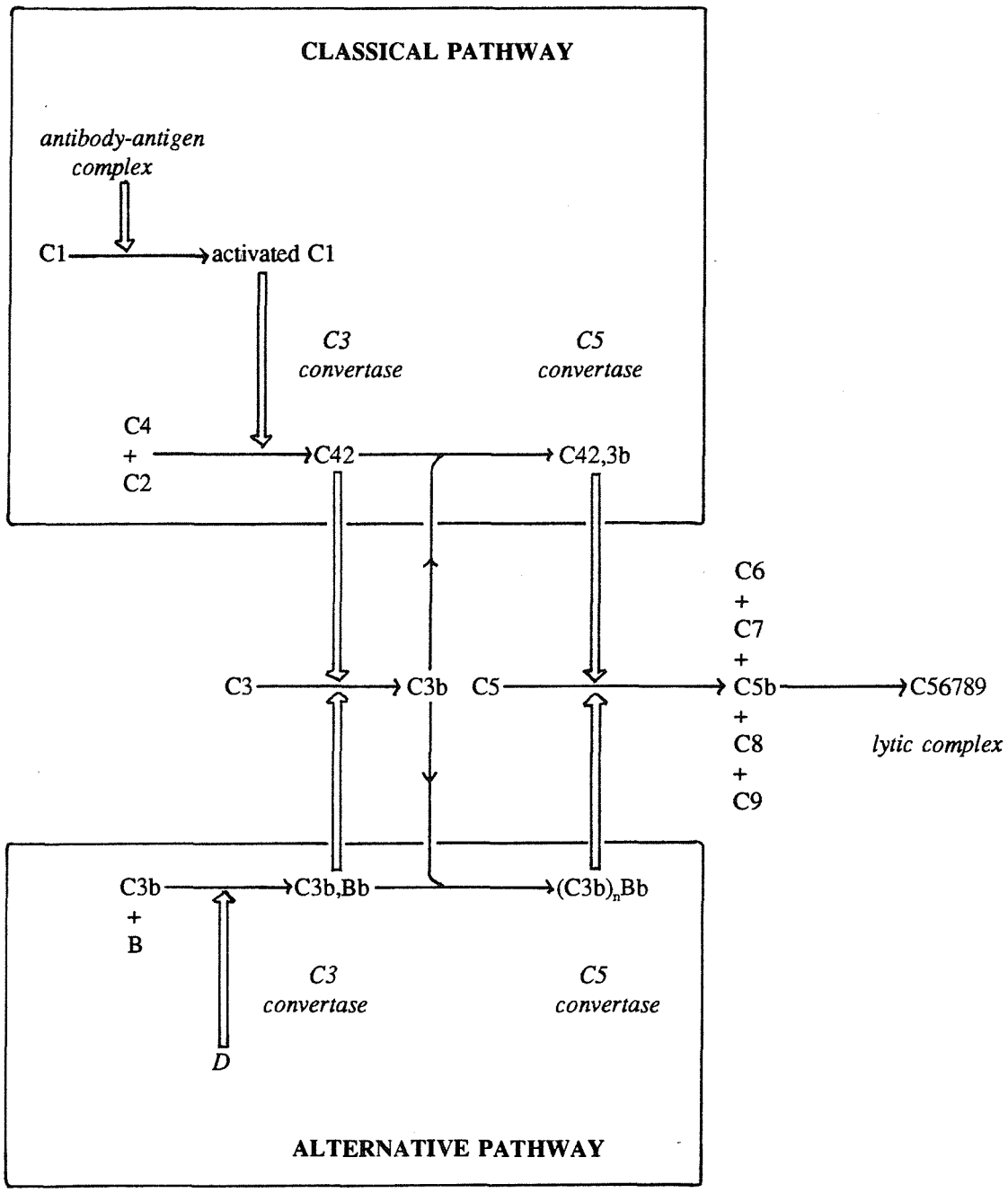
1.2.6.2. Serum Resistance.

The TraT protein also confers resistance to the bactericidal activities of human serum complement on host cells, thereby increasing their survival rates (Fietta et al., 1977; Ogata & Levine, 1980). The serum complement system (shown in Figure 1.8), which is a primary host defence, can be activated by either the classical pathway (as the result of the formation of an antigen-antibody complex, between the invading bacteria's antigens and the antibodies pre-existing in the host) or by the alternative pathway (which is stimulated by the cell envelope of the invading bacteria). The complement system is a protein cascade whose activation leads to deposition of complement component C3b, a major serum opsonin, on the surface of activating structures and subsequently, the formation of a lytic complex. This lytic complex inserts into the bilayer and destroys its integrity and functioning, ultimately causing cell lysis. Complement also helps fight infection by attracting phagocytic cells to the site of infection and by enhancing the ability of these cells to ingest and destroy the invading organism. The phagocytes (e.g. macrophages and polymorphonuclear leucocytes) are stimulated by the complement system because they have specific receptors for Fc, C3b and C5b degradation products.

In general, the observed increase in serum resistance caused by a F-like plasmid is only moderate and the resistance level of rough strains (e.g. *E. coli* K-12) carrying such plasmids is not as high as that for smooth *E. coli* strains (Taylor & Hughes, 1978). Therefore, to detect any increase in resistance, the serum concentration needs to be adjusted in accordance with the basic sensitivity level of the bacterial strain being studied. The serum source is another important factor that needs to be taken into account. Ogata & Levine, (1980) found that the plasmid R100 had very little effect on the resistance of *E. coli* K-12 cells with human serum. However, they observed a marked increase in resistance of the same strain when incubated with rabbit or guinea pig sera.

FIGURE 1.8.

Complement Cascade.



Schematic drawing comparing the generation of C3 and C5 convertase by the classical and alternative pathways. While the classical pathway is triggered by antibody-antigen complexes, the alternative pathway is triggered both by the presence of C3b and by cell-wall polysaccharides and other activators (e.g. factors B and D).

It was later shown that it was the TraT protein of the F-like plasmids R100 (Ogata *et al.*, 1982) and R6-5 (Moll *et al.*, 1980) that were responsible for mediating serum resistance on the cells harbouring them. A second determinant for serum resistance, *iss* (for increased survival in serum), has been identified in the colicinogenic ColV plasmids carried by many clinical isolates of *E. coli* (Chuba *et al.*, 1986). The serum resistance of ColV plasmids seems to be due mainly to the *iss* gene product, with the TraT protein acting synergistically. The effects of the TraT protein could only be seen when the *iss* gene was deleted or the *traT* gene was on a multicopy plasmid.

More recently, the involvement of the TraT protein, specified by the virulence plasmids of *Salmonella*, in serum resistance has been confirmed (Sukupolvi *et al.*, 1992 - Microb. Pathogen., in press). The serum resistance of plasmid-carrying and plasmid-less *Salmonella* strains from different laboratories were compared under identical conditions. Also, to eliminate the background resistance of wild-type, smooth *Salmonella* the plasmids were transferred to a rough serum-sensitive *E. coli* strain. It was found that inactivation of the *traT* gene present on the *Salmonella* plasmids eliminated serum resistance, suggesting that *traT* was indeed the gene responsible for the observed plasmid-mediated increase in serum resistance.

The mechanism for serum resistance conveyed by the TraT protein, however, is not yet fully understood. It is thought that the protein mediates resistance by interfering with the correct assembly, functioning or insertion of the complement membrane attack complex (Timmis *et al.*, 1985).

traT^{+} cells containing the plasmid R6-5 are also resistant to uptake by mouse peritoneal macrophages (Agüero *et al.*, 1984). The TraT protein is a passive inhibitor of phagocytosis as it interferes with opsonization by the alternative complement pathway. Expression of the *traT* gene product results in the restriction of the complement cascade component, C3 deposition and also affects opsonin distribution such that complement deposition is diffuse and irregular. This idea supports the theory that homogeneous deposition of complement on the bacterial cell surface is required for the 'zipper effect' which facilitates phagocytosis (Griffin *et al.*, 1975).

1.3. APPLICATIONS OF OUTER MEMBRANE PROTEINS AS CARRIERS FOR FOREIGN PEPTIDES AND PROTEIN SEQUENCES.

With the characterisation of outer membrane proteins at the molecular level, there has been considerable interest in using the proteins as carriers for foreign peptide and protein sequences. The potential and actual applications of such systems are quite varied and important in biological terms. Firstly, such systems can be used for the determination of the topology of the outer membrane proteins (Charbit *et al.*, 1986). They can also be used to expose the functional domains of receptors at the cell surface for the study of receptor-ligand interactions (Marullo *et al.*, 1989). Using this approach, it may also be possible to tether antibodies to the cell surface, thereby allowing the capture of antigens of interest. Finally, and above all, such systems have applications in the design of novel vaccine strains.

In the first experiments demonstrating the feasibility of the approach, Charbit *et al.*, (1991) probed the topology of the LamB protein using a reporter epitope. Previously, the main genetic method for study of location and folding of proteins was to produce fusions with entire reporter enzymes. For example, ß-galactosidase, alkaline phosphatase and ß-lactamase have all been used in this way (Manoil & Beckwith, 1986; Manoil, 1990). However, for topological studies, such fusions have limitations. Firstly, they can lead to deletion of the carboxyl end of the vehicle protein (this end being critical for biogenesis of many proteins). Secondly, the size and sequence of the fused enzyme may alter the location or folding of the protein under investigation as is the case with LamB (Boulain *et al.*, 1986). To circumvent these problems, the genetic insertion of a reporter peptide corresponding to a foreign antigenic determinant into the LamB protein of *E. coli* K-12 was tested (Boulain *et al.*, 1986; Charbit *et al.*, 1986). A model for the folding of this protein in the outer membrane, based on the protein sequence and the sites of alterations causing resistance to phage λ, was already available (Charbit *et al.*, 1984). Hence, certain regions were identified as likely to be surface-exposed. To test these assumptions experimentally, the C3 epitope from the VP1 protein of poliovirus was inserted into 3 defined sites of LamB (at amino acids 146, 153 and 374) located in regions predicted to be present on the outside of the cell.

It was found that insertion of 13 extra amino acids did not affect the stability, location or functioning of the LamB protein. Additionally, in two cases (sites 153 and 374) the epitope was recognised on whole cells by specific antibodies, strongly suggesting that these two regions of the LamB protein were at the cell surface. In addition to the topographical information obtained with this method it is also possible to obtain information on regions involved in subunit:subunit interactions or, for multi-functional proteins, to derive information on regions directly involved in specific functions.

Agterberg *et al.*, (1987) used the same technique to probe the topology of the PhoE protein of *E. coli* K-12. Previously, a model for the folding of the PhoE protein in the outer membrane, based on mutants of the protein that have altered antibody binding properties, was proposed (Van der Ley *et al.*, 1985). The C-terminal region of VP1 protein of foot-and-mouth disease virus was therefore inserted into a region of the protein predicted to be cell-surface-exposed. The level of expression of the resultant hybrid protein was normal and it was also incorporated into the outer membrane. The VP1 epitope was also recognised by a monoclonal antibody raised against the virus in whole cells, demonstrating that it was highly likely to be exposed to the outside of the cell.

Marullo *et al.*, (1989) genetically inserted part of the human ß1- and ß2-adrenergic receptor into the LamB protein. They expressed the hybrid receptor in *E. coli* and measured its affinity and selectivity for several compounds. *E. coli* cells expressing the hybrid receptor retained the properties of the corresponding receptors in mammalian tissues. Potentially, such an approach greatly simplifies experimental procedures required for pharmacological studies on active receptors. In the bacterial system, receptor-binding assays display low background and high reproducibility and hence the use of such hybrids in bacteria provides an appropriate tool for rapidly screening the binding properties of certain synthetic compounds to different receptor subtypes.

Perhaps the most important application of outer membrane carrier proteins is in the exposure of foreign antigenic determinants at the cell surface for the development of

new types of vaccines or serum diagnostic reagents. Since the TraT protein shows considerable promise in this respect and since the ultimate aim of these studies is to explore such possibilities, it is worthwhile reviewing the current status of vaccine research and the systems currently under development.

1.3.1. Types of Vaccine.

Any vaccine to be considered by the World Health Organisation's Expanded Program for Immunisation must be (Bloom, 1989):-

- inexpensive;
- safe;
- extremely effective (protection in 90 to 100% of recipients);
- able to engender lifelong immunity;
- heat stable and not needed to be kept cold at all stages;
- administrable in one shot and compatible with other vaccines;
- simple to give, preferably by a non-invasive route;
- able to be given at birth.

At present there are four kinds of vaccine available (Bloom, 1989). Despite their excellent success record, many of these vaccines have their limitations and disadvantages:-

1.3.1.1. Killed vaccines.

These are the simplest and least expensive to prepare. The causal agent is grown in large amounts and then inactivated under conditions that ensure the retention of the immunogenic activity of the protective antigens. However, such vaccines can vary in reproducibility and require careful monitoring to ensure that no viable organisms are present. Moreover, the handling of large volumes of virulent organisms is a potential hazard to the personnel involved and the immediate environment.

1.3.1.2. Anti-idiotype vaccines.

These are based on the principle that because antibody active sites (the idiotype) are complementary to the specific antigenic determinant to which they bind, some antibodies raised against a particular idiotype may indeed mimic the antigen. However, it has proved difficult to achieve high levels of immunisation or immunological memory with these vaccines because T-cells are not developed against the pathogen itself.

1.3.1.3. Subunit vaccines.

These are prepared from the individual components of a pathogen, by chemical synthesis or recombinant DNA technology. These vaccines are chemically defined, reproducibly prepared and assayed and are usually inexpensive to manufacture. However, several immunisations and boosters are often needed.

1.3.1.4. Live attenuated vaccines.

For the preparation of these vaccines the virulent organism, obtained from the infected host, is weakened by growth on an unnatural host or under conditions such that the product will then proliferate in the natural host without causing disease. The great advantage of live vaccines is that entry of the challenge inoculum into the tissues allows antigen persistance and also accelerated recall of the earlier cell-mediated immunity which is able to control the further growth of the organism before the infection can assume clinically significant proportions. Those currently in use have the advantages of generally inexpensive production, persisting immunity and a good safety record. However, their disadvantages include the fact that they have to be stored at refrigerated temperatures, there maybe adventitious agents in the cells or medium used for production, and, as always, there is the hazard of reversion to virulence.

1.3.2. Improvement of these Vaccines.

The aim is clearly to produce vaccines by methods that will allow greater control of their biological properties and eliminate, as far as possible, their side effects. Such goals have now been brought within reach by advances in our understanding of the structure of the genomes of many different organisms, added to our knowledge of the structure and function of protective antigens and the mechanisms of bacterial and viral pathogenicity (Brown, 1990).

Considering this and the various criteria for new vaccines, the most appealing are genetically engineered live attenuated vaccines. Those that are especially sought after are those that can become multivaccine vectors and can immunise simultaneously against multiple antigens. It has long been recognised that living micro-organisms are often superior to inactivated or sub-unit vaccine preparations in their ability to stimulate protective immune responses. In the past, live vaccines were a problem because they were derived either from a virulent species that was antigenically closely related to a pathogen, or from variants of the pathogen derived by passage through an unnatural host or an artificial medium (Charles & Dougan, 1990). As a direct consequence, the genetic lesions responsible for attenuation remained uncharacterised, leading to problems of quality control of vaccines in large-scale production and of reversion to virulence. The increasing availability of information about the genes that enable pathogens to survive within the host make feasible the deliberate construction of genetically attenuated strains suitable for use as live vaccines.

1.3.3. Live Attenuated Vaccine Strains.

Two basic strategies are being developed for use as live attenuated recombinant multivaccine vehicles, one using viral vaccines and the other using bacterial vaccines (Bloom, 1989):-

1.3.3.1. Viral vaccines.

These can express antigens in eukaryotic cells with correct folding, proteolytic processing, glycosylation, secretion and subunit assembly. They can stimulate the production of cytotoxic T-lymphocytes as well as antibodies. An example of an extensively used viral vaccine is Jenner's smallpox vaccine, where a naturally occurring attenuated strain of the cowpox virus (vaccinia) is used.

1.3.3.2. Bacterial vaccines.

These have a special ability to immunise for long periods and elicit both local immunity (e.g. in the gut) and cell-mediated immunity. One important point is that attenuated strains of bacteria have lower virulence for a particular host than a fully infectious pathogen and can establish limited infections which stimulate natural immune responses.

There are currently two attenuated bacterial species that are being studied intensively for the expression of foreign genes. The Bacille Calmette-Guérin (BCG) vaccine strain is the oldest and most widely used live bacterial vaccine in use today (Brown, 1990). It is an attenuated bovine tubercle bacillus and is used to immunise against tuberculosis. This mycobacterial vaccine requires only a single shot to engender cell mediated immunity for periods of 5 to 50 years, with a low frequency of complications. It is known to be an effective adjuvant, enhancing immune responses. Like most bacterial vaccines it is very inexpensive. Recently it has been possible to introduce foreign DNA into the BCG strain and hence express foreign antigens. Initial results of experimental immunisation studies suggest that modified BCG strains show great promise for the future (Snapper *et al.*, 1988, Barletta *et al.*, 1990).

The other main type of bacteria being investigated for use in the expression of heterologous antigens are the *Salmonellae*. The popularity of *Salmonella* species (e.g. *S. typhi* and *S. typhimurium*) for the use as live attenuated vaccines has rapidly increased in the last ten years. For this reason, and because one such strain was

chosen for use in this work, I shall review this type of vaccine in more detail.

1.3.4. Attenuated *Salmonella* Vaccine Strains.

1.3.4.1. The *Salmonella* Pathway in Infection.

Salmonellae give rise to a variety of disease conditions in humans known collectively as salmonellosis. These diseases have been subclassed into four pathological conditions: gastroenteritis, enteric fever, bacteraemia and the asymptomatic carrier state.

The pathogenic *Salmonella* serotypes once within the hosts ileum must adhere to the epithelial cell membrane. Microbial adherence requires the participation of two factors: a receptor, which usually comprises of specific carbohydrate residues on the eukaryotic cell surface and a bacterial adhesin, which is typically a protein structure on the bacterial cell surface. Chemotaxis may also contribute to *Salmonella* entry into eukaryotic cells (Uhlman & Jones, 1982).

The bacteria, once adhered to the eukaryotic cell surface, then have to penetrate the eukaryotic cell surface and gain entry into (invade) the epithelial cells through the apical membrane. This invasive property of the bacteria is an essential step for pathogenesis. Each invading organism enters into a separate vacuole several of which later coalesce so most of the intracellular organisms are found within a single large vacuole. The bacteria utilise the eukaryotic host structures in this process. For example, *Salmonella* require functional host microfilaments for entry and the invading bacteria are surrounded by polymerised actin during internalisation. Finlay & Falkow, (1988) demonstrated that the *Salmonella* replicate inside the vacuoles within epithelial cells, and that bacterial entry and intracellular replication does not require endosome acidification.

The bacteria now within the eukaryotic epithelial cells have two options. Serotypes causing gastroenteritis survive and probably proliferate in this cell type. By contrast,

enteric serotypes continue through the cell and transcytose the opposite surface (the basolateral membrane) of the epithelial cell (Finlay *et al.*, 1988). This *Salmonella* adherence to and invasion is an active event requiring bacterial proteins and RNA synthesis, but not DNA replication (Finlay *et al.*, 1989). Later work suggested that *Salmonella* may not need to produce new proteins prior to internalisation, however, the growth phase is very important for infection (Lee & Falkow 1990). Once through the basolateral membrane, the *Salmonella* travel to their principal target of infection, the macrophage. These cells are one type of a range of 'professional' phagocytic cells that form an important line of defence against invading micro-organisms. Macrophages engulf the bacteria in membrane-bound vacuoles, or phagosomes, which then fuse with cytoplasmic granules, lysosomes, to form phagolysosomes. The lysosomes contain an arsenal of proteins or peptides with anti-microbial activity. The *Salmonella*, thus, have several mechanisms for survival within the macrophages. These include inhibition of the respiratory burst (preventing the production of toxic metabolites of oxygen, such as hydrogen peroxide and superoxide), inhibition of lysosomal fusion, escape from the phagolysosome and resistance to granular anti-microbial peptides (such as the defensins). Hence, the pathogenic *Salmonella* can proliferate within these cells.

The molecular basis of intracellular survival within phagocytic cells is presently unknown although several of the genetic loci involved have been identified (Fields *et al.*, 1986; 1989; Groisman *et al.*, 1989; Groisman & Saier, 1990). For example, *Salmonella* virulence, survival in mouse macrophages and sensitivity to the bactericidal action of defensins are all controlled by a single transcription regulatory protein termed PhoP. Further analysis of this and other virulence regulons should lead to a better understanding of the molecular basis of *Salmonella* pathogenesis.

1.3.4.2. Types of Attenuation.

Salmonella typhimurium is closely related to *E. coli* and, as a consequence, genetic manipulation of this organism is relatively easy. Although few surface-associated virulence factors have been characterised in *Salmonella*, mutations in a variety of genes have been shown to attenuate virulent strains. The genetic strategy to produce

these disabled *Salmonellae* is to delete genes required for pathogenesis or survival. Such mutations include those affecting galactose metabolism (*galE*); aromatic compound metabolism (*aroA, C & D*); purine metabolism (*purA, E & H*); phosphate metabolism (*phoP*); cyclic adenosine monophosphate regulation (*crp & cya*) and transcriptional activation of porin synthesis under different osmotic conditions (*ompR*) [Dougan & Tite, 1990]. Mutations in these genes give rise to *Salmonella* strains displaying different degrees of attenuation (O'Callaghan *et al.*, 1988b) and hence immunogenicity.

galE mutants are characterised by a block in the enzyme uridine diphosphate-galactose-4-epimerase (Germanier & Fürer, 1971). Without any external supply of galactose, these mutants cannot synthesise UDP-galactose (see Figure 1.11) and, because galactose is incorporated in the LPS via UDP-galactose, only incomplete LPS is formed (see Figure 1.2). This incomplete LPS structure, lacking the O-specific oligosaccharide repeat unit, makes the *galE* mutants resistant to phage P22 and sensitive to rough-specific phages, such as C21. *galE* mutants are peculiar in that their LPS phenotype changes markedly depending on the substrate provided. For example, when they are grown on media containing galactose they can synthesise complete LPS and become phenotypically smooth, expressing the LPS O-antigen which may be important in immunity. Unlike other rough mutants of *S. typhimurium*, *galE* mutants are endowed with an outstanding protective capacity. This is probably due to the capacity of the strains to undergo phenotypic reversion *in vivo* (i.e. in the presence of galactose). However, prolonged contact with galactose brings about a dramatic lysis of cells. The reason for reduced virulence of *galE* strains with incomplete LPS seems to be due to an increased sensitivity of the mutant cells to intracellular killing within the phagolysosome. However, even after phenotypic reversion (to smooth LPS) the avirulence of the *galE* mutant (over any other epimerase-negative strains tested) can be explained by the mutant not being able to metabolise the galactose and it accumulating in the galactose-1-phosphate and UDP-galactose forms, thereby causing lysis of growing cells. Thus, the properties of the *galE* mutants *in vivo* are dependent on two mechanisms acting in opposite directions: virulence- and immunogenicity-increasing biosynthesis of cell wall LPS and virulence-lowering galactose-induced

bacteriolysis. One disadvantage associated with these mutants is their high tendency to change their properties by secondary mutation in the structural or regulator genes of the galactose operon.

Other critical attenuating lesions are in the *aro* genes which encode enzymes involved in the pre-chorismate biosynthetic pathway (e.g. *aroA* encodes 5-enolpyruvylshikimate-3-phosphate synthase and *aroC* encodes chorismate synthase) responsible for synthesis of aromatic compounds including para-aminobenzoic acid (PABA), dihydroxybenzoic acid (DHB) and amino acids. These compounds in turn are required for the synthesis of folate (needed for DNA and RNA synthesis), enterochelins (iron-binding proteins) and protein synthesis, respectively (see Figure 1.9). It is the limited availability of these compounds *in vivo* that inhibits the growth of the *aro* mutant bacteria inside its host, making them avirulent (Hoiseth & Stocker, 1981; O'Callaghan *et al.*, 1988b). Such strains, however, can still penetrate cells and reach the liver and spleen, where they may persist for a few weeks, similar to cured *Salmonella* strains. Dougan *et al.*, (1988) found that mutations in *aroA*, *aroC* and *aroD* all reduce virulence to a similar degree, unlike strains with *pur* mutations. This makes these mutants attractive candidates for vaccine development and indeed they have already been shown to confer good protection against the corresponding parental pathogen. *aroA*$^-$ *Salmonella* have already been shown to induce immune responses and, in some cases, protect against challenge with a virulent isolate of *Salmonella* when orally administered to several species including mice (Hoiseth & Stocker, 1981; O'Callaghan *et al.*, 1988b), calves (Stocker *et al.*, 1983; Smith *et al.*, 1984), sheep (Mukker *et al.*, 1987) and humans (Stocker, 1988; Levine *et al.*, 1987).

The *pur* genes encode enzymes which affect the biosynthesis of purines. Mutations that act at different points within this pathway can, however, have widely differing affects on attenuation. For example, purine-dependant mutants with lesions in *purA* (LD_{50} of 10^8) are much more attenuated than mutants with lesions in *purE* (LD_{50} of 10^3) [O'Callaghan *et al.*, 1988b]. These differences might be explained by the fact that the *purE* and *purA* gene products act before and after, respectively, the inosine

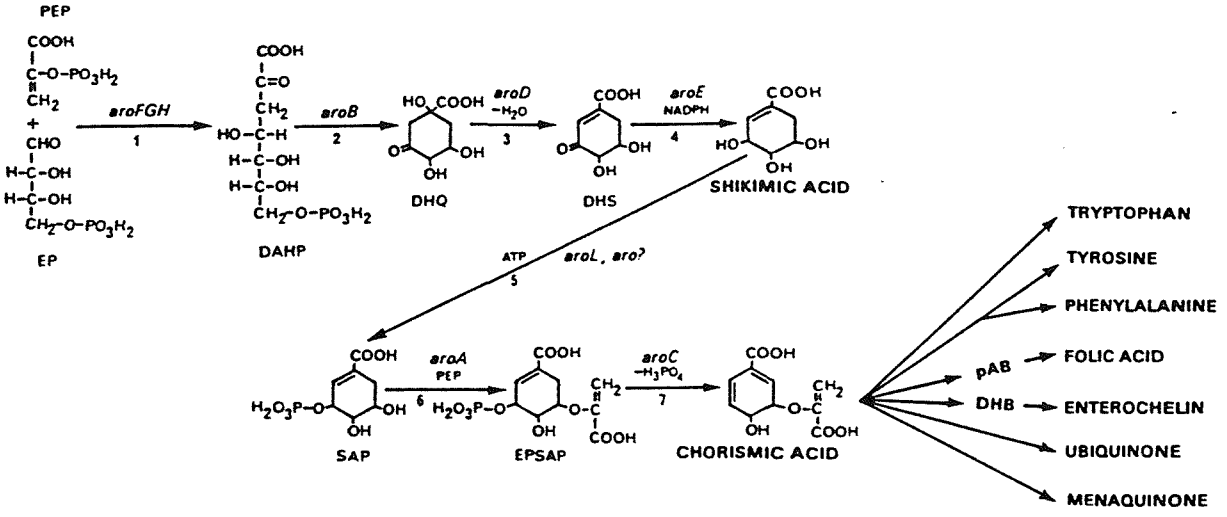
monophosphate branch point of the purine biosynthetic pathway (see Figure 1.10). Despite their attenuating effect *pur*⁻ mutants appear to be less promising as candidates for vaccine strains (Miller *et al.*, 1990). For example, when the *aroA* or *purA* or *aroA* + *purA* auxotrophic derivatives of the same strain of *S. typhimurium* were tested by intravenous infection of BALB/c mice it was found that only the *aroA* mutant was able to induce long lasting effective immunity against virulent *Salmonella* challenge (Dougan & Tite, 1990). This was due to the persistence of the *aroA* strains in the RES, as this strain is far more resistant to killing in the first 24 hours after infection than the other strains tested. Thus, the *aroA* strain has a period of potential replication which enables it to persist in the mice. The *aroA* strains were also found, in the early post-immunisation stages, to efficiently activate macrophages which may therefore lead to the induction of further cellular immune responses protecting against salmonellosis.

Salmonella phoP mutants have also been found to be avirulent but can survive within macrophages (Galán & Curtiss III, 1989). They, therefore, afford some protection when used as live vaccines by the intraperitoneal route (Miller *et al.*, 1989). Animals injected with a wild-type pathogenic strain have a 1000-fold increase in LD_{50} if they had been previously immunised with *phoP* mutants. An example of one such attenuating lesion is a mutation in the *phoP* locus that renders the organism constitutive in the production of *pag* products (phenotype $PhoP^C$). Such $PhoP^C$ mutants of *S. typhimurium* are very effective when used as a live vaccine against mouse typhoid fever and are superior to $PhoP^-$ bacteria. As few as 15 $PhoP^C$ bacteria protect mice against 10^5 LD_{50} of wild-type organisms by the intraperitoneal route (Miller *et al.*, 1990). Therefore the *pag* gene products may be important immunogens for protective immunity against mouse typhoid. Miller *et al.*, (1990) investigated various strains with *aroA* mutations combined with *phoP* regulon mutations for virulence attenuation and immunogenicity. The $PhoP^-$ or $PhoP^C$ mutants were shown to further attenuate *aroA* mutant *Salmonella typhimurium* by at least 100-fold and that, at least at high levels of vaccinating organisms, immunogenicity was retained. Therefore, *phoP* regulon mutations may increase the safety of *aroA* live preparations.

FIGURE 1.9.

Aromatic Biosynthesis Pathway.

(adapted from Stocker, 1988)



All aromatic metabolites are synthesised by bacteria from a single intermediate, chorismic acid. This and the final products of the aromatic biosynthesis pathway are shown.

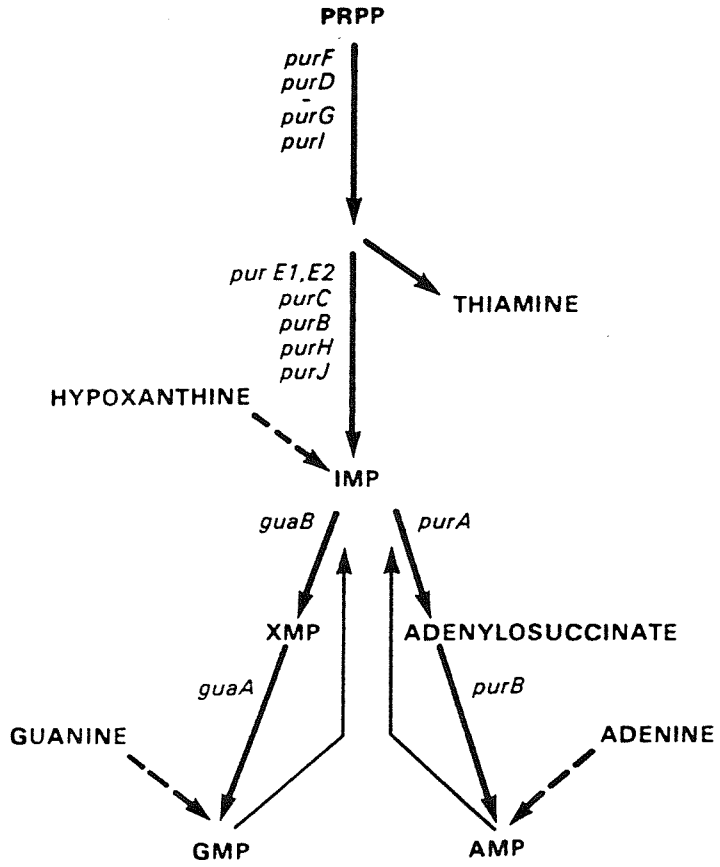
The name and order of the key genes, that code for the enzymes responsible for each step of the pathway, are shown above each arrow.

PEP	phosphoenolpyruvate
EP	erythrose 4-phosphate
DAHP	3-deoxyarabinoheptulosonate-7-phosphate
DHQ	5-dehydroquinate
DHS	5-dehydroshikimate
SAP	shikimate-5-phosphate
EPSAP	3-enolpyruvylshikimate-5-phosphate

FIGURE 1.10.

Purine Biosynthesis Pathway.

(adapted from Stocker, 1988)



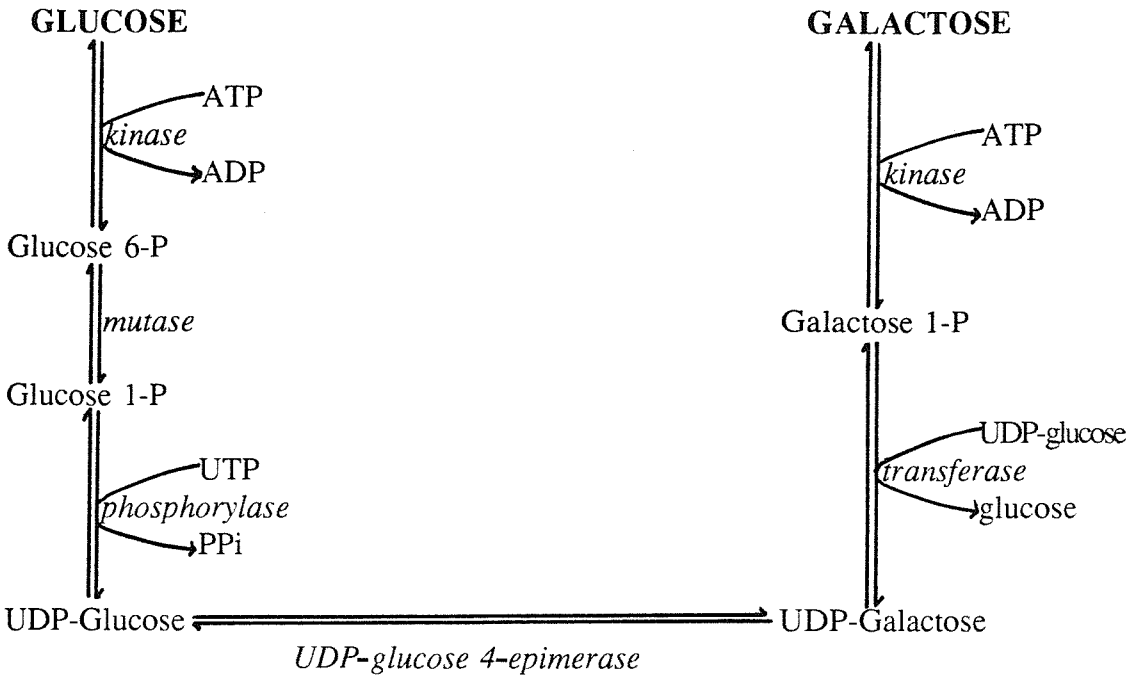
The thick lines represent the main reactions of the *de novo* purine biosynthesis pathway and the dashed lines represent those of the salvage pathways of exogenous purines. The thin lines represent the utilisation of adenine as a source of guanine (or *vice versa*).

The genes encoding the enzymes responsible for each step of the pathway are shown on each arrow.

PRPP	5-phosphoribosyl-1-pyrophosphate
IMP	inosine monophosphate
XMP	xanthine monophosphate

Figure 1.11.

UDP-Galactose Pathway.



BOLD CAPITALS are unmodified sugars.

Boxed residues are nucleotide sugars which are suitably acyivated for polymerisation.

Glucose acts as a precursor for the synthesis of galactose (and other hexoses, not shown here).

The *galE* gene encodes the enzyme UDP-glucose 4-epimerase which catalyses the isomerisation of UDP-galactose form UDP-glucose, using glucose form the central hexose monophosphate pool found within the cells. Thus, *galE* mutants are unable to produce UDP-galactose *in vivo* from glucose or from galactose as galactose is not a member of the hexose pool. However, external addition of galactose overrides this.

Other avirulent strains that retain their immunogenic properties are pleiotropic regulatory mutants such as *cya* and *crp* mutants, which lack adenylate cyclase and the cAMP receptor protein, respectively. The products of these two genes are necessary for the transcription of many genes and operons concerned with the transport and breakdown of catabolites; hence strains carrying mutations in these genes (*cya* or *cya crp* mutants) were found to protect mice against oral challenge with wild-type *Salmonella typhimurium* (Curtiss & Kelly, 1987). In chickens a Δ*cya* Δ*crp* mutant of *S. typhimurium* (χ3985) derived from the virulent strain χ3761 (LD_{50} of $2x10^3$ CFU for one-day-old chicks) is completely avirulent for chicks of the same age (LD_{50} of $4x10^9$ CFU) (Hassan & Curtiss III, 1990). As with the *aroA* and *galE* mutants, the *cya* and *crp* phenotypes could not be corrected either by diet or by the animal host. Although cAMP is present in mammalian cells, the amount present (1.0 to 0.1 μM) in the tissues is well below the concentration needed (0.1 to 1.0 mM) to allow the *cya* mutants to display a wild-type phenotype *in vitro*. Furthermore, the inclusion of a *crp* mutation abolishes any benefit that could accrue from uptake of cAMP *in vivo* by *cya* mutants (Curtiss III & Kelly, 1987).

Mutations in *ompR*, a gene which encodes the regulatory protein that controls expression of outer membrane porins (OmpC and OmpF) in response to osmolarity changes, also results in avirulence of *Salmonella* when mice were inoculated orally or intravenously (Dorman *et al.*, 1989), but not when they were inoculated intraperitoneally (Miller *et al.*, 1989) with such a strain. In contrast, mutations in either *ompC* or *ompF* did not lead to the same level of attenuation of *Salmonella* virulence. The phenotypic effects of insertional inactivation of *ompR* would not be expected to be identical to those of *ompC* and *ompF* because the latter genes are not the only ones under the control of *ompR*. Other environmental factors, such as changes in pH, temperature, and oxygen availability (on *S. typhimurium* after oral infection) also play a role in altering the expression of the porin genes *ompC* and *ompF*.

