UNIVERSITY OF SOUTHAMPTON

BipA: A new ribosome accessory protein that regulates *Escherichia coli* virulence

By

Andrew James Grant Bsc (Hons)

A thesis presented for the degree of

Doctor of Philosophy

Division of Biochemistry and Molecular Biology

Faculty of Science

April 2001

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE
DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Doctor of Philosophy

BipA: A NEW RIBOSOME ACCESSORY PROTEIN THAT REGULATES *ESCHERICHIA COLI* VIRULENCE

By Andrew James Grant

Enteropathogenic *Escherichia coli* (EPEC) are a major source of diarrhoeal disease and cause significant infant mortality and morbidity in the developing world. The capacity of EPEC to cause disease is linked to their ability to adhere tightly to epithelial cells of the small intestine and to trigger complex cytoskeletal rearrangements resulting in the formation of the attaching and effacing (A/E) lesion. The A/E lesion results in the formation of a pedestal – a cup-shaped structure that surrounds each bacterium and raises it above the surface of the host cell. The precise reason for pedestal formation is unknown although it appears to be an important determinant of diarrhoeal disease.

This thesis describes the functional characterisation of BipA, a member of the ribosome-binding GTPase superfamily, that is widely distributed in bacteria and plants and that regulates the characteristic ability of EPEC to rearrange the actin cytoskeleton of infected host cells. This process involves a 35 kb cluster of genes in EPEC known as the Locus of Enterocyte Effacement (LEE) that codes for a type III secretion system and associated effector proteins. To study the role of BipA in EPEC pathogenesis, a series of mutant derivatives were produced using targeted mutation of the chromosomal *bipA* gene. Analysis of a *bipA* null mutant of EPEC strain E2348/69 showed that the synthesis of the key effector proteins EspA, EspB, EspD, Tir and intimin was blocked. Moreover, BipA also regulated the proteolytic degradation of intimin. Analysis of further mutants indicates that BipA does not operate via three known regulators of the EPEC genes involved in A/E lesion formation (Integration Host Factor, H-NS and Per). However, transcription of the *ler* gene, coding for a LEE-encoded regulator, was abolished in the *bipA* null mutant, indicating that BipA directly or indirectly controls Ler expression.

Other studies indicated that BipA regulates a range of virulence and stress processes. Although growth of EPEC at 37°C was unaffected in the *bipA* null mutants, BipA was required for growth at low temperatures. This finding was exploited in conjunction with a novel transposable promoter system to construct strains of *E. coli* in which growth at low temperature was dependent on the sugar L-arabinose. Additionally, BipA negatively regulated cell motility in EPEC. Thus BipA has all the hallmarks of a global regulator. In contrast to other global regulators, however, BipA has striking sequence similarity to Elongation Factor G, suggesting that it operates at the level of the ribosome.

CONTENTS

FIGURES

TABLES

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Dr David O'Connor (DO'C) for initiating my interest in microbial pathogenesis and being a constant source of inspiration.

I would like to express my appreciation to Dr Michele Farris, and acknowledge the SEM photos, confocal photos and the *ler* transcript map as her work. Special thanks to Karen Platt, for the relentless sequencing of pBipA constructs, and for running the gel for the *luxS* transcript map. I am grateful to Frank Ebel, Gad Frankel, Jay Hinton and Brendan Kenny for the antibodies they kindly donated.

I would like to say a BIG THANKS to members of the DO'C group past and present for invaluable assistance and expert tuition. In particular, a special mention to Dr Phil Adams and Dr Niamh Kinsella, for helping me along in the early days. Thanks also to everyone in the coffee club and to my friends. In particular Black, Susie, Buckland, Ash and Jo for keeping me sane. I am grateful to Andrea for her support and sympathetic ear at the end.

Finally to my family for continued support and encouragement throughout all of my studies, it was much appreciated. Thank-you.

This work was supported by a grant from the BBSRC.

PUBLICATIONS

Below is a list of my publications and papers in press/preparation at the time of producing this thesis.

Farris M., **Grant A.,** Richardson T.B. and O'Connor C.D. (1998) BipA: a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. *Molecular Microbiology.* **28**, 265-279

Farris M., **Grant A.,** Jane S., Chad J. and O'Connor C.D. (1998) BipA affects Ca^{++} fluxes and phosphorylation of the translocated intimin receptor (Tir/Hp90) in host epithelial cells infected with enteropathogenic *Escherichia coli. Biochemical Society Transactions.* **26**, S225-S225

Owens R.M., **Grant A.,** Davies N. and O'Connor C.D. (2001) Copurification of the lac repressor with polyhistidine-tagged proteins in immobilized metal affinity chromatography. *Protein Expression and Purification.* **21**, 352-360

Grant A. J., Haigh. R., Williams P. and O'Connor C.D. (2001) An *in vitro* transposon system for highly regulated gene expression: construction of *E. coli* strains with arabinose-dependent growth at low temperatures. *Gene.* (In Press)

Grant A. J., Farris M., Williams P., Alefounder P. and O'Connor C.D. A member of the ribosome binding GTPase superfamily controls multiple virulence factors in enteropathogenic and enterohaemorrhagic Escherichia coli via the locus of enterocyte effacement (LEE)-encoded regulator. (In Preparation)