An alternative method of vaccine strain construction involves systematically inactivating genes necessary for *Salmonella* pathogenesis, such as those required for passage through the epithelial cells or survival within macrophages. Transposon

mutants of *Salmonella choleraesuis* that are unable to transcytose a polarised Madin Darby Canine Kidney monolayer have been identified (Finlay *et al.*, 1988). One of these mutants, strain 6A, is markedly deficient in its abilities to adhere to and invade the monolayer, yet it increases its permeability. This strain still seems to be motile, with normal LPS and does not have any mutations in the genes encoding type 1 pili (Finlay *et al.*, 1988). However, when administered to BALB/c mice (orally and i.v.) the LD_{50} of this strain is 2 logs higher than that for its virulent parent. This occurs even though it persists in the liver, spleen and gut for several days after challenge (Wilson *et al.*, 1990). In view of this persistence and avirulence the strain 6A shows some potential for vaccine use, perhaps in combination with other attenuating lesions.

In principle, any bacterial pathogen strain harbouring mutation(s) in specific genes which are known to cause some degree of attenuation (i.e. which reduce the virulence of the pathogen) can be considered for use as a vaccine strain. Ideally, however, the vaccine strain should harbour:-

a) mutations in more than one gene, preferably located in different regions of the chromosome to minimise the risk of reversion due to genetic exchange mechanisms;
b) mutations carrying defined and substantial genetic lesions again to minimise reversion;
c) mutations that cannot be suppressed by secondary mutations in other genes (Charles & Dougan, 1990).

Hence, some combination of the various mutants described above would probably be appropriate for fulfilling these criteria. The *aro* mutants are particularly promising in this respect. Doses of 10^4 of virulent *S. typhimurium* organisms reproducibly kill mice within several days of infection, indeed as few as 10 organisms have been seen to kill susceptible mice. However, the corresponding *aroA* mutants do not kill mice when fed orally at doses of more than 10^{10} organisms. Moreover, these strains can establish self-limiting infections in the mice, being detectable in low numbers, in deep tissues including the liver and spleen. The attenuated *Salmonella* strains were found to immunise both locally and at the T-cell level, systemically stimulating humoral,

secretory and cellular immune responses in the host (Dougan *et al.*, 1987a). The ease of administration of these oral *S. typhimurium* vaccines is another obvious advantage in their favour, but also the concept of a common mucosal immune system postulates that lymphocytes, primed at one mucosal surface, traffic preferentially to other mucosal surfaces, thus, inducing a general state of immunity at these primary defence barriers (Mestecky, 1987). Hence, oral delivery of vaccines may also lead to immunity which is effective against infections which are not gut-borne (e.g. respiratory infections). Thus, there are three major advantages of using these recombinant *Salmonella* vectors (Dougan & Tite, 1990): they are closely related to *E. coli*, they are potently immunogenic and they can be used as oral vaccines.

1.3.5. Expression of Heterologous antigens in *aroA*⁻ *Salmonella* Vaccine Strains.

Recent developments in vaccine construction have allowed avirulent strains of *Salmonella* to be used as carriers of cloned genes that specify foreign proteins. In this way, an immune response to the heterologous protective antigens can be obtained.

Several heterologous antigens have now been expressed in *Salmonella* vaccine strains. Most of the antigens were introduced on recombinant plasmids using expression systems designed primarily for use in *Escherichia coli* K-12. *Salmonella* and *E. coli* are sufficiently closely related that most promoters active in *E. coli* will also function in *Salmonella*. However, in many cases the relevant genetic control systems are not present in *Salmonella* (e.g. *Salmonella* is *lac* negative), so heterologous antigens under study have, on the whole, been expressed constitutively.

The following are a few examples of heterologous antigens that have been expressed in attenuated *Salmonella* vaccine strains and tested in mammals.

Heterologous proteins can be expressed as cytoplasmic (as with ß-galactosidase), secreted (see section 1.3.6.) or as surface proteins in *Salmonella*. Brown *et al.*, (1987) used an attenuated *aroA*⁻ *S. typhimurium* vaccine strain to carry a pBR322-based plasmid that directs the expression of a cytoplasmic protein, ß-galactosidase, as an

intracellular antigen. The ß-galactosidase constituted around 1% of the total cell protein and mice intravenously vaccinated with the *Salmonella typhimurium aroA*$^-$ construct mounted a significant immune response to ß-galactosidase, suggesting the immune system can recognise intracellular antigens. Of even greater significance is the fact that mice vaccinated in this way develop a delayed-type hypersensitivity response to ß-galactosidase. This suggests that there is stimulation of cell-mediated immunity to the cloned antigen. Protective immunity to certain diseases requires cell-mediated immunity and thus this route of immunisation is a highly attractive option. The intracellular antigen also elicited a humoral immune response. This has since been substantiated using other proteins (Leclerc *et al.*, 1990).

There is still no clear evidence whether secretion is required in order to obtain good recognition by the immune system. Initial studies with secreted proteins have centred on the B-subunit of the heat-labile enterotoxins (LT-B) of *E. coli* and *V. cholerae.* These bacteria secrete immunologically-related enterotoxins which are composed of an A-subunit (with enzymatic and toxic activity) and an immunodominant B-subunit (which itself is non-toxic, but serves to deliver the A-subunit to the eukaryotic cell). Anti-B subunit antibodies can neutralise activity of the holotoxin. Maskell *et al.*, (1987) orally immunised mice with an avirulent *aroA*$^-$ mutant *Salmonella typhimurium* strain carrying a pBR322-derived plasmid encoding the gene for *Escherichia coli* LT-B. They found that the *aroA* mutant strain of *S. typhimurium* was capable of delivering the heterologous antigen to the murine systemic and secretory systems to elicit both humoral and secretory toxin-neutralising antibodies, without altering their efficacy against Salmonellosis. Schödel & Will, (1990) constructed a vector that expressed several hepatitis B viral (HBV) B- and T-cell epitopes as carboxy-terminal fusion proteins with LT-B (non-toxic subunit B) in *aroA*$^-$ *Salmonella.* These recombinant *Salmonella* were found to elicit anti-LT-B B- and T-cell immune responses and anti-HBV nucleocapsid antigen T-cell responses when fed orally to mice.

Stevenson & Manning, (1985) introduced a plasmid encoding the cloned K88ab gene cluster encoding K88 fimbrial antigens from porcine enterotoxigenic *E.*

coli) into a *Salmonella typhimurium galE* strain. This derivative was shown to efficiently express the K88 pilus and oral immunisation with the strain produced a significant antibody response in the gut. Dougan *et al.*, (1986) have also studied a strain, *Escherichia coli* strain of serotype o9:K36:H19, that expresses the K88 antigen *in vivo*. This strain was able to efficiently colonise the small bowel of young piglets after oral infection. They also produced an *aroA*$^-$ *S. typhimurium* strain that expressed the K88 antigen. This strain elicited anti-K88 antibodies in mice immunised both orally and intravenously. The mice were also well-protected against subsequent challenge with the highly virulent *S. typhimurium* parent strain. Thus, these *Salmonella* strains can be used to elicit humoral and secretory anti-K88 responses.

O'Callaghan *et al.*, (1988a) introduced a pBR322-based plasmid encoding a determinant for the *Escherichia coli* K1 polysaccharide into a *Salmonella typhimurium aroA*$^-$ vaccine strain. They found that the cells harbouring the plasmid expressed the K1 polysaccharide at their cell surface. Mice that had been infected with this vaccine strain intravenously retained it in their liver and spleen for several weeks and were protected against challenge with virulent *S. typhimurium*. The vaccinated mice, however, failed to produce detectable levels of humoral anti-K1 polysaccharide antibodies.

Poirier *et al.*, (1988) investigated the protective immunogenicity of the M5 protein of *Streptococcus pyrogenes* (infections of this organism can trigger rheumatic fever and rheumatic heart disease), again using an attenuated *aroA*$^-$ strain of *Salmonella typhimurium*. A pSC101-based plasmid directed the expression of M5 protein, which accumulated in the periplasmic space of the vaccine strain. They found that oral administration of this recombinant strain elicited an immune response that protected the mice against subsequent intranasal or intraperitoneal challenge with a mouse-virulent *S. pyrogenes* M5 strain. The mice were also well-protected against Salmonellosis after a single oral dose. Immunological examination of the mice demonstrated a significant humoral and salivary antibody response to M5.

Sadoff *et al.*, (1988) described a method of inducing protective cell-mediated immunity to sporozoites by immunisation with attenuated *S. typhimurium* transformed with the *Plasmodium berghei* circumsporozoite (C3) gene on a pUC-based plasmid. They found that when this system was used to immunise mice orally, the C3-expressing *Salmonella* colonised the liver, induced antigen-specific cell-mediated immunity and protected the mice against sporozoite challenge in the absence of antisporozoite antibodies. This work emphasises the importance of cell-mediated immunity in the protective response to certain pathogens.

Tite *et al.*, (1990) used a plasmid encoding the nucleoprotein of influenza A virus expressed in an *aroA*$^-$ strain of *S. typhimurium* to investigate both humoral and cell-mediated immunity to this viral gene product. Parenteral and oral immunisation of mice with these nucleoprotein-expressing bacteria generated strong virus-specific T-cell responses detected in lymph node assays. The fine specificity of the T-cell responses after immunisation with this construct was found to be identical to that observed after immunisation with either purified recombinant nucleoprotein or infectious influenza virus.

These examples demonstrate the promise of *aroA*$^-$ *Salmonella typhimurium* strains for use as carriers of heterologous antigens. However, much work remains to be done routinely to obtain a solid protective immune response to a particular foreign antigen.

1.3.6. Immunogenicity of Foreign Peptides Expressed at the Bacterial Cell Surface.

It is already well-known that the immune response to an epitope depends critically on the way the epitope is presented to the immune system. Moreover, there is much work suggesting that cell surface components of bacteria are highly immunogenic. For these reasons several surface proteins have now been employed as carriers of foreign antigenic determinants. These include type 1 fimbriae of *Escherichia coli*, the flagellin protein of *Salmonella* and certain outer membrane proteins of *Escherichia coli*.

Insertion of small peptide sequences, known to represent antigenic determinants, into specific regions of surface proteins has several advantages. Firstly, it is easier to export such antigen molecules to the cell surface in high numbers, if inserted into the correct region of the protein. Secondly, the hybrid protein may retain the function of the original protein in its immunogenicity. An additional consideration is that insertion of the peptide into the carrier protein and subsequent transport to the cell surface may prevent its degradation by cytosolic peptidases. Finally, and perhaps most importantly, the carrier proteins themselves may be strongly immunogenic, which may be important for the stimulation of an immune response directed against the inserted epitope. Hence, the immune response of a host to a peptide antigen may be greatly enhanced when it is genetically fused to a bacterial cell surface component.

1.3.6.1. Examples of Surface Proteins Employed for this Purpose.

Adhesins, in many cases, are part of type 1 fimbriae which are filamentous appendages produced on the cell surface of a range of bacteria. Hedegaard & Klemm, (1989) inserted nucleotide sequences coding for different viral epitopes (e.g. hepatitis B surface antigens, C3 epitope of VP1 capsid protein of type 1 poliovirus and the epitope of VP1 capsid protein of foot-and-mouth disease virus) into selected points of the fimbrial structural protein *fimA* gene. They showed that functionally normal type 1 fimbriae carrying the foreign epitope sequences were produced which could be recognised by antibodies directed against the foreign parent protein.

Similarly, the flagellin proteins of *Salmonella muenchen* have been used to express epitopes at the bacterial surface of a flagellin-negative *aro S. dublin* vaccine strain. For example, Newton *et al.*, (1989) genetically inserted a 15 amino acid sequence for an epitope of cholera toxin subunit B into the central hypervariable region of flagellin, the major polypeptide of the filamentous part of *Salmonella* flagella. The chimeric flagellin functioned normally and the epitope was expressed on the flagellar surface. Parenteral administration to mice of an *aroA*$^-$ flagellin-negative strain of *S. dublin* expressing the chimeric flagellin gene evoked antibodies to cholera toxin. The immune

response to the epitope appears to be heightened by the exposure of the epitope to the outside of the cell, the potent immunogenicity of bacterial flagellin and the humoral and cellular immune responses evoked by *aro Salmonella*. Similarly, two epitopes of surface proteins of hepatitis B virus (Wu *et al.*, 1989); epitopes gp120 of human immunodeficiency disease virus type 1 (Stocker & Newton, unpublished observations - cited Newton *et al.*, 1989); an epitope of the M protein of *Streptoccus pyogenes* type 5 (Beachy *et al.*, unpublished - cited Newton *et al.*, 1990); immunogenic determinants of hen egg-white lysozyme (Kuwajima *et al.*, 1988) and epitopes of *Plasmodium berghei* circumsporozoite protein (Majarian *et al.*, 1989) have been expressed with this system. In all these investigations an immune response to the foreign epitope was generated in mice, rabbits, or guinea pigs after administration of either an *aroA* live vaccine strain carrying the recombinant flagellin gene or killed flagellate bacteria or flagella of such a strain.

Agterberg & Tommassen, (1991) used the PhoE porin protein (an abundant transmembranous outer membrane protein of *Escherichia coli* K-12) as an exposure system to transport foreign antigenic determinants to the cell surface. The system was found to be very flexible, since insertions varying in length and nature could be made in different cell-surface-exposed regions of PhoE without interfering with the assembly process of the mutant proteins in the outer membrane (Agterberg *et al.*, 1987). Two antigenic determinants of the structural VP1 protein of foot-and-mouth disease virus were inserted in different combinations in four cell-surface-exposed regions of PhoE (Agterberg & Tommassen, 1991). The epitopes were exposed at the bacterial cell surface and they kept their antigenic and immunogenic properties in this PhoE-associated conformation. Immunisation of guinea pigs with one of these constructs gave complete protection against challenge with the virus.

Charbit *et al.*, (1986) developed a procedure to expose and present a foreign epitope at the surface of *Escherichia coli* by using the LamB protein as a carrier. A number of 'permissive' sites within the LamB protein (i.e. sites that allow the insertion of a foreign peptide without the loss of all the biological properties of the protein) have been identified (Charbit *et al.*, 1991). When the DNA encoding the C3 epitope of VP1

coat protein of type 1 poliovirus was genetically inserted into one of these sites, a stable, non-toxic hybrid protein was synthesised. Moreover, the epitope was exposed on the cell surface and the biological properties and immunological functions of the LamB protein were conserved (Charbit *et al.*, 1986). When the *E. coli* strain expressing the corresponding hybrid protein was used directly as an immunogen in rabbits it elicited a humoral response to the inserted epitope (Charbit *et al.*, 1987, 1988b).

The same procedure was also used to express different regions of the envelope protein of HIV1 at the surface of *Escherichia coli* (Charbit *et al.*, 1990). Recombinant bacteria expressing 8 peptides from gp110 (pep1 to pep8), which are conserved between HIV1 and HIV2, were used as live immunogens in rabbits by the intravenous route. The 8 constructions elicited anti-LamB antibodies, showing that the hybrid proteins were immunogenic and one of them, LamB-pep8, generated antibodies able to react with gp160 and to neutralise HIV1 *in vitro*. Recombinant clones were also used to test sera of seropositive individuals and to characterise specific antibodies.

The high antibody titres obtained with some LamB hybrids suggests that the mode of presentation (the LamB protein or its environment) induces T-cell help (Leclerc *et al.*, 1989). Also, the route of administration appears to be a critical determinant of the response obtained. For example, subcutaneous injection of the bacteria gave no response to the inserted peptide sequences in mice. Leclerc *et al.*, (1989) showed that the position of the inserted peptide correlated with the immunogenicity of the LamB protein. Therefore, differences in the response obtained after intravenous or subcutaneous immunisation could be related to the differences in LamB protein accessibility to the immune system. The behaviour of the LamB hybrids in *S. typhimurium aro* derivatives is less clear, as most of the work to date has been with *E. coli* laboratory strains. Also, it remains to be seen if LamB hybrids are accessible to antibody molecules in bacteria with smooth LPS (such as the *Salmonella* vaccine strains). Nevertheless, the LamB outer membrane porin protein has been used successfully to express epitopes on the outer surface of at least some bacteria and to present them to the immune system.

Pistor & Hobom, (1990) and Schorr *et al.*, (1991) both developed systems using the OmpA outer membrane protein of *E. coli* as a carrier to express foreign antigens at the cell surface of an attenuated *Salmonella typhimurium* strain. Pistor & Hobom (1990) inserted two different oligonucleotides coding for either the influenza A virus monomeric haemagglutinin or its major antigenic epitope A into different outer domains of the OmpA protein. Recombinant *asd S. typhimurium* (Nakayama *et al.*, 1988) were found to express the hybrid proteins in the outer membrane of the cell. The recombinant strains were orally administered to mice. Although no serum antibodies could be detected the mice were found to be partially protected against a lethal dose of mouse-adapted pathogenic influenza A virus. This may be explained, along with the results observed by Sadoff *et al.*, (1988), by cell-mediated immunity. Schorr *et al.*, (1991) used the OmpA vectors of Pistor & Hobom to express *Plasmodium falciparum* blood stage malarial antigens (SERP and HRPII) in an attenuated *S. typhimurium cya crp* strain. In this strain, the hybrid OmpA proteins were also found to integrate into the bacterial outer membrane and expose the malarial antigens at the cell surface. Mice orally immunised with these strains showed a humoral immune response to the SERP and HRPII epitopes. This OmpA system, in contrast to the expression system of flagellin (which can only present around 20 amino acids), or LamB (which can present only around 60 amino acids), seems to allow the stable surface-expression of foreign antigens of at least 730 residues and may therefore be used for the surface-expression of entire proteins or different epitopes of one or more antigens (Schorr *et al.*, 1991). A potential disadvantage, however, is that the hybrid OmpA proteins may compete with the native protein, which is a major component of the outer membrane, and hence reduce the overall concentrations of both proteins.

1.3.6.2. Suitability of the TraT Protein for Presentation of Antigenic Determinants to the Immune System.

Ideally, any carrier protein used for the presentation of foreign antigenic determinants to the immune system should be:-

(a) non-essential to the normal functioning of the cell;
(b) produced and tolerated in large amounts at the cell surface;
(c) contain sites suitable for insertion of a foreign antigenic determinant;
(d) capable of enhancing the immunogenicity of the inserted epitope;
(e) capable of stimulating cell-mediated immunity to the inserted epitope;
(f) potentially highly exposed to the cell surface, even in smooth strains.

The TraT protein, an oligomeric outer membrane lipoprotein specified by plasmids of the IncF incompatibility group, is a protein that potentially fulfils all of these criteria and hence appears to be a promising candidate for presenting foreign antigenic determinants to the immune system.

TraT is very highly exposed at the cell surface (Manning *et al.*, 1980), see 1.2.5., and is produced and tolerated in large amounts. For example, the TraT protein is normally present at concentrations of between 10,000 to 20,000 copies per cell (Achtman *et al.*, 1977). However, it is seen that mutants that over-produce the TraT protein by at least a ten-fold excess tolerate the protein at the cell surface (Manning *et al.*, 1984; O'Connor & Timmis, 1987). TraT, being plasmid-encoded, is also non-essential to the cell and to normal functioning of the outer membrane. Hence, disruption of the TraT protein by epitope insertion should be less detrimental to cell function. The TraT protein has also already been cloned and sequenced and a suitable *traT*$^{+}$ plasmid is available for insertion of epitope coding sequences. In this study, the plasmid pDOC23 (see 5.1.1.1.) was used, as it has several potential unique cloning sites for the insertion of foreign oligonucleotides.

Very recently it has been established that the TraT protein is also capable of enhancing both humoral immunogenicity and stimulating cell-mediated immunity to an inserted epitope, making it an especially attractive candidate for antigen presentation. The purified protein has been shown to have powerful adjuvant activity when chemically conjugated to proteins and peptides, provoking both B- and T-cell responses (Croft *et al.*, 1991). In the latter case, the covalently-attached lipid moiety present at the amino terminus of the mature protein may be responsible. Evidence

supporting this idea was reported by Deres *et al.*, (1989) who covalently attached a lipid moiety (identical to that of the TraT lipoprotein) to peptide epitopes and demonstrated that this allows efficient priming of cytotoxic T-cell responses *in vivo*. It is well known that, *in vitro*, short synthetic viral peptides at picomolar concentrations (and without lipid attachment) can mimic naturally processed antigens in T-cell recognition assays. However, *in vivo* such peptides do not prime cytotoxic T-lymphocytes at any concentration and hence do not have a protective effect. This failure of the peptides to prime the cytotoxic T-cells *in vivo* is thought to be due to either a missing signal, consisting of cytokines released by antigen presenting cells (APC), or to the non-physiological form of antigen presentation (as cytotoxic T-cells usually recognise fragments derived from proteins produced endogenously in the target cell). In contrast, Deres *et al.*, (1989) found that peptides covalently linked to tripalmitoyl-S-glyceryl-cysteinyl-seryl-serine (P_3CSS), the immunologically active N-terminal sequence of Braun's Lipoprotein, were able to prime cytotoxic T-lymphocytes *in vivo*. They found that these lipopeptides mediated attachment to the cell membranes, internalisation into the cytoplasm (where it associates with MHC class I glycoprotein) and the activation of macrophages to secrete cytokines. All of these responses led to a virus-specific cytotoxic T-cell (MHC class I - restricted) response as strong as that induced by the infectious virus itself. Since the TraT protein contains the same attached lipid moiety, it is highly probable that it too will mediate cytotoxic T-cell responses (as reported by Croft *et al.*, 1991). The TraT protein therefore possesses several properties that suggest it may be particularly suited for the task of antigen presentation.

1.4. AIMS OF THE PRESENT WORK.

The goal of the project was to investigate the suitability of the TraT protein for the presentation of foreign antigenic determinants at the bacterial cell surface, using two complementary approaches.

Firstly, to obtain structural information on the protein following purification to apparent homogeneity of the R6-5 TraT protein in a biologically active form. With the availability of the purified protein the functioning and specificity of TraT in the processes of surface exclusion and serum resistance could also be investigated. It was hoped this, in turn, would yield information useful in the design of TraT hybrid proteins.

The second approach was to construct derivatives with a foreign epitope inserted at different sites of the protein. Again, this would allow the structure of the TraT protein to be investigated and, additionally, should establish the feasibility of such constructs for presenting antigenic determinants to the immune system of mice.

CHAPTER 2:

MATERIALS & METHODS.

2. MATERIALS & METHODS.

2.1. MATERIALS, REAGENTS AND MEDIA USED IN THIS WORK.

2.1.1. Materials Used.

The following specialist materials were used (the suppliers are listed in parenthesis):-

Antibiotic discs (Oxoid).

Filter holders (sterile/pyrogen free and disposable, grade FP030/2 with 0.45 μm pore size); nitrocellulose circles (grade BA 85/20 with 0.45 μm pore size); and nitrocellulose filter paper (grade BA85 with 0.45 μm pore size) (Schleicher and Schuell).

Filter paper, 3MM (Whatman).

Mono-Q FPLC column (Pharmacia).

Photographic film 667 (Polaroid).

Oligonucleotide Purification Cartridges (Applied Biosystems).

X-ray film, blue-sensitive (Genetic Research Instrumentation Ltd.).

2.1.2. Reagents Used.

The following reagents were used:-

Acrylamide; N,N'-methylene-bis-acrylamide; Coomassie Brilliant Blue R-250 (Bio-Rad).

Agar No.1; tryptone; yeast extract (Oxoid Ltd.).

Agarose NA (Pharmacia AB).

AMPLITAQ™ recombinant Taq DNA polymerases (Perkin Elmer Cetus).

Bradford reagent (Bio-Rad).

Calcium chloride; Ethidium bromide (BCL).

Developer LX24; Fixer FX40 (Kodak).

DNA 1 kb ladder size marker (BRL).

dNTPs (Pharmacia).

GENECLEAN™ kit (Bio-101).

Immunoglobulins, rabbit anti-mouse, conjugated with horse-radish peroxidase (Dako Patts, Denmark).

Phenol, water-saturated (Rathburn Chemicals Ltd).

Phosphoramidites and chemicals for DNA synthesis (Applied Biosystems and Cruachem).

Radiolabelled α-[^{35}S]-ATP (10 mCi/ml) (Amersham International plc.).

Restriction enzymes along with their buffers, T4 DNA ligase, calf intestinal alkaline phosphatase (Promega, BCL, New England Biolabs and NBL).

SEQUENASE™ DNA sequencing kit (US Biochemical Corp).

SEQUIGEL™ sequencing system (National Diagnostics).

Triton X-100, specially pure for membrane research (Boehringer).

X-Gal (Northumbria Biologicals Ltd).

Zwittergent 3-14 (Calbiochem-Behring).

All other chemicals were purchased from Sigma or BDH (Merck) Ltd. and, where possible, were of the highest grade available.

2.1.3. Media Used.

The media used in this work are listed in Table 2.1.

Agar No.1 was added to give a final concentration of 1% for plates and 0.6% for top agar. The LB medium used for the growth of the *aroA*$^-$ strain SL3261 was supplemented with DHB (0.01%, final concentration). Agar plates used for the growth of this strain were pre-spread with 30 µl of 0.1% DHB.

All media were sterilised by autoclaving (15 PSI, 20 min).

TABLE 2.1.

Media Used:

Medium	Composition	Use / Reference
LB	tryptone 1.0% yeast Extract 0.5% NaCl 0.5% pH adjusted to 7.2	Growth of bacterial strains
2x TY	tryptone 1.6% yeast extract 1.0% NaCl 0.5% pH adjusted to 7.2	Growth of MV1190
Minimal Medium Plates	M9 Salts: (per litre) Na_2HPO_4 6 g KH_2PO_4 3 g NaCl 0.5 g NH_4Cl 1 g pH adjusted to 7.2, autoclaved, cooled and added to it filter sterilised: 1 M $MgSO_4$ 1 ml 20% glucose 10 ml 1 M $CaCl_2$ 0.1 ml 1 M thiamine HCl 1 ml	Growth of MV1190
H	tryptone 1.0% NaCl 0.5% pH adjusted to 7.2	Propagation of M13 bacteriophage
BBL	BBL soybean trypticase 1.0% NaCl 0.5% Autoclaved and added sterile: $MgSO_4$ (10 mM final concentration) maltose (0.2% final concentration)	Growth of Y1090 (for lambda phage propagation).

TABLE: 2.1. (cont^{d}.)

Media Used:

Medium	Composition	Use / Reference
Defined Salts Medium	(per 250 ml) trisodium citrate $2H_2O$ 112.5 mg $(NH_4)_2SO_4$ 250 mg K_2HPO_4 1.75 g KH_2PO_4 750 mg $MgSO_4.7H_2O$ 25 mg pH adjusted to 7.2, autoclaved, cooled and added to it filter sterilised: 0.31% Histidine 1.25 ml 20% glucose 2.5 ml For the growth of *aroA*$^{-}$ strains the following filter sterilised (unless stated otherwise) supplements were added: 0.99% phenylalanine (in 0.01 M HCl), autoclaved 1.25 ml 0.41% tryptophan 1.25 ml 0.36% tyrosine 1.25 ml 25 mM PABA 1 ml 25 mM DHB 1 ml	Davis & Mingioli, (1950). Growth of *aroA*$^{-}$ *S. typhimurium* strains

TABLE 2.2.

Salmonella typhimurium Strains Used:

Strain.	Genotype / Phenotype.	Source / Reference.
LB5000	*metA22 metE551 trpC2 ilv-452 H1-b H2-e,n,x* (cured of Fels 2) *fla-66 rpsL120 xyl-404 leu hsdL6 hsdSA29 hsdSB*	Bullas & Ryu, (1983)
LB5010	*metA22 metE551 ilv-452 leu-3121 trpC2 xyl-404 galE856 hsdL6 hsdSA29 rpsL120 H1-b H2-e,n,x fla-66 nml(-)Fel-2(-)*	Bullas & Ryu, (1983)
SL1344	*hisG46*	Hoiseth & Stocker, (1981)
SL3261	*hisG46 ΔaroA554*	Hoiseth & Stocker, (1981)
SL696	*metA22 trpB2 H1-b H2-e,n,x flaA66 rpsL120* LT2 smooth	Wilkinson *et al.*, (1972)
SH5014	*ilv-1178 thr-914 his-6116 metA22 trpB2 H1-6 H2-e,n,x flaA66 rpsL120 xyl-404 metE551 rfaJ4041* LT2 rough	Nurminen *et al.*, (1976)
SH6749	*ilv-1178 his-6165 metA22 trpB2 H1-6 H2-e,n,x flaA66 rpsL120 xyl-404 metE551 hsdL6 hsdS129 galE496* LT2	Palva & Mäkelä, (1980)
AA3007	*polA2 ara-9*	Whitfield & Levine, (1973)

TABLE 2.3.

Escherichia coli Strains Used:

Strain.	Genotype / Phenotype.	Source / Reference.
M72	*rpsL lacZam Δbio-uvrB ΔtrpEA2 (λNam7 Nam53 cI857 ΔH1)*	Bernard *et al.*, (1979)
LE392-λ	*galT22 hsdR514 lacY1 metB1 supE44 supF58 trpR55* λ^+	Murray *et al.*, (1977); O'Connor & Timmis, (1987).
LE392-λ (*rif*)	A rifampicin-resistant derivative of LE392-λ	
HB101	*F⁻ hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^R) *xyl-5 mtl-1 supE44* λ^-	Boyer & Roulland-Dussoix, (1969)
EH1529	HB101 carrying the *Salmonella typhimurium* virulence plasmid with the wildtype SS-A gene	Sukupolvi *et al.*, (1987)
EH1316	HB101 carrying the *Salmonella typhimurium* virulence plasmid with the SS-A mutation and Tn5	Sukupolvi *et al.*, (1987)
MH1160	MC4100 *ompR101*	T. Silhavy
SM1005	*F⁻ lacU169 rpsL relA thiA fibB gyrA ompC ompF14*	Nogami *et al.*, (1985)
SG480 Δ900	Deleted *ompB* locus derived from MC4100	T. Silhavy
DH1	*F⁻ recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44* λ^-	Hanahan, (1983)
C600	*F⁻ thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ^-	Appleyard, (1954); Young & Davis, (1983)
MV1190	*Δ(lac-proAB) thi supE Δ(srl-recA) 306::Tn10* (tet^R) *[F': traD36 proAB lacI^qZ M15]*	Bio-Rad laboratories
Y1090	F' *Δ(lacU169) proA⁺ Δ(lon) araD139 strA supF [trpC22::Tn10(*tet^R*)] (pMc9) hsdR*(r_K^- m_K^+)	Promega Corporation, (1991)

TABLE 2.4.

Plasmids Used:

Plasmid	Important Characteristics.	Source / Reference.
pCI857	pMC931 with λcI857 gene replacing the Ap^R gene, Km^R	Remaut *et al.*, (1983)
pDOC23	pPLc245 carrying R6-5 *traT* gene, Ap^R	O'Connor & Timmis, (1987)
pDOC55	pDOC23 derivative with the *traT* gene replaced with *lacZ* α-peptide gene and *EcoRI* site removed, Ap^R	O'Connor & Timmis, (1987)
pDOC40	*XbaI* linker mutant of pDOC23 causing supersensitive phenotype	Sukupolvi & O'Connor, (1987)
pJH1	pDOC23 with C3 epitope inserted in *StuI* site (837) of *traT*	Taylor *et al.*, (1990)
pIT6	pDOC23 with C3 epitope inserted in *HpaI* site (1062) of *traT*	
pIT9	pDOC23 with C3 epitope inserted in *EcoRV* (643) site of *traT*	
pIT10	pDOC23 with C3 epitope inserted in *BalI* (1108) site of *traT*	
pIT11	pDOC23 with C3 epitope inserted in *ScaI* (1002) site of *traT*	
pKT134	Mutants of pKT107 (Moll *et al.*, 1980) that constitutively over-produces TraT, Tc^R	Manning *et al.*, (1982)
pKT135		
pKT138		
pKT150		
R6-5	Tetracycline-sensitive derivative of R6 *traT*$^+$ Sfx IV Sm^R Spc^R Cm^R Km^R Su^R Fd^R Hg^R	Timmis *et al.*, (1978)
ColB::Tn5 *seq1*	ColB-K98 tagged with Tn5*Seq1* (Nag *et al.*, 1988). Sfx II Km^R	Frydman & Meynell, (1969)
R1	62 MDa plasmid which carries Tn3 & Tn4 *traT*$^+$ Sfx III Ap^R Cm^R Km^R Sm^R Su^R	Meynell & Datta, (1966)

TABLE 2.4. (Cont^{d}.)

Plasmids Used:

Plasmid	Important Characteristics.	Source / Reference.
R100-1	Conjugative antibiotic resistance plasmid derepressed for transfer *traT*$^{+}$ Sfx II Cm^{R} Hg^{R} Sm^{R} Su^{R} Tc^{R}	Chandler *et al.*, (1977); Hoar, (1970)
F::Tn5	F plasmid tagged with Tn5 (Km^{R}) Sfx I	M. Russell
F::Tn9	F plasmid tagged with Tn9 Sfx I Cm^{R}	Laboratory stock

TABLE 2.5.

Phage Used:

Bacteriophage	Important Characteristics	Source / Reference
M13mp18	Cloning vector, produces single-stranded DNA	Bio-Rad laboratories
lambda 649	lambda phage clone (number 649) of the *Escherichia coli* chromosome (94.4 minutes)	Kohara *et al.*, (1987)
P22 HT *int*	High frequency of transduction, deficient in prophage integration	Schmieger, (1972)
Br60	Rough LPS specific	Wilkinson *et al.*, (1972)
C21	Specific for host cells with rough Rc or Rd chemotypes	Wilkinson *et al.*, (1972)

2.1.4. Antibiotics Used.

Where necessary antibiotics were added to the agar (after sterilisation and cooling to below 50°C) at the following final concentrations:-

Ampicillin:	100 μg/ml
Kanamycin:	50 μg/ml
Tetracycline:	12.5 μg/ml
Rifampicin:	50 μg/ml
Chloramphenicol:	25 μg/ml
Nalidixic acid:	20 μg/ml

For liquid cultures the concentrations of the antibiotics used were halved.

2.2. STRAINS, PLASMIDS AND PHAGE USED IN THIS WORK.

The bacterial strains used are shown in Tables 2.2 (*E. coli*) and 2.3 (*S. typhimurium*), the plasmids used are shown in Table 2.4 and the phage used are shown in Table 2.5.

2.3. DNA PREPARATION.

2.3.1. Purification of Plasmid DNA using Alkaline Lysis.

2.3.1.1. Rapid Preparation of Plasmid DNA.

Microgram quantities of plasmid DNA were prepared by the alkaline lysis method of Close & Rodriguez, (1982). Cells from an overnight culture (1-2 ml) were washed with 0.6 ml SET Buffer (50 mM Tris-HCl, pH 8; 50 mM Na_2EDTA, pH 8; 20% sucrose); resuspended in 150 μl of SET Buffer and combined with 5 μl DN'ase-free RN'ase (a 5 mg/ml stock being made up in sterile 0.1 mM Tris-HCl, pH 7.5; 0.3 mM

NaCl, heated to 100°C and allowed to slowly cool to r.t.) and 350 µl of Lysing Solution (0.2 M NaOH; 1% SDS). After gently mixing, the solution was incubated on ice (5 min) before 250 µl of Neutralisation & Renaturation Solution (3 M KAc, pH 4.8, the pH being adjusted with glacial acetic acid) was added. Following incubation on ice (5 min) the lysate was precipitated by centrifugation (6 min). The supernatant was transferred to a fresh microcentrifuge tube and mixed with -20°C isopropanol (0.7 ml). The plasmid DNA was pelleted by centrifugation (6 min) and washed with -20°C, 75% ethanol before being air dried. The DNA pellet was resuspended in 50 µl of sterile TE Buffer (10 mM Tris-HCl, pH 8; 1 mM Na_2EDTA, pH 8).

This method yielded approximately 1 to 3 µg of plasmid, when cultures bearing multicopy plasmids were used. The DNA was pure enough to be used directly for transformation, but had to be phenol extracted (see 2.4.1.) before it could be digested by restriction enzymes.

Plasmids prepared by this method were occasionally used for sequencing using the following modification of the standard procedure (Riley, 1989). Prior to precipitation with isopropanol, 4 µl of DEPC was added to the plasmid solution. The tubes were capped, mixed vigorously and incubated at r.t. (4 min); 65°C (15 min) and on ice (5 min). The tubes were vented occasionally to release the CO_2 produced. The denatured proteins in the plasmid solution were then removed by centrifugation (4 min) and the plasmid DNA purification continued as described (Close & Rodriguez, 1982), by precipitating the plasmid DNA with -20°C isopropanol.

2.3.1.2. Large Scale Purification of DNA.

A combination of the methods of Close & Rodriguez, (1982) and Mirendorf & Pfeffer, (1987) was followed:-

The plasmid-bearing strains were grown to late exponential phase and pelleted at 6,500g (5k rpm in a Sorvall RC-3B centrifuge, using a 4 x 1,000 ml rotor) and 4°C for 15 min. The cells pelleted from a 1 litre culture were resuspended in SET Buffer

(50 ml) and gently mixed with Lysing Solution (50 ml). After incubation on ice (5 min) Neutralisation & Renaturation Solution (25 ml) was added and the solution mixed and incubated on ice (5 min). Following centrifugation at 40,000g (18k rpm in a Beckman J2-21 centrifuge with a JA-20 rotor) and 4°C for 15 min, the supernatant was poured through a tea strainer to remove all the precipitate and -20°C isopropanol (0.6 times its volume) was added. The solution was recentrifuged, as before, and the pellet washed with -20°C, 75% ethanol before being resuspended in sterile TE Buffer (5.7 ml). To this was added ribonuclease A (40 μg/ml final concentration) and after incubation at 37°C (15 min), exactly 6.0 g of caesium chloride and 10 mg/ml ethidium bromide (0.3 ml) were added. The preparation was centrifuged at 240,000g (50k rpm in a Beckman L7 ultracentrifuge, using an 12 x 13.5 ml titanium rotor) and 20°C overnight.

The plasmid band was taken and the ethidium bromide removed by extraction (4 times) with caesium chloride saturated isopropanol. The sample was diluted and precipitated with r.t. 96% ethanol. The DNA pellet was resuspended in sterile TE buffer and reprecipitated with -20°C 96% ethanol before being washed with 75% ethanol, allowed to air dry and resuspended in sterile TE buffer (200 μl).

2.3.1.3. Estimation of DNA Concentration.

DNA concentrations were measured in quartz cuvettes against a suitable blank. The absorbance at 260 nm (to find the DNA concentration) and 280 nm (to check for protein contamination) were measured. A DNA solution with an absorbance of 1 at 260 nm was assumed to be equivalent to 50 μg/ml (Maniatis *et al.*, 1982). If the concentration of protein present in the plasmid preparation was too high (the A_{260}:A_{280} was less than 1.8) the DNA was further purified by extraction with phenol and chloroform (see section 2.4.1.).

2.3.2. Extraction of M13 Single Stranded DNA.

Single-stranded DNA was prepared for sequencing as follows: 2 x TY (1.5 ml) was

inoculated with 15 µl of an overnight culture of MV1190 and a M13 phage plaque. After incubation at 37°C with shaking for 6 hours, the culture was spun down in a microcentrifuge (5 min) and the phage containing supernatant carefully removed. The supernatant was then respun to remove residual cells and transferred to a fresh tube. 200 µl of PEG/NaCl (20% PEG 8000; 2.5 M NaCl, filter-sterilised) was mixed with the supernatant and the solution allowed to stand at r.t. for 15 min. The phage particles were collected by centrifugation for 5 min and then a further 2 min, each time the supernatant being removed fully. The phage were resuspended in TE buffer (100 µl), phenol extracted (as in 2.4.1.) and then ethanol-precipitated (as in 2.4.2.).

2.3.3. Synthesis and Purification of Oligonucleotides.

The required oligonucleotide DNA (0.2 µM scale) was synthesised on an Applied Biosystems 381A DNA synthesiser in the 'trityl-on' mode, using the ß-cyanoethyl phosphoramidite method (Brown & Brown, 1991). The oligonucleotides were then purified using Oligonucleotide Purification Cartridges, following the instructions supplied by the manufacturer.

To obtain double stranded oligonucleotide, two complementary strands were annealed by mixing 100 pmol of each purified DNA strand together, heating to 95°C in a water bath and allowing the DNA to cool slowly to r.t.

2.4. DNA MANIPULATIONS.

2.4.1. Phenol Extraction.

The phenol was prepared by dissolving it in $CHCl_3$ (1:1, w/v) and adding 0.1% 8-hydroxyquinoline. This was extracted 3x with 1M Tris-HCl, pH 8.0 to equilibrate the pH of the phenol to above 7 and 3x with an equal volume of TE buffer, pH 8.0. The phenol was stored under TE buffer in the dark at 4°C. The DNA was extracted by mixing with an equal volume of phenol/$CHCl_3$, vortexing and then centrifuging (3

min) to separate the phenol and aqueous phases. The top (aqueous) phase was carefully taken, avoiding the protein at the interface and was transferred to a clean microcentrifuge tube. The phenol was removed from the DNA solution by extracting twice with an equal volume of chloroform. The chloroform was removed from the preparation by extracting 3 times with water-saturated ether, removing the top (ether) layer each time. The ether was then evaporated from the sample by incubating at 68°C for 10 min.

2.4.2. Ethanol Precipitation.

DNA was routinely precipitated by adding 0.1 x the volume of 3 M NaAc (pH 5.6) and 2.5 x the volume of -20°C 96% ethanol and incubating in a dry-ice/methanol bath (approx. -70°C) for 10 min. The DNA was pelleted by centrifugation (10 min), washed with 100 µl -20°C 75% ethanol, air dried at r.t. and resuspended in sterile TE Buffer.

2.4.3. Restriction Enzyme Digestion.

All restriction digestions were carried out as described by Maniatis *et al.*, (1982) or by following the instructions supplied by the manufacturer. Generally, a control tube lacking any restriction enzyme was set up to ensure that no additional nucleases were present.

2.4.4. DNA Ligation.

The ligations were carried out in small volumes (10-20 µl) with 1-5 units of T4 DNA ligase. In each case, an experimental and a control tube were set up, the tubes being the same except that the control tube did not contain insert DNA.

Recircularisation of the linearised plasmid (vector) DNA was selected against by recutting the ligation mixture with one of the original restriction enzymes whose site was destroyed upon ligation with the insert DNA. If such a site was not available then

the vector DNA was pretreated with alkaline phosphatase (see 2.4.5.).

Blunt-ended ligations were carried out (Wu *et al.*, 1987) by adding to a microcentrifuge tube the following:- linearised plasmid DNA; 2x the amount of insert DNA; 1x Hexamine Cobalt Chloride Ligation Buffer (10x: 660 mM Tris-HCl, pH 7.6; 100 mM $MgCl_2$; 100 mM DTT; 10 mM spermidine-HCl; 3 mM ATP, pH 7; 10 mM hexamine cobalt chloride, filter-sterilised and stored at -20°C) (Rusche & Howard-Flanders, 1985); 0.2 mg/ml nuclease-free BSA and T4 DNA ligase. The ligation was carried out at 15°C overnight.

Sticky-ended ligations were carried out by adding equal amounts of vector and insert DNA together along with:- 1x Ligation Buffer (10x: 500 mM Tris-HCl, pH 7.4; 100 mM $MgCl_2$; 200 mM DTT; 10 mM ATP, pH 7; 50 ug/ml nuclease-free BSA, filter-sterilised and stored at -20°C) and T4 DNA ligase. The ligation was carried out either at r.t. for 2-4 hours or in an r.t. water-bath placed at 4°C overnight, so that the temperature drops slowly.

2.4.5. Alkaline Phosphatase Treatment of DNA.

This was used to prevent self-ligation of DNA fragments. Following digestion of DNA by restriction enzymes the DNA was phenol/$CHCl_3$ extracted (see 2.4.1.) and ethanol-precipitated (see 2.4.2.) and resuspended in 1x Alkaline Phosphatase Buffer (10x: 500 mM Tris-HCl, pH 9; 10 mM $MgCl_2$; 1 mM $ZnCl_2$; 10 mM Spermidine). Calf intestinal phosphatase was added (1 unit per μg DNA) and incubated at 37°C for 30 min; 47°C for 15 min and then 57°C for 15 min. After phosphatase treatment, the mixture was again phenol/$CHCl_3$ extracted at least three times and ethanol-precipitated.

2.4.6. Agarose Gel Electrophoresis of DNA.

Horizontal agarose gels were prepared essentially as described by Sharp *et al.*, (1973) and McDonnell *et al.*, (1977). Gels (of concentrations between 0.5 and 1.5%, depending on the size of the DNA fragments to be visualised) were run in 1x Tris-Acetate EDTA Buffer (50x TAE stock:- 242 g tris base; 1 mM Na_2EDTA, pH 8; 51.7 ml glacial acetic acid in 1 litre) containing ethidium bromide (0.6 μg/ml, final concentration). The samples (0.3 to 0.5 μg DNA) were mixed with 1x Agarose Gel Loading Buffer (10x GLB:- 3 ml glycerol; 30 mg bromophenol blue in 10 ml sterile TE buffer) and electrophoresed at a constant voltage of 40 to 150 volts (depending on the percentage of the gel used), until the bromophenol blue dye front had reached the end of the gel.

The bands on the gel were visualised using a 300 nm ultra-violet transilluminator. and the gel was photographed using polaroid 667 film. The DNA size markers used for the electrophoresis were SPPI DNA digested with *EcoRI* (Ratcliff *et al.*, 1979) and 1 kb ladder from BRL (see Table 2.7).

2.4.7. Extraction of DNA from Agarose Gels.

A large preparative (200 ml) agarose gel, of the required percentage, was run overnight at 40 mV as described above. The required band was excised from the gel under ultra-violet light. The DNA was then purified from the agarose gel slice using GENECLEAN™, following the instructions supplied by the manufacturer (Bio101 Inc., USA). The DNA was then further purified by phenol/$CHCl_3$ extraction (see 2.4.1.) and ethanol-precipitation (see 2.4.2.).

2.4.8. DNA Sequencing.

2.4.8.1. Preparation of single-stranded DNA.

2.4.8.1.1. Plasmid DNA.

Plasmid DNA was converted to a single-stranded form by alkaline denaturation as described by Chen & Seeburg, (1985). The plasmid DNA (2-4 μg) was mixed with 2 M NaOH (2 μl) and incubated at r.t. (5 min), before adding 5 M ammonium acetate, pH 7.5 (8 μl) and -20°C absolute ethanol (100 μl). The DNA was then precipitated by incubation at -70°C (5 min) in a dry-ice/methanol bath and centrifugation (10 min). The pellet was washed with 75%, -20°C ethanol, air-dried and dissolved sterile water (7 μl).

2.4.8.1.2. M13 DNA.

If primers were not available for sequencing a plasmid directly then the segment of DNA to be sequenced was cloned into M13mp18 plasmid DNA using the methods described in 2.4.3. and 2.4.4. The DNA was then transformed into MV1190 using the method described in 2.5.1.5. Single-stranded M13 DNA was extracted using the method described in 2.3.2.

2.4.8.2. Sequencing Reactions.

DNA sequencing was performed using a modified form of T7 DNA polymerase, as described in the manual accompanying the SEQUENASE™ DNA sequencing kit. Briefly, DNA was annealed to the appropriate primer in 1x Sequencing Buffer (5x Buffer:- 200 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl; 100 mM DTT)

before the labelling mixture [containing 6.6 mM DTT; 7.5 μM of each dGTP, dCTP, dTTP; α-[^{35}S]-dATP (5 μCi, 400 Ci/mmol) and SEQUENASE™ enzyme] were added. The tubes were incubated at r.t. (5-10 min) prior to the addition of the Termination Mixes (containing 80 μM of each dATP, dCTP, dGTP & dTTP; and 8 μM of the appropriate dideoxynucleotide) to samples of the labelled DNA. Each reaction mix was incubated at 37°C (5 min) before Stop Solution (95% formamide; 20 mM Na_2EDTA, pH 8.0; 0.06% bromophenol blue; 0.05% cyanol FF) was added and the samples stored at -20°C until needed.

2.4.8.3. The Polyacrylamide Gel for Sequencing.

The gel plates (21 cm x 40 cm) were assembled and the gel poured as per the SEQUI-GEN Sequencing Cell (Bio-Rad) instructions. The gel was made from a 6% acrylamide solution (made from a 40% acrylamide / 1.6% N,N'-methylene bisacrylamide stock solution) containing: 8 M urea; made up with 1x TBE, Tris-borate EDTA Buffer, (89 mM Tris-HCl, pH 8 ; 89 mM boric acid; 2.5 mM Na_2EDTA, pH 8) and helped to set with 1/100th the vol of 10% fresh APS and 15 μl of TEMED. The gel was left for at least 3 hours to polymerize and then pre-run for 30 min at 1600 volts to warm the gel to 50°C.

Samples were denatured by heating at 95°C (3 min) before being loaded (3 μl per well) onto the gel. The gel was run at 50°C for 1 to 4 hours (depending on the distance between the primer and the sequence to be read) and fixed by soaking in Fixing Solution (10% glacial acetic acid; 12% methanol) for 20 min. Following transfer to Whatman 3MM paper and drying (1 to 2 hours at 80°C), the gel was placed in contact with X-ray film for 2 days, in a light-proof cartridge. The film was developed using standard conditions.

2.4.9. PCR Amplification of DNA.

The polymerase chain reaction (PCR) was carried out on a Techne programmable DRI-BLOCK™ PHC-1 PCR machine using sterile solutions and equipment.

To a sterile 0.5 ml microcentrifuge tube was added:-

10x Reaction Buffer [100 mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, 0.1% (w/v) gelatin, autoclaved and stored at -20°C]	10 µl
1.25 mM stock of each dNTP	16 µl
20 µM primer 1	5 µl
20 µM primer 2	5 µl
Template DNA	1 µl
sterile water to give a total volume of:	100 µl
sterile mineral oil	50 µl

A control tube was set up which contained all of the above except for the template DNA. The tubes were heated at 95°C (4 min) to denature the template DNA to single strands and then 0.5 µl of AmpliTaq (2.5 units) was added to each tube. The DNA was amplified using the following sequence of temperatures for 20 cycles:

94°C for 2 min (to denature the template DNA)
52°C for 2 min (to anneal the primers to template)
72°C for 2 min (strand elongation)

When all 20 cycles had finished the reaction mixes were chloroform extracted and the samples were analysed by electrophoresis on 1.5% agarose gels.

2.5. GENETIC TECHNIQUES.

2.5.1. Transformation.

Standard procedures were used to introduce plasmid DNAs into *E. coli* host strains by transformation. If a plasmid DNA containing the lambdaP_L promoter was transformed into an *E. coli* strain producing the temperature-sensitive lambda repressor (e.g. K-12ΔH1Δ*trp*), then care was taken to ensure that cells were not exposed to temperatures above 30°C during the procedure and hence protein synthesis was not induced. In each experiment, competent cells were also plated on selective media to test for potential contamination.

2.5.1.1. The Method of Chung & Miller, (1988).

Cells (50 ml), grown to an A_{600} of 0.3 to 0.6, were harvested and resuspended in 5 ml of ice-cold Transformation & Storage Buffer (10% PEG 8000; 5% dimethylsulphoxide; 10 mM $MgCl_2$; 10 mM $MgSO_4$; filter-sterilised and stored at -20°C). After incubation on ice (10 min), plasmid DNA (100-500 pg) was added to 100 µl aliquots of the competent cells. Following incubation on ice (5 min), Transformation & Storage Buffer (0.9 ml) and 20% sterile glucose (2 µl) were added. The cells were incubated at 37°C (1 hour) and plated on selective media before being incubated overnight at 37°C.

2.5.1.2. The Method of Mandel & Higa, (1970).

Cells (50 ml), grown to a density of around 5 x 10^7 cells/ml (A_{600} of 0.2-0.3; or A_{600} of 0.5-0.6 for *rec*$^-$ strains) were chilled on ice (5 min) and harvested. The cells were resuspended in ice-cold sterile 50 mM $CaCl_2$ (50 ml) and incubated on ice (20 min) before being resuspended in 50 mM $CaCl_2$ (2 ml) again. Plasmid DNA (40 ng) was

added to 200 µl aliquots of the competent cells and incubated on ice (40 min), before being heat shocked to 42°C (2 min) and returned to ice (5 min). Fresh LB (0.8 ml) was added and the cells were incubated at 37°C (1 hour) before being plated on selective media.

2.5.1.3. The Method of Kushner, (1978).

Cells grown to a cell density of around 5 x 10^7 cells/ml were harvested (2 ml aliquots) and resuspended in 1 ml of sterile Solution I (10 mM MOPS, pH 8; 10 mM RbCl). The cells were then pelleted, resuspended in 1 ml sterile Solution II (100 mM MOPS, pH 6.5; 50 mM $CaCl_2$; 10 mM RbCl) and incubated on ice (15 min). After harvesting the cells again they were resuspended in sterile Solution II (200 µl). To this was added 3 µl of spectroquality, unoxidised DMSO and 1 to 200 ng of plasmid DNA (in 10 µl of TE buffer). Following incubation on ice (30 min) the cells were heat shocked to 42°C (90 sec) and made up to 5 ml with prewarmed LB. The cells were then incubated at 37°C (1 hour), plated on selective LB plates and incubated overnight at 37°C.

2.5.1.4. The Method of Lederberg & Cohen, (1974).

For transformation of *S. typhimurium*, the method of Lederberg & Cohen, (1974) was followed. Cells (50 ml), grown to an A_{600} of 0.6 were chilled on ice and harvested. After resuspension in ice-cold sterile 100 mM $MgCl_2$ (50 ml), the cells were pelleted and resuspended in ice-cold sterile 100 mM $CaCl_2$ (25 ml). Following incubation on ice (20 min), the cells were pelleted and resuspended ice-cold sterile 100 mM $CaCl_2$ (2.5 ml). To 0.2 ml aliquots of the competent cells was added, 40 ng of plasmid DNA (in 100 µl of TE Buffer). The cells were incubated on ice (30 min) before being heat-shocked to 42°C (2 min) and diluted 10-fold with pre-warmed fresh LB. Following incubation at 37°C (90 to 120 min), the cells were pelleted,

resuspended in LB (200 µl), spread on a selective LB plate and incubated overnight at 37°C.

2.5.1.5. Transformation of M13 DNA.

M13 DNA was transformed into MV1190 competent cells using the following modification of the method described in 2.5.1.2. After heat shock, the following were mixed together in the order given:-

Fresh MV1190 cells	200 µl
Transformed cells	300 µl
100 mM IPTG	40 µl
2% X-gal (dissolved in dimethyl formamide)	40 µl
Molten H-top agar	3 ml

The above contents were mixed, poured onto a prewarmed H plate, allowed to set and then incubated at 37°C overnight.

2.5.2. Transduction with P22 Bacteriophage.

P22 phage stocks were serially diluted (10^0 to 10^{-8}) with sterile λ dil (10 mM Tris/Cl, pH 7.5; 10 mM $MgSO_4$) and titred as per Davis *et al.*, (1980), against the required indicator strain to find the phage dilution where the plaques were just confluent.

Phage lysates were prepared on the indicator strain containing the plasmids to be transduced by adding 10 µl of a phage dilution giving confluent plaques to 100 µl of each overnight culture. The mixtures were vortexed and incubated at 30°C (30 to 45

min) prior to the addition of molten H-top agar (3 ml per tube) and pouring onto prewarmed, dry selective LB plates. The plates were allowed to dry, before being incubated at 30°C overnight.

The next day, the confluent plaques were harvested by flooding the plates with 4 ml of SM Phage Buffer (100 mM NaCl; 10 mM $MgSO_4$; 50 mM Tris/Cl, pH 7.5; 0.1% gelatin) and incubating at 4°C for 3 hours. The phage containing buffer was then collected from the plates and stored at 4°C (after the addition of a few drops of chloroform).

The plasmids were transduced into the smooth *aroA*$^-$ *S. typhimurium* host strain by adding various amounts of the phage stocks (1; 5; 10; 20 or 50 µl) to 100 µl of an overnight culture. The tubes were incubated at 30°C (1 hour) before being plated on selective LB plates additionally supplemented with EGTA (5 mM); DHB (30 µl of a 0.1% solution spread on each plate) and incubated at 30°C overnight. Transductants were sequentially restreaked onto the same selective medium until they were free from phage.

2.5.3. Conjugal Transfer of F-like Plasmids and *in vitro* Surface Exclusion Assay.

Plasmid donor strains were constructed by a routine procedure described by Miller, (1972). The surface exclusion activity due to purified TraT protein was measured essentially as described by Minkley & Willetts, (1984). The donor strains used are given in Table 2.6 and the recipient strain was LE392-λ(*rif*).

TABLE 2.6.

Donor Strains Used for Conjugal Transfer.

Donor Strains Used.	Antibiotic Resistance used to select for plasmid.	Surface Exclusion Group. [a]
DHI (R6-5)	kanamycin	IV
DHI (R100-1)	chloramphenicol	IV
DHI (pCJ105 = F::Tn9)	chloramphenicol	I
DHI (F::Tn5)	kanamycin	I
DHI (R1)	kanamycin	III
DHI (ColB-K98::Tn5 *seq1*)	kanamycin	II

[a] The surface exclusion groups are those defined by Willetts & Maule, (1986).

Donor and recipient strains grown in LB supplemented with the appropriate antibiotic without shaking at 32°C overnight were diluted 1:25 with fresh LB and allowed to stand for 2 hours at 37°C. The cultures were chilled on ice and then mixed in equal proportions to form a master mating mixture.

A series of microcentrifuge tubes were set up containing the purified TraT protein (0; 1; 2; 4; 8; 10 μg/ml, final concentration) made up to a final volume of 80 μl with sterile PBS (0.8% NaCl; 0.02% KCl; 0.215% $Na_2HPO_4.2H_2O$; 0.2% KH_2PO_4; at pH 7.2 and autoclaved). Aliquots (0.2 ml) of the mating mixture were then added to the TraT-containing tubes, mixed and incubated at 37°C for 40 min on a slowly rotating wheel.

0.1 ml samples of the cells were serially diluted with PBS and plated on LB plates supplemented with rifampicin (to select for recipients, which is equivalent to donors) and rifampicin + kanamycin or chloramphenicol (to select for transconjugants). A control was set up using the equivalent dilution of the TraT protein buffer, instead of the TraT protein, to ensure that the detergent or azide in the buffer had no effect on conjugation. Following incubation at 37°C overnight the plates were counted and the number of colony forming units per ml of undiluted conjugated cells (CFU/ml) was worked out. From this (and the fact that the number of donors in the control and test matings were identical) the Surface Exclusion Index was worked out, using the expression shown below:

$$\text{Surface Exclusion Index} = \frac{\text{Transconjugants for control matings (no TraT protein)}}{\text{Transconjugants for test matings (with TraT protein)}}$$

2.5.4. Assay for Outer Membrane Permeability to Hydrophobic Antibiotics.

Various *traT* derived plasmids were transformed into the *E. coli* EH1316 and EH1529 cells and the sensitivity of the transformants to hydrophobic antibiotics tested using the disc-diffusion-assay essentially described by Sukupolvi *et al.*, (1984).

Cultures of the strains to be tested were grown in LB, containing Ap for plasmid selection, at 37°C overnight. The next day the cultures were diluted 1:500 with fresh LB and spread (100 µl) on LB + Ap plates. Antibiotic-containing discs (6 mm in diameter) impregnated with the following amounts of antibiotic:- erythromycin (30µg), fusidic acid (10µg), novobiocin (30µg), rifampicin (2µg) and vancomycin (30µg) were placed on each plate. Following incubation overnight at 37°C, the diameter of the zone of inhibition of growth around each disc was measured.

2.5.5. Temperature-Induction of Protein Synthesis from λP_L Expression Vectors.

The relevant plasmid with the cloned gene downstream of the λP_L promoter in the correct orientation was transformed into the *E. coli* strain M72. Induction of protein synthesis of the cloned gene from the promoter was tested by shifting the cultures from 30°C to 42°C.

Cultures (5 ml), supplemented with the appropriate antibiotics were grown overnight without shaking at 30°C and then with shaking at 30°C (90 min). A sample (1 ml) of each culture was taken and, following measurement of the absorbance at 600 nm, the cells were collected, resuspended in 100 µl of 1x Final Sample Buffer, FSB (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol and 0.004% bromophenol blue), and immediately boiled for 5 min. Meanwhile the cultures were shaken vigorously at 42°C (30 min) and then 37°C (2 hours). The A_{600} of the induced cultures was measured and another 1 ml sample was taken, as before, for SDS-PAGE. An equivalent of 0.2 A_{600} units of each protein sample was run on a 13% polyacrylamide gel (see section 2.6.1.) and, after staining with Coomassie Blue, the protein bands of each strain before and after temperature-induction were compared.

2.5.6. Test for Bacterial Resistance to Serum in the Presence of Purified TraT Protein.

The resistance of *E. coli* cells LE392-λ(*rif*) to serum was measured in the presence of the purified R6-5 TraT protein.

Overnight cultures, grown without shaking at 32°C, were diluted 1:25 with fresh LB and grown to late exponential phase. The cells were harvested, washed and finally resuspended in PBS. The absorbance of the cells was measured and the suspension was diluted with PBS to give approx. 5 x 10^6 cells/100 µl to which were added 15 µg of the purified TraT protein along with 100 µl of pooled adult guinea-pig serum (10% final concentration). The cells were mixed and incubated on a slowly rotating wheel at 37°C (30 min) before being serially diluted with PBS, plated on LB medium and incubated overnight at 37°C. The next day the surviving cells were counted.

2.6. PROTEIN TECHNIQUES.

2.6.1. SDS Polyacrylamide Gel Electrophoresis of Proteins.

Size fractionation of proteins by SDS-PAGE was carried out following the method of Laemmli, (1970). The Bio-Rad mini-gel electrophoresis apparatus was used according to the manufacturers instructions. The protein standard size marker used is shown in Table 2.7.

The resolving gel consisted of 13% acrylamide (13% acrylamide; 0.35% N,N'-methylene bisacrylamide; 0.375 M Tris-HCl, pH 8.8; 0.1% SDS; APS and TEMED) and the stacking gel of 4.5% acrylamide (4.5% acrylamide; 0.12% N,N'-methylene bisacrylamide; 0.253 M Tris-HCl, pH 6.8; 0.1% SDS; APS and TEMED). The protein samples were boiled in 1x FSB for 5 min before being loaded onto the gel and run with Gel Running Buffer (25 mM Tris; 192 mM glycine; 0.1% SDS) at a constant voltage of 60 V until the bromophenol blue dye front had almost reached the end of the gel.

TABLE 2.7.

Size Markers Used:

Fragment	DNA markers (kb)		Protein marker	
	BRL 1kb size marker [a]	SPPI/*EcoRI* size marker [b]	Dalton Mark VII-L™ [c]	
			(kDa)	Protein
1	12.21	8.51	66	Albumin, bovine
2	11.19	7.35	45	Albumin, egg
3	10.81	6.11	36	Glyceraldehyde-3-phosphate dehydrogenase
4	9.16	4.84	29	Carbonic anhydrase
5	8.14	3.59	24	Trypsinogen
6	7.12	2.81	20.1	Trypsin inhibitor
7	6.10	1.95	14.2	α-lactalbumin
8	5.09	1.86		
9	4.07	1.51		
10	3.05	1.39		
11	2.03	1.16		
12	1.63	0.98		
13	1.01	0.72		
14	0.51	0.48		
15	0.50	0.36		
16	0.39			
17	0.34			
18	0.29			
19	0.22			
20	0.20			
21	0.15			
22	0.13			
23	0.07			

[a] Purchased from BRL.
[b] Prepared by C.D. O'Connor (Ratcliff *et al.*, 1979).
[c] Purchased from Sigma.

Gels were stained (Webber & Osborne, 1969) with Coomassie Blue Stain (0.25% Coomassie Brilliant Blue R-250 dissolved in 9% acetic acid; 45% methanol) at 37°C (30 min) before destaining in 7.5% acetic acid; 5% methanol with gentle agitation in the presence of a few scraps of plastic foam to soak up the stain.

Silver staining was carried out by a modification of Eschenbruch & Bürk, (1982). The gel (in a clean, protein free container) was placed in Fixing Solution (50% MeOH; 12% acetic acid) for 1 hour and then soaked in a large volume of deionised distilled water overnight, with several changes of water, to remove the methanol. A solution of 5-fold diluted 0.880 sp. gr. NH_4OH was used to titrate 18.4 ml of 1 N HCl to pH 4.7 and the volume noted. To 0.4 x this volume of NH_4OH was added 0.36% (w/v) NaOH (8.4 ml) then 19.4% (w/v) $AgNO_3$ solution (1.6 ml) was slowly added, while stirring vigorously. The solution was made up to 40 ml with deionised distilled water and used to stain the gel (15 min). The gel was then washed in deionised distilled water for 15 min with at least 3 changes of water. Once the excess silver had been washed off, the stain was developed with a solution of 0.005% citric acid; 1.9% formaldehyde. The reaction was stopped, before the background started to darken, by rinsing the gel with deionised distilled water and soaking it in 0.05% citric acid; 0.0175% methylamine for 30 min. The gel was photographed and stored in deionised distilled water.

2.6.2. Rapid Preparation of Cell Envelope Proteins.

This method was based on that described by Morona & Reeves, (1982). The cells from an overnight culture (10 ml) were harvested, washed with PBS (10 ml) and resuspended in 10 ml of 50 mM Tris-HCl, pH 8.5; 2 mM Na_2EDTA. After sonication in a MSE SONIPREP 150 using a cycle of 15 seconds on with 30 seconds off for cooling in an ice-water bath, the unbroken cells were pelleted 4,400g (6k rpm in a Beckman J2-21 centrifuge) and 4°C for 10 min and the protein-containing supernatant was taken. The cell envelope fraction was then pelleted at 40,000g (18k rpm) and 4°C for 20 min. The pellet was resuspended in 1 ml of 1x FSB, boiled for 5 min and samples run on an SDS polyacrylamide mini-gel (see 2.6.1). If the amount of

outer membrane protein produced by different strains was to be directly compared then the OMP pellets were resuspended in 300 µl of Tris-HCl, pH 8.5 and the A_{280} of a 1:10 dilution measured using quartz cuvettes. The equivalent of A_{280} of 0.1 for each of the samples was then loaded onto the gel.

2.6.3. Purification of Biologically Active TraT Protein.

The TraT protein was purified by modifying the porin purification method of Gehring & Nikaido, (1989):-

2.6.3.1. Isolation of Outer Membrane Proteins.

E. coli MH1160 (pKT135) cells, grown at 37°C with shaking for 8 hours in LB supplemented with tetracycline, were harvested in 1 litre batches in a Sorvall RC-3B centrifuge at 6,500g (6k rpm) for 15 min at 4°C. The cells were washed with 30 mM Tris-HCl, pH 7.5 and pelleted again at 4,400g (6k rpm in a Beckman J2-21 centrifuge) and 4°C for 15 min. The cell pellets were resuspended in 30 mM Tris-HCl; 1 mM $MgCl_2$, pH 7.5 (at approximately 30 ml per gram wet weight of cells), and Pancreatic DN'ase I and RN'ase A were added, each to a final concentration of 500 µg/ml. The cells were then broken open by sonicating the suspension in a MSE SONIPREP 150 in 30 ml batches, at an amplitude of 8 µm, for 30 seconds with equal time for cooling on ice for at least 8 cycles. The unbroken cells were removed by low speed centrifugation, 3,000g (5k rpm in a Beckman J2-21) for 5 min at 4°C.

The supernatant was carefully decanted off and SDS [0.5% (w/v) final concentration] was added to solubilise the inner membrane and the non-porin and non-TraT outer membrane proteins. The extract was incubated at 20°C for 20 min before being centrifuged at 100,000 x g (34k rpm in a MSE PREPSPIN 50 with an 8 x 50 ml aluminium rotor) for 1 hour at 4°C. The pellet was gently rinsed with distilled water and TraT was extracted by resuspending it in Buffer A [1% SDS; 175 mM NaCl (as much NaCl was added dropwise to the solution until no more could be added without precipitating out); 30 mM Tris-HCl, pH 7.5; 10 mM Na_2EDTA, pH 8] by sonicating

in small batches of 750 µl at an amplitude of 8 µm, 5 sec on with 5 sec off for cooling on ice. The suspension was incubated at 37°C (30 min) before being centrifuged at 100,000g (36.5k rpm in a MSE PREPSPIN with a 10 x 10 ml aluminium rotor) at 4°C for 15 min. The supernatant, containing the TraT protein, was collected.

2.6.3.2. Gel filtration of Outer Membrane Protein Oligomers.

A Sephacryl S-300 column, with a final bed volume of 75 ml, was poured and then packed down at a flow rate of 30 ml/cm^2/hr using a peristaltic pump. The void volume of the column was measured using blue dextran and the column was equilibrated with 300 ml of Buffer B [0.1% SDS; 0.4 M LiCl; 50 mM Tris-HCl, pH 7.5; 0.1 M Na_2EDTA, pH 8; 3 mM NaN_3], before applying 2 ml of the protein sample to the top of the column. The sample was passed through the column at a flow rate of 24 ml/cm^2/hr using the same buffer. A total of 100 ml was collected in 3 ml fractions.

The absorbance at 280 nm was measured to see which fractions appearing just after the void volume contained the protein. The protein-containing fractions were run on a 13% SDS polyacrylamide gel (see 2.6.1.) to confirm the presence of TraT. The relevant fractions were pooled and dialysed overnight at 4°C, against 4 litres of 10 mM Tris-HCl, pH 7.5; 3 mM NaN_3. The protein solution was then concentrated down, by ultra-filtration using an Amicon YM5 membrane.

2.6.3.3. Fast Protein Liquid Chromatography (FPLC) Purification of the TraT protein.

About 0.5 to 1.0 mg of protein was loaded onto a Mono-Q FPLC column using a 1 ml or 2 ml injection loop as necessary. The best conditions for the separation of the proteins with FPLC were found to be:-

a) Buffers:

(i) NO SALT: 25 mM Tris/SO_4;
0.1 mM Na_2EDTA;
0.1% Zwittergent 3-14.
Adjusted to a final pH of 8.0 with conc. H_2SO_4.

(ii) SALT: As NO SALT buffer, but also containing 1 M Na_2SO_4.

b) Sample: containing 0.1% Zwittergent 3-14.

c) Gradient:

(i) An approximation to a concave ($y=x^2$) salt gradient (0 to 300 mM) in 40 min (see Table 2.8 and Figure 2.1).

(ii) 1 M Na_2SO_4 for 6 min (41 to 47 min)

(iii) 0 mM Na_2SO_4 for 6 min (47 to 53 min).

One ml fractions were collected throughout the FPLC run and were analysed on a 13% SDS polyacrylamide gel. The TraT-containing fractions were pooled and the protein was concentrated to approximately 2 ml using an Amicon 8010 concentrating cell prior to desalting with a Sephadex G25 (10 x 1 cm) PD10 column (equilibrated with the no salt buffer to be used in the next FPLC run).

The TraT was eluted on the second FPLC run using the following conditions:-

a) Buffer:

(i) NO SALT: 25 mM MOPS;
0.1 mM Na_2EDTA;
0.1% Zwittergent 3-14.
Adjusted to a final pH of 6.5, with NaOH.

(ii) SALT: As NO SALT buffer but also contains 1 M Na_2SO_4.

b) Sample: containing 0.1% Zwittergent 3-14.

c) Gradient: as for first FPLC run.

TABLE 2.8.

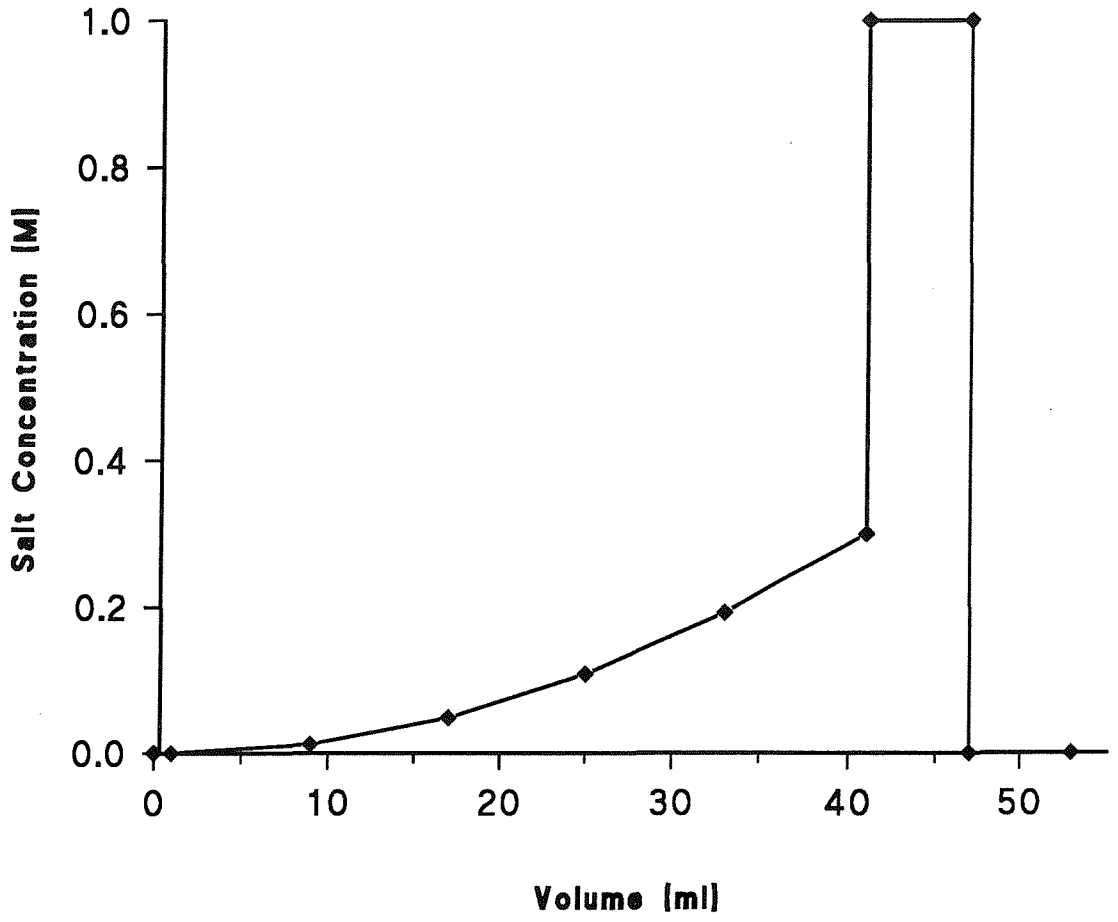
The FPLC Concave Salt Gradient (0-300 mM) used for TraT Purification.

time in min (or volume in ml)	$[Na_2SO_4]$ in mM
0	0
1 [a]	0
9	12
17	48
25	108
33	192
41	300

[a] This is the injection volume (either 1, 2 or 4 ml).

FIGURE 2.1.

Salt Gradient throughout the FPLC Runs.



The injection volume shown here is 1 ml.

The salt gradient is a concave gradient ($y=x^2$) from 0 to 300 mM in 40 minutes and then 1M for 6 minutes, followed by 0 mM for a further 6 minutes.

The fractions that contained the pure TraT protein were pooled and dialysed overnight at 4°C against 4 litres of 25 mM Tris-HCl, pH 7.5, containing 3 mM NaN_3. The purified protein was then concentrated to between 1 and 3 ml, using an Amicon CENTRIPREP 10 concentrating cell and stored at 4°C.