ABBREVIATIONS

A/E	Attaching and Effacing
AI-2	Autoinducer
Ap	Ampicillin
ATP	Adenosine triphosphate
BFP	Bundle Forming Pilus
BipA	BPI inducible protein A
BPI	Bactericidal/Permeability-Increasing
cAMP	adenosine 3'5'-cyclic monophosphate
cat	Chloramphenicol acetyltransferase
CFTR	Cystic Fibrosis Transmembrane Receptor
cGMP	guanosine 3'5'-cyclic monophosphate
Cm	Chloramphenicol
DA	Diffuse Adherence
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EAF	EPEC Adherence Factor
EAST1	EAEC heat-stable enterotoxin 1
EF-G	Elongation Factor G
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Esc	*Escherichia coli* secretion genes
Esp	*Escherichia coli* secreted protein
ETEC	Enterotoxigenic *Escherichia coli*
FAS	Fluorescent-actin staining
FITC	Fluorescein isothiocyanate
FSB	Final Sample Buffer
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GNRP	Guanine nucleotide release factor
GTP	Guanosine triphosphate
H-NS	Histone-like nucleoid structuring protein

Hrp	Horse radish peroxidase
IgA	Immunoglobulin A
IHF	Integration host factor
IL-8	Interleukin-8
IP_3	Inositol triphosphate
Isc	Short circuit current
kb	kilobase
kDa	kiloDalton
Km	Kanamycin
LA	Localised Adherence
LB	Luria Bertani
LEE	Locus of Enterocyte Effacement
Ler	LEE encoded regulator
LPS	Lipopolysaccharide
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MOPS	Morpholinopropane sulphonate
mRNA	messenger Ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	Nuclear Factor kappa B
OD	Optical Density
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Per	Plasmid-encoded regulator
PIP_2	Phosphatidylinositol diphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PMN	Polymorphonuclearleukocyte
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
TEER	TransEpithelial Electrical Resistance
Tc	Tetracycline
Tir	Translocated intimin receptor
tRNA	transfer Ribonucleic acid
WASP	Wiskatt-Aldrich Syndrome Protein

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 General Introduction

With the discovery of new pathogens, the re-emergence of old ones and the inexorable development of antibiotic resistance, the study of -'microbial pathogenesis'- has never been so timely. It is now clear that diverse bacterial pathogens employ common mechanisms to adhere, invade or interact with host cells and ultimately cause disease (Finlay and Falkow, 1997). These mechanisms include pili and fimbrial adhesins for adherence to host cells, conserved machinery for the secretion of virulence factors, and the production of toxins to subvert key cellular processes. Recent scientific advances have enabled us to better understand how microbes cause disease; such studies will ultimately allow the development of new and possibly more precise therapeutics to be used in combating infection.

In recent years, considerable progress has been made in genome analysis, and to date the complete genome sequences of over forty microorganisms, including several pathogens have been determined (Hommais *et al.*, 2001). These studies highlight the fact that diverse pathogens often share common virulence mechanisms. Furthermore, they establish that many, essential virulence factors are commonly found in blocks of genes known as pathogenicity islands or islets. These blocks of genes are often flanked by repetitive sequences or transposable elements suggesting that the DNA segment was acquired through horizontal gene transfer (Hentschel *et al.*, 2000). The sequences of the pathogenicity islands often differ in fundamental ways to the rest of the genome. For example, the G + C content of pathogenicity islands typically differs from the rest of the genome (Hacker and Kaper, 2000). Pathogenicity islands are also often inserted into hot spots on the chromosome such as tRNA loci (Finlay and Falkow, 1997; Hacker and Kaper, 2000). As the number of bacterial pathogens that are sequenced increases, genomics in concert with appropriate bioinformatic analysis will undoubtedly uncover many additional virulence factors through homology

searches with existing factors and by comparison between the genomes of pathogenic and non-pathogenic strains

The virulence determinants that have been studied to date are rarely expressed constitutively. Rather, they are induced once the pathogen detects environmental stimuli that are indicative of a host environment. The precise reasons for the regulated expression of virulence determinants remain unknown. However, one plausible explanation is that, as unicellular organisms, bacterial pathogens need to avoid wasting ATP on the inappropriate synthesis of virulence proteins. Additionally, pathogens are more likely to escape detection by the host immune system if they delay the expression of highly immunogenic virulence determinants until they have reached an appropriate location in the host.

In addition to dedicated virulence determinants, whose sole reason for synthesis appears to be to cause disease, certain other genes involved in virulence may also be expressed in the non-host environment. Chief among these are genes that allow the bacteria to survive a specific physiological stress e.g. exposure to low pH. It is becoming increasingly clear that, in some cases, these genes are co-regulated with dedicated virulence determinants, such that their basal levels of expression are appropriately adjusted to the host. In many instances, co-regulation is achieved by use of a so-called global regulatory protein, which controls the expression of multiple genes in parallel.

This dissertation describes the characterisation of a GTPase that has all the hallmarks of a novel global regulator. The protein, termed BipA is unusual because it is unrelated to other known virulence or global regulatory proteins, but shares sequence similarity to GTPases that interact with ribosomes. Indeed, recent unpublished studies show that BipA has GTPase activity that is stimulated in the presence of 70S ribosomes (R. Owens and C. D. O'Connor, unpublished results), suggesting that it may regulate target proteins by a novel mechanism operating at the level of the ribosome.

As this thesis concentrates on the role of BipA in one form of pathogenic *Escherichia coli*, it is pertinent to first briefly review these bacteria and the diseases they cause.

1.2 Classification of virulent *Escherichia coli*

The gram-negative bacterium *Escherichia coli* (*E. coli*) is the predominant species among the facultative anaerobic normal flora of the intestine, where it plays an important role in normal gastrointestinal function. *E. coli* was first implicated as a cause of diarrhoea in the late 1940s by Kauffman, (1947). Since there are many different types of *E. coli*, most of which do not cause disease (although those that do, do so by a range of means), it is necessary to have a system for classifying these bacteria. There are presently two methods of classification serotyping and virotyping.

Serotyping is based on the differences in antigenic properties of bacterial surface molecules. Two surface molecules form the basis of this classification, the O antigen of lipopolysaccharide (LPS) and H antigen formed by flagella. Specific combinations of O and H antigens define the serotype of an isolate (Levine and Edelman, 1984; Salyers and Whitt, 1994). Serotypic markers have limited sensitivity and specificity in identifying diarrhoeagenic bacteria from non-pathogenic bacteria, and the classification of bacteria has moved largely towards identifying characteristics that determine the specific pathogenicity of a strain, a process known as virotyping.

Virotyping is associated with the disease process caused by the bacteria and takes into account the patterns of bacterial attachment to host cells, the effects of attachment and production of toxins (Levine, 1987; Salyers and Whitt, 1994). Classification by virotyping currently results in five pathogenic virotypes of *E. coli*. These are enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC). There are also new emerging groups of pathogenic *E. coli*, namely, DAEC (diffusely adherent *E. coli*) and CDEC (cell-detaching *E. coli*). However, since little

is known about the pathogenic mechanisms of these bacteria they will not be considered in this study.

1.3 Virotypes of *E. coli*

Diarrhoeagenic bacteria frequently share common strategies for infection (Finlay and Falkow, 1997). These include colonisation of a mucosal location, subversion of host defences, multiplication within the host, and generation of host damage (Nataro and Kaper, 1998). Moreover, each virotype of diarrhoeagenic *E. coli* possesses particular strategies for colonisation of the host and for the subsequent generation of disease. These will be summarised in the following passages.

1.3.1 Enterotoxigenic *E. coli* (ETEC)

ETEC strains colonise the proximal small intestine and promote fluid efflux from the intestinal mucosa by releasing toxins that modulate cyclic nucleotide monophosphate levels, producing a secretory state that causes diarrhoea (Baldwin *et al.*, 1990). For ETEC to cause disease they must first adhere to the epithelial cells. This is achieved by the means of colonisation factors (CFAs), filamentous organelles on the surface of the *E. coli* that mediate interactions with host cells (Levine and Edelman, 1984; Helander *et al.*, 1997). These adhesion fimbriae allow ETEC to bind to specific receptors on proximal enterocytes of the small intestinal, and represent an early event in colonisation and the development of diarrhoeal disease (Knutton *et al.*, 1987b). Three major types of CFA have been reported, these include rigid rods, bundle-forming flexible rods, and thin flexible wiry structures (Nataro and Kaper, 1998).

ETEC cause diarrhoea by elaborating two types of toxin, and may express one or both of these toxins. The toxins in question are heat labile toxin (LT), and heat stable toxin (ST) (Levine and Edelman, 1984; Tsen and Jian, 1998). LT-I is endocytosed after binding to the host cell membrane, and acts by ADP-ribosylating a G protein, thereby stimulating an adenylate cyclase on the basolateral surface of the mucosal

cell. The result is permanent activation of adenylate cyclase, leading to the uncontrolled release of cyclic adenosine monophosphate (cAMP). This in turn activates a cAMP dependent protein kinase. The net result is phosphorylation of chloride channels (such as the Cystic Fibrosis Transmembrane Receptor - CFTR) situated in the apical membrane of the epithelial cell. This stimulates chloride ion secretion from secretory crypt cells and also inhibits NaCl absorption by villus tip cells. This increase in the luminal ion concentration promotes the passive movement of water through the paracellular pathway, leading to osmotic diarrhoea (Levine and Edelman, 1984; Nataro and Kaper, 1998). The predominant heat-stable toxin STa (ST-I), binds to guanylate cyclase located in the apical membrane of the host cell causing an activation of guanylate cyclase, resulting in an increase in cGMP, which has the same effect as the uncontrolled release of cAMP (Levine and Edelman, 1984; Nataro and Kaper, 1998).