2.6.3.4. Estimation of the TraT Protein Concentration.

The approximate protein concentration in each sample was then determined using Bradford Reagent (Bradford, 1976). A 1 mg/ml stock solution of bovine serum albumin (Pentax Fraction V) was made up using the same buffer that the protein was dialysed against (25 mM Tris-HCl, pH 7.5; 3 mM NaN_3). As the Bradford assay is sensitive to some detergents another BSA 1 mg/ml stock solution was also made up with Zwittergent 3-14 added at a final concentration 0.001% (The concentration of the detergent in the protein buffer). Dilutions of the BSA and TraT solutions were then made and to 200 µl of each dilution was added 1 ml of 5-fold diluted Bradford Reagent. The solutions were mixed thoroughly and incubated at r.t. (10 min). The absorbance of each tube was then measured at 595 nm against the sample containing no protein. A standard curve was then plotted and the TraT protein concentration estimated.

A more accurate protein concentration was determined using amino acid analysis (kindly performed by L. Hunt, Protein Sequencing Unit, Southampton University). The protein was passed through an Applied Biosystems 420A Derivatizer with automated hydrolysis. Separation of the PTH-amino acids was then achieved on an Applied Biosystems 130A Separation System, using norleucine as an internal standard. Integration of peaks was performed using the Protein Sequencing Unit's own integration package.

2.6.4. Circular Dichroism of the TraT Protein.

Circular dichroism (CD) spectroscopy of TraT was carried out, in collaboration with Dr. A.F. Drake (SERC Molecular Spectroscopy Unit, Department of Chemistry,

Birkbeck College, London) using a Jasco spectropolarimeter. The undiluted TraT protein in a 25 mM Tris, 0.0025% Zwittergent 3-14 buffer was scanned from 190 to 260 nm in a 0.1 cm light path quartz cell at r.t. The final spectrum was obtained by averaging four such scans. The α-helical structure was calculated using a program developed at the SERC Centre for Molecular Spectroscopy.

2.6.5. Electron Microscopy of the TraT Protein.

Dilutions of the pure TraT protein were negatively-stained with uranyl acetate on copper grids coated with Formvar and examined by high resolution transmission electron microscopy. This was carried out in collaboration with P. Hawtin, senior microbiologist at Southampton General Hospital.

2.7. IMMUNOLOGICAL TECHNIQUES.

2.7.1. *In situ* Colony Immunoblotting.

Colonies (including positive controls for the antibodies to be used) were streaked onto sterile nitrocellulose filters placed on LB plates supplemented with the appropriate antibiotic(s). The filters were incubated at 30°C overnight.

The next day the expression of the gene of interest was induced by either: (i) incubating the filters at 42°C for two hours (for genes under the control of the λP_L promoter); or (ii) by transferring the filters to LB plates containing IPTG (for genes under the control of the *lac* promoter).

The colonies were lysed and neutralised as described by Voordouw *et al.*, (1985), by placing the filters sequentially on Whatman 3MM paper soaked in 0.5 M NaOH (1 min); 1 M Tris-HCl, pH 7.4 (1 min); 300 mM NaCl (1 min) and 30 mM trisodium citrate, pH 7.0. Membrane lipids were then extracted by treating the filters as described by Henning *et al.*, (1979a), by placing them on 3MM paper soaked in

phenol : chloroform : n-heptane, 2:5:8 (1 min) and then rinsing the filters with chloroform : methanol, 2:1 to remove the phenol. The filters were finally placed on Whatman 3MM paper soaked in 75% ethanol and air-dried.

The filters were then incubated at r.t. for one hour in Blocking Buffer (10 mM Tris-HCl, pH 7.5; 1.875 mM Na_2EDTA, pH 8.0; 150 mM NaCl; 0.5% BSA; 0.05% Triton X-100) to saturate the non-specific binding sites. The filters were incubated with the required antibodies:-

Antibodies Used:

1) **Anti-polio (C3) Monoclonal Antibody** (1 in 400 dilution). This antibody was raised against purified core particles of type I poliovirus (Horaud *et al.*, 1987) and was a gift from S. van der Werf (Institut Pasteur, Paris). The antibody recognises an epitope corresponding to residues 93-103 of the VP1 coat protein.

2) **Anti-TraT Monoclonal Antibody 867** (Bitter-Suermann *et al.*, 1984) (1 in 400 dilution). This antibody was obtained from the immunisation of mice with the purified TraT protein (Bitter-Suermann *et al.*, 1984) and was a gift from D. Bitter-Suermann (University of Mainz, Germany). This monoclonal antibody binds to amino acids 184 to 200 of the mature TraT protein (Taylor *et al.*, 1990).

Immunoblotting of the hybrid proteins was performed as described by Towbin *et al.*, (1979). The filters were incubated at r.t. (2 hours) with gentle agitation, with one of the above antibodies (diluted 1:400 with Blocking Buffer). The filters were then washed (for 30 min at r.t. with gentle agitation, with at least 3 changes of Blocking Buffer), prior to incubation for 1 hour with a rabbit anti-mouse immunoglobulins conjugated to horse-radish peroxidase (1 in 400 dilution in Blocking Buffer). The second antibody was removed by washing the filters for 30 min in TS Buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl), with three changes of solution.

Binding of monoclonal antibodies to the colonies on the filter was detected as

described by Hawkes *et al.*, (1982), developing the colour with 4-chloro-1-naphthol and hydrogen peroxide. The stain was preserved by rinsing in water. The filters were then photographed, using polaroid 667 film, dried and stored in the dark.

2.7.2. Western Blotting of Proteins.

The proteins to be immunoblotted were first fractionated on a 13% SDS polyacrylamide gel (see 2.6.1.) and then transferred to a nitrocellulose filter using the TE 70 SEMIPHOR semi-dry transfer unit (Hoefer Scientific Instruments, USA). Immediately after electrophoresis the gel was trimmed and soaked in Transfer Buffer (25 mM Tris; 192 mM glycine; 20% (v/v) methanol) for 5 min and assembled into a gel sandwich (as shown in the manufacturers instructions) along with blotting papers and a nitrocellulose filter that were similarly soaked in transfer buffer.

The gel sandwich was placed in the blotting apparatus with the filter nearest the anode and the proteins were transferred at 100 mA for 30 min. Following transfer the gel was stained with Coomassie Blue and destained (see 2.6.1.), to ensure that efficient transfer had occurred, and the filter was incubated in 150 ml blocking buffer at r.t. for 1-2 hours. The filter was then blotted as described in 2.7.1.

2.7.3. Enzyme Linked Immunosorbant Assay (ELISA) of Serum Samples.

Two peptides (synthesised within the department by Dr. R. Sharma) were used to coat the wells of polystyrene micro-ELISA plates. One of the peptides was a C3 peptide with the following amino acid sequence:- D P N A S T T N K D K L. The other peptide was a control peptide with a sequence that would not bind the C3 antibodies, if present. The control peptide had the following sequence:-
T E P V P D P R A V N Q D K K C

The peptides were dissolved in 0.1 M carbonate/bicarbonate buffer, pH 9.6 to give a final concentration of 1 mg/ml and 200 µl of the required peptide was placed in each well of the micro-ELISA plates. The ELISA was carried out in duplicate, with

both peptides, for each serum sample. The plates were left in a sealed lunch box, containing a piece of damp paper to create a humid environment, at 4°C overnight. The next day the peptide was removed from the wells and stored at 4°C for reuse.

The specificity of the two peptides for C3 IgGs was tested by carrying out ELISAs using the C3 monoclonal antibody (described in 2.7.1.) in place of the serum samples.

The mouse serum samples to be assayed were thawed slowly and centrifuged (15k rpm and 4°C for 10 min in a Jouan bench top centrifuge) to pellet any red blood cells that may have remained in the serum. The supernatant was carefully taken off.

The ELISA plates were treated as per Harlow & Lane, (1988) and were washed 4 times with PBS containing Tween 20 (0.05%). The serum samples were then added to the first well in a 1:15 dilution with PBS/Tween and then in a 1:3 serial dilution across the plate to give the following serum dilutions:- 1:15 1:45 1:135 1:405 1:1215 1:3645 1:10935 1:32805

The plates were incubated in a humid box at 37°C for 60 min and then the unbound antibodies were removed with 4 washes of PBS/Tween as before. A 1:400 dilution of the secondary rabbit anti-mouse HRP-conjugated monoclonal antibody was made with PBS/Tween and 200 µl added to each well. The plates were incubated again in a humid box at 37°C for 60 min and then washed 4 times, as before.

Antibody binding was detected (Maskell *et al.*, 1987) by adding to each well 200 µl of a HRP substrate (10 mg/ml solution of ortho-phenylene diamine in methanol, diluted 1:100 with PBS and with 8 x 10^{-3} times the volume of 30% H_2O_2 added). The plates were incubated again at 37°C for 30 min. After this time, 50 µl 3.6 M H_2SO_4 was added to stop the reaction and the plates were immediately read at 492 nm using a micro-ELISA plate reader (with a calibration of 1.0 and a threshold of 1.7). The reader was blanked on a well that contained only the substrate and the H_2SO_4.

2.8. IMMUNISATION OF BALB/c MICE WITH VACCINE STRAINS.

2.8.1. Verification of the Characteristics of Vaccine Strains.

Prior to inoculation, the strains were tested to ensure that the C3 and TraT 867 epitopes were present using the method described in 2.7.1. The strains were also tested for induction of synthesis of the relevant protein using the methods described in 2.5.5. and 2.6.1.

Additionally, bacteria were cross-streaked with phage P22, BR60 and C21 (see Table 2.5) to ensure that they had the correct (smooth) LPS chemotype. The plates were incubated overnight at 30°C prior to checking for lysis at the cross-point with the SL strains.

The *aroA*$^-$ character of SL3261 and its derivatives was verified using a defined salts medium (Davis & Mingioli, 1950) with and without the aromatic amino acid supplements mentioned in Table 2.1.

2.8.2. Growth of Vaccine Strains for Immunisation.

10 ml overnight standing cultures (LB; Ap, 100 μg/ml; Km, 50 μg/ml; DHB, 0.01%) of the vaccine strains were grown at 30°C. The next day the cultures were diluted 1:25 with the same medium and grown at 30°C and 130 rpm to an A_{600} of around 0.3 (i.e.about 1 x 10^8 CFU/ml). Once the cultures had reached the required density they were immediately chilled on ice and pelleted at 6,000g (7k rpm in a Beckman J2-21 centrifuge) for 15 min at 4°C. The cells were washed with 30 ml of PBS, and resuspended in the amount of PBS necessary to give a concentration of 10^6; 10^8; 10^9 or 10^{10} CFU/ml (as appropriate).

2.8.3. Administration of Vaccine Strains to Mice and Collection of Serum Samples.

BALB/c mice (12 weeks old at the start of the experiment and fed normally) were inoculated orally with various dilutions of the *Salmonella* strains, each in a final volume of 0.2 ml, using a gavage needle. Serum samples were collected from mice by tail tipping as described by Harlow & Lane, (1988), using heparinised capillary tubes to collect approximately 100 µl of blood from each mouse per bleed. The blood was allowed to clot at 37°C (60 min) and then incubated at 4°C overnight. The tubes were spun at 15k rpm, 4°C for 10 min in a Jouan bench top centrifuge and the serum removed. Samples were stored at -20°C until analysed.

2.8.4. Autopsy of Mouse Livers.

Mice were killed by cervical dislocation and their livers dissected out. Following homogenisation in 2 ml of sterile water, serial dilutions of the liver homogenate were plated on LB plates supplemented with DHB or additionally ampicillin. Plates were incubated at 30°C and the resulting colonies counted.

CHAPTER 3:

PURIFICATION OF THE R6-5 TRA'F PROTEIN.

3. PURIFICATION OF THE R6-5 TRAT PROTEIN.

3.1. INTRODUCTION.

The TraT protein has several physico-chemical properties in common with other major outer membrane proteins and is especially similar to the porin proteins. For example, both the porins and TraT are oligomeric, very heat stable, highly resistant to proteases and insoluble in many detergents. However, despite these similarities the proteins share essentially no regions of sequence similarity (see 1.2.2.). For this reason and the fact that the TraT protein appears to be multi-functional and may be suitable as a carrier for foreign antigens, data on its structure is of obvious interest. However, to date very little is available. Accordingly, it was decided to purify and characterise a representative TraT protein in order to obtain some basic information about its structure and function.

Previous work by Minkley developed a method for the purification of the F TraT protein (Minkley & Willetts, 1984), that might have provided a starting point for characterisation of the protein. Unfortunately, his procedure involved solubilisation in a detergent (Cutscum) that is no longer obtainable and being a laboratory cleaning agent it is poorly defined in chemical terms. Taylor, (1990) also purified the TraT protein using a modified method of Minkley & Willetts, (1984), digesting away all the non-porin or TraT proteins with pronase and then extracting the TraT proteins from an SDS polyacrylamide gel. This method, however, does not produce biologically active TraT protein.

For these reasons it was decided to develop an alternative procedure for the purification of the protein. Since TraT shares many properties with the porins, the approach adopted was to modify the protocol of Gehring & Nikaido, (1989) for porin purification to allow the isolation of TraT. This chapter reports the development of a procedure for the purification of biologically active TraT protein to apparent homogeneity.

3.2. RESULTS.

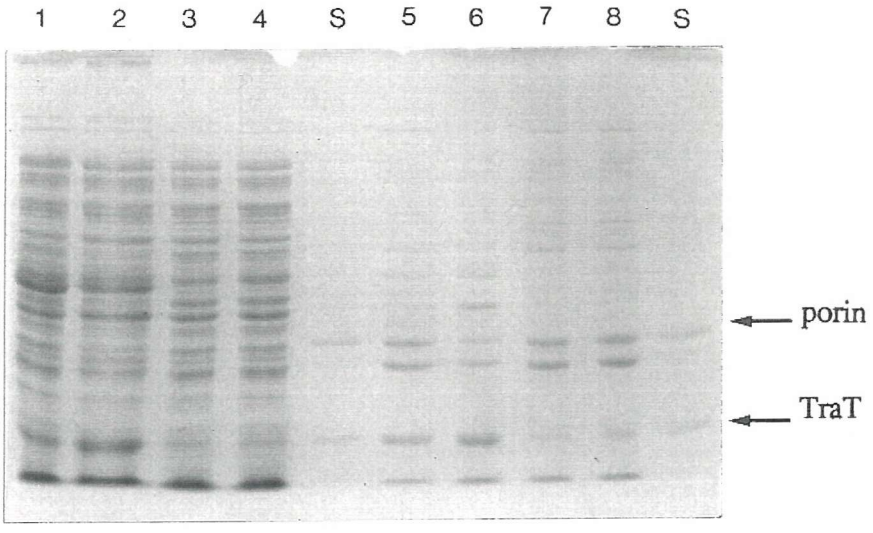
3.2.1. Choice of TraT Over-Producing Strain.

The TraT protein is normally present at a concentration of 10 000 to 20 000 copies per cell (Achtman *et al.*, 1977). However, mutants producing at least ten-fold excess of this amount have been described (Manning *et al.*, 1984; O'Connor & Timmis, 1987). The use of these strains greatly aided attempts to purify the protein on a large scale. A survey of several of the over-producing TraT derivatives (pKT134, pKT135, pKT138 and pKT150) was carried out by running identical amounts of crude cell envelope preparations from each strain on an SDS polyacrylamide gel. Comparison of each strain indicated that the strain C600*rif* (pKT135) was the most suitable TraT over-producing strain for the purification (see Figure 3.1). Preliminary experiments with strains carrying pDOC23, in which *traT* is under the control of λP_L (the strong leftward promoter of phage λ) indicated that, while large amounts of TraT could be produced, much of it was present in the precursor form (data not shown).

Experiments with the strain C600*rif* (pKT135) showed that, while the TraT protein could be readily separated from OmpF, using a modification of the procedure of Gehring & Nikaido (1989), attempts to separate it from OmpC (the main *E. coli* porin in cells grown in LB at 37°C) were unsuccessful. Minkley & Willetts (1984) also found that porin co-purifies with TraT, but did not identify which one. This co-purification of the TraT and OmpC proteins is potentially interesting as the iso-electric points, sub-unit molecular masses and amino acid sequences of the TraT and porin proteins differ drastically, and may be indicative of a specific interaction. For the purposes of purification, therefore, it was necessary to employ a strain lacking OmpC (and preferably OmpF as well). Three 'porin-deficient' strains were constructed by transforming the pKT135 plasmid into the strains SM1005 (*ompC*$^-$, *ompF*$^-$); SG480Δ900 (*ompB*$^-$) and MH1160 (*ompR*$^-$). Testing of these strains showed that the strain MH1160 (pKT135) produced constitutively the most TraT protein and the least OmpC and OmpF proteins, under the specified growth conditions (see Figure 3.2). Thus, this strain was employed for the large scale purification of the TraT protein.

FIGURE 3.1.

Comparison of Outer Membrane Proteins Produced by the pKT (TraT Over-Producing) Plasmids.



13% polyacrylamide gel, stained with Coomassie Brilliant Blue, of the whole cell proteins (tracks 1 to 4) and cell envelope preparations (tracks 5 to 8) of *E. coli* C600*rif* carrying various pKT plasmids (Manning *et al.*, 1982).

Cell envelope preparations were prepared as described in Section 2.6.2. Equal absorbances (A_{600} of 0.2, for whole cell samples and A_{280} of 0.1, for cell envelope samples) were run along side each other so the relative amounts of protein produced by the different strains could be compared.

Track S: TraT and porin protein standard.

Tracks 1 & 5: C600*rif* (pKT134)

Tracks 2 & 6: C600*rif* (pKT135)

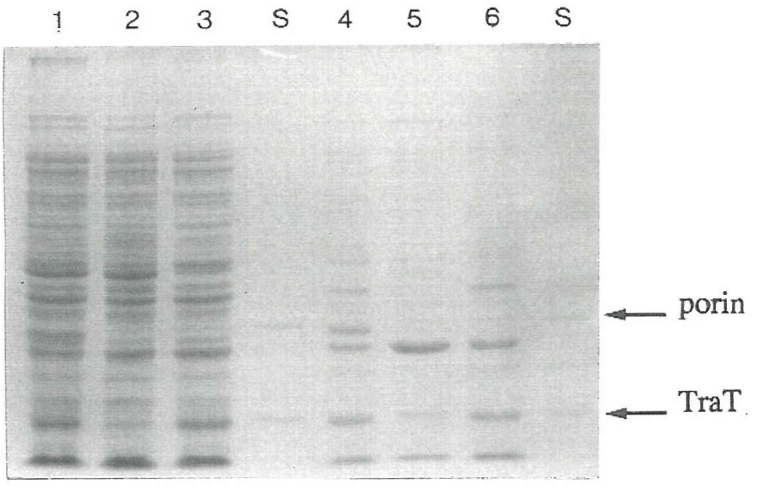
Tracks 3 & 7: C600*rif* (pKT138)

Tracks 4 & 8: C600*rif* (pKT150)

The strain C600*rif* (pKT135) was seen to produce the most TraT protein and was chosen for the purification.

FIGURE 3.2.

Comparison of the Outer Membrane Proteins Produced by Different Porin-Deficient Strains.



13% polyacrylamide gel, stained with Coomassie Brilliant Blue, of the whole cell proteins (tracks 1 to 3) and cell envelope preparations (tracks 4 to 6) of the plasmid pKT135 in different porin-deficient host cells.

Cell envelope preparations were prepared as described in Section 2.6.2. Equal absorbances (A_{600} of 0.2, for whole cell samples and A_{280} of 0.1, for cell envelope samples) were run along side each other so the relative amounts of protein produced by the different strains could be compared.

Track S: TraT and porin protein standard.
Tracks 1 & 4: SG480Δ900 (pKT135) Tracks 2 & 5: SM1005 (pKT135)
Tracks 3 & 6: MH1160 (pKT135)

The strain MH1160 (pKT135) was seen to produce the most TraT protein and the least porin protein and hence was chosen for the purification.

3.2.2. Purification of the TraT Protein.

A modified method for the purification of the porin proteins (Gehring & Nikaido, 1989) was developed as follows:

3.2.2.1. Extraction and Gel Filtration of Outer Membrane Proteins.

The strain MH1160 (pKT135) was grown at 37°C as it has been shown that the TraT protein is present in reduced amounts in the outer membrane when grown at temperatures of 30°C or less (Sowa *et al.*, 1983). Additionally, the cells were grown for 8 hours, as the over-producing strains had a longer lag-phase, grew more slowly and tended to lyse after mid-exponential phase, relative to strains that did not over-produce the TraT protein (Manning *et al.*, 1982).

Following sonication of cells and extraction of TraT and other proteins from the cell envelopes with SDS, the proteins were passed down a sephacryl S-300 gel filtration column. Using a buffer containing 0.1% SDS, the TraT protein was found to elute from the column directly after the void volume indicating that it remained in the oligomeric form. The TraT-containing fractions, which had mainly porin protein contaminants (see Figure 3.3), were collected and prepared for Mono-Q FPLC.

3.2.2.2. Fast Protein Liquid Chromatography (FPLC) of the TraT Protein.

The best conditions for the separation of the TraT protein from the porins, using Mono-Q FPLC, were found to be as follows:-

a) Buffers:

(i) NO SALT: 25 mM Tris/SO_4;
0.1 mM Na_2EDTA;
0.1% Zwittergent 3-14.
At pH 8.0.

(ii) SALT: As NO SALT buffer, but also containing 1 M Na_2SO_4.

b) Sample: containing 0.1% Zwittergent 3-14.

c) Gradient:

(i) An approximation to a concave ($y=x^2$) salt gradient (0 to 300 mM) in 40 minutes (see Table 2.8).

(ii) 1 M Na_2SO_4 for 6 minutes

(iii) 0 mM Na_2SO_4 for 6 minutes.

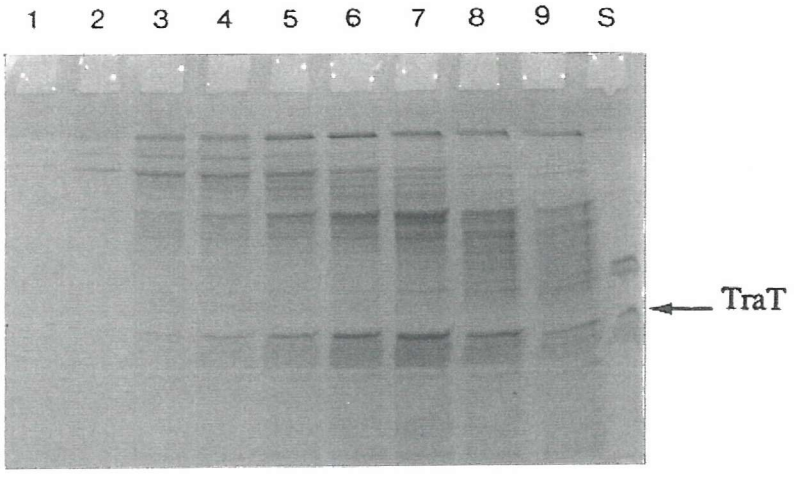
A profile of the salt gradient throughout the FPLC run can be seen in Figure 2.1 and a typical elution profile of the TraT protein, using the above system is shown in Figure 3.4.

After SDS-PAGE of the peak fractions (see Figure 3.5) it was found that the TraT protein peaks at this stage were not pure (two minor contaminants, and a trace of the porin proteins were present). To remove these, the pooled sample was desalted and rechromatographed through the Mono-Q FPLC column at pH 6.5. The conditions used in the first FPLC run were repeated except that the MOPS buffer system given in section 2.6.3.3. was used. A typical elution profile of this can be seen in Figure 3.6.

Using this method the TraT protein was purified to the extent that only a single band, corresponding to a molecular weight of 24 kDa, was seen on silver stained SDS polyacrylamide gels (see Figure 3.7). This also indicated that the purified protein was essentially devoid of LPS. The TraT protein concentration was calculated, using amino acid analysis, to be 144.8 μg/ml and the overall yield was found to be around 0.2 mgs of purified TraT protein per litre of bacterial culture.

FIGURE 3.3.

Sephacryl S-300 TraT-Containing Column Fractions.



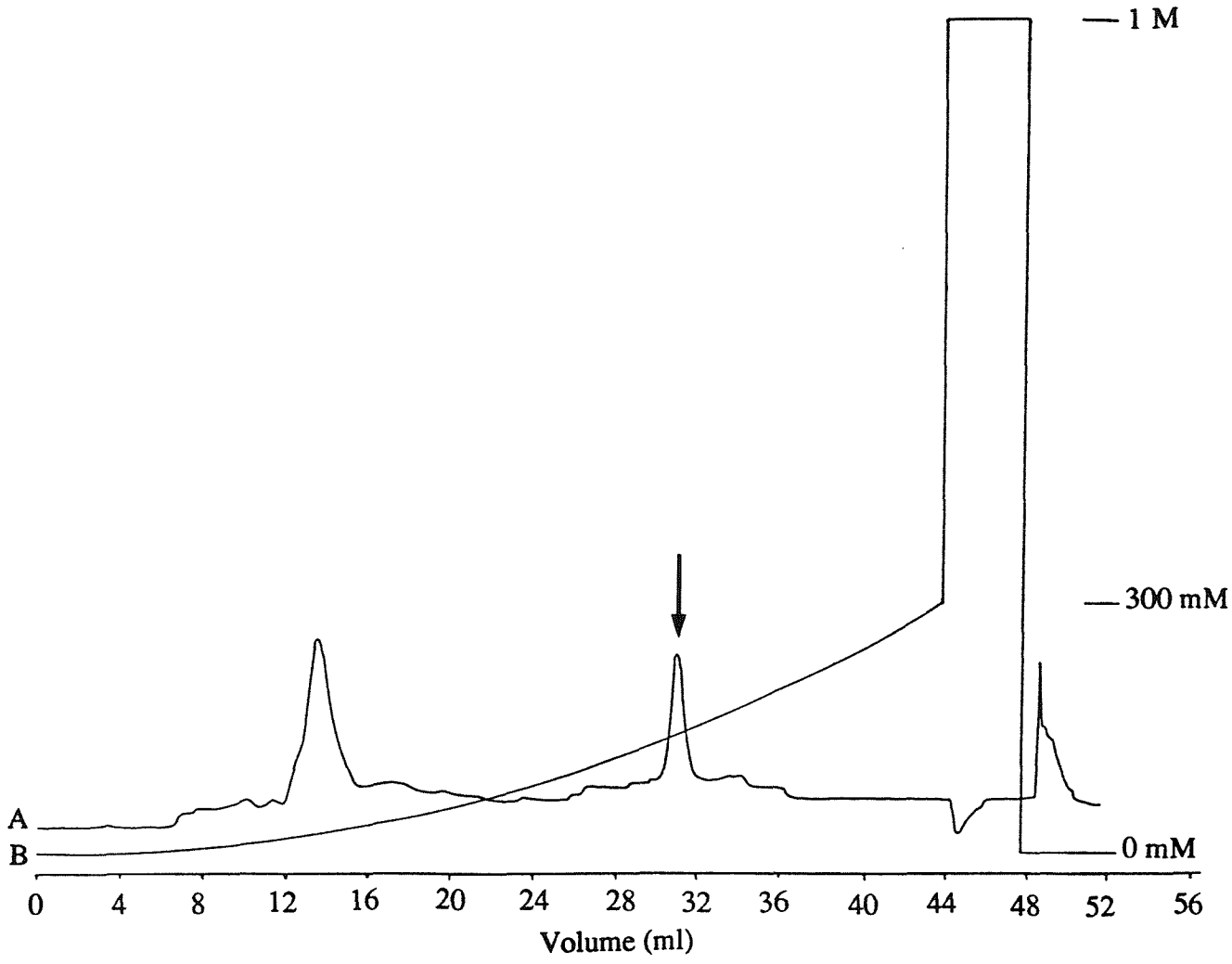
13% SDS polyacrylamide gel, stained with Coomassie Brilliant Blue, of the TraT protein containing Sephacryl S-300 column fractions that eluted just after the void volume.

Tracks 1 to 9: Sephacryl column fractions 11 to 19.
Track S: TraT and porin protein standard.

Fractions 11 to 19 inclusive (27 ml total volume) were pooled and used to further purify the TraT protein.

FIGURE 3.4.

Typical FPLC Elution Profile, at pH 8.0, of the TraT Protein Preparation.



Injection volume = 4 ml

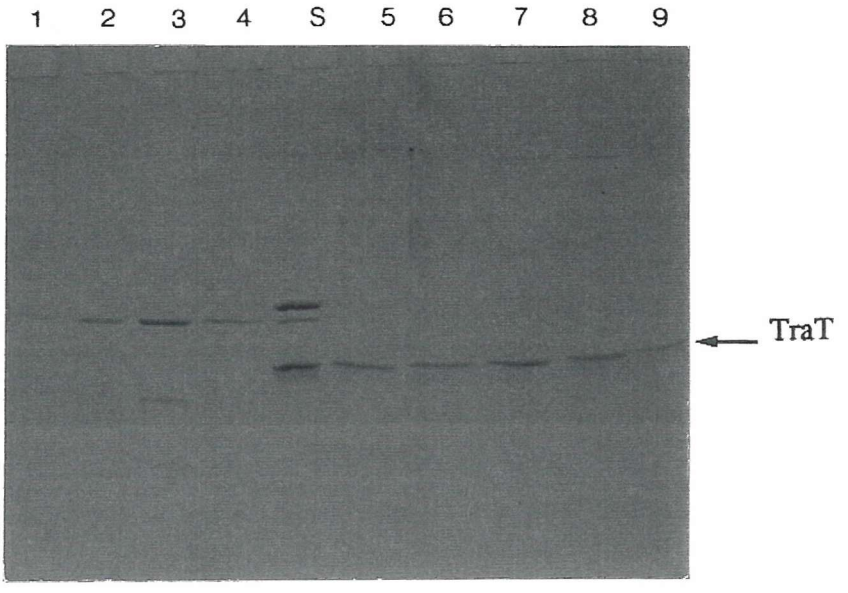
Line A: Represents the A_{280} of the sample being eluted throughout the FPLC run. Scale of x-axis: 0.6 mm = 2 ml; y-axis: 1.2 cm = 0.05 units.

Line B: Represents the salt concentration throughout the FPLC run. Scale of x-axis: 0.6 mm = 2 ml; y-axis: 1.2 cm = 100 mM.

The TraT protein peak is marked with an arrow.

FIGURE 3.5.

Mono-Q FPLC Peak Fractions.



13% SDS polyacrylamide gel, stained with Coomassie Brilliant Blue, of the Mono-Q FPLC (pH 8) peak fraction shown in the trace in Figure 3.4.

Track S: TraT and porin protein standard.

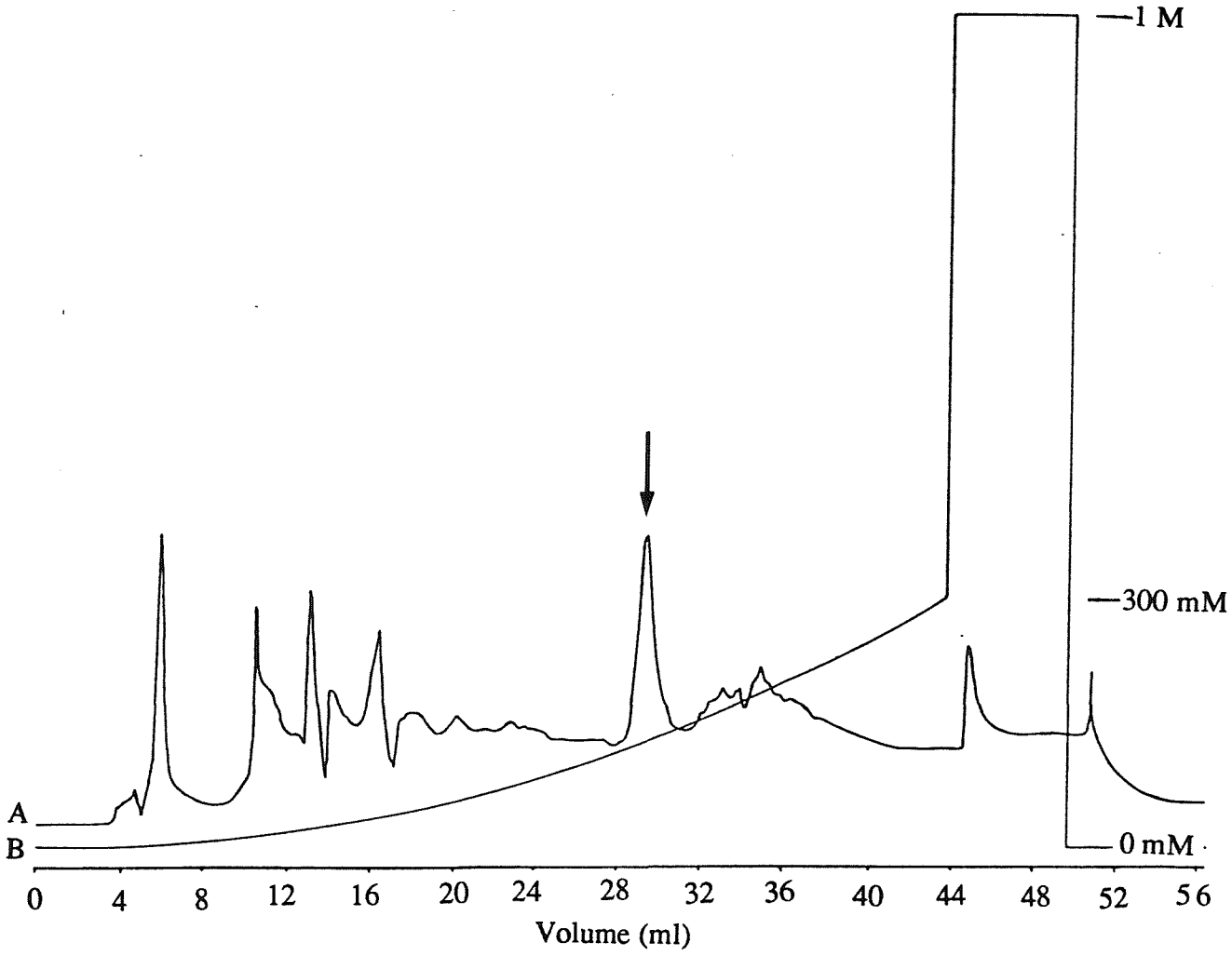
Tracks 1 to 4: FPLC fractions 12 to 15 (corresponding to a salt concentration of 0.5 mM to 2.5 mM).

Tracks 5 to 9: FPLC fractions 30 to 34 (corresponding to a salt concentration of 12.5 mM to 17.5 mM).

TraT-containing fractions (30 to 34) were pooled and further purified by an FPLC run at pH 6.5.

FIGURE 3.6.

Typical FPLC Elution Profile, at pH 6.5, of the TraT Protein Preparation.



Injection volume = 4 ml

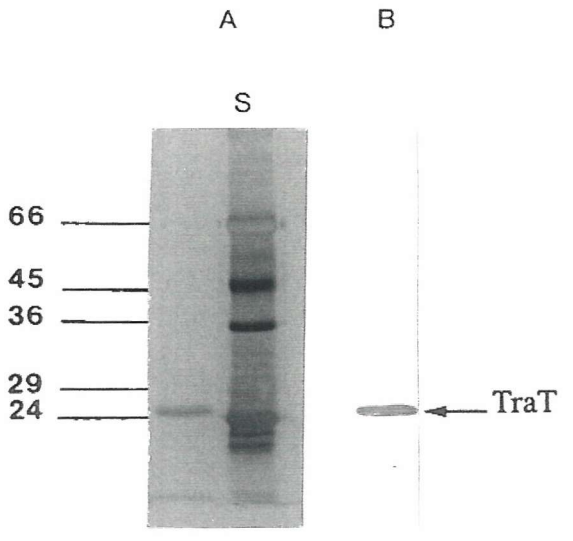
Line A: Represents the A_{280} of the sample being eluted throughout the FPLC run. Scale of x-axis: 0.6 mm = 2 ml; y-axis: 1.2 cm = 0.05 units.

Line B: Represents the salt concentration throughout the FPLC run. Scale of x-axis: 0.6 mm = 2 ml; y-axis: 1.2 cm = 100 mM.

The TraT protein peak is marked with an arrow.

FIGURE 3.7.

Purified TraT Protein.



TraT protein after Mono-Q FPLC (pH 6.5), purified to apparent homogeneity and run on a 13% SDS polyacrylamide gel that was (A) silver-stained or (B) Western blotted.

Silver staining was carried out as described in Section 2.6.1. Western blotting was carried out as described in Section 2.7.2. using the anti-TraT monoclonal antibody mAb867. Antibody binding was detected using anti-mouse IgG conjugated to HRP and the chromogenic indicator 4-chloro-1-napthol.

Track S: Sigma SDS-VII protein standard (sizes in kDa are shown).

3.3. DISCUSSION.

The TraT protein was successfully purified to apparent homogeneity using the protocol shown in Figure 3.8.

The strain MH1160 (pKT135) was ultimately chosen for the purification procedure because it minimised the co-purification of the porins and contained relatively large amounts of the mature form of TraT in the outer membrane. Following extraction of outer membrane proteins, samples were passed down a Sephacryl S-300 column, a hydrophilic matrix of a cross-linked copolymer of allyl dextran and N,N'-methylene bisacrylamide that minimises non-specific adsorption and maximises recovery. The S-300 gel has an exclusion size of between 10 and 1,500 kDa and hence was of an appropriate size for the collection of TraT oligomers. The gel filtration step was also important for the removal of the bacterial lipopolysaccharide from the protein. Gehring & Nikaido, (1989) showed that this step was important in the purification of the porin proteins and that elution of the proteins during Mono-Q chromatography was delayed if LPS was present.

To purify the TraT protein from the porins the high resolving power of a fast protein liquid chromatography column was used. Mono-Q, a strong anionic exchanger (using a quaternary amine as the charged group) on a beaded hydrophilic resin with one of the narrowest particle sizes (10 μm) was chosen. Two runs were carried out using buffers at two different pHs (pH 8 and then pH 6.5), to remove contaminating proteins with different isoelectric points.