1.3.2 Enteroaggregative *E. coli* (EAEC)

EAEC were previously referred to as enteroadherent-aggregative *E. coli*, which was shortened to enteroaggregative *E. coli* (EAggEC), which was further abbreviated to EAEC. EAEC have a distinctive pattern of aggregative adherence (AA) to cultured human epithelial cells *in vitro* (Wai *et al.*, 1996), and adhere in small clumps on the surface of the small intestinal mucosa in a 'stacked brick' lattice-like pattern (Savarino *et al.*, 1991). A number of EAEC strains possess 2-3 nm diameter bundle-forming fimbriae, named aggregative adherence fimbriae I (AAF/I). These are thought to be involved in the initial adherence of bacteria to the intestinal mucosa. The fimbriae are encoded on a 60 MDa plasmid termed pAA that is required for AA (Nataro *et al.*, 1994; Rich *et al.*, 1999).

Following adherence, EAEC enhance mucus secretion from the mucosa, producing a bacterium-mucus biofilm (Nataro and Kaper, 1998). EAEC may then elaborate a cytotoxin, which damages the epithelial cells. A candidate for this toxin is the low molecular weight, heat-stable, plasmid-encoded enterotoxin named; EAEC heat-

stable enterotoxin 1 (EAST1) (Savarino *et al.*, 1991; Savarino *et al.*, 1993). EAST1 has homology with the enterotoxic domain of heat-stable enterotoxin STa of enterotoxigenic *E. coli*, and is postulated to stimulate the particulate form of guanylate cyclase through the same receptor-binding region as STa (Savarino *et al.*, 1993). EAEC also produce a 108 kDa heat-labile enterotoxin and cytotoxin named Pet (plasmid-encoded toxin) (Eslava *et al.*, 1998). Pet is encoded on the pAA virulence plasmid and is suggested to induce enterotoxic effects, tissue damage, inflammation and mucus secretion (Navarro-García *et al.*, 1998).

1.3.3 Enteroinvasive *E. coli* (EIEC)

EIEC are closely related to *Shigella* in their virulence (Beutin *et al.*, 1997). EIEC penetrate and destroy the colonic epithelium by invading intestinal mucosal cells, whereupon they proliferate in the epithelial cell and spread laterally form cell to cell, which results in inflammation and ulceration of the colonic mucosa (Levine, 1987; Echeverria *et al.*, 1992; Berlutti *et al.*, 1998). EIEC possess a large 140 MDa invasivity (inv) plasmid that contains the genes for epithelial cell invasion (Beutin *et al.*, 1997). The transcription of the virulence plasmid pINV is controlled by environmental stimuli and temperature, being induced at 37°C and repressed at 30°C (Berlutti *et al.*, 1998). EIEC resemble *Shigella*, but do not produce Shiga toxin (Levine and Edelman, 1984). However, they are expected to produce one or more enterotoxins that elicit the secretory diarrhoea, one such enterotoxin may be *Shigella* enterotoxin (Sen) (Nataro *et al.*, 1995).

1.3.4 Enterohaemorrhagic *E. coli* (EHEC)

EHEC are a subset of STEC (Shiga toxin-producing *E. coli*), and have previously been termed VTEC (verotoxigenic *E. coli*). EHEC colonise the intestinal mucosa, and, by subverting the intestinal epithelial cell function, produces a characteristic histopathological feature known as the 'attaching and effacing' (A/E) lesion (Moon *et al.*, 1983). EHEC are distinguished from EPEC by the production of high levels of

Shiga toxin, and a plasmid that encodes fimbriae (Levine, 1987; Liu *et al.*, 1999). EHEC can cause acute gastroenteritis and haemorrhagic colitis, and produce severe renal and neurological complications as a result of the translocation of Shiga toxins (Stx) across the gut (Frankel *et al.*, 1998). It has been postulated that EHEC cause disease by killing or irreversibly harming sensitive cells by a non-specific blockade of mRNA translation, resulting in cytotoxicity due to the lack of production of critical molecules required to maintain cell integrity (Thorpe *et al.*, 1999). Work by Thorpe *et al.* (1999) has suggested that Stx provokes increased synthesis and secretion of the chemokine IL-8. EHEC infection is associated with bloody diarrhoea that may be attributable to alterations in the architecture of the intestinal epithelial cells. Infection by EHEC alters the distribution of ZO-1, a tight junction-associated protein (Philpott *et al.*, 1998). Tight junctions act as a barrier to intracellular diffusion across the epithelium; disruption of this barrier would affect the permeability of these cells.

A cartoon representation of the mechanisms of disease of these four virotypes of *E. coli* is shown in Figure 1.1.