During purification SDS was used at concentrations up to 1%, which was found to be too low to dissociate the TraT oligomers. However, since we wished to subsequently use the protein in a surface exclusion assay (see Chapter 4) and it is well known that pili are very sensitive to SDS, after the gel filtration stage the detergent was changed to Zwittergent 3-14 (N-tetradecyl-N,N-dimethyl-3-amino-1-propane-sulphonate) which was less likely to cause dissociation of pili. Until recently the majority of surfactants used in the solubilisation of membrane proteins were either

anionic (e.g. SDS) or non-ionic of the polyoxyethylene type (e.g. alkylphenol ethoxylates such as the Triton X series). Both of these classes have their drawbacks due to their variable binding affinities for proteins. For example, proteins extracted from membrane preparations with SDS usually exhibit a near total loss of biological activity. In contrast, the non-ionic detergents, while they do not interfere with the biological activity of the solubilised proteins, tend to have a low solubilising efficiency. The zwitterionic detergent was also selected because the biological activities of membrane proteins extracted with these detergents are generally not affected (Gonenne & Ernst, 1978). Zwittergent is also capable of retaining its zwitterionic character over a wide pH range, due to its strong quaternary ammonium ion and acidic sulphonate ion both of equal strength (see Figure 3.9). Thus, Zwittergent is an ideal choice for use on ionic columns.

Subsequent to the purification of TraT by this method another protocol for the purification of the TraT protein was published (Croft *et al.*, 1991). Here, the integral membrane proteins were extracted from the cells using a buffer containing 20% ethanol. The TraT and OmpF proteins were precipitated from the supernatant by the addition of ethanol to 50% and OmpA was precipitated by the addition of ethanol to 80%. The proteins were then resuspended in 1% Zwittergent (type unspecified) and eluted from a DEAE-Sepharose column at pH 6.5 using a linear salt gradient of 0 to 1 M NaCl. The eluted integral membrane proteins were precipitated again with ethanol, resuspended in 10% SDS and size-purified on a Sephacryl S-300 column, eluting with a buffer containing 2% SDS. This method, however, in contrast to the method described in this chapter, does not produce the biologically active TraT protein as the monomeric form of the protein is collected.

The characterisation of the purified R6-5 TraT protein is described in the next chapter.

FIGURE 3.8.

Flow Chart Summary of the TraT Protein Purification Protocol.

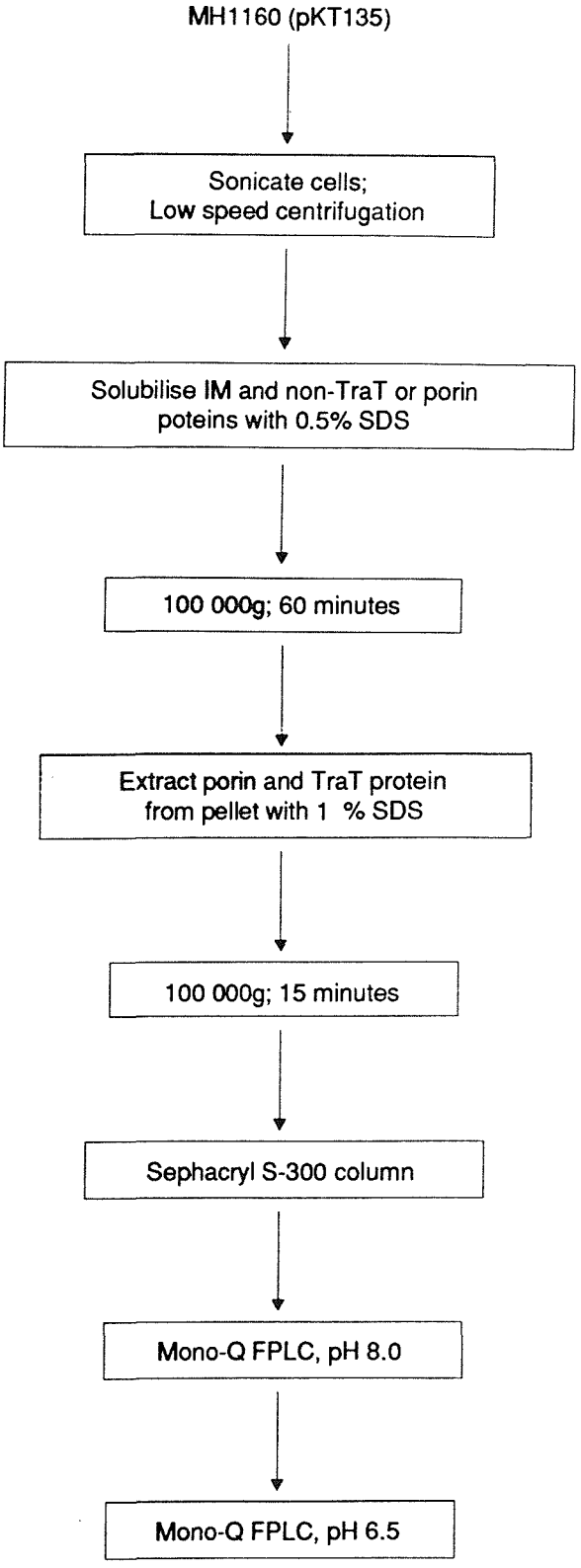
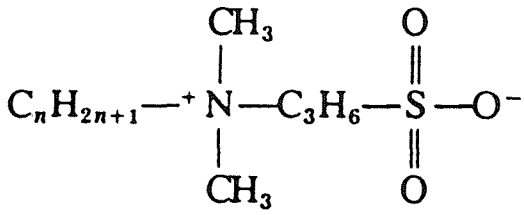


FIGURE 3.9.

Diagram of Zwitterionic Sulfobetaine Surfactants.

(Gonenne & Ernst, 1978)



Where *n* can range from 8 to 18.

Sulfobetaines are synthetic amphiphilic 3-(alkyldimethylammonio)-1-propanesulfonates. They are able, unlike other surfactants, to retain their zwitterionic character over the entire pH range. This property is attributed to the presence of both a strongly basic quarternary ammonium and an acidic sulfonate ion (i.e. groups of equal strength). It is assumed that this 'ionic balance' prevents the irreversible binding of the sulfobetaines to anion and cation-exchange resins and also underlies their general compatibility with other classes of surfactants.

CHAPTER 4:

PROPERTIES OF THE PURIFIED TRAT PROTEIN.

4. PROPERTIES OF THE PURIFIED TRAT PROTEIN.

4.1. INTRODUCTION.

Ten years ago essentially nothing was known about the structure of TraT and its role in surface exclusion had only recently been discovered (Achtman *et al.*, 1977). Since then significant information on its molecular organisation has become available, although much still remains to be learnt about its biological functions and structure.

Studies on the structure and function of the TraT protein have increased significantly with the unexpected finding that the *traT* gene occurs, separate from the other *tra* genes, in the virulence-associated plasmids of *Salmonella typhimurium* (Sukupolvi *et al.*, 1990) and *Yersinia enterocolitica* (China *et al.*, 1990). Although several components are thought to contribute to serum resistance in these bacteria, very recently it has been established that *traT* is the major factor in plasmid-determined serum resistance in the former case (Sukupolvi *et al.*, 1992 - Microb. Pathogen., in press).

The mechanism of action of the TraT protein in serum resistance is unknown. However, it is thought that the TraT protein increases the resistance of the bacteria to the lytic actions of complement (Moll *et al.*, 1980; Ogata *et al.*, 1982; Rhen & Sukupolvi, 1988). This type of resistance is one of the major factors associated with the virulence of invasive bacteria that cause generalised infections (Joiner, 1988; Timmis *et al.*, 1985) and hence any further understanding of the action of the TraT protein in serum resistance is of general relevence.

The mechanism of the TraT protein in surface exclusion has been under debate for many years and hence this is also an area worthy of investigation using the biologically active, purified TraT protein. Current thinking suggests that TraT prevents the unfavourable mating of two cells carrying F-like plasmids of the same surface exclusion group by the pili of the *donor* preferentially binding the TraT protein present on the cell surface of the acting *recipient* (Minkley & Willetts, 1984). This

prevents the sex pilus from binding to a receptor, thought to be on the cell surface of the *recipient* (Willetts & Maule, 1986), thereby preventing the *donor-recipient* cell contacts that trigger the depolymerisation of the pili. As the two cells are not brought into close contact, conjugation does not occur.

Willetts & Maule, (1974) proposed a model in which F-like plasmids of different surface exclusion groups expressed slightly different pili and TraT proteins. As surface exclusion was only abolished between two cells carrying F-like plasmids of the SAME surface exclusion group, it was thought that the sex pili specified by a plasmid in one surface exclusion group will only bind to the TraT protein specified by plasmids of the same surface exclusion group. Specificity of interaction between the TraT protein and sex pilus was further suggested by experiments in which a *donor* cell containing two plasmids of different surface exclusion groups was mated with a *recipient* cell containing a plasmid of the same group as one of the plasmids in the *donor*. As conjugation occurred here and both plasmids were transferred from the *donor* to the *recipient* it was suggested that such *donor* cells express mixed pili, and hence only some of the pili can interact with the TraT protein in the *recipient*. This leaves the remaining pili free to interact productively with the receptor on the surface of the *recipient* cell (Willetts & Maule, 1974). This model has therefore been interpreted as indicating that the TraT protein acts specifically in the surface exclusion event. However, sequence analysis indicates that the differences in the primary sequences of the TraT proteins are only very slight (Sukupolvi & O'Connor, 1990). For example, the difference between the amino acid sequences of the F (Sfx_I) and R6-5 (Sfx_{IV}) TraT proteins is a single amino acid change from Gly to Ala at residue 120 (see Figure 1.7 and section 1.2.3.). As effectively the only difference between the TraT proteins here is a single methyl group, it was thought very unlikely that such a defined specificity in surface exclusion could be attributed to this. Therefore, it was important to establish unambiguously whether the purified, biologically active R6-5 TraT protein could selectively inhibit the conjugation of a *recipient* cell with specific *donors* that contain F-like plasmids of different surface exclusion groups.

Finally, as very little is known about the structure of the TraT protein, it was decided to see if the protein has structural similarities with the other outer membrane proteins since it shares so many of their physico-chemical properties. Accordingly, circular dichroism studies were carried out on the purified protein to determine its α-helical content and electron microscopy was carried out to try to obtain insights into its native structure.

4.2. RESULTS.

4.2.1. Assay for Surface Exclusion Specificity.

To determine whether the purified TraT protein was still biologically active and had surface exclusion specificity, the degree of inhibition of transfer obtained when plasmids of different surface exclusion groups were mated in its presence was measured.

The *in vitro* assay for surface exclusion activity due to TraT was carried out as described in section 2.5.3., using *donors* carrying a range of F-like plasmids of different surface exclusion groups (see Table 2.7) and the purified R6-5 TraT protein. No surface exclusion effect was observed if buffer alone was added to the mating cells of LE392(λ)*rif* and DHI (R6-5), hence the trace amounts of Zwittergent 3-14 (tested up to a final concentration of 0.1%) and azide in the buffer had no effect on conjugation. In contrast, addition of the purified R6-5 TraT protein to a mixture of DHI (R6-5) and LE392-λ(*rif*) inhibited transfer of the plasmid in a dose-dependent manner (see Table 4.1). For example, at the maximum concentration of 10 μg/ml, the TraT protein inhibited transfer of the R6-5 plasmid 9-fold. Thus, it appears that the protein, purified by the procedure described in Chapter 3, retains surface exclusion activity *in vitro*.

TABLE 4.1.

Surface Exclusion Index for LE392-λ(*rif*) mated with DHI (R6-5).

Final Concentration of TraT Added.	Apparent Surface Exclusion Index ± Standard Deviation. [a]
1 μg/ml	1.5 ± 0.67
2 μg/ml	1.5 ± 0.37
4 μg/ml	2.8 ± 1.36
8 μg/ml	5.5 ± 2.31
10 μg/ml	8.6 ± 2.31

[a] The apparent SURFACE EXCLUSION INDEX (Achtman *et al.*, 1980) was calculated as the frequency of transfer of the *donor* plasmid to the *recipient* [LE392(λ)*rif*] without the addition of any purified R6-5 TraT protein, divided by the frequency of the same transfer in the presence of a specified concentration of the purified R6-5 TraT protein. The indices represent the means (plus standard deviations) of at least three separate assays with the indices of each experiment not varying by more than 0.54 units.

CONTROL EXPERIMENT.

Volume of TraT Storage Buffer added equivalent to that added in the:	Apparent Surface Exclusion Index:
4 μg/ml of TraT experiment	0.78
10 μg/ml of TraT experiment	1.16

TABLE 4.2.

Surface Exclusion Index for LE392-λ(*rif*) mated with DHI (R100-1).

Final Concentration of TraT Added.	Apparent Surface Exclusion Index ± Standard Deviation.
1 μg/ml	1.3 ± 0.32
2 μg/ml	1.5 ± 0.37
4 μg/ml	1.7 ± 0.31
8 μg/ml	2.7 ± 1.14
10 μg/ml	3.8 ± 0.69

TABLE 4.3.

Surface Exclusion Index for LE392-λ(*rif*) mated with DHI (pCJ105 = F::Tn9).

Final Concentration of TraT Added.	Apparent Surface Exclusion Index ± Standard Deviation.
1 μg/ml	1.0 ± 0.30
2 μg/ml	1.1 ± 0.14
4 μg/ml	1.2 ± 0.28
8 μg/ml	1.3 ± 0.11
10 μg/ml	1.8 ± 0.23

TABLE 4.4.

Surface Exclusion Index for LE392-λ(*rif*) mated with DHI (F::Tn5).

Final Concentration of TraT Added.	Apparent Surface Exclusion Index ± Standard Deviation.
1 μg/ml	2.1 ± 0.46
2 μg/ml	1.6 ± 0.77
4 μg/ml	0.8 ± 0.34
8 μg/ml	1.6 ± 0.37
10 μg/ml	2.0 ± 1.51

TABLE 4.5.

Surface Exclusion Index for LE392-λ(*rif*) mated with DHI (R1).

Final Concentration of TraT Added.	Apparent Surface Exclusion Index ± Standard Deviation.
1 μg/ml	0.5 ± 0.27
2 μg/ml	0.8 ± 0.12
4 μg/ml	1.0 ± 0.16
8 μg/ml	1.6 ± 0.20
10 μg/ml	1.9 ± 0.33

TABLE 4.6.

Surface Exclusion Index for LE392-λ(*rif*) mated with DHI (ColB2-K98::Tn5*seq*1).

Final Concentration of TraT Added.	Apparent Surface Exclusion Index ± Standard Deviation.
1 μg/ml	1.0 ± 0.02
2 μg/ml	0.8 ± 0.01
4 μg/ml	0.9 ± 0.19
8 μg/ml	1.3 ± 0.12
10 μg/ml	2.9 ± 0.54

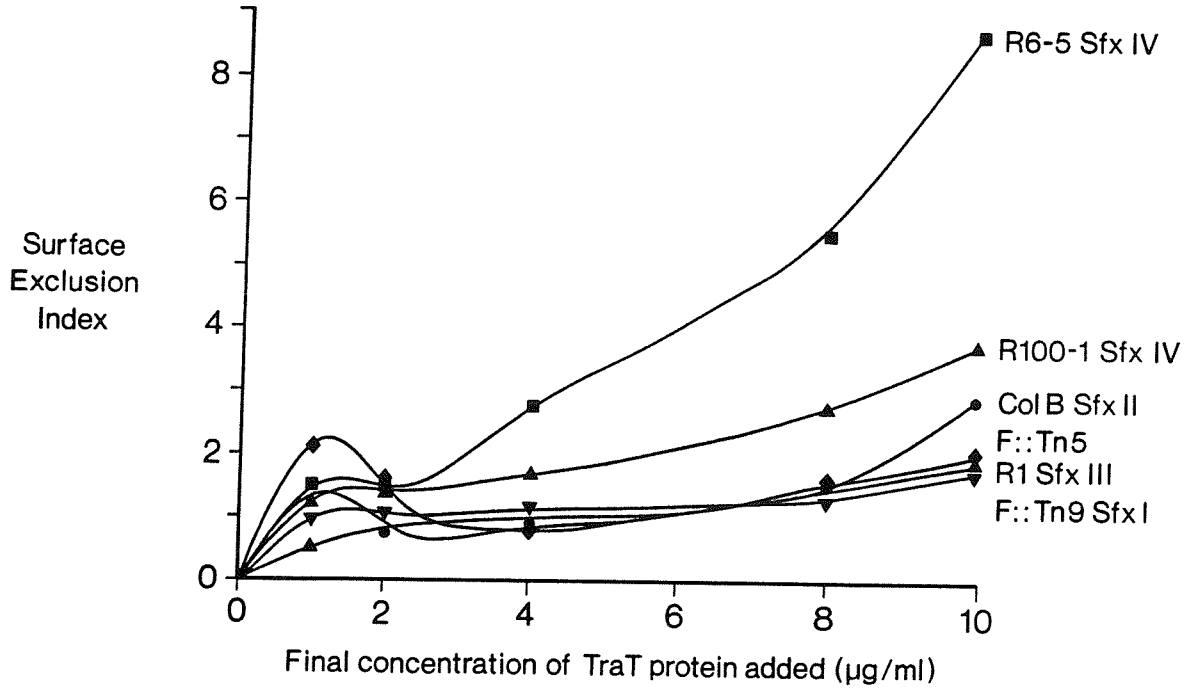
TABLE 4.7.

Comparison of the Surface Exclusion Indices (with the Maximum Amount of TraT Added) for the Different Donor Strains.

Donor Strain.	Sfx Group	Apparent Surface Exclusion Index (with 10 μg/ml of TraT Added) ± Standard Deviation.
DHI (R6-5)	IV	8.6 ± 2.31
DHI (R100-1)	IV	3.8 ± 0.69
DHI (F::Tn9)	I	1.8 ± 0.23
DHI (F::Tn5)	I	2.0 ± 1.51
DHI (R1)	II	1.9 ± 0.33
DHI (ColB)	III	2.9 ± 0.54

FIGURE 4.1.

Graph Showing the Specific Inhibition of Transfer of Plasmids of Surface Exclusion Group IV by the Purified R6-5 TraT Protein.



The results are the average of at least three experiments.

It was found that the R6-5 TraT protein inhibited transfer of plasmids belonging to the same surface exclusion group (IV) to a far greater extent than it inhibited the transfer of plasmids of the other groups (Tables 4.2 to 4.6 and Figure 4.1). The R6-5 plasmid showed a 9-fold reduction in transfer in the presence of the purified R6-5 TraT protein, at a final concentration of 10 μg/ml. However, the conjugation in the strains carrying the plasmids of a different Sfx group to R6-5 was barely affected by the addition of the R6-5 TraT protein: their maximum surface exclusion indices being less than a quarter of that found with the R6-5 plasmid (see Table 4.7). Hence, it was concluded that the purified protein is able to discriminate between cells harbouring a plasmid in surface exclusion group IV and those carrying plasmids of other surface exclusion groups, implying that the TraT protein does show specificity in its surface exclusion activity.

Inhibition of transfer of the plasmid R100-1, which is in the same Sfx group (Sfx_{IV}) as the plasmid R6-5 and has a *traT* gene with an identical DNA sequence (Ogata *et al.*, 1982), was also found. However, the effect was less marked than for R6-5 (Table 4.2). This may be due to the fact that, unlike R6-5, the R100-1 plasmid is derepressed for transfer (see Discussion).

4.2.2. Serum Resistance Conferred by the TraT Protein.

The biologically active, pure R6-5 TraT protein was tested to see if it could confer serum resistance activity on *E. coli*, LE392(λ) cells. The cells were incubated with the purified TraT protein (15 μg) in the presence or absence of pooled adult guinea pig serum (10%, final concentration) at 37°C for 30 min.

It was found, from at least three independent experiments, that an average of 81% (± 8.9%, standard deviation) of the LE392(λ) cells incubated with serum survived in the presence of the TraT protein (75 μg/ml, final concentration). In contrast, only an average of 5.5% (± 1.8%, standard deviation) of the cells survived in the absence of the added protein. Hence, the addition of the purified R6-5 TraT protein to the serum resistance assay causes an approximately 14-fold increase in the survival of cells in

serum.

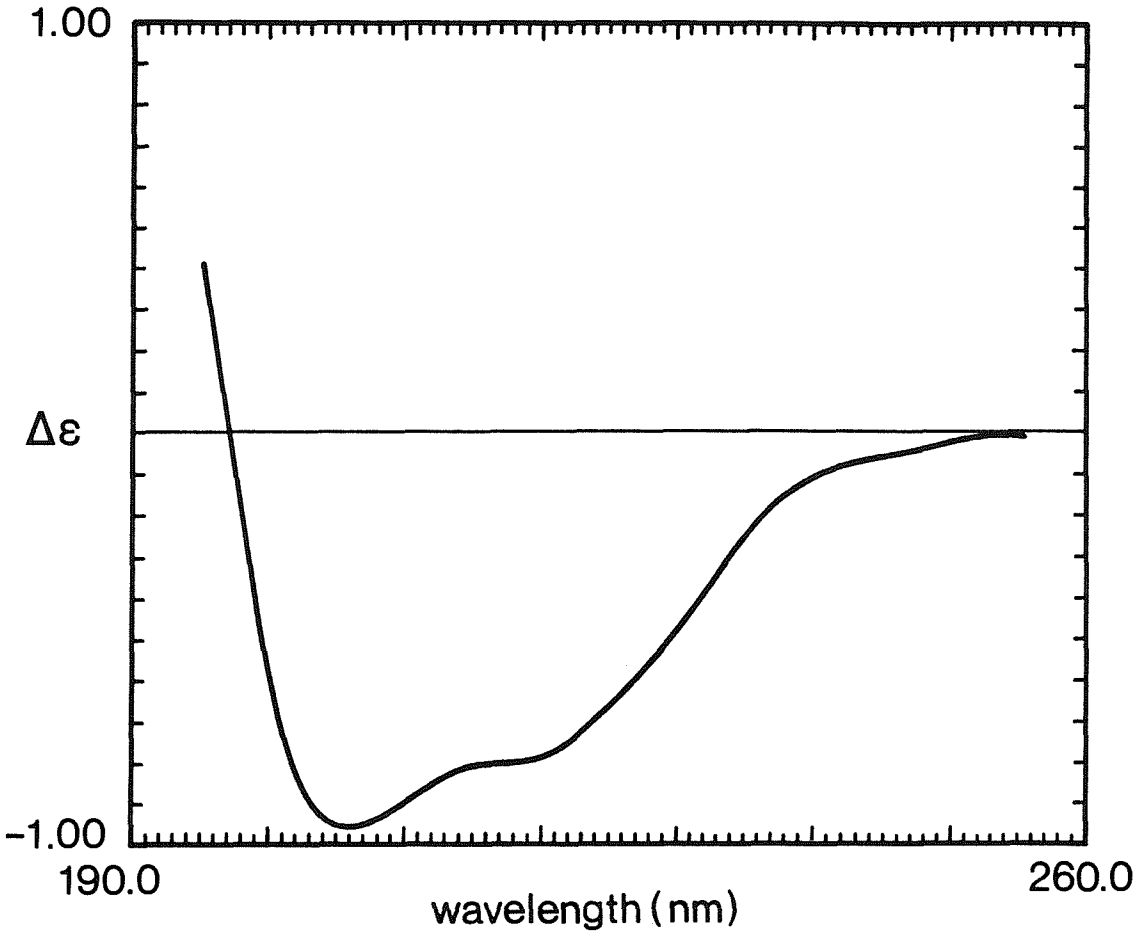
4.2.3. Structural Studies on the TraT Protein.

To obtain information on TraT secondary structure, the purified protein was analysed by circular dichroism. The CD spectrum indicated that approximately 20% of the protein had α-helical structure (see Figure 4.2). Although the spectrum also indicated that a substantial amount of ß-structure was also present, it was not possible to calculate a precise value as the margins of error are greater in such cases (Johnson, 1988). While this result gives us a good guideline to the α-helical content of the protein it cannot be taken as absolute because the TraT protein is an oligomeric membrane protein and most of the derivation of the prediction rules are for globular hydrophilic proteins.

High resolution electron microscopy of the purified protein proved inconclusive. At a magnification of x100,000 a few irregularly shaped particles were visible. These had a diameter of about 9 nm, but they did not resemble the doughnut structures reported by Minkley (cited Ippen-Ihler & Minkley, 1986). Since our TraT preparations were purified to apparent homogeneity and retained biological activity, the possibility remains that the doughnut structures were due to some contaminant or due to aggregation of oligomers. More detailed analysis of the protein by electron microscopy and other biophysical techniques will be required to resolve this issue. Preliminary analytical ultracentrifugation experiments indicate that the TraT protein purified by the present method is mono-disperse, i.e. does not show aggregation of the oligomer to higher order forms (JLH, J. Rosenbusch & C.D. O'C - unpublished results).

FIGURE 4.2.

Circular Dichroic Spectrum of the Purified R6-5 TraT Protein.



The spectrum was measured in a 0.1 cm path length cell at room temperature.

4.3. DISCUSSION.

The TraT proteins and pili specified by plasmids belonging to different surface exclusion groups have subtle differences in their sequences (Ogata *et al.*, 1982; Finlay & Paranchych, 1986; Jalajakumari *et al.*, 1987; Sukupolvi & O'Connor, 1990). The experiments reported above indicate that, even though the differences are rather slight, the purified TraT protein can discriminate between cells carrying plasmids of different surface exclusion groups and that the TraT protein interacts specifically with the pilus (Willetts & Maule, 1986; Forst *et al.*, 1989). It is envisaged that when male cells interact in conjugation, the purified TraT protein binds to pili specified by plasmids of the SAME surface exclusion group better than to pili specified by plasmids of a different surface exclusion group. The binding of the TraT protein to the pilus tip will prevent the pilus binding a receptor which is thought to be on the cell surface of the *recipient* (Willetts & Maule, 1986). Thus, pilus interaction with TraT prevents the formation of stable mating aggregates between the donor and recipient cells and consequently prevents conjugation. Surface exclusion was quantified in the present experiments so that the extent of surface exclusion between the plasmids in different surface exclusion groups could be compared.

The conjugal transfer of the R6-5 plasmid was inhibited by the addition of the purified TraT protein in a dose-dependent manner, showing an 11% reduction in transfer with the addition of the purified R6-5 TraT protein to the conjugating cells. This surface exclusion effect was specific for the surface exclusion group of the plasmid being transferred. For example F-like plasmids belonging to surface exclusion groups I, II, or III showed at least a four-fold increase in conjugation relative to that of the R6-5 plasmid (surface exclusion group IV). The conjugal transfer of the R100-1 F-like plasmid (Sfx_{IV}) was also inhibited by the addition of the R6-5 TraT protein, but not to the same extent as R6-5. As R100-1 is derepressed for transfer it expresses far more pili on its cell surface than a plasmid that is not derepressed, like R6-5 (Grossman & Silverman, 1989). This excess of sex pili means that the fixed concentration of R6-5 TraT protein added will be titrated out leaving unbound pili free

to interact with the pilus receptor on the *recipient* cell and hence will allow conjugation to occur. The results, therefore, provide some support for the idea that TraT interacts with the pilus.

These results lead to the conclusion that a single amino acid difference in the TraT proteins of F and R100-1 is responsible for the observed differences in their surface exclusion specificities. Although, at first glance, it seems difficult to reconcile such a subtle difference in primary sequence (essentially a single methyl group) with the dramatic alteration in specificity, secondary structure predictions suggest that the presence of Gly rather than Ala at position 120 of the F TraT protein creates an additional ß-turn, see Table 4.8, (Chou & Fasman, 1979). Consequently, the structure of the protein may be radically altered in this region. An additional consideration is that, since TraT is oligomeric, such small differences will be amplified in the native form of the protein. Recently, the sequence of the ColB2-K98 *traT* gene (Surface exclusion group II) has been determined (Harrison *et al.*, 1992 - submitted). Interestingly, the mature portion of the deduced ColB TraT protein sequence differs from that of the R6-5 TraT protein sequence at only one residue (Asn_{116} to Ser_{116}) which is located only 5 amino acids away from the Gly to Ala change noted above. Taken together, these results strongly suggest that single alterations in a 5 amino acid region of the TraT protein (residues 116-120) determine its specificity in surface exclusion.

This conclusion was further endorsed by domain-swapping experiments where DNA encoding the presumptive R6-5 (Sfx_{IV}) TraT specificity region was replaced by the corresponding *traT* gene segment of ColB-K98::Tn5*seq*1 (Sfx_{II}). The surface exclusion indices of the host cells expressing the hybrid protein for R6-5 and ColB-K98::Tn5*seq*1 were then determined. Substitution of the ColB fragment into the R6-5 TraT protein switched the surface exclusion specificity from Sfx_{IV} to Sfx_{II} (Harrison *et al.*, 1992). This confirms the importance of this single region in specific recognition.

TABLE 4.8.

Predicted Beta-Turns in the Specificity Regions of the R6-5 and F TraT Proteins.

Residues.		TraT protein	Tetrapeptide with predicted ß-turn.	Pt (x 10^4)
From	To			
112	115	F & R6-5	Gly-Tyr-Asn-Ser	1.34
114	117	F & R6-5	Asn-Ser-Asn-Ser	4.53
116	119	R6-5	Asn-Ser-Ala-Gly	1.19
117	120	F	Ser-Ala-Gly-Gly	2.63

The ß-turn probabilities were calculated for tetrapeptides using the BETATURN program of PC-GENE. A ß-turn is defined as a region of four consecutive amino acids where the polypeptide chain folds back on itself by nearly 180°.

Key:

Pt the probability of bend occurrence.

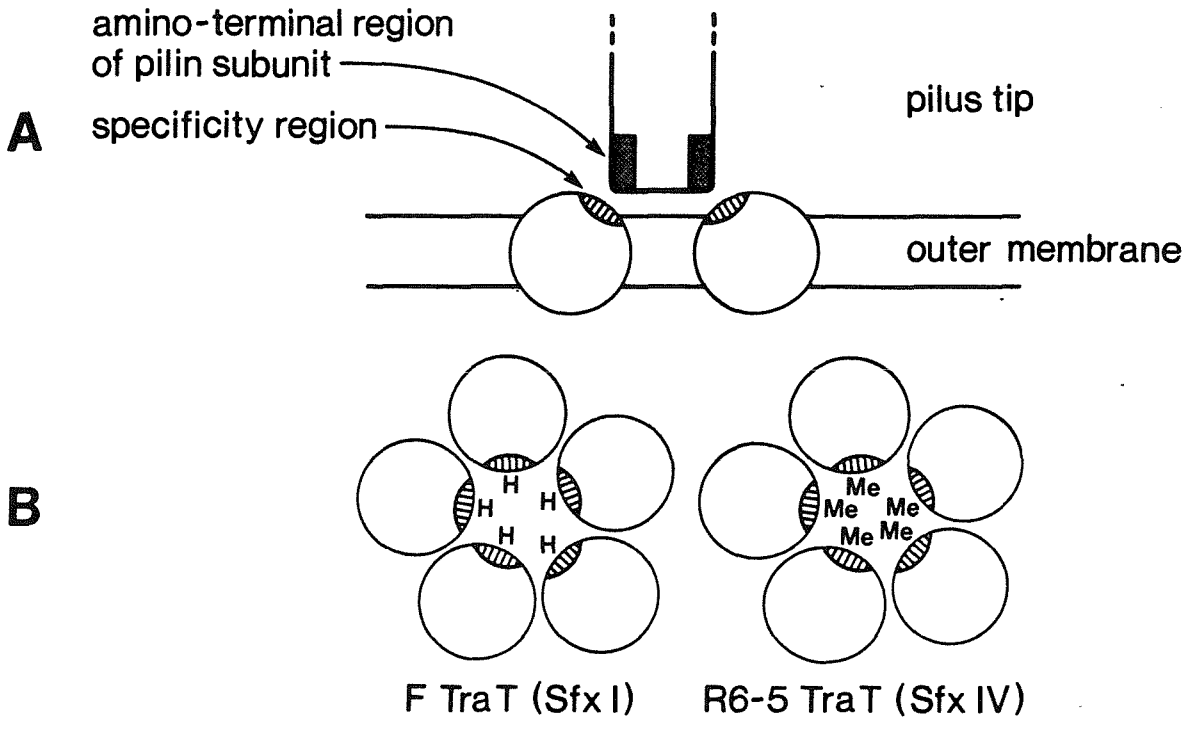
A model that would explain how this specificity region interacts with the pilus protruding from a donor cell is shown in Figure 4.3. In this model it has been assumed that the TraT protein monomers assemble into a doughnut-type structure, as observed by Minkley (Ippen-Ihler & Minkley, 1986). Additionally, the TraT oligomer is represented as consisting of 5 monomers, as pilin subunits are related by a five-fold rotation axis around the helix axis in the assembled pilus (Marvin & Folkhard, 1986). However, it should be stressed that, as of yet, the number of TraT monomers in the oligomer has not been determined. The model shown also assumes that the amino acids in the specificity region (amino acids 116-120) face into the central core of the TraT oligomer. If this were the case then the projection of five methyl groups into the central core of the protein (in the case with the F TraT protein) is likely to alter substantially interactions between this region and the pilus tip. In other words, the additional groups in the central core will change the shape of the binding site for the tip of the sex pilus. It is proposed that only a sex pilus with the correct amino-terminal sequence will be able to bind to its cognate TraT protein and that the other pili will be prevented from doing so by steric hinderance. In this way the surface exclusion specificity may well be attributed to the amino acid differences in the TraT and pilin proteins of the different F-like plasmids.

Unfortunately, our electron microscopy data does not prove or disprove the doughnut structure observed by Minkley (Ippen-Ihler & Minkley, 1986) for TraT oligomers. Hence, further structural studies are required.

The circular dichroism studies revealed that the TraT protein contained an unusually high proportion of α-helical structure (approximately 22%) compared with other major outer membrane proteins (Vogel & Jähnig, 1986). This is quite surprising as the TraT protein shares many properties with other outer membrane proteins. Since outer membrane proteins are exposed to a rather harsh environment, containing a variety of chaotropic agents and proteases, it is possible that TraT and, for example, the porins have undergone convergent evolution. Whatever the explanation, it is likely that TraT differs functionally in its structural organisation in the outer membrane.

FIGURE 4.3.

Proposed Model Explaining the Surface Exclusion Specificities of the F-like Plasmids.



This model is based on the assumption that the sex pilus interacts with the TraT protein to prevent conjugation.

A) Schematic representation of a cross-section through the outer membrane showing the proposed interaction between the specificity regions (hatched) in the TraT oligomer and the tip of a pilus from the donor cell.

B) Schematic representation of a perpendicular view of the oligomer with the side chains of amino acids of the specificity region (Gly_{120} or Ala_{120}) projecting into the central binding site for the pilus tip.

The biologically active R6-5 TraT protein was found to confer significant resistance on the *E. coli* LE392(λ) cells to the bactericidal action of guinea pig serum. It has been suggested that TraT protein affects serum resistance by acting on the terminal complement complex on the bacterial cell surface. Hence, TraT either disrupts the assembly, deposition and/or functioning of the terminal complex. Ogata & Levine, (1980) proposed that this could through some region of the protein interact with the complement components. The consensus to date has been that TraT needs to be membrane bound to have the required protective effect against the complement complex. In addition to this, Manning and co-workers suggested that the TraT protein must interact with the surface structures in a highly specific manner in order to provide protection against serum (cited Moll *et al.*, 1980). However, from the results presented here it is seen that this is not strictly the case, as exogenous TraT protein can neutralise the lytic actions of serum complement. This result, therefore, underlines the need for further research into the interactions of the TraT protein with the specific components of complement.

CHAPTER 5:

INSERTION OF THE C3 EPITOPE OF POLIOVIRUS INTO THE TRAT PROTEIN.

5. INSERTION OF THE C3 EPITOPE OF POLIOVIRUS INTO THE TRAT PROTEIN.

5.1. INTRODUCTION.

It is now well established that the genetic insertion of a defined foreign antigenic determinant into different regions of an outer membrane protein can be used to probe the topology of the protein. Following insertion, antibodies directed against the inserted determinant are used to find out which regions of the protein are exposed at the cell surface. This approach, which was pioneered by Charbit *et al.*, (1986), using the LamB protein, has subsequently been used by several other groups [e.g. Agterberg *et al.*, (1987) on the PhoE protein] as described in Section 1.3.

Genetic insertion of a foreign antigenic determinant into specific regions of a carrier protein also shows promise as a way of improving the immune response to the epitope and indeed several cell surface proteins have now been employed for this purpose with encouraging results (Hedegaard & Klemm, 1989; Newton *et al.*, 1989; Schorr *et al.*, 1991; Agterberg & Tommassen, 1991; Charbit *et al.*, 1987; 1988b) [see Chapter 1].

The TraT protein may be particularly suitable for this task (see Chapter 1) and recent evidence that this is the case has been obtained with the purified TraT protein which, when chemically conjugated to proteins and peptides, strongly stimulated both B- and T-cell responses (Croft *et al.*, 1991). Thus, the TraT protein is a very attractive candidate as a carrier of foreign antigenic determinants to the immune system.

This chapter describes work in which the genetic insertion of a well-characterised foreign antigenic determinant was used to probe the topology of the TraT protein. Since it was anticipated that insertions into TraT were likely to perturb the structure of the protein (especially in the case of large insertions or insertions into a transmembranous region of the protein) the degree of distortion caused by the insertion was assessed. The hybrid constructs produced were also tested for their ability to transport epitopes to the bacterial cell surface and hence for their suitability

for vaccine design.