1.4 Enteropathogenic *E. coli* (EPEC)

EPEC infection is not generally thought to cause severe diarrhoea after the neonatal period (Hill *et al.*, 1991). However, EPEC remain a major cause of infantile morbidity and mortality in developing countries, killing hundreds of thousands of children per year worldwide (DeVinney *et al.*, 1999b), while outbreaks in more developed countries are sporadic. EPEC exerts its pathogenic effects by initially adhering to the surface of enterocytes in small. Where bacteria have adhered, the microvilli on the epithelial cell are absent, and in areas of no bacterial adherence the microvilli become extended, producing the characteristic 'attaching and effacing' (A/E) lesion (Moon *et al.*, 1983). The A/E lesion is the hallmark of EPEC infection and is formed when EPEC adhere to epithelial cells *in vitro* or *in vivo* (Donnenberg *et al.*, 1997). It is characterised by localised destruction of the brush border microvilli, intimate bacterial adhesion and cytoskeletal reorganisation, with a proliferation of

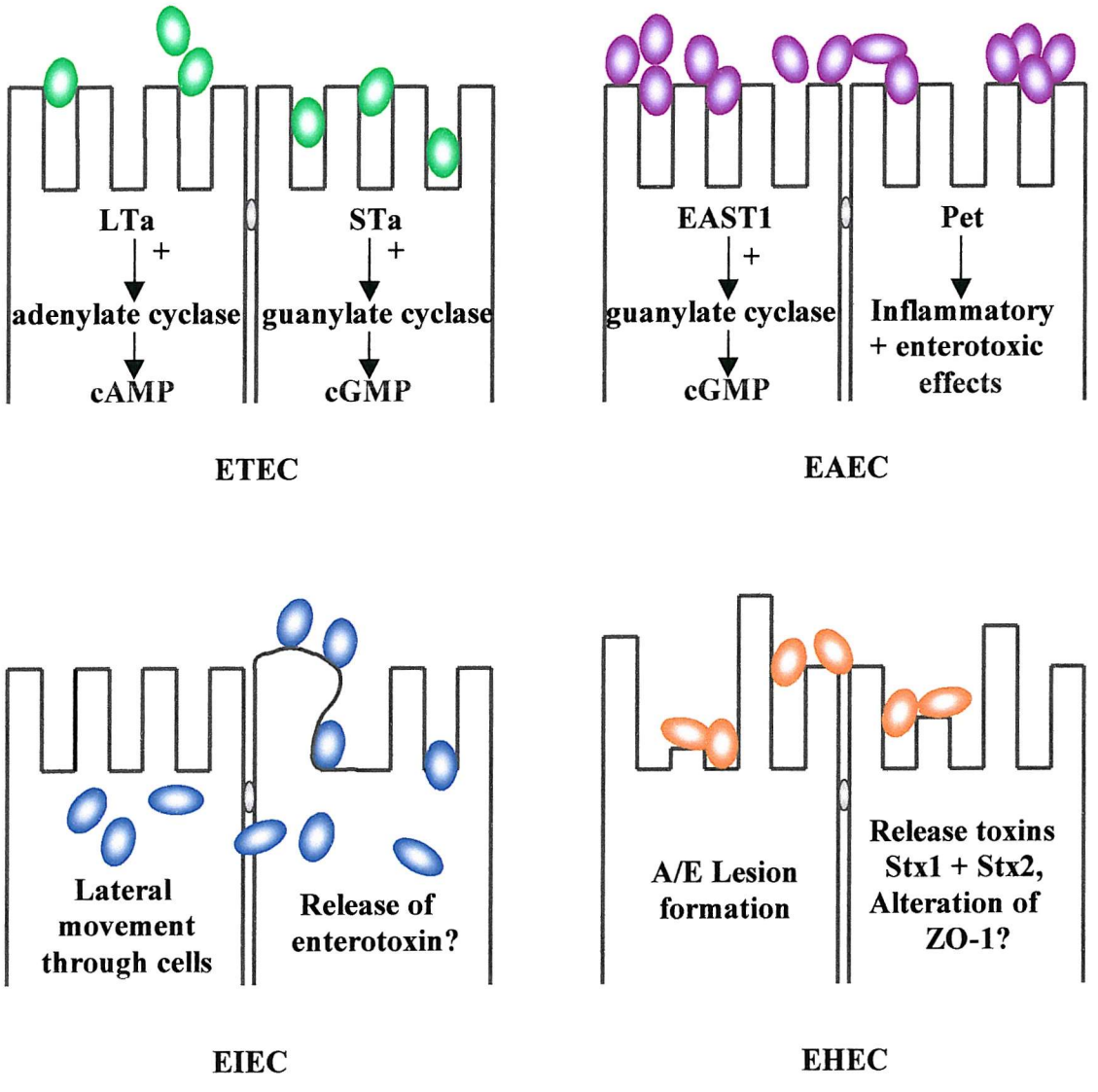


Figure 1.1. Summary diagram showing the disease mechanisms of ETEC, EAEC, EIEC and EHEC. The apical surface of the intestinal epithelial cells is shown; the coloured spheres represent adherent *E. coli*.

actin beneath the adherent bacteria resulting in the formation of a cup-like structure - the pedestal - to which the bacteria become intimately attached. The characteristic accumulation of actin beneath the attached bacterium in A/E lesions is diagnostic and has given rise to the fluorescent-actin staining (FAS) test Knutton *et al.* (1989). (Fluorescein isothiocyanate (FITC)-conjugated phalloidin is used to identify filamentous actin that accumulates beneath the adherent bacteria). The exact events that lead to fluid secretion and diarrhoea during EPEC infection are still unclear. A possible mechanism will be reviewed in later sections of this chapter.

EPEC strains are considered to belong to 12 different O serotypes, which have diverged into two major groups of clones - EPEC clones 1 and 2. Within each clone a number of O antigens are present, while the H antigens are conserved (Adu-Bobie *et al.*, 1998). The bacterial strains used in the present studies were E2348/69, the prototype O127:H6 EPEC strain, and JPN15, a derivative of E2348/69 that lacks the EAF plasmid (Levine *et al.*, 1985). Since this virotype is the principal concern in this study, the mechanism by which EPEC strains cause disease is explained in further detail within this introduction. One proposed model of EPEC infection is summarised in Figure 1.2.

1.5 EPEC attachment to host cells

Bacterial adhesion to host cells is one of the first steps in infection, and is believed to be a prerequisite for pathogenic events. Rather than uniformly covering cells (diffuse adherence), incubation of EPEC with cultured epithelial cells leads to the formation of distinct microcolonies of attached bacteria to the epithelial surface. This distribution is termed localised adherence (LA) (Scaletsky *et al.*, 1984) and is mediated by the bundle-forming pilus (BFP), encoded on the EPEC adherence factor (EAF). BFP are produced within the microcolony of adherent bacteria and form a meshwork of interbacterial fibres that stabilises the colony, subsequently they are involved in the dispersal of bacteria from bacterial microcolonies (Knutton *et al.*, 1999).

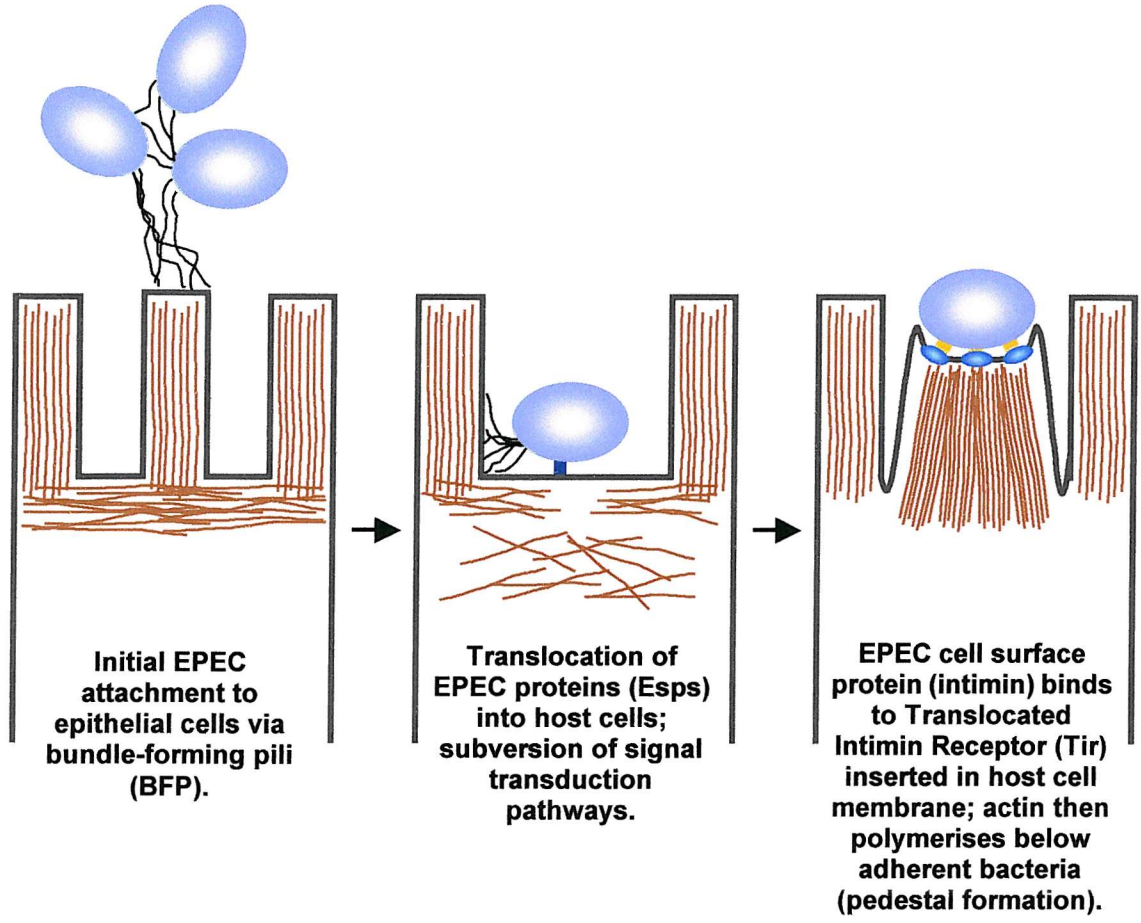


Figure 1.2. A cartoon representation of the three stages of EPEC infection, involving adherence, signal transduction and intimate attachment, has been proposed by Donnenberg and Kaper, (1992b).