5.1.1 The System Used.

5.1.1.1. The Plasmid pDOC23.

The plasmid used for the genetic insertion of DNA, coding for the C3 epitope, into the *traT* gene was pDOC23 (O'Connor & Timmis, 1987), which is schematically represented in Figure 5.1. Several features of this plasmid make it useful for this purpose, for example it contains:-

a) the wild-type R6-5 *traT* gene under the control of λP_L, the strong leftward promoter from bacteriophage lambda. Transcription is therefore strongly repressed in the presence of the lambda repressor;

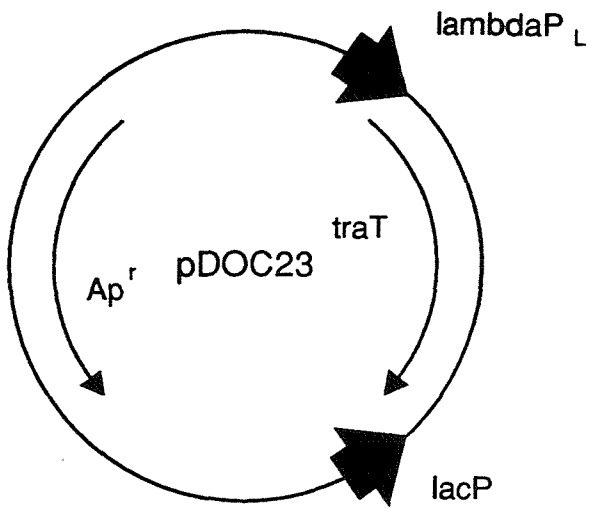
b) a *lac*P promoter situated at the 3'-end of *traT* and oriented towards λP_L. The reverse-orientation *lac* promoter, which can be induced with IPTG, further reduces the expression of the *traT* gene by two mechanisms. Firstly, the anti-sense mRNA produced from the *lac*P is complementary to the *traT* mRNA produced from the upstream λP_L. Thus, the two complementary RNAs may bind preventing translation into protein. Secondly, RNA polymerase molecules travelling in opposite directions along the gene will collide thereby inhibiting the transcription of the *traT* gene. Hence, the two promoters together prevent the undesired over-expression of the cloned *traT* gene, as it has been shown that, like other outer membrane proteins, excess TraT protein can be deleterious to the cell (Manning *et al.*, 1982). The system also allows for large quantities of the TraT protein to be produced when needed. For example, when the pDOC23 plasmid is in a host strain expressing a temperature-sensitive lambda repressor (cI857), it is found that a temperature shift from 30°C to above 33°C inactivates the cI repressor, and in so doing induces the lambda promoter upstream of the *traT* gene.

c) an ampicillin resistance gene, which acts as a selectable marker for the plasmid.

FIGURE 5.1.

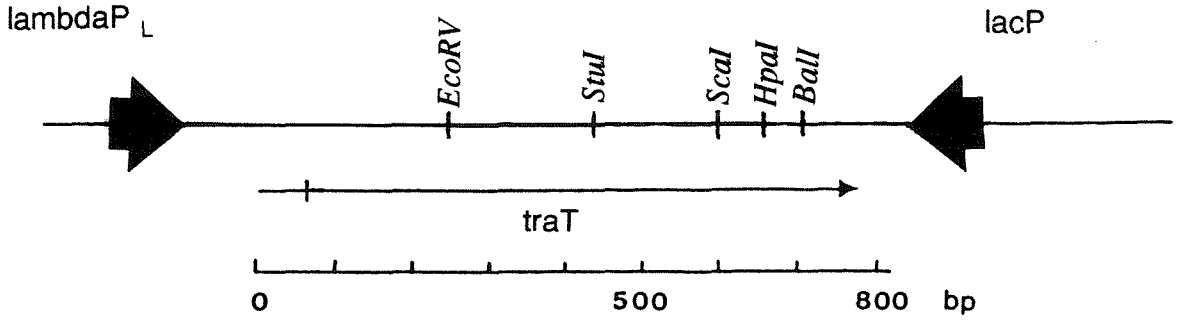
A) The Plasmid pDOC23.

(O'Connor & Timmis, 1987)



The plasmid is approximately 3.8 kb in size. The filled arrows represent the convergent promoters (and operators) in the expression/repression system. LambdaP$_L$ is the strong leftwards bacteriophage promoter and LacP is the lactose promoter. The long arrows represent the directions of transcription of the ampicillin resistance (Ap^R) gene and *traT* gene.

B) Schematic Representation of the *traT* Gene.



An exploded linear map of the *traT* gene showing the C3 DNA insertion sites used (Taylor *et al.*, 1990). The DNA base pair coordinates of the *traT* gene are shown underneath the gene.

d) several unique cleavage sites (see Figure 1.4) within the *traT* gene. For example: *EcoRV*, *StuI*, *HpaI* and *BalI* (corresponding to residues 61, 125, 200 and 216, respectively) into which oligonucleotides can be cloned. Additionally, a *ScaI* site (at residue 180) can be used if pDOC23 is partially digested, as the only other such site resides in the ampicillin resistance gene.

5.1.1.2. The C3 Epitope of Poliovirus.

The epitope chosen for insertion into the TraT protein was the well-characterised C3 epitope from VP1 coat protein of type 1 poliovirus. The C3 epitope is a hydrophilic peptide that corresponds to a turn, located at amino acid residues 93 to 103 of the VP1 coat protein (see Figure 5.2A). It is the major immunogenic peptide of the virus (Wimmer & Jameson, 1984) and antibodies recognising the epitope can be raised against heat-inactivated virions (Horaud *et al.*, 1987).

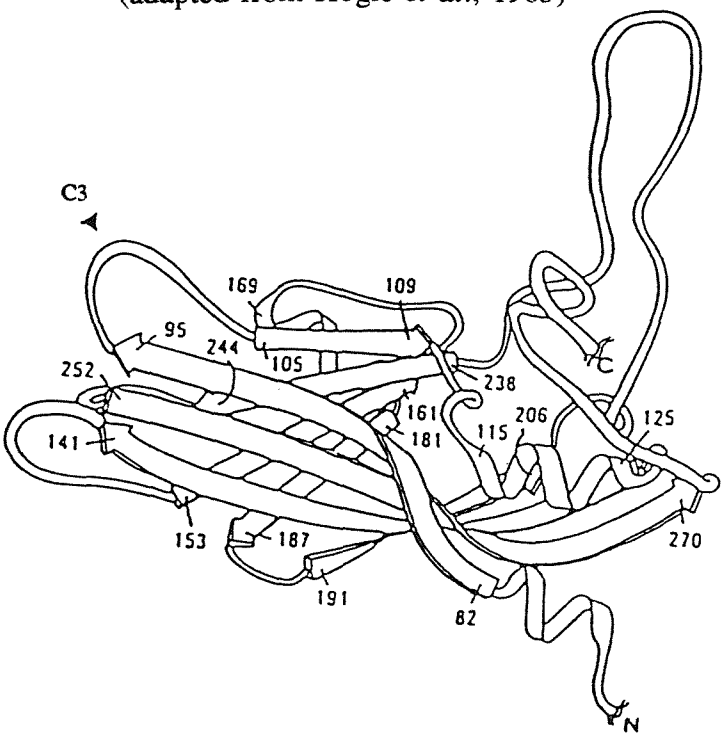
The C3 epitope was chosen for the study of TraT protein topology and to determine the suitability of the TraT protein as a carrier of foreign antigenic determinants because:-

(a) it is small (only 13 amino acids) and hence it is less likely to distort the TraT protein structure;
(b) it is a well-characterised continuous epitope;
(c) it is not normally present in *E. coli* cells and hence is easy to detect with the high affinity monoclonal antibodies that have been raised against it (Horaud *et al.*, 1987);
(d) it does not appear to have any important secondary structural requirements, which otherwise might be distorted on insertion into the TraT protein;
(e) it has previously been shown to be a suitable candidate for insertion and presentation by outer membrane proteins (e.g. the LamB protein used by Charbit *et al.*, 1986; 1987; 1988a; Leclerc *et al.*, 1990).

FIGURE 5.2.

A) The VP1 Coat Protein of Type 1 Poliovirus.

(adapted from Hogle *et al.*, 1985)



The position of the 11 amino acid C3 epitope (between residues 93-103), in the loop region, is shown.

B) DNA and Amino Acid Sequence of the C3 Epitope.

SalI

5'-T CCG GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG GA-3'

3'-A GGC CTA TTG GGC CGC AGC TGG TGA TTG TTC CTA TTC CT -5'

Pro **Asp** Asn Pro Ala Ser Thr Thr Asn Lys Asp **Lys**

The upper two arms show the DNA sequence of the double-stranded synthetic oligonucleotide encoding the C3 epitope (a 39'mer). The lower arm corresponds to the amino acid sequence, the middle section **Asp** to **Lys**, corresponds to amino acids 93 to 103 of the VP1 protein of poliovirus serotype 1. The unique *SalI* restriction endonuclease site (underlined bases), and the potential trypsin cleavage sites within the C3 peptide (arrows) are shown.

5.1.2. Choice of Site in the TraT Protein for Insertion of the C3 Epitope.

The *StuI* site was chosen for the insertion of synthetic DNA coding for the C3 epitope because it lies in the central region of the *traT* gene (see Figure 1.4). Hence, the corresponding region in the protein (around residue 125) has an overall hydrophobic nature (see Figure 1.5). The region may therefore constitute a membrane-spanning portion of TraT. Close inspection of the sequence suggests that the adjacent region (residues 114-117) forms a ß-turn [In fact these residues have the second highest probability within the whole R6-5 protein of adopting a ß-turn configuration (see Figure 5.3)]. As such ß-turn regions are likely to be exposed on the external or internal face of the outer membrane (Paul & Rosenbusch, 1985) it was of interest to see the effects of C3 insertion nearby. Additionally, the region determining surface exclusion specificity also maps here (see Chapter 4), only 5 amino acids away from residue 125. Since surface exclusion is a surface event and is thought to involve interactions with the pili from *donor* cells, it is possible that this region is exposed at the cell surface.

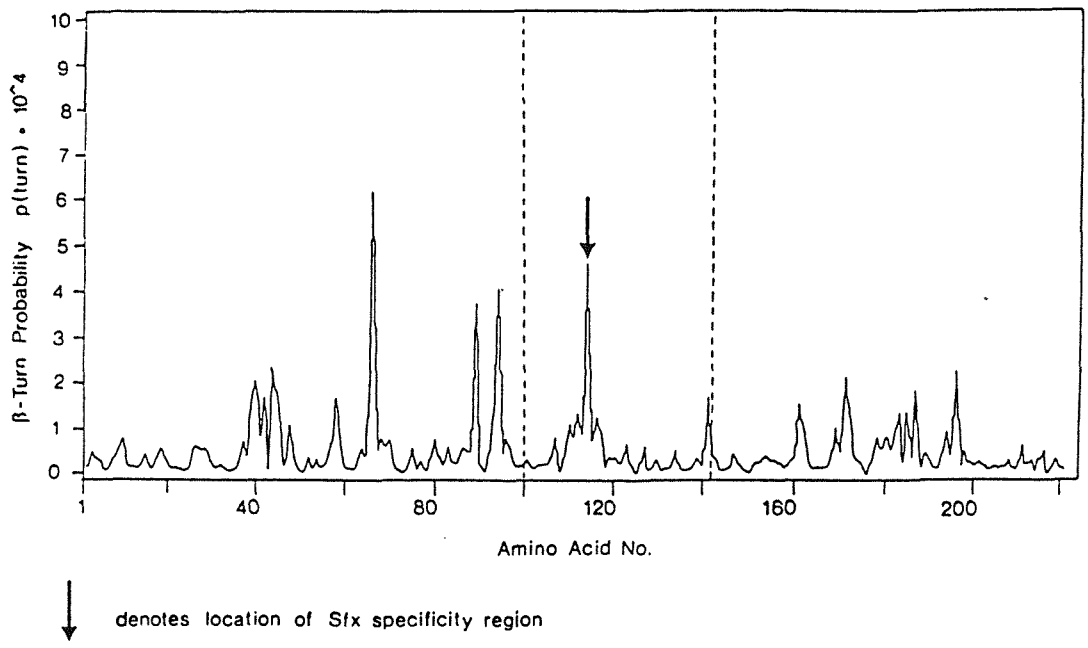
5.2. RESULTS.

5.2.1. Design and Synthesis of a DNA Fragment Specifying the C3 Epitope.

A double-stranded (39'mer) oligonucleotide coding for the C3 epitope (see Figure 5.2B) was designed and synthesised using the codons most frequently used in bacterial outer membrane proteins (Grosjean & Fiers, 1982). Additionally, a unique restriction site (*SalI*) was added (without altering the coding sequence) for ease of detection of the inserted DNA. The bases of the first and last codon were adjusted so that, when inserted, the *traT* gene still read in-frame (i.e. the epitope coding sequence was in reading frame 3, which is appropriate for its insertion into the *StuI* site of the *traT* gene).

FIGURE 5.3.

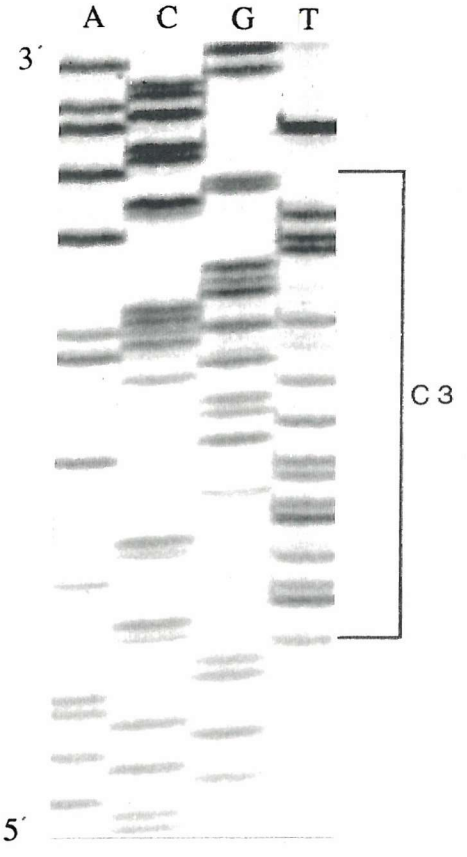
Plot of the ß-turn Probability Profile for the Mature R6-5 TraT Protein.



Beta-turn probabilities were calculated for tetrapeptides using the BETATURN program of PC-GENE. A ß-turn is defined as a region of four consecutive amino acids where the polypeptide chain folds back on itself by nearly 180°. The region between the dotted lines represents the main hydrophobic region of the protein. Region 114-117 (Asn-Ser-Asn-Ser), in the specificity region of the protein, is marked with an arrow.

FIGURE 5.4.

Autoradiogram showing the DNA Sequence of pJH1 around the C3 Insertion Site.



Portion of the sequencing gel of pJH1 showing the cloned C3 fragment and flanking regions.

Sequencing was carried out as described in Section 2.4.8. The reverse primer L2R (which hybridises to nucleotides 910-928 of the *traT* gene) was used.

pJH1 contains the C3 epitope in reading frame 3 inserted into the *traT* gene of pDOC23 at the *StuI* restriction site, between nucleotides 836 and 837 (coordinates referring to those given in Figure 1.4 and as used by Ogata *et al.*, 1982).

The sequence encoding the C3 epitope and the 5' to 3' direction of sequencing are shown.

5.2.2. Cloning the C3 Oligonucleotide into the *traT* Gene.

The plasmid pJH1 was constructed by blunt-end ligation of the double-stranded C3 oligonucleotide into *StuI*-cut pDOC23. The ligated plasmids were amplified in *E. coli* LE392-λ and a representative plasmid containing the insert was found by restriction digestion analysis. This plasmid was shown, by sequencing (see Figure 5.4), to contain a single C3 oligonucleotide fragment inserted in the correct orientation without any mutations and was designated pJH1 (which expresses the hybrid protein TraT/$C3_{125}$).

Other TraT/C3 constructs were made, in collaboration with I.M. Taylor, by inserting oligonucleotides encoding the C3 epitope into the *EcoRV*, *ScaI*, *HpaI* and *BalI* sites of the *traT* gene to produce the hybrid constructs TraT/$C3_{61}$, TraT/$C3_{180}$, TraT/$C3_{200}$ and TraT/$C3_{216}$, respectively (Taylor *et al.*, 1990).

5.2.3. Expression of the TraT/$C3_{125}$ Fusion Protein.

The plasmid pJH1 was shown to express a TraT protein with a molecular weight larger than that of the wild-type TraT protein, showing that it expresses the hybrid TraT/C3 protein. This was further confirmed by immunoblots of whole cell lysates of strains expressing the hybrid protein, using the anti-TraT monoclonal antibody mAb 867 and the anti-C3 monoclonal antibody (for gels see Taylor *et al.*, 1990). The λP_L promoter was also shown to be functional, as temperature shift of the strain, M72 (pJH1), to 42°C induced synthesis of the TraT/$C3_{125}$ protein.

5.2.4. Properties of the TraT/$C3_{125}$ Fusion Protein.

5.2.4.1. The C3 Epitope of pJH1 is Not Exposed to the Cell Surface.

The induced hybrid protein, specified by pJH1, was also detected by immunoblotting of lysed and whole colonies (as described in 2.7.1.) with the anti-TraT mAb 867 and the anti-C3 mAb. Table 5.1 summarises the immunoblotting results and Figure 5.5 shows the filters after immunoblotting with the two monoclonal antibodies.

TABLE 5.1.

In Situ Immunoblot Results of the Hybrid Protein Encoded by pJH1.

Plasmid present in M72 cells.	Cross-reaction with mAb 867		Cross-reaction with anti-C3 mAb	
	lysed	unlysed	lysed	unlysed
pDOC23 (*traT*$^+$, C3$^-$)	yes	no	no	no
pIT9 (*traT*$^+$, C3$^+$)	yes	yes	yes	yes
pJH1 (test plasmid)	yes	no	yes	no

FIGURE 5.5.

In Situ Colony Immunoblots of TraT/$C3_{125}$ Hybrid Protein.

E. coli M72 carrying the appropriate constructs were streaked onto nitrocellulose filters, grown overnight at 30°C and induced by temperature shift to 42°C for 2 hours. Colonies, in both the intact and lysed states, were probed (as described in Section 2.7.1.) with (A) the anti-TraT monoclonal antibody, mAb867 or (B) an anti-C3 monoclonal antibody.

E. coli M72 (pIT9) was a positive control for anti-C3 mAb (Taylor *et al.*, 1990) and M72 (pDOC23) was the negative control for the anti-C3 mAb and a positive control for anti-TraT mAb867.

The results are summarised in Table 5.1.

The colonies were streaked onto each filter in the following pattern:-

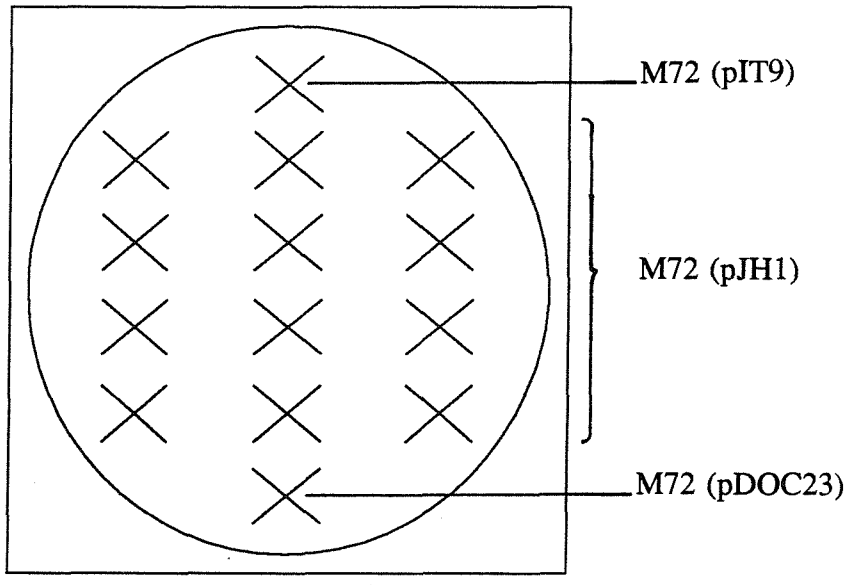
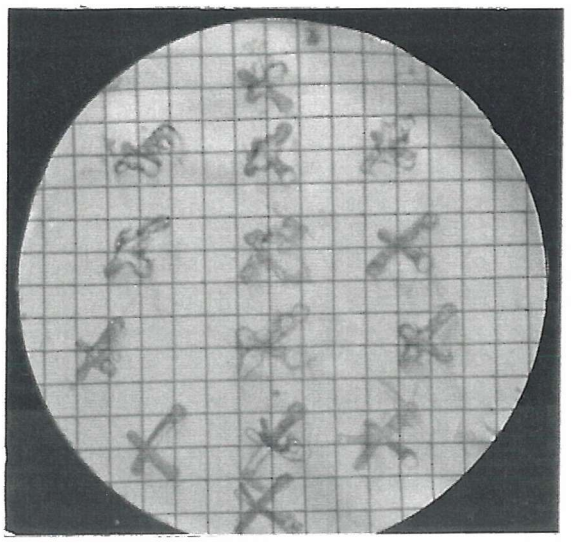


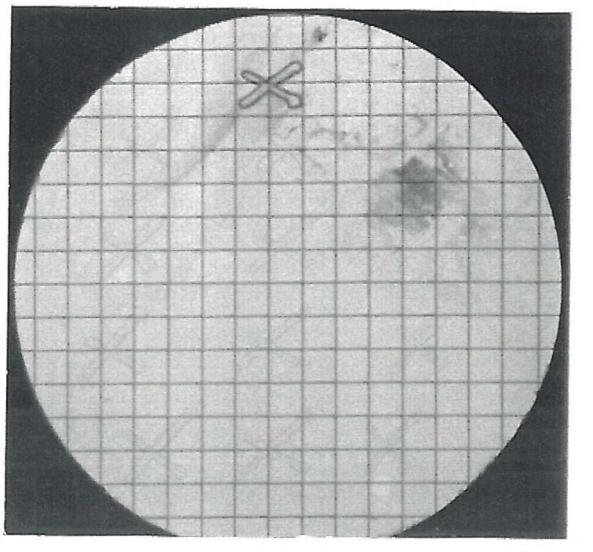
FIGURE 5.5.

(A) Anti-TraT Monoclonal Antibody, mAb867.

Lysed Cells.

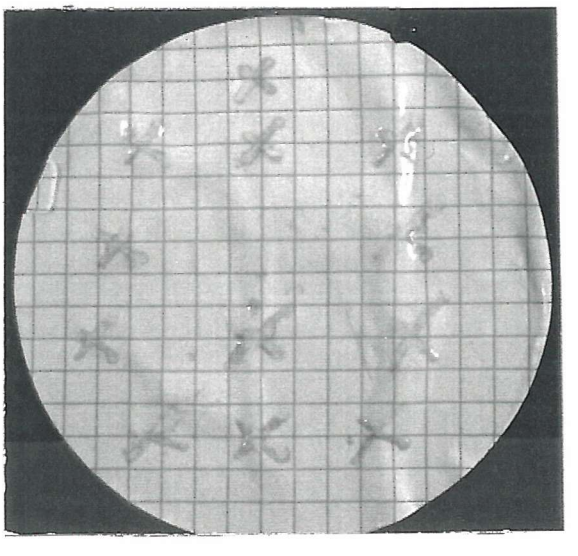


Unlysed Cells.

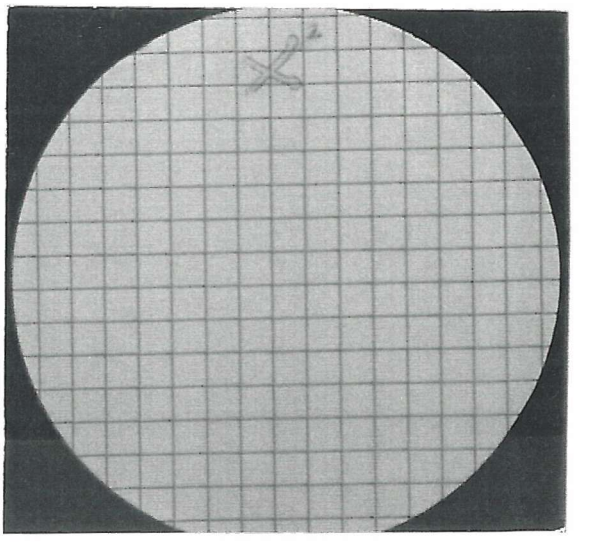


(B) Anti-C3 Monoclonal Antibody.

Lysed Cells.



Unlysed Cells.



The lysed M72 (pJH1) colonies all reacted with both the anti-TraT mAb and the anti-C3 mAb, indicating that the pJH1 constructs expressed both the TraT and C3 proteins. The pDOC23 and pIT9 controls, expressing wild-type and TraT/$C3_{61}$ proteins, respectively, proved that this was not due to non-specific binding of the antibodies.

In contrast, the anti-TraT mAb did not bind to unlysed cells expressing the hybrid TraT/$C3_{125}$ protein, suggesting that the corresponding epitope was not surface-exposed. The finding was in marked contrast to the TraT/$C3_{61}$ protein expressed by the positive control M72 (pIT9), which strongly bound the mAb in unlysed cells, indicating that this hybrid protein had major conformational alterations to that of the wild-type TraT protein.

Equally, however, intact cells expressing the TraT/$C3_{125}$ hybrid did not bind the anti-C3 mAb, again in contrast to M72 (pIT9). This suggested that the C3 epitope in the TraT/$C3_{125}$ protein was either not located in the outer membrane or that it was inserted in a part of the protein that is on the inside of the cell or that sterically hindered it from reacting with the mAb (e.g. by the folding of the TraT protein). The first possibility was ruled out following cell fractionation and immunoblotting experiments which confirmed that the hybrid protein was located in the outer membrane (Taylor *et al.*, 1990).

5.2.4.2. Potentiation of Antibiotic Supersensitivity Phenotype.

Previous studies suggested that the R6-5 TraT protein can form hetero-oligomers with the pSLT TraT protein of *S. typhimurium* (Rhen *et al.*, 1988). It was therefore of interest to see if this was the case for the TraT/$C3_{125}$ protein. To test this, the TraT/$C3_{125}$ protein was expressed in SS-A mutant (EH1316) and wild-type (EH1529) *E. coli* cells (see Table 2.3) and the supersensitivity to hydrophobic antibiotics was determined (as described 2.5.4.). The results of the antibiotic disc-diffusion-tests are shown in Table 5.2.

TABLE 5.2.

Effect of Different TraT Proteins on the SS-A Mutant Phenotype.

Strain.	TraT protein	Diameter of Zones of Inhibition (mm)[a]				
		E_{30}	FD_{10}	NV_{30}	RD_2	VA_{30}
SS-A (λ) wt strain (EH1529)	None	7	-[b]	7	10	7
SS-A (λ) mutant strain (EH1316)		22	8	16	23	17
EH1529 (pDOC23)	Wild-type	13	-	10	14	16
EH1316 (pDOC23)		13	-	10	14	16
EH1529 (pDOC40)	Permeability Mutant[d]	16	-	9	16	15
EH1316 (pDOC40)		25	10	16	24	21
EH1529 (pJH1)	TraT/$C3_{125}$	**20**	**-**	**8**	**17**	**18**
EH1316 (pJH1)		**28**	**15**	**25**	**18**	**ND[c]**

KEY:

[a] The diameter of the antibiotic-impregnated disc (6 mm) has not been subtracted. The numbers subscripted to the antibiotic symbols refer to the amount (in μg) of the antibiotic in each disc.

[b] No zone of inhibition was observed.

[c] Not determined, as the growth of the colonies around the antibiotic disc was too sparse to be measured accurately.

[d] The *traT* gene on this plasmid has a linker insertion mutation causing increased outer membrane permeability to hydrophobic agents (Sukupolvi *et al.*, 1987)

E Erythromycin
FD Fusidic acid
RD Rifampicin
NV Novobiocin
VA Vancomycin

As expected, the wild-type control R6-5 TraT protein (expressed by the plasmid pDOC23) corrected the SS-A mutant phenotype, restoring normal levels of antibiotic sensitivity. In contrast to this, the TraT/$C3_{125}$ hybrid protein did not abolish the SS-A phenotype, but rather potentiated the effect: EH1316 (pJH1) was much more sensitive to antibiotics than the EH1316 host (SS-A mutant) strain.

A potentiating effect similar to that of TraT/$C3_{125}$ was also found when pDOC40, which also causes a permeability phenotype, was introduced into the SS-A mutant strain (Sukupolvi & O'Connor, 1987). However, comparison of the relative sizes of the zones of inhibition around the antibiotic discs showed that the effect is much greater with the TraT/$C3_{125}$ hybrid protein than with the mutant TraT protein encoded by pDOC40.

5.2.4.3. Ability to Assemble into Oligomers.

Samples of a cell envelope preparation of the TraT/$C3_{125}$ hybrid protein from induced cells were run on a 10% SDS polyacrylamide gel in Final Sample Buffer (containing SDS) but without prior heat denaturation. It was seen (see Taylor *et al.*, 1990) that the hybrid TraT/$C3_{125}$ protein was still able to assemble into an oligomeric form which was resistant to dissociation with SDS (2% (w/v) final concentration).

The TraT/$C3_{125}$ oligomers were also tested for resistance to trypsin (data not shown, see Taylor *et al.*, 1990). The hybrid was completely resistant, like the wild-type protein, to high concentrations of trypsin (1 mg/ml final concentration), even though the inserted C3 epitope contained extra, potential trypsin cleavage sites (see Figure 5.2B).

5.3. DISCUSSION.

A foreign antigenic determinant (the C3 epitope of poliovirus) was successfully inserted into the TraT protein at position 125. Analysis of inner and outer membrane

fractions from cells expressing TraT/$C3_{125}$, following sucrose-gradient centrifugation, indicated that the protein resides in the outer membrane (Taylor *et al.*, 1990). This is supported by the observation that the protein increases outer membrane permeability to hydrophobic antibiotics (see Table 5.2). Hence, the insertion of the foreign epitope did not block export of TraT to the outer membrane.

Although present in the outer membrane, whole cells expressing the hybrid TraT/$C3_{125}$ protein did not react with the anti-C3 mAb. This suggests that the C3 epitope in the TraT/$C3_{125}$ protein was either inserted into a region of the protein that is not cell-surface-exposed or that it was not accessible to the antibody molecules on the cell surface. The results presented in Chapter 4 suggest that the specificity region, 5 residues away from the site of insertion, is located on the external face of the outer membrane. However, as it was not possible to detect the C3 epitope in unlysed cells, the most likely explanation is that although the epitope is present externally, it is masked, i.e. the anti-C3 mAb cannot gain access to it due to steric hinderance of the surrounding TraT residues.

The immunoblot results also indicate that, like the wild-type protein, TraT/$C3_{125}$ does not expose the endogenous mAb 867 epitope at the cell surface (Jurs *et al.*, 1984). This is in marked contrast to several other TraT hybrid proteins which appear to have grossly altered conformations (Taylor *et al.*, 1990). This implies that it is unlikely that the C3 epitope in TraT/$C3_{125}$ is inserted into a transmembranous region of the protein which would greatly distort the TraT conformation. Additionally, the TraT/$C3_{125}$ protein retained the ability to assemble into an oligomeric form and was resistant to high concentrations of trypsin. Taken together, these observations suggest that the conformation of the TraT/C3 protein is not very different from that of the native protein and that the C3 epitope has been inserted into a region not involved in subunit:subunit interactions necessary for oligomer formation.

Sukupolvi, (1987) showed that the introduction of the *traT*-containing plasmid F_o*lac* of *S. typhi* into the SS-A mutant strain did not reverse the mutant phenotype. This implies that, even though the primary sequence of the F_o*lac* TraT protein is highly

similar to that of R100, it cannot form functional oligomers with the SS-A mutant TraT protein. There is one major region of the F_o*lac* TraT protein where the amino acid residues are altered from those of the R100 protein. This region, amino acid residues 52-71 (see Figure 1.7), may therefore be important for subunit:subunit interactions. The TraT/C3 constructs described here and by Taylor *et al.*, (1990) rule out the regions around residues 125, 180 and 200 as being important for oligomer assembly and are consistent with the involvement of a region in the amino-terminal third of the protein.

Although the TraT/$C3_{125}$ protein conformation was not distorted enough to expose the 867 epitope and the protein still assembled into oligomers, it was not able to correct the permeability phenotype due to the SS-A TraT mutant specified by the pSLT plasmid of *S. typhimurium*. Rather it potentiated the effect in a similar fashion to the TraT protein expressed by the plasmid pDOC40. The latter TraT protein has two additional residues, one of which is charged, inserted at residue 127 of the protein (i.e. just two residues away from the insertion site used here). The fact that the TraT protein, which is not normally needed for outer membrane integrity, may be harmful if present in a mutated form (as in TraT/$C3_{125}$) in the outer membrane, suggests that the integrity and the permeability barrier function of the outer membrane are dependent on its correct interactions with other critical components. This, therefore, emphasises the importance of choosing a suitable site for epitope insertion.

Hence, while these results provide some information on the structure of the TraT protein (particularly with respect to the regions involved in oligomer formation), it would appear that the region around residue 125 is not suitable for cell surface exposure of foreign epitopes. Nonetheless the hybrid protein constructed may still be of interest in vaccination studies as it is not yet clear if cell surface exposure is required for enhancement of the immunogenicity of a foreign epitope. For example, it has recently been found that insertion of guest epitopes into MalE, a protein located in the periplasm of *E. coli*, stimulates an immune response to the epitope when live bacteria expressing the hybrid protein are administered to mice (Leclerc *et al.*, 1990).

CHAPTER 6:

EXPRESSION OF TRAT/C3 DERIVATIVES IN AN *AROA*⁻ *SALMONELLA TYPHIMURIUM* VACCINE STRAIN.

6. EXPRESSION OF TRAT/C3 DERIVATIVES IN AN *AROA⁻ SALMONELLA TYPHIMURIUM* VACCINE STRAIN.

6.1. INTRODUCTION.

Recent studies have rekindled interest in the use of attenuated live bacteria as vaccine strains and the new generation of rationally-attenuated bacterial vaccines that are under development show considerable promise in the effort to combat key infectious diseases. This is principally because they confer high levels of protection against the corresponding pathogen, are convenient to administer (e.g. by the oral route) and appear to have insignificant levels of reversion due to the nature of the attenuating mutations employed. However, a further crucial advantage is that they permit the expression of heterologous proteins and peptides that may be used to obtain a protective immune response to other pathogens. This raises the possibility that a single vaccine strain might be used to immunise against more than one pathogen, or more particularly, against those which cannot be converted into vaccines by other means.

A number of foreign proteins and peptides have been expressed in rationally-attenuated bacterial vaccine strains, particularly those derived from *Salmonella* and, in a number of cases, have been shown to elicit solid protection against the pathogen in an experimental situation (see Section 1.3.3.). However, results with other recombinant proteins have been disappointing, for example, Hayes *et al.*, (1991) found that immunisation of mice with a *S. typhimurium* vaccine strain expressing LamB/*Chalmydia trachomatis* epitopes B1 and B2 fusion proteins produced only a weak immune response. The *aroA⁻ S. typhimurium* vaccine of Salas-Vidal *et al.*, (1990), expressing a fusion protein of ß-galactosidase and VP7 (A 37 kDa glycoprotein of rotavirus SA11, rotavirus being the single most important cause of severe infantile gastroenteritis), showed similar results. The vaccine strain produced comparable titres of circulating antibodies against *S. typhimurium* LPS and ß-galactosidase, but, antibodies against SA11 could not be detected. Additionally, O'Callaghan *et al.*, (1988a), Sadoff *et al.*, (1988) and Pistor and Hobom, (1990) have

had similarly disappointing immune responses to the inserted antigens of their vaccine constructs (see Chapter 1). In some of these cases, it is likely that the poor response was due to the low immunogenicity of the antigen used or its sub-optimal presentation to the immune system. In other cases, however, it is possible that the antigen was not adequately expressed (or was degraded) in the bacterial strain on administration to the host animal. For all of these reasons, the development of systems that not only improve the immunogenicity of the antigen in question but also ensure that it is adequately expressed *in vivo*, have become a critical goal in vaccine design.

Previous studies suggest that the use of surface-exposed proteins as a carrier of a foreign antigen may lead to an enhanced immune response against the antigen, although the evidence to date is more suggestive than definitive. For example, studies with flagellin-cholera fusions showed that the chimeric flagellin functioned normally and the epitope was expressed on the flagelar surface. Parenteral administration of an *aroA*$^-$ flagellin-negative *S. dublin* strain expressing the chimeric flagellin to mice evoked anti-cholera antibodies (Newton *et al.*, 1989). Similar promising results have been found using the other epitope-specifying oligonucleotides inserted into the antigen-determinant part of the cloned flagellin gene and an *aro*$^-$ *Salmonella* vaccine strain (Newton *et al.*, 1990; Wu *et al.*, 1989; Kuwajima *et al.*, 1988; Majarian *et al.*, 1989). In addition to this, both the LamB (Charbit *et al.*, 1988b; 1990) and PhoE (Agterberg & Tommassen, 1991) outer membrane proteins have been used to transport foreign antigenic determinants to the cell surface and to elicit an immune response against the antigen. Hence, the available evidence suggested that it was worthwhile exploring the potential immunostimulatory properties of such cell surface proteins.

As outlined previously, several properties of the TraT protein make it an important candidate for such studies (see Chapter 1). With the avialability of defined TraT/C3 constructs it was therefore of interest to investigate the behavior of such fusion proteins in a *S. typhimurium* vaccine strain. Accordingly, this Chapter describes work aimed at optimising the expression and stability of such fusions in an *aroA*$^-$ strain of *S. typhimurium.*

6.2. RESULTS.

Based on analysis of the properties of the TraT/C3 constructs (Taylor *et al.*, 1990) two constructs were selected and transduced into the *aroA*$^-$ *S. typhimurium* strain, orally administered to BALB/c mice and tested for their ability to elicit an immune response. Following the results of this work, the expression system was redesigned to allow stable integration and regulated expression of the *traT/C3* genes in the *Salmonella* chromosome.