Previous studies using cultured epithelial cells of non-intestinal origin implicated a type IV pilus – BFP, as the initial EPEC attachment factor (Giron *et al.*, 1991). However, recent studies by Hicks *et al.* (1998), using *in vitro* organ culture (IVOC) of paediatric small intestinal tissue, suggest that BFP-mediated localised adherence may be more tissue specific. EPEC strains interact differently with intestinal tissue from children than with cell lines in culture. The findings of Hicks *et al.* (1998) suggest that in human intestinal organ culture, BFP appears not to be involved in the initial stages of EPEC non-intimate adherence, but rather is involved in the formation of complex 3D structures permitting bacteria-bacteria interactions. This

indicates that non-intimate adhesion to the host cell is mediated via adhesin(s) other than BFP. BFP remain important for the formation of microcolonies on the cell surface. EPEC strains harbouring the EAF plasmid have the ability to form microcolonies on the epithelium and on cultured epithelial cells (Levine *et al.*, 1985). Studies using human gut tissue also indicate the formation of dense microcolonies, suggesting that LA occurs *in vivo* in the host (Yanomoto *et al.*, 1992). The EAF plasmid is required for full virulence of EPEC strains possessing this plasmid, as exemplified by human volunteer studies (Levine *et al.*, 1985). The EAF plasmid is not essential for the A/E phenotype; however, as plasmid cured strains can still cause the A/E lesion. Nonetheless, strains cured of the EAF plasmid have a reduced ability to adhere to epithelial cells and are less virulent than plasmid containing strains (Jerse and Kaper, 1991; Knutton *et al.*, 1997). Non-adherent *E. coli* K-12 expressing the plasmid become HEp-2 adhesive. However, unlike the EPEC strain, they do not show intimate attachment or cytoskeletal rearrangements (Knutton *et al.*, 1987c).

The EAF plasmid contains a 12 kb region that encompasses the *bfp* operon, a cluster of 14 genes coding for proteins necessary for BFP biogenesis. The operon includes *bfpA*, which encodes pilin, the major structural subunit of the EPEC bundle forming pilus (Donnenberg *et al.*, 1992a). BfpA is initially expressed as a pre-protein, which is cleaved to its mature form by the product of *bfpP* a pre-pillin peptidase (Zhang *et al.*, 1999). The formation of a disulphide bond in the C-terminus of pilin, confers protein stability, and is crucial for the biogenesis of type IV fimbriae (Zhang and Donnenberg, 1996).

Initially, BFP consists of 4 to 7 nm diameter fimbrial structures that aggregate to form a filament of 10-100 laterally aligned filaments (Figure 1.3A). These filaments form a network of fibres that promotes links between bacteria within microcolonies attached to cultured epithelial cells. The net effect is to promote bacteria-bacteria interactions and the aggregation of bacteria to the epithelial cell, i.e. microcolony formation (Figure 1.3B) (Girón *et al.*, 1991; Knutton *et al.*, 1999). Subsequently, BFP undergo structural changes resulting in the formation of longer and thicker (100

nm diameter) bundles, which have the propensity to develop into thicker bundles. The development of thick bundles causes a disruption of bacteria-bacteria interactions resulting in the dispersal of the bacteria from their aggregates (Bieber *et al.*, 1998; Knutton *et al.*, 1999).

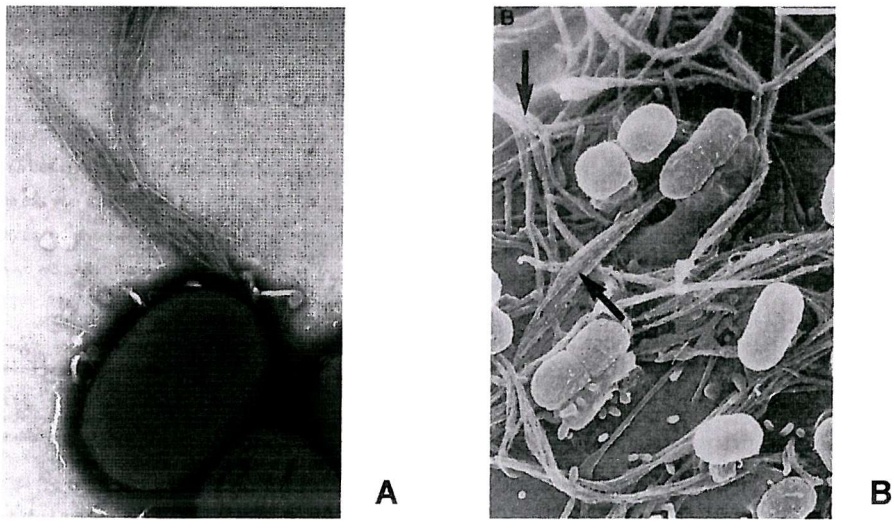


Figure 1.3. (A) Transmission electron microscopy revealing BFP expression by wild-type EPEC strain E2348/69. Reprinted by permission from Blackwell Science (Stone *et al.*, 1996. *Molec. Microbiol.* **20**, 325-337) copyright (1996) Blackwell Science Ltd. (B) Scanning electron micrograph showing HEp-2 cells infected with EPEC strain E2348/69 for 6 h, indicating a three-dimensional network of BFP bundles above A/E bacteria. Arrows indicate aggregated filaments twisted around each other to form thick bundles, scale bar 0.5 μm. Reprinted by permission from Blackwell Science (Knutton *et al.*, 1999. *Molec. Microbiol.* **33**, 499-509) copyright (1999) Blackwell Science Ltd.