6.2.1. Expression System and *S. typhimurium* Vaccine Strain Used.

It is generally agreed that it is better to regulate the expression of a heterologous antigen in a vaccine strain as constitutive expression may place the bacterium at a selective disadvantage leading to rapid clearance of the strain from the host (Charles & Dougan, 1990). Since the *traT/C3* hybrid genes were under the control of the λP_L, the strong leftward promoter of phage λ, we reasoned that such a system might be suitable for inital studies on the TraT/C3 proteins in *S. typhimurium*, as expression could be temperature-regulated. By use of a temperature-sensitive λcI repressor, high levels of synthesis of the hybrid protein would only be obtained at temperatures around or above 33°C due to heat-inactivation of the repressor. It might, therefore, be anticipated that the *traT/C3* genes would be switched on by the temperature of the host animal. Without this induction of synthesis the basal level of the TraT protein might be too low to stimulate an immune response.

For these reasons, before the plasmids containing the *traT* or *traT/C3* genes were transformed into *S. typhimurium*, the plasmid pcI857 [which expresses the cI857 temperature-sensitive allele of the λcI repressor (Remaut *et al.*, 1983)] was introduced into the cells by transformation (see Section 2.5.1.4.). The presence of cI857 was essential to repress the expression of the protein from λP_L, which, due to the strength of the promoter and because the TraT protein is an outer membrane protein, would otherwise be deleterious to the cell (Manning *et al.*, 1982).

S. typhimurium possesses several restriction-modification systems [e.g. *hsdSA*, *hsdSB* and *hsdLT* (sometimes called *hsdL*) genes (Colson & Van Pel, 1974)], hence, all plasmids were first transformed into LB5010, a modification-plus, restriction-minus strain of *S. typhimurium*. The plasmids were then moved by transduction, using phage P22 HT *int*⁻ (as described in Section 2.5.2.), into the final *S. typhimurium* vaccine strain, SL3261 (see Table 2.2).

6.2.2. Choice of Hybrid TraT/C3 Proteins for Expression in *S. typhimurium*.

The hybrid TraT/C3 proteins chosen for study in the *S. typhimurium* vaccine strain were decided on by comparing the properties of the TraT/C3 proteins in *E. coli* (Taylor *et al.*, 1990).

The TraT/$C3_{180}$ protein (specified by pIT11) was chosen because it retains surface exclusion activity, supresses the SS-A permeability mutant phenotype and assembles into trypsin-resistant oligomers (indicating that its overall conformation is not grossly distorted from that of the wild-type TraT). Additionally, the C3 epitope in the construct does not appear to be cell-surface-exposed, as shown by immunoblotting studies. It was, therefore, of interest to measure the immune response to C3 in this case, especially as recent work suggests that surface exposure is not a prerequisite to obtain an antibody response to the C3 epitope (Leclerc *et al.*, 1990).

By contrast, the C3 epitope in the TraT/$C3_{61}$ protein (specified by pIT9) was chosen because it appears to be highly cell-surface-exposed in *E. coli* K-12. However, the available evidence also suggests that the conformation of the TraT/$C3_{61}$ protein is severely disrupted from that of the wild-type (as the hybrid protein reacts with anti-TraT 867 monoclonal antibodies added to the outside of unlysed cells). Hence, it was of interest to see which of the two hybrid proteins gave the best (if any) immune response.

Three other strains were used as controls. These were:- the SL3261 host strain, [which does not contain the R6-5 *traT* gene or the C3 epitope]; SL3261 (pcI857)

(pDOC55), [which contains everything the experimental strains contain except for either of the test genes]; and SL3261 (pcI857) (pDOC23), [which contains the R6-5 *traT* gene but not the C3 epitope].

6.2.3. Expression of the TraT/C3 Proteins in the *S. typhimurium* Vaccine Strain.

Following P22 transduction of pIT9 and pIT11 from LB5010 (pcI857) into SL3261 (pcI857), the expression of the corresponding TraT/C3 proteins was monitored. On shifting the temperature from 30°C to 37°C it was found that high-level synthesis of each hybrid protein was obtained (see Figure 6.1). Comparison of the protein profiles of induced and non-induced strains by densitometer scanning of SDS-polyacrylamide gels indicated that the synthesis of the TraT/$C3_{61}$, TraT/$C3_{180}$ and TraT proteins increased around 3.4-, 4.8- and 5.9-fold, respectively, after temperature induction for 2 hours.

Further characterisation of the strains by streaking them onto minimal media plates (see Table 2.1) showed that the vaccine strains still retained the *aroA*$^-$ phenotype. The strains were similarly shown to still be smooth (as judged by their sensitivity to phage P22 and resistance to phages Br60 and C21, see Table 2.5). The strains were all sensitive to P22 and thus retained the O-antigen portion in their LPS. Hence, the attenuating lesion or LPS chemotype had not been affected by the introduction of the *traT/C3* plasmids by P22 transduction.

The vaccine strains (and additionally the *galE*, LB5010, strains) were also tested to see if the C3 epitope and/or the TraT protein were exposed at the cell surface and could be accessed by the relevant antibody molecules. In the case of the LB5010 strains, cells were grown in the presence or absence of added glucose and galactose. Under the former conditions the cells were able to synthesise complete LPS molecules containing the O-antigen, whereas cells grown without added galactose had rough LPS (Germanier & Fürer, 1971).

FIGURE 6.1.

Temperature Induction of TraT Protein Synthesis from the λP_I in the *S. typhimurium* Vaccine Strains.

Whole cell proteins of the SL3261 vaccine strains, before (A) and after (B) temperature induction of TraT protein synthesis, run on a 13% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue.

Temperature induction from 30°C to 37°C for 2 hours was carried out as described in Section 2.5.5. Identical amounts of protein were run in each track by loading an A_{600} of 0.2 of each sample.

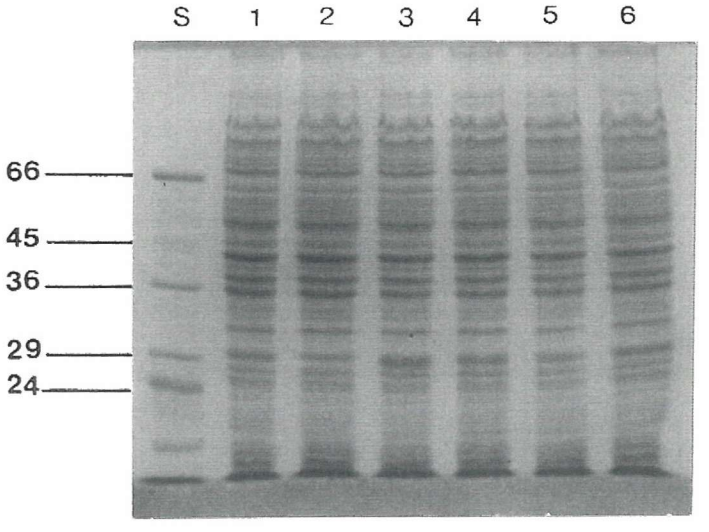
Track S: Sigma SDS-VII protein standard.

Track 1: SL3261 (pcI857) (pDOC23)	Track 2: SL3261 (pcI857) (pDOC55)
Track 3: SL3261 (pcI857) (pIT6)	Track 4: SL3261 (pcI857) (pIT9)
Track 5: SL3261 (pcI857) (pIT10)	Track 6: SL3261 (pcI857) (pIT11)

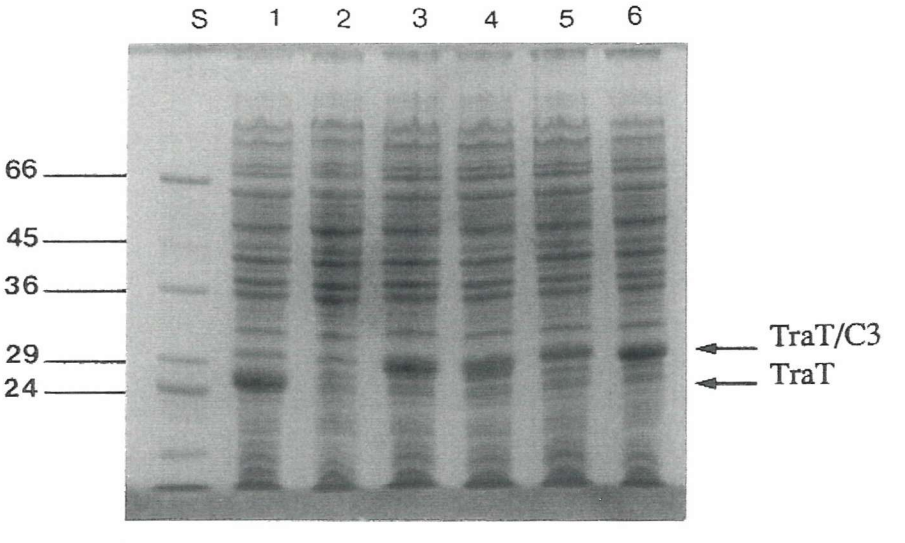
Gel (B) shows that TraT protein synthesis was induced on temperature shift of the cells from 30°C to 37°C. The induced TraT and hybrid TraT/C3 proteins are shown.

FIGURE 6.1.

(A) Before Temperature Induction (30°C, overnight).



(B) After Temperature Induction (37°C, 2 hours).



The surface exposure of the TraT protein and of the C3 epitope inserted in the specific sites of the TraT protein was investigated qualitatively with both strains, using the filter immunoblotting assay described in Section 2.7.1. On incubation with the two monoclonal antibodies the results shown in Figures 6.2 & 6.3 (and summarised in Tables 6.1 & 6.2) were obtained.

The immunoblot results of the rough and smooth *galE* strains showed that the presence of long chain LPS had only a small effect on recognition of the TraT 867 epitope by its antibody. The LB5010 (pcI857) (pIT6), (pIT9) and (pIT10) strains all reacted strongly with anti-TraT mAb 867, in agreement with the immunoblot results seen with these plasmids in *E. coli* cells (Taylor *et al.*, 1990). The LB5010 (pcI857) (pJH1) and (pIT11) strains, however, also showed a faint reaction with the anti-TraT mAb 867 in smooth cells. This reaction was more apparent in the smooth cells, implying that the 867 epitope in these constructs is exposed to the outside of the cells when grown in the presence of galactose, possibly due to cell lysis (Germanier & Fürer, 1971). Similarly, with the anti-C3 mAb it is seen [as also seen with *E. coli* cells (Taylor *et al.*, 1990)] that the C3 epitope is only exposed to the outside of the cell in the strains LB5010 (pcI857) (pIT6), (pIT9) and, to a small extent (pIT10), in the rough strains. However, in the smooth strains the reaction with C3 mAb added to the unlysed cells is far weaker, indicating that the O-antigen of the LPS partiallly masks of the exposure of the C3 epitope to the outside of the cell.

From the immunoblot results of the SL strains it appears that the C3 epitope, in three strains: SL3261 (pcI857) (pIT6), (pIT9) and (pIT10), is still exposed to the outside of the cell (i.e. reacted with the monoclonal antibody directed against it in both lysed and unlysed cells). The other strains SL3261 (pcI857) (pIT11) and (pJH1) did not react with the C3 monoclonal antibody in intact cells and hence the C3 epitope in these cases is not accessible to its cognate antibody in *S. typhimurium* SL3261 cells. Taylor *et al.*, (1990) observed the same pattern of results when they immunoblotted the *E. coli* cells, M72, harbouring the same set of plasmids.

TABLE 6.1.

In Situ Immunoblot Results of λP_L Constructs in *S. typhimurium galE* Rough and Smooth Strains.

Plasmid present in LB5010 (pcI857) cells.	Hybrid protein expressed.	Cross-reaction with mAb 867.		Cross-reaction with anti-C3 mAb.	
		- gal	+ gal	- gal	+ gal
pDOC55	-	-	-	-	-
pDOC23	wt TraT	-	-	-	-
pIT6	$TraT/C3_{200}$	++	+++	+++	++
pIT9	$TraT/C3_{61}$	++	+++	++	+
pIT10	$TraT/C3_{216}$	++	+++	+	+/-
pIT11	$TraT/C3_{180}$	+/-	+	-	-
pJH1	$TraT/C3_{125}$	+/-	+/-	-	+/-

FIGURE 6.2.

In Situ Colony Immunoblots of TraT/C3 Hybrid Proteins Expressed in *Salmonella typhimurium galE* Strains with and without complete LPS.

All the TraT/C3-expressing constructs in *S. typhimurium* LB5010 cells, also harbouring the plasmid pcI857 (which expresses the temperature sensitive λ repressor), were streaked onto nitrocellulose filters, grown overnight at 30°C and induced by temperature shift to 37°C for 2 hours. Colonies, grown in the presence or absence of glucose and galactose (to produce cells with and without complete LPS chains, respectively), were probed (as described in Section 2.7.1.) with (A) the anti-TraT monoclonal antibody, mAb867 or (B) an anti-C3 monoclonal antibody.

The results are summarised in Table 6.1.

The colonies were streaked onto each filter in the following pattern:-

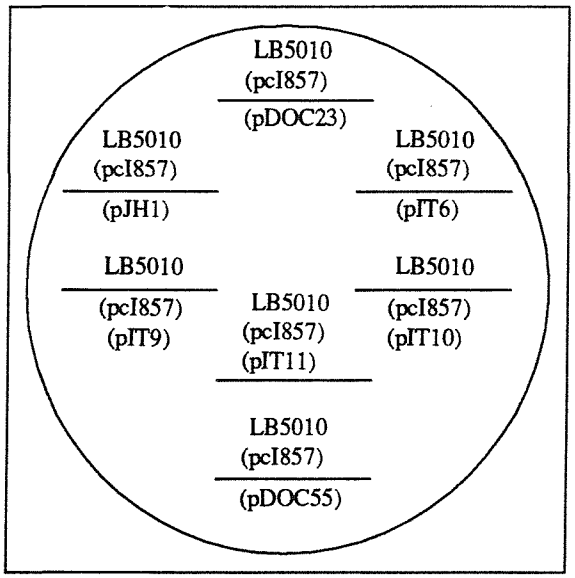
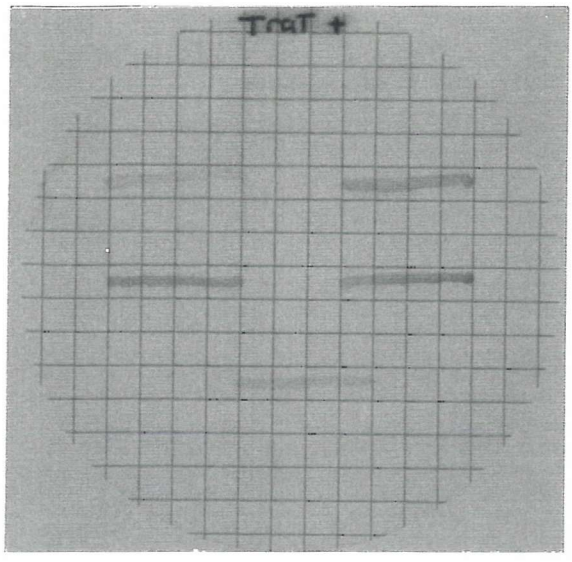


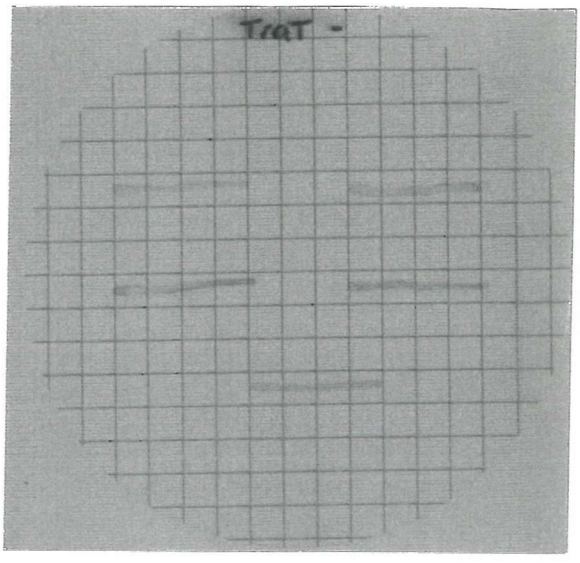
FIGURE 6.2.

(A) Anti-TraT Monoclonal Antibody, mAb867.

Grown with Galactose.

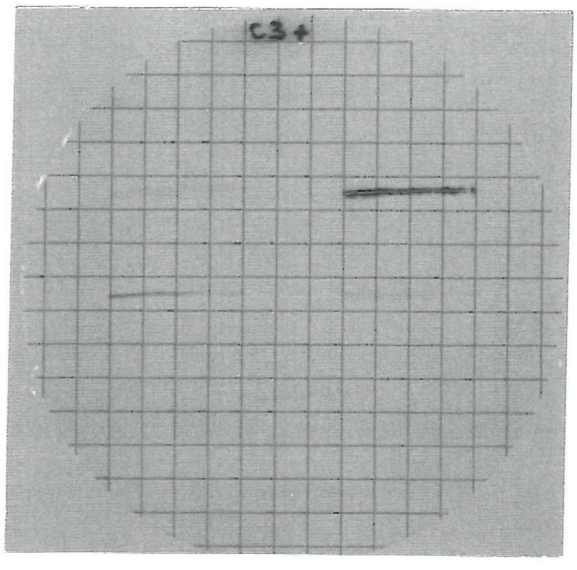


Grown without Galactose.



(B) Anti-C3 Monoclonal Antibody.

Grown with Galactose.



Grown without Galactose.

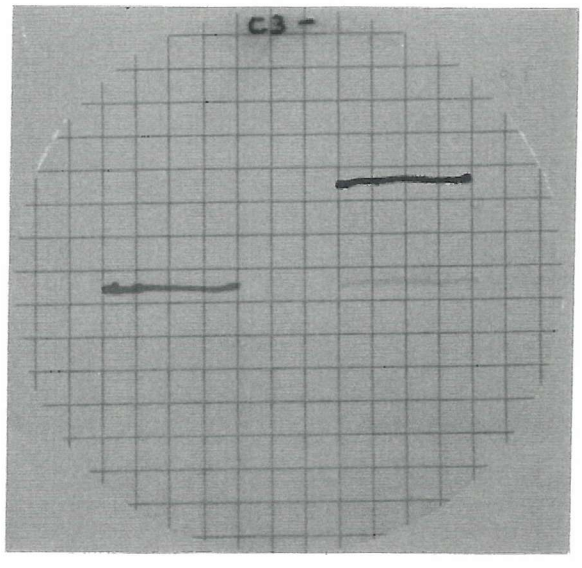


TABLE 6.2.

In Situ Immunoblot Results of λP_L Constructs in the *S. typhimurium aroA* Strain.

Plasmid present in SL3261 (pcI857) cells.	Hybrid protein expressed.	Cross-reaction with mAb 867.		Cross-reaction with anti-C3 mAb.	
		lysed	unlysed	lysed	unlysed
pDOC55	-	no	no	no	no
pDOC23	wt TraT	yes	no	no	no
pIT6	$TraT/C3_{200}$	yes	yes	yes	yes
pIT9	$TraT/C3_{61}$	yes	yes	yes	yes [a]
pIT10	$TraT/C3_{216}$	yes	yes	yes	yes [a]
pIT11	$TraT/C3_{180}$	yes	no	yes	no
pJH1	$TraT/C3_{125}$	yes	no	yes	no

[a] reaction was faint.

FIGURE 6.3

In Situ Colony Immunoblots of TraT/C3 Hybrid Proteins Expressed in *Salmonella typhimurium aroA* Strain.

All the TraT/C3-expressing constructs in *S. typhimurium* SL3261 cells, also harbouring the plasmid pcI857 (which expresses the temperature sensitive λ repressor), were streaked onto nitrocellulose filters, grown overnight at 30°C and induced by temperature shift to 37°C for 2 hours. Colonies, in both the intact and lysed states, were probed (as described in Section 2.7.1.) with (A) the anti-TraT monoclonal antibody, mAb867 or (B) an anti-C3 monoclonal antibody.

The results are summarised in Table 6.2.

The colonies were streaked onto each filter in the following pattern:-

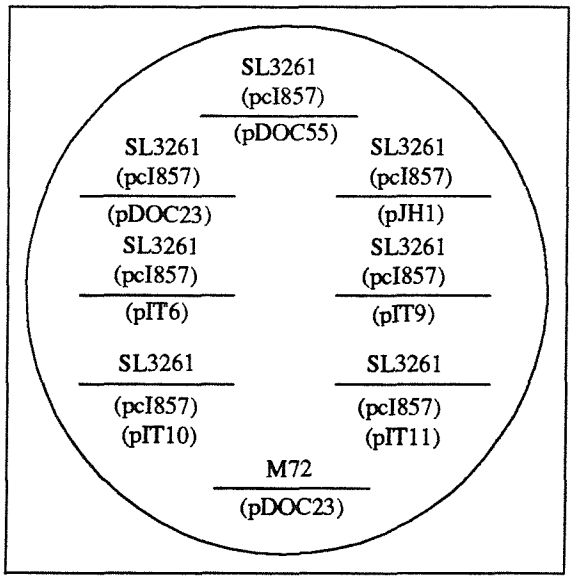
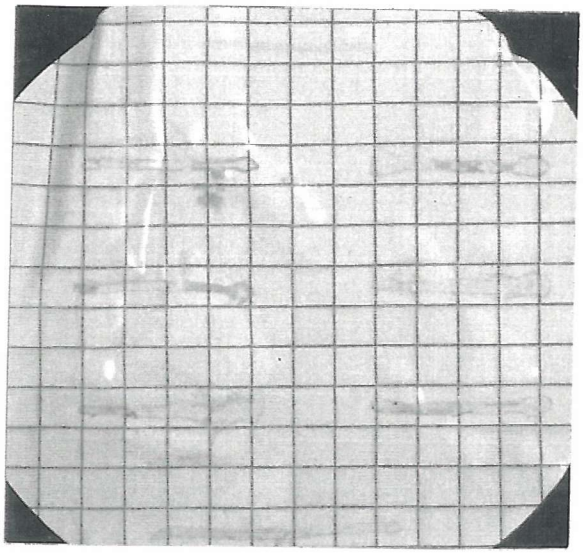


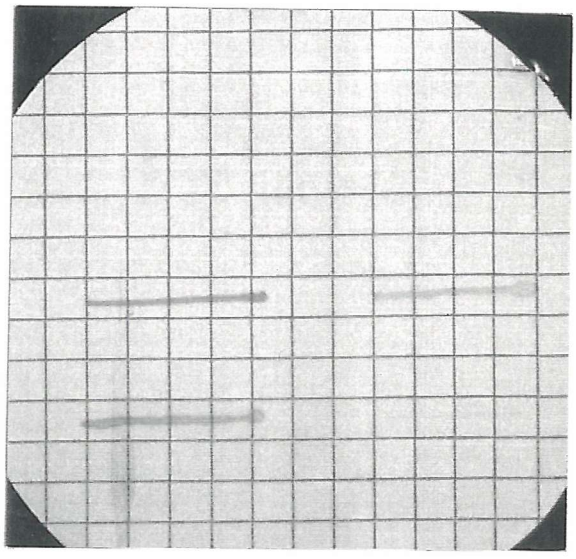
FIGURE 6.3.

(A) Anti-TraT Monoclonal Antibody, mAb867.

Lysed Cells.

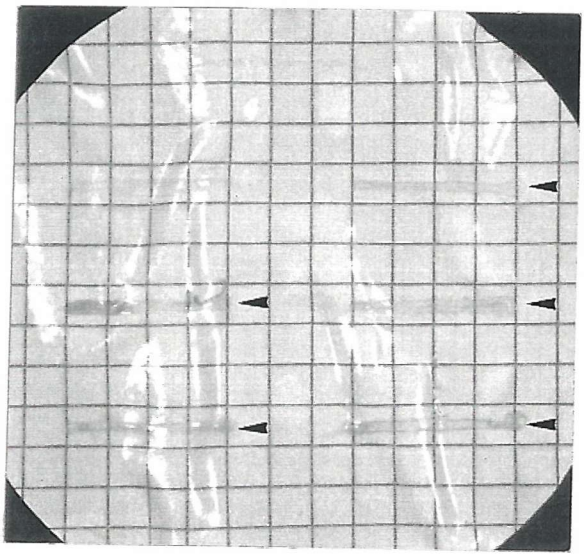


Unlysed Cells.

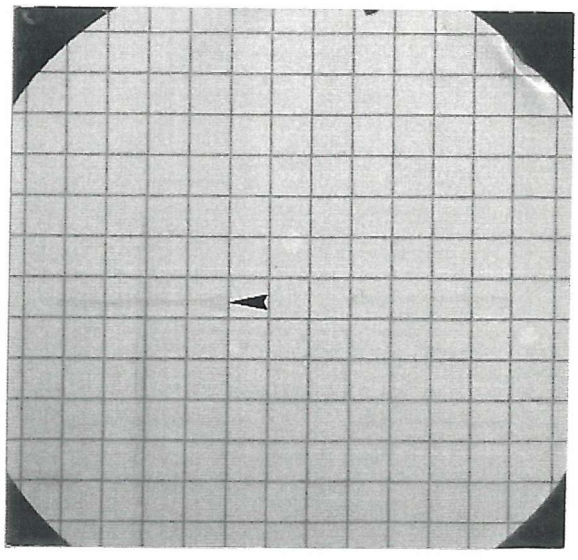


(B) Anti-C3 Monoclonal Antibody.

Lysed Cells.



Unlysed Cells.



6.2.4. Oral Administration of the Strains to BALB/c Mice.

Before administration of the strains to mice, the precise amount of each strain needed to give the required dose was determined. The strains were grown (as described in Section 2.8.3.) to an A_{600} of around 0.3 (which corresponded to 0.9-1.2 CFU/ml) and the doses administered to mice were based on the experiments previously carried out by Maskell *et al.*, (1987).

Preliminary experiments demonstrated that oral administration of up to 10^{10} CFU of SL3261 (pcI857) (pDOC23) caused no ill-effects (data not shown). Hence, over-expression of the TraT protein did not appear to cause increased virulence of the attenuated *Salmonella* strain.

Test and control strains were administered orally according to the immunisation schedule shown in Table 6.3 and control mice were sacrificed 1, 7 and 14 days after inoculation to check for the presence of bacterial cells. Liver autopsies (carried out as described in Section 2.8.4.) indicated that the strains colonised this organ after day 1 and were still increasing in number after day 14. Due to the small numbers of bacteria re-isolated it was difficult to give a quantitative estimate of the rate of increase of numbers in the liver. However, very few of the re-isolated bacteria were ampicillin or kanamycin resistant indicating that the plasmids were very unstable and had been lost from the *Salmonella* cells upon inoculation. This was supported by ELISA (carried out as described in Section 2.7.3.) of the serum samples from the mice inoculated with bacteria containing the *traT/C3* constructs. No cross-reaction was detected with the synthetic C3 peptide (1 mg/ml) whereas, the anti-C3 monoclonal antibody, at a 1 in 65,610 dilution, readily detected the C3 peptide used in the assay (data not shown here).

After this work was completed, several other groups reported similar findings of plasmid instability *in vivo* (O'Callaghan *et al.*, 1990; Salas-Vidal *et al.*, 1990; Agterberg *et al.*, 1991).

TABLE 6.3.

Immunisation Schedule for the Experimental Mice.

Cage	No. & Sex	Dose (CFU)	Strain administered	Administered on days	Blood taken on days
A	4 males	10^9	SL3261 (pcI857) (pIT9)	1, 65 & 70	0, 7, 14, 21, 28 & 72
B	4 males	10^9	SL3261 (pcI857) (pIT11)	1, 65 & 70	0, 7, 14, 21, 28 & 72
C	4 males	10^9	SL3261 (pcI857) (pDOC23)	1, 65 & 70	0, 7, 14, 21, 28 & 72
D	4 males	10^9	SL3261 (pcI857) (pDOC55)	1, 65 & 70	0, 7, 14, 21, 28 & 72
E	4 males	10^9	SL3261 host strain	1, 65 & 70	0, 7, 14, 21, 28 & 72

6.2.5. Construction of Modified Plasmids for the Stable, Regulated Expression of TraT/C3 Derivatives in *S. typhimurium* SL3261.

In view of the extreme instability observed upon administration of the TraT/C3 strains to mice, it was decided to modify the strains to obtain stable and appropriately-regulated expression of the TraT/C3 hybrid proteins upon oral administration to an animal host.

Recently, Strugnell *et al.*, (1990) stabilised antigen expression by introducing the genes encoding the foreign protein directly into the *Salmonella* chromosome, using the *aroC* locus. They used a system based on *E. coli* plasmid vectors which contain a DNA fragment, encoding the *aroC* gene, cloned from the chromosome of *S.typhimurium* C5. The *aroC* gene was modified using synthetic oligodeoxyribonucleotides so that it contained several unique restriction enzyme sites into which DNA, encoding heterologous antigens, could be cloned. DNA was integrated into the *S. typhimurium* chromosome at *aroC* by transferring the vectors into *S. typhimurium polA* mutants and allowing homologous recombination to occur between the cloned and chromosomal *aroC* genes. Strugnell *et al.*, (1990) used this system to integrate nucleotide sequences, encoding tetanus toxin fragment C and the *Treponema palladium* lipoprotein, into the *Salmonella* chromosome. The expression of both antigens was shown. In view of these results, it was decided to adapt the pDEL2 system to allow integration of the *traT/C3* genes into the *S. typhimurium* chromosome.

Since the λP_L inducible promoter has not specifically been developed for *in vivo* use it may well be inappropriate in *Salmonella* and hence contribute to the instability of the plasmid, making it unsuitable for use in the vaccine strain. However, if chromosomal integration is employed then the integrated gene is only likely to be present in one, or at most a few copies, so a relatively strong promoter needs to be employed. For these reasons, the λP_L promoter was replaced by the *groEL* promoter in each of the *traT* constructs. Recently, Buchmeier & Heffron, (1990) have shown that the product of the *groEL* gene undergoes a massive induction when *S.*

typhimurium enters macrophages. Additionally, when the cells are shifted from low to high temperatures, the synthesis of the heat shock proteins increases transiently. Hence, by placing the *traT/C3* genes under the control of the *groEL* promoter, strong and appropriately-regulated expression should be acheived.

6.2.5.1. Insertion of the *groEL* Promoter Upstream of the *traT/C3* Genes.

DNA isolated from lambda phage 649 (Kohara *et al.*, 1987) of the *Escherichia coli* chromosome (94.4 minutes) was used as template DNA to amplify the *groEL* promoter gene using PCR (see section 2.4.9.). The PCR primers used were designed such that:-

a) they corresponded to the two ends of the *groEL* promoter. Primer 1 corresponded to coordinates 5 to 24 and primer 2 to coordinates 287 to 306 inclusive (the coordinates referring to those described by Miki *et al.*, 1988);
b) they were rich in G and C bases at their 3'-ends to aid binding to the target sequences, but were not complementary to each other or to themselves;
c) they contained restriction endonuclease sites (*KpnI* for primer 1 and *BglII* for primer 2) at their 5'-ends, to allow subsequent cloning into the appropriate DNA vectors;
d) 3 extra bases (AAA) were present at the 5'-ends to aid subsequent digestion by the relevant restriction enzyme (New England Biolabs, 1989).

The DNA sequences of the two primers used are shown in Figure 6.4 and the *groEL* promoter sequence amplified using PCR is shown in Figure 6.5.

The amplified 320 bp *groEL* DNA fragment was purified, cut with *KpnI* and *BglII* and ligated into *KpnI*- and *BamHI*-digested pDEL2. A schematic representation of the plasmid pDEL2 showing the cloning sites used is given in Figure 6.6. Agarose gels showing the DNA fragments throughout this cloning procedure are shown in Figure 6.7. A plasmid with the *groEL* insert was detected by restriction enzyme analysis and the whole *groEL* sequence, along with the flanking cloning sites, was shown to be as expected by placing the segment into M13 and sequencing through the promoter

region (as described in Section 2.4.8.). A plasmid with the correct *groEL* gene sequence was designated pJH2.

6.2.5.2. Genetic Insertion of *traT/C3* Hybrid Genes into pJH2.

The *traT* or *traT/C3* genes were excised from pIT9, pIT11 and pDOC23 using *EcoRI* and ligated into *EcoRI*-cut, phosphatase-treated pJH2 (as described in Sections 2.4.4. and 2.4.5.). The clones isolated from the respective ligations were analysed by restriction digestion (*KpnI & HpaI*; *KpnI & StuI* and *KpnI & BstEII* double digestions) to find plasmids of the correct size and containing inserts in the correct orientation. Agarose gels showing the cloning of these fragments and restriction digestion analysis of the resultant plasmids are shown in Figure 6.8. The plasmids isolated with the correct inserts were designated pJH9, pJH11 and pJH23, respectively. A schematic diagram showing the construction of one of the plasmids, pJH11, is shown in Figure 6.9.

6.2.5.3. Expression of TraT/C3 Constructs from the *groEL* Promoter.

The exposure of the TraT and C3 proteins of the pJH9, pJH11 and pJH23 plasmids in *E. coli* cells (LE392-λ) was tested by carrying out *in situ* immunoblots of the lysed and unlysed strains (as described in 2.7.1.). All the constructs (except pJH2) were found to express the TraT protein in lysed cells as expected. However, the pJH9 construct, and to a lesser extent the pJH11 construct, expressed the TraT 867 epitope to the outside of the cells. Since this epitope is not normally accessible when its antibody is added to intact cells, it appears that the TraT proteins encoded by pJH9 and pJH11 are distorted, as is seen with the pIT9 and pIT11 constructs (Taylor *et al.*, 1990). pJH9 and pJH11 both expressed the C3 epitope in lysed cells, whereas pJH9 was the only construct to express the C3 epitope in unlysed cells. Hence, the immunoblot results (shown in Figure 6.10 and summarised in Table 6.4) show that the hybrid TraT/C3 proteins retained their characteristic patterns of cross-reactivity when expressed in the new *groEL* constructs.

FIGURE 6.4.

The *groEL* Promoter PCR Primers.

Primer 1:

5′- AAAGGTACCTCGATAGCAGGCCAATGCCC -3′

KpnI

Primer 2:

3′- GGCCGCAGTGGGTATTGTCTATCTAGAAAA -3′

BglII

Primer 1 correspond to coordinates 5 to 24 and primer 2 corresponds to coordinates 287 to 306 of the *groEL* gene (Miki *et al.*, 1988). The unique restriction enzyme sites (*KpnI* and *BglII*), added to allow cloning of the PCR fragment into the plasmid pDEL2, are shown.

FIGURE 6.5.

Nucleotide Sequence of the *groEL* Promoter Region.

(Miki *et al.*, 1988)

Primer 1

5′- GAC**GTCGATA** **GCAGGCCAAT** **GCCC**TGGGCC AGCCCCAGTT

CTTGTTTGAG TCCACTCATG GGTTGATGTC CGATTGCGCC

CAAATTTTGG GCAACTGCGT AGATTTTCGA TGGTAGCACA

ATCAGATTCG CTTATGACGG CGATGAAGAA ATTGCGATGA

AATGTGAGGT GAATCAGGGT TTTCACCCGA TTTTGTGCTG

ATCAGAATTT TTTTTCTTTT TCCCCCTTGA AGGGGCGAAG

−35

CCTCATCCCC ATTTCTCTGG TCACCAGCCG GGAAACCACG

−10 +1

TAAGCT**CCGG** **CGTCACCCAT** **AACAGAT**ACG −3′

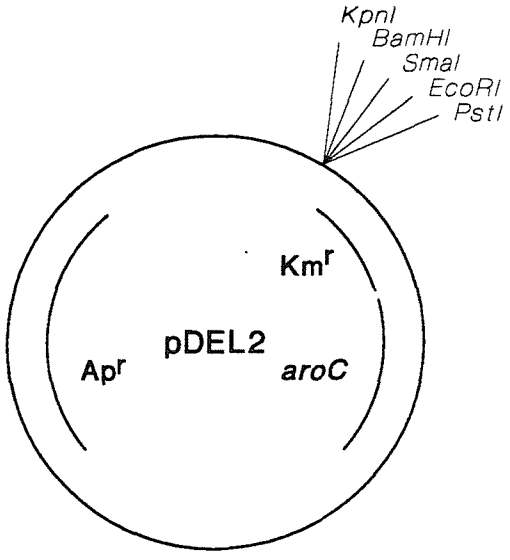
Primer 2

The nucleotides are numbered from the first nucleotide of the *AatII* site (the first base shown here) at 2.58 kb. The PCR primer annealing sites are shown (bold and underlined), along with the promoter -10 and -35 boxes (underlined).

FIGURE 6.6.

The Plasmid pDEL2.

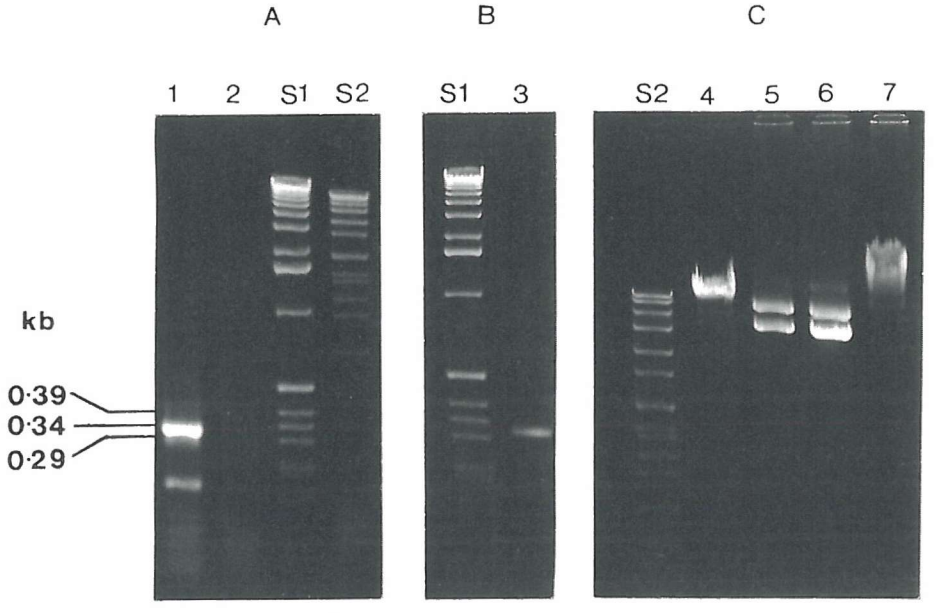
(Strugnell *et al.*, 1990)



The plasmid is approximately 8 kb in size. The unique restriction enzyme sites in the multiple cloning region are shown. The relative positions of the kanamycin and ampicillin resistance genes are shown, along with the *aroC* region of homology to the *Salmonella* chromosome.

FIGURE 6.7.

Construction of the Plasmid pJH2.



1.5% (A & B) and 0.8% (C) agarose gels of DNA throughout the stages of construction of the plasmid pJH2.

Tracks S1: BRL 1 kb DNA standard.

Tracks S2: SPPI/*EcoRI* DNA standard.

Track 1: *groEL* PCR DNA of lambda clone 649 using the primers shown in Figure 6.4.

Track 2: *groEL* PCR control (no template DNA added to the reaction).

Track 3: *groEL* PCR DNA (around 300 bp) after extraction from a large scale preparative gel and digestion with *KpnI* and *BglII*.

Track 4: pDEL2, cut with *KpnI* and *BamHI*.

Track 5: pDEL2 restriction digestion control (no enzymes added to the reaction).

Track 6: pDEL2 DNA, uncut.

Track 7: *groEL* PCR DNA fragment ligated into *KpnI* and *BamHI*-cut pDEL2 to produce the plasmid pJH2.

FIGURE 6.8.

Cloning of the TraT/C3 Constructs into the Plasmid pJH2 and Restriction Analysis of the Resulting Plasmids.

(A) 0.8% agarose gels of DNA throughout the stages of construction of the representative plasmid pJH9.

Tracks S: BRL 1 kb standard.

Track 1: pIT9 DNA, uncut.

Track 2: pIT9, *EcoRI*-digested.

Track 3: $traT/C3_{61}$ DNA band extracted from a large scale preparative gel (approx. 1 kb in size).

Track 4: pJH2 DNA, uncut.

Track 5: pJH2, *EcoRI*-digested.

Track 6: $traT/C3_{61}$ fragment ligated into *EcoRI*-cut pJH2 to produce the plasmid pJH9.

(B) DNA restriction fragments of the plasmids pJH9, pJH11 and pJH23 run on a 0.8% agarose gel.

Tracks S: BRL 1 kb standard.

Tracks 1; 2; 3: pJH9, digested with *KpnI* & *StuI*; *KpnI* & *BstEII*; *KpnI* & *HpaI*, respectively.

Tracks 4; 5; 6: pJH11, digested with *KpnI* & *StuI*; *KpnI* & *BstEII*; *KpnI* & *HpaI*, respectively.

Tracks 7; 8; 9: pJH23, digested with *KpnI* & *StuI*; *KpnI* & *BstEII*; *KpnI* & *HpaI*, respectively.

FIGURE 6.8.

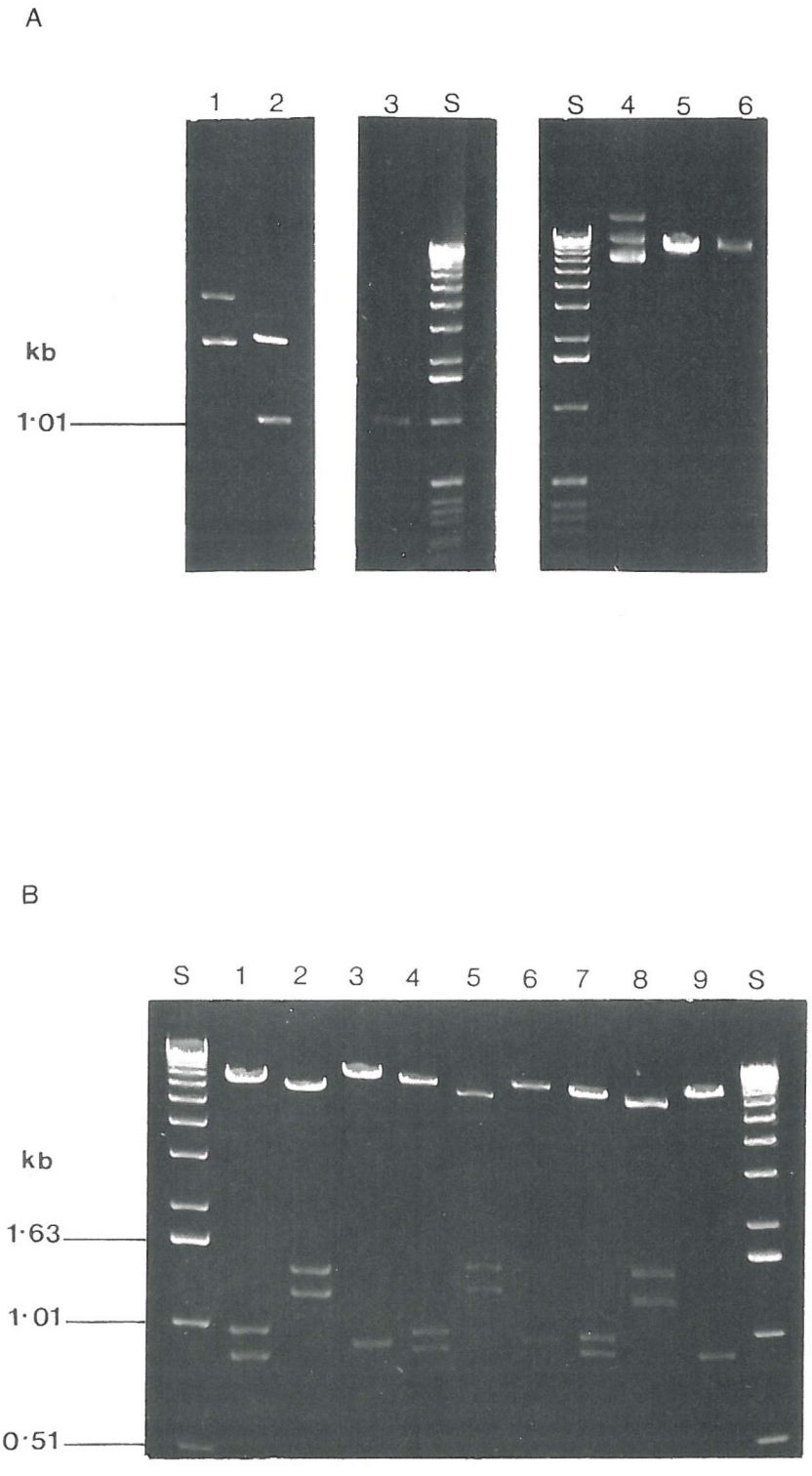


FIGURE 6.9.

Scheme showing the Construction of the Representative Plasmid pJH11.

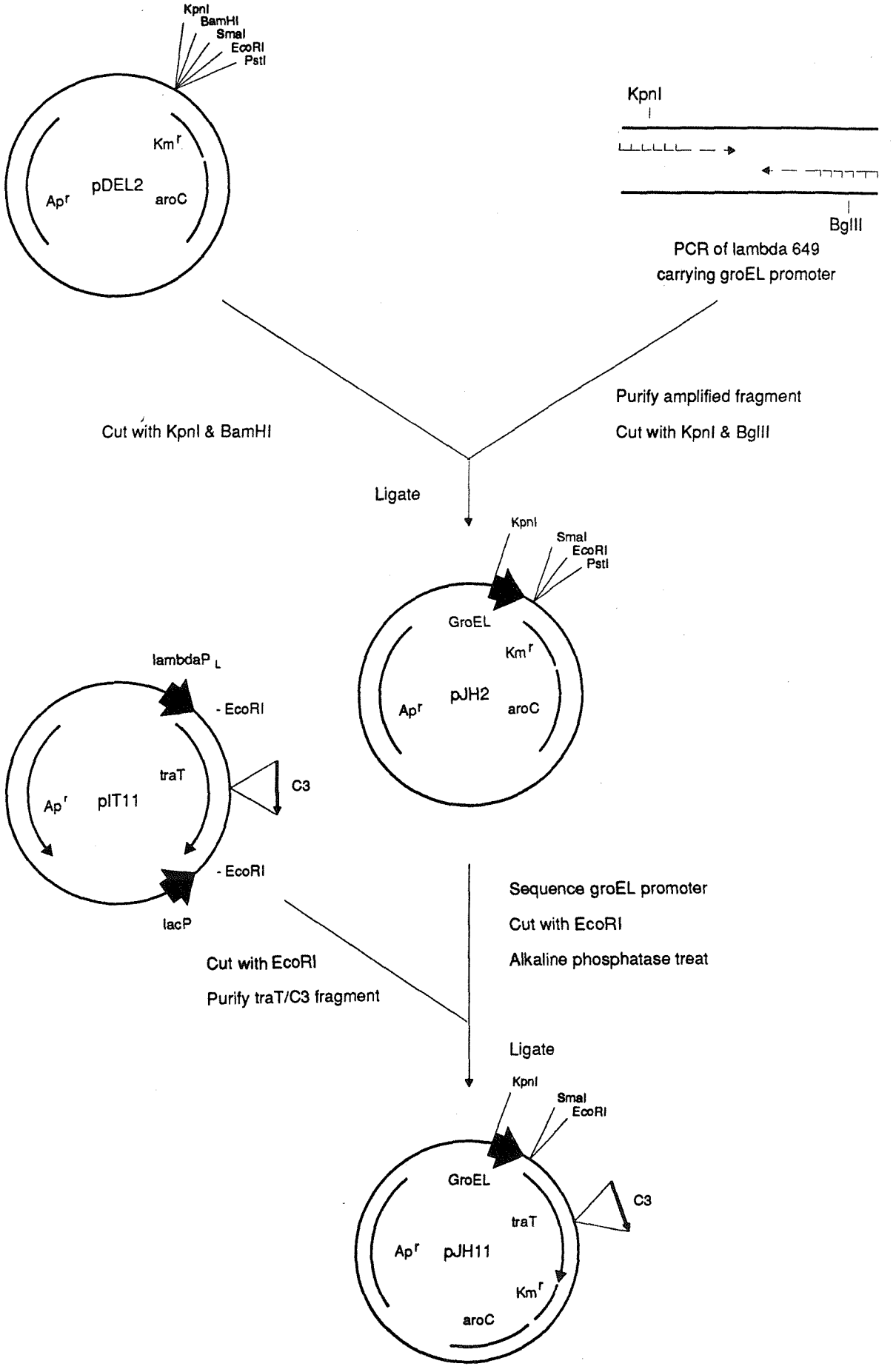


TABLE 6.4.

In Situ Immunoblot Results of *groEL* Constructs in the *E. coli* Strain LE392-λ.

Plasmid present in LE392-λ cells.	Hybrid protein expressed.	Cross-reaction with mAb 867.		Cross-reaction with anti-C3 mAb.	
		lysed	unlysed	lysed	unlysed
pJH2	-	no	no	no	no
pJH9	TraT/$C3_{61}$	yes	yes	yes	yes [a]
pJH11	TraT/$C3_{180}$	yes	no	yes	no
pJH23	wt TraT	yes	no	no	no
pJH23r	wt TraT in reverse orientation	yes	no	no	no

[a] reaction was faint

FIGURE 6.10.

In Situ Colony Immunoblots of TraT/C3 Hybrid Proteins Expressed from the pJH Plasmids in *E. coli.*

All the TraT/C3-expressing constructs in *E. coli* LE392-λ cells were streaked onto nitrocellulose filters, grown overnight at 30°C and induced from the *groEL* promoter by temperature shift to 37°C for 2 hours. Colonies, in both the intact and lysed states, were probed (as described in Section 2.7.1.) with (A) the anti-TraT monoclonal antibody, mAb867 or (B) an anti-C3 monoclonal antibody.

The results are summarised in Table 6.4.

The colonies were streaked onto each filter in the following pattern:-

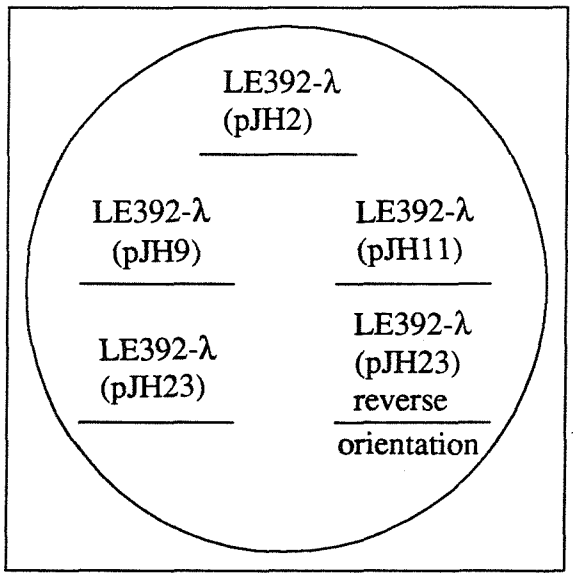
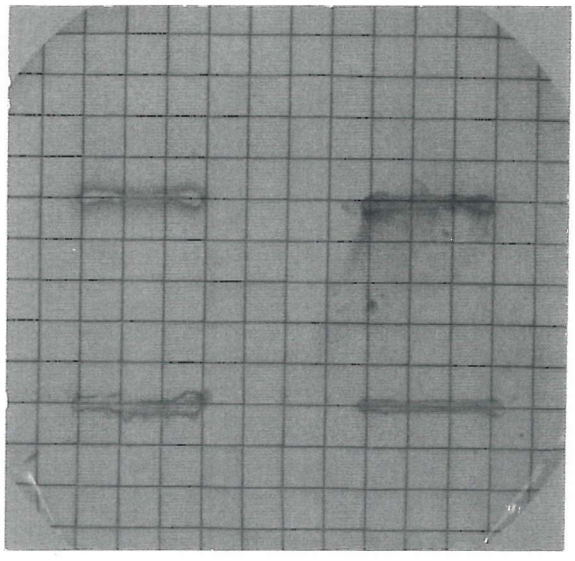


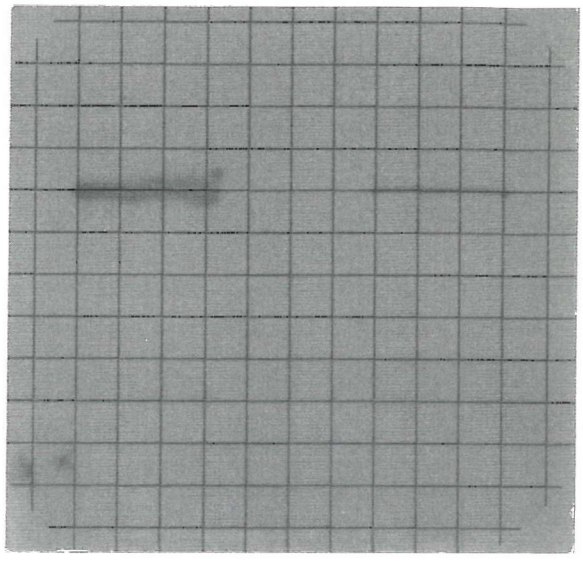
FIGURE 6.10.

(A) Anti-TraT Monoclonal Antibody, mAb867.

Lysed Cells.

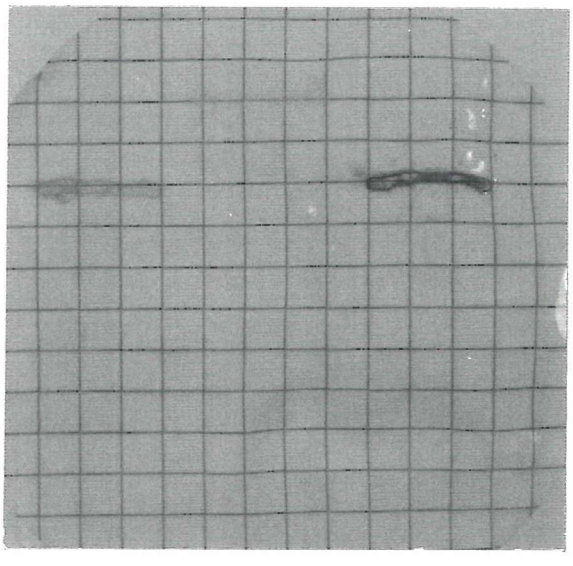


Unlysed Cells.



(B) Anti-C3 Monoclonal Antibody.

Lysed Cells.



Unlysed Cells.

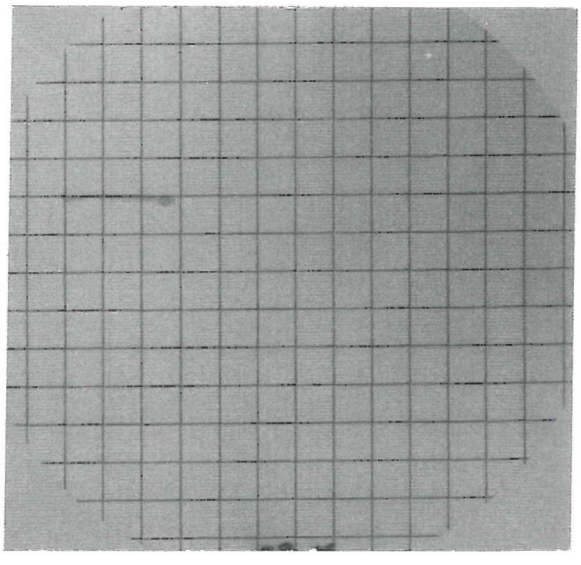


FIGURE 6.11.

Western Blotting of the Whole Cell Proteins Expressed from the pJH Plasmids in *E. coli* and *S. typhimurium galE* Strains.

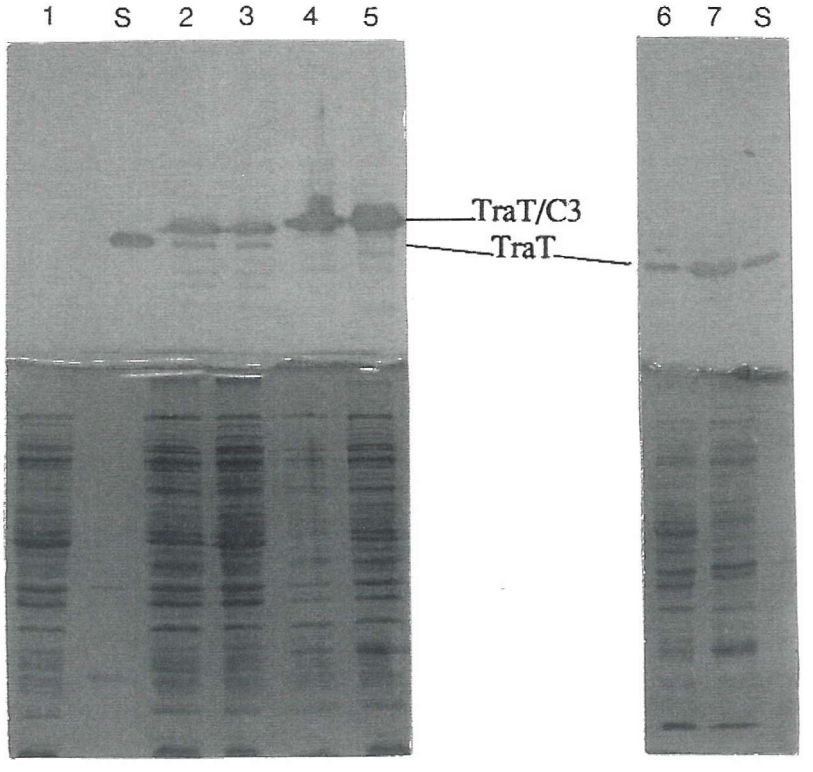
Total protein extracts from cells carrying the pJH plasmids were run on a 13% SDS polyacrylamide gel and (i) stained with Coomassie Brilliant Blue or (ii) Western blotted (as described in Section 2.7.2.) with (A) the anti-TraT monoclonal antibody, mAb867 or (B) an anti-C3 monoclonal antibody.

Tracks S: TraT and porin protein standard. Track 1: LE392-λ (pJH2)
Track 2: LE392-λ (pJH9) Track 3: LB5010 (pJH9)
Track 4: LE392-λ (pJH11) Track 5: LB5010 (pJH11)
Track 6: LE392-λ (pJH23) Track 7: LB5010 (pJH23)

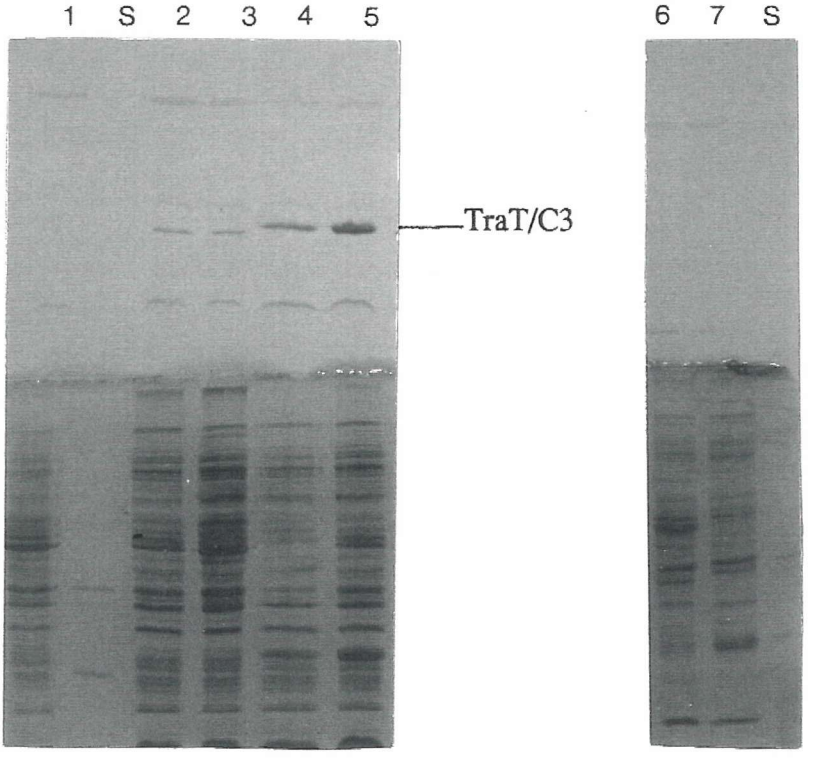
The wild-type and hybrid TraT proteins (as indicated) were of the predicted molecular weight and were highly expressed in all the constructs.

FIGURE 6.11.

(A) Anti-TraT Monoclonal Antibody, mAb867.



(B) Anti-C3 Monoclonal Antibody.



Immunoblotting of whole cell-lysates of cells carrying pJH9, pJH11 or pJH23 showed the presence of large quantities of each TraT/C3 protein (see Figure 6.11). Densitometer-scanning of the Coomassie Blue-stained gel indicated that the $TraT/C3_{61}$, $TraT/C3_{180}$ and TraT proteins corresponding to around 4, 13 and 16%, respectively, of the total SDS-soluble protein.

6.2.5.4. Expression of pDEL2/*groEL/traT/C3* Derivatives in *S. typhimurium* Strains.

Following their analysis in *E. coli*, the pJH plasmids were transformed into the intermediatory *S. typhimurium* strain, LB5010. Rapid, small-scale preparations of plasmid DNA from these strains were made and restriction enzyme analysis confirmed that no gross alteration in the *traT/C3* and *groEL* genes had occurred. Additionally, the *E. coli groEL* promoter directed the synthesis of large amounts of the corresponding proteins in *Salmonella*, as demonstrated by immunoblotting (see Figure 6.11). Hence, the modified constructs are now ready for integration into the *S. typhimurium* chromosome and subsequent immunisation studies. Work on these aspects is currently in progress.

6.3. DISCUSSION.

The work presented in this chapter shows that the R6-5 TraT protein and its TraT/C3 derivatives can be expressed at high levels in *S. typhimurium* without altering any of the important characteristics of the vaccine strain. It seems, therefore, that the presence of the endogenous TraT protein, specified by the pSLT plasmid in *S. typhimurium*, has no significant effect on the biosynthesis of the R6-5 TraT protein.

Although the strains initially constructed produced high levels of each TraT/C3 protein upon induction *in vitro*, no immune response was obtained *in vivo*. The results of the liver autopsy experiments indicated that this was due to the very rapid rate at which the plasmids were lost from the cells upon administration to the animal host. Additionally, the high level expression of the hybrid protein may have also affected

the ability of the *Salmonella* to invade Peyer's patches and survive in the reticuloendothelial system (O'Callaghan *et al.*, 1990) and thus may explain the lack of an immune response seen here. Subsequently, several other groups have reported similar observations suggesting that the problem may be of more general importance (Salas-Vidal *et al.*, 1990; Agterberg *et al.*, 1991). Another limitation of this system is that it does not eliminate the possibility of transfer of the plasmid from the hybrid *Salmonella* strain to the mammalian bacterial commensals, or environmental bacteria.

For all of these reasons, some other method of improving the genetic stability of the extrachromosomal vectors carrying the genes of interest, once the antibiotic selection was removed, was required. A number of different approaches have been taken recently in an effort to prevent plasmid segregation (Meacock & Cohen, 1980; Nakayama *et al.*, 1988; Hone *et al.*, 1988; Strugnell *et al.*, 1990).

The approach taken here in an attempt to stabilise the *traT/C3* constructs *in vivo* was to modify the strategy developed by Strugnell *et al.*, (1990). Integration of heterologous genes at a specific chromosomal locus such as *aroC* greatly increases the probability that the foreign sequences will be passed on to daughter cells and additionally creates a further attenuating mutation in an *aroA*$^-$ *S. typhimurium* strain. However, a potential disadvantage of the method is that the level of expression of the gene in question is likely to be lower overall, due to gene dosage effects.

To circumvent this potential problem a promoter that is strongly induced when *Salmonella* cells are located within macrophages was placed upstream of the *traT/C3* genes. Immunoblotting experiments (see Figure 6.11) show that very large quantities of each corresponding protein are produced when the non-integrated plasmid form is present. Hence, it is likely that sufficient quantities of each TraT/C3 protein will be produced when the corresponding genes are present on the *Salmonella* chromosome.

The availability of *Salmonella* strains expressing a well-characterised TraT protein to which monoclonal antibodies are available will also allow experiments to investigate the degree of surface exposure of the protein in cells expressing smooth

LPS. Bentley & Klebba, (1988) showed that it is the O-antigen and core structure of the LPS that controls antibody accessibility to certain porin surface epitopes in *E. coli* and *S. typhimurium*. They found for, both strains, that an intact O-antigen completely blocked antibody binding to the porin surface epitopes. However, as the LPS chemotype became rougher more monoclonal antibodies were able to recognise the epitopes. For this reason it was important to test the binding of the smooth vaccine strains used to monoclonal antibodies directed against the C3 epiotope and TraT protein. Preliminary results using a qualitative assay indicate that, in contrast to other outer membrane proteins (Leclerc *et al.*, 1989), TraT may still be accessible to antibody molecules in intact cells derived from smooth strains. While these results need to be confirmed using a quantitative assay (e.g. the fluorescence-cell sorting assay developed by Bentley & Klebba, 1988), they suggest that TraT may be particularly useful for the cell surface exposure of heterologous peptides.

CHAPTER 7:

GENERAL DISCUSSION.

7. GENERAL DISCUSSION.

The highly surface-exposed nature of the TraT lipoprotein makes it a promising candidate for the presentation of foreign antigenic determinants to the immune system. This in turn raises some fundamental questions about its structure and function(s). In the present study several of these aspects of the TraT protein have been investigated taking two complementary approaches. The first approach was to purify the TraT protein in a biologically active form such that its structure and biological function could be investigated. Secondly, the topology and suitability of the protein as a carrier of foreign epitopes was investigated by genetic insertion of the C3 epitope of poliovirus into the *traT* gene.

This chapter summarises and discusses the results obtained with the aim of identifying important topics for future investigation.

7.1. TRAT PROTEIN STRUCTURE AND FUNCTION.

Before the TraT protein can be employed as a carrier of protective foreign antigenic determinants by vaccine strains further information on its structure and biological roles needs to be determined. For this reason, the TraT protein was successfully purified to apparent homogeneity allowing several of its properties to be investigated in more detail.

Initial attempts to purify the TraT protein showed that it was difficult to purify TraT from the porin protein, OmpC, even after Mono-Q FPLC at two pHs. As the iso-electric points, sub-unit molecular masses and amino acid sequences of these two proteins differ drastically, this observation strongly suggests that there may be a specific interaction between the proteins. While the region(s) involved remain to be identified, it will probably be important to avoid inserting foreign antigenic determinants into such portions of the TraT protein as this is likely to perturb the stability and permeability properties of the outer membrane. Indeed, one of the five

sites chosen for insertion of the C3 epitope (around residue 125) did lead to increased outer membrane permeability to hydrophobic compounds. It remains to be seen, however, if this is due to perturbation of interactions between TraT and OmpC or between TraT and some other membrane component.

Circular dichroic measurements showed that TraT, contrary to other characterised outer membrane proteins, contains a relatively high proportion of α-helical structure. Taken together with the lack of amino acid similarity with other known outer membrane proteins, this result suggests that the protein may have undergone convergent evolution to develop the physico-chemical properties it shares with the other proteins (i.e. heat stability; resistance to proteases-digestion and chaotrophic agents and insolubility in many detergents). Thus, from the results described here it seems likely that TraT has a very different functional and structural organisation in the outer membrane in comparison with other characterised major outer membrane proteins.

The purified R6-5 TraT protein was incorporated into an *in vitro* surface exclusion assay to determine whether TraT acts SPECIFICALLY in inhibiting conjugation. The results obtained demonstrate that this is the case and hence support the idea that the protein is able to discriminate between donor cells harbouring different IncF plasmids. This is a somewhat surprising result in view of the subtle differences between the TraT proteins specified by different F-like plasmids. However, the data indicates that amino acid alterations mapping to a small portion of the protein (residues 116-120) are responsible for the specificity effect.

These results also provided support for the theory that TraT interacts specifically with the pilus to prevent conjugation. For example, the derepressed R100-1 plasmid, although in surface exclusion group IV, was not inhibited to the same extent as the R6-5 plasmid which is repressed for transfer. From this information the model shown in Figure 4.3 was proposed. Further experiments to directly prove a specific interaction between the TraT protein and the tip of the sex pilus would provide further support for this model. This could, in principle, be shown using affinity techniques,

for example, by demonstrating that TraT bound to a support matrix selectively purifies pili from a homogenate made from donor cells. Alternatively, immune-electron microscopy could be used, by adding TraT labelled with colloidal gold to pilated cells in the hope that the protein would specifically decorate pili. A further important question that needs to be addressed is the number of TraT subunits present in the native oligomeric form. Analytical ultracentrifugation experiments are under way to investigate this (J.L.H., J.P. Rosenbusch, C.D. O'C. - unpublished work). Current thinking, based on its shared physico-chemical properties with the porins, is that TraT is trimeric. We, however, favour the suggestion that it consists of 5 monomers, based on the elution characteristics of the native protein on Sephacryl columns (see Chapter 3) and the fact that pili with which it is believed to interact have a five-fold rotational axis (Marvin & Folkhard, 1986).

The purified TraT protein was also found to confer significant resistance to the bactericidal actions of guinea pig serum when added to *E. coli* cells. This indicates that the protein does not have to be present in the outer membrane to confer serum resistance. However, further work needs to be carried out into the interactions between TraT and the specific components of complement to evaluate how TraT confers serum resistance on bacterial cells.

Some topographic studies on the TraT protein were carried out by inserting the C3 epitope of poliovirus into a region of the protein predicted to be cell-surface-exposed. The region of the TraT protein chosen as the insertion site was only 5 residues away from the specificity region of the protein (a five amino acid region of the protein implicated in the specificity in surface exclusion of different TraT proteins encoded by different F-like plasmids) which was thought to be cell-surface-exposed. However, the C3 epitope inserted in this region was not recognised by antibodies added to whole cells, indicating that this part of the protein is either not cell-surface-exposed, or which is more likely, the folding of the TraT protein on the outside of the cell somehow masks the exposure of the epitope.

7.2. USE OF TRAT FOR THE PRESENTATION OF EPITOPES TO THE IMMUNE SYSTEM.

Two TraT/C3 constructs were expressed in an *aroA*⁻ *Salmonella typhimurium* vaccine strain. One of the constructs exposed the C3 epitope to the outside of the cell, but its overall native protein structure was disrupted and biological activity lost. The other construct did not expose the C3 epitope on the outside of the cell, but did display properties similar to the native TraT protein. This construct was chosen because surface exposure of the epitope is not necessarily essential to elicit an immune response (Brown *et al.*, 1987; Leclerc *et al.*, 1990). The TraT/C3 derivatives were expressed at high levels in *Salmonella*, without detectably altering any of the important characteristics of the vaccine strain. Thus, synthesis of the R6-5 TraT was not affected by the presence of the endogenous TraT protein specified by the *Salmonella* pSLT plasmid and expression of the foreign epitope was at the same level as that found in *E. coli* cells.

However, on administration to mice, and thus removal of the antibiotic selection, the TraT/C3 expressing plasmids were found to be unstable and hence no immune response was elicited. Consequently, an expression system was constructed so that plasmid segregation could be prevented by integrating the *traT/C3* genes directly into the *Salmonella* chromosome at the *aroC* locus. To overcome any possibility of the λP_L promoter (which was not specifically developed for such use) contributing to the plasmid instability and to ensure that the integrated gene was expressed in sufficient amounts to elicit an immune response, an alternative strong promoter (*groEL*) was employed. The *groEL* promoter allowed the production of large quantities of the TraT/C3 hybrid protein in *S. typhimurium* LB5010, indicating that strong and appropriately regulated expression of the genes should be achieved when the genes are integrated into the *Salmonella* chromosome.

The constructs are now ready for integration into the *Salmonella* chromosome and assuming they prove useful in subsequent immunisation studies, there are a number of interesting questions that can be addressed.

Firstly, the maximal length of the epitope inserted into TraT can be investigated. A similar epitope expression system employing the flagellin protein as a carrier only seems to be able to present single epitopes (around 20 residues) of well-defined antigens (Newton *et al.*, 1989; Wu *et al.*, 1989; Majarian *et al.*, 1989; Kuwajima *et al.*, 1988), before its ability to assemble into functional flagella is lost. The LamB protein of *E. coli*, however, allows the insertion of up to 60 amino acids and similarly, PhoE can accommodate insertions of upto 50 residues without affecting the biogenesis of the proteins (Charbit *et al.*, 1988a; Agterberg & Tommassen, 1991). Recently, the OmpA protein has been shown to allow the stable surface expression of at least 730 amino acids residues, although the functional state of the carrier protein was not reported (Pistor & Hobom, 1990; Schorr *et al.*, 1991). Such information about the TraT protein would enable us to evaluate its potential for the development of a multivalent vaccine system.

Hofsta *et al.*, (1979) have shown that the majority of antibodies induced by immunisation against intact cells of *E. coli* are against the LPS constituent of the cell wall. Thus, outer membrane proteins such as TraT could be at least partially masked to the immune system by envelope components. Physical treatments of the LamB protein have been shown to lead to some unmasking of the protein facilitating the processing by macrophages (Leclerc *et al.*, 1989). Thus, studies of the effect of smooth LPS on presentation of the epitope, which have been touched on in this thesis, need to be followed up.

It is not yet clear if the functionality of the carrier protein is important *per se* or if it is merely that a hybrid protein must avoid disturbing the outer membrane. If the latter is the case then the TraT lipoprotein has a considerable potential advantage over the other proteins in that the attached lipid is probably the only essential element required for stable anchorage to the membrane. Additionally, only the first ten or so amino acids of the mature portion of a lipoprotein are required for its targeting to the outer membrane (Yamaguchi *et al.*, 1988), suggesting that loss of large parts of the TraT polypeptide will not affect the ultimate destination of the hybrid polypeptide.

Recent work tentatively suggests that surface exposure of the foreign epitope is not necessarily essential for an antibody response (Brown *et al.*, 1987; Leclerc *et al.*, 1990). Because the targeting of lipoproteins to the inner or outer membranes appears to be determined by which residue is present at the +2 position of the protein, they constitute an ideal system to answer this question definitively. Additionally, lipoprotein mutants that lack attached lipid, due to the alteration of the lipid attachment signal, e.g. the Cys at +1 of the protein (Yamaguchi *et al.*, 1988), should be invaluable in determining the importance of the lipid moiety for eliciting a cytotoxic T-cell response in addition to production of specific antibodies (Deres *et al.*, 1989; Croft *et al.*, 1991).

The addition of T-cell sites may also help maximise the immune response. Cell-mediated immunity involves antigen processing and is critical for long-term protective immunity against infection, and hence insertion of a foreign epitope in association with supplementary flanking T-cell determinants is one possible refinement that can be investigated. Such a system has already been studied by Agterberg & Tommassen, (1991) who showed that both B- and T-cell epitopes can be successfully expressed at the cell surface, with the epitopes keeping their antigenic and immunogenic properties.

Finally, many foreign proteins and peptides expressed in bacteria are subject to degradation by proteases and limited proteolysis of the heterologous antigen may help produce a T-cell response, due to the processing requirements of such epitopes (Townsend *et al.*, 1988). Thus, studies to investigate the ways in which bacterial proteases affect the immune response to well-defined foreign proteins and peptides should help clarify this issue.

It is hoped that the work described here will permit at least some of these studies to be accomplished in the future.

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