The expression of BFP is not constitutive but regulated at the transcriptional level during the exponential phase of growth and is controlled by conditions such as the concentration of ammonium and calcium ions, as well as the temperature (Girón *et al.*, 1991; Donnenberg *et al.*, 1992a; Puente *et al.*, 1996; Ramer *et al.*, 1996; Tobe *et al.*, 1996). The transcription of the *bfp* operon is positively regulated by the expression of a second operon, the *bfpTVW* operon - also known as *perABC*. This

operon is located as a separate locus on the EAF plasmid downstream of the *bfp* operon (Gomez-Duarte and Kaper, 1995; Tobe *et al.*, 1996).

1.6 The Locus of Enterocyte Effacement (LEE)

The ability of EPEC to induce A/E lesions is dependent on the production of a secretion system that delivers specific proteins into host cells. This system is classified as a type III secretion system and operates when bacteria make contact with host cells. EPEC possess a 35 kb pathogenicity island termed the LEE, for Locus of Enterocyte Effacement (McDaniel *et al.*, 1995). This region when transferred to avirulent *E. coli* is sufficient to confer the ability to induce A/E lesions, suggesting that all of the pathogen specific genes necessary for inducing A/E lesions are encoded within this pathogenicity island (McDaniel and Kaper, 1997). The LEE is not unique to EPEC and similar pathogenicity islands are found in EHEC, RDEC-1, *Citrobacter rodentium* and diarrhoeagenic *Hafnia alvei*, suggesting a common pathway underlying A/E lesion formation.

The complete LEE region of EPEC has been sequenced. Analysis of the sequence suggests that it contains 41 predicted open reading frames of 50 or more amino acids arranged in at least five polycistronic operons (Elliott *et al.*, 1998). The complete region of the LEE is 35,624 bp with an average G + C content of 38.4%, which is significantly lower than the G + C content of the *E. coli* chromosome (50.8%), suggesting that it was acquired by horizontal gene transfer from another source (Blattner *et al.*, 1997; Elliott *et al.*, 1998; Wieler *et al.*, 1997). Genes necessary for virulence in a number of species are believed to have been acquired by horizontal gene transfer, and such determinants are often located on mobile genetic elements such as phages, plasmids or transposons, which can be transferred from one cell to another (Karch *et al.*, 1999). The LEE of the prototype EPEC strain E2348/69 is inserted into the *E. coli* K-12 chromosome at 82 min immediately adjacent to the *selC* tRNA locus encoding the tRNA for selenocystine. This is also the site of insertion for a different pathogenicity 'island' in uropathogenic *E. coli* and suggests that this

region of the *E. coli* chromosome may be a 'hot-spot' for the insertion of genes encoding virulence factors (McDaniel and Kaper, 1997; Nataro and Kaper, 1998).

The LEE of EPEC can be broadly divided into three regions, a large region encoding the genes for the production of the type III secretion system, a middle region containing the genes for intimate attachment, and a region encoding the secreted proteins (See Figure 1.4.) (Elliott *et al.*, 1998). These three regions are organised as five major polycistronic operons *LEE1* through to *LEE4* and *LEE5* (previously known as the Tir operon). *LEE1*, *LEE2* and *LEE3* encode components of the type III secretion apparatus, the *esc* (*E. coli* secretion genes) and *sep* (secretion of *E. coli* protein) genes, while the *LEE5* operon contains the genes for Tir, CesT (a chaperone for Tir), and intimin. *LEE4* encodes the secreted Esps (*E. coli* secreted proteins) (McDaniel and Kaper, 1997; Elliot *et al.*, 1998; Mellies *et al.*, 1999). The LEEs of EPEC O127:H6 and EHEC O157:H7 are highly conserved at the DNA and protein level for components of the type III secretion system, but show variability for the *esp*, *eae* and *tir* genes (Frankel *et al.*, 1998; Sperandio *et al.*, 2000).

Expression and secretion of the type III secretion system and other genes on the LEE is influenced by a number of environmental conditions including pH, temperature and media (Haigh *et al.*, 1995; Rosenshine *et al.*, 1996a; Kenny *et al.*, 1997b). Maximal LEE gene expression occurs with conditions similar to those of the intestine. Thus secretion is maximal at 37°C, pH7, and physiological osmolarity and requires the presence of sodium bicarbonate and calcium (Kenny *et al.*, 1997b). Kenny *et al.* (1997b), suggest that the activation of expression of virulence factors in the small intestine may in part be triggered by the high levels of sodium bicarbonate released by the intestinal mucosa to neutralize the acidic contents from the stomach. Moreover, the increased concentration of ammonia in the colon could reduce BfpA expression and protein secretion, (events shown to be down-regulated in the presence of ammonia), switching off virulence gene expression after passage through optimal colonization sites (Puente *et al.*, 1996; Kenny *et al.*, 1997b).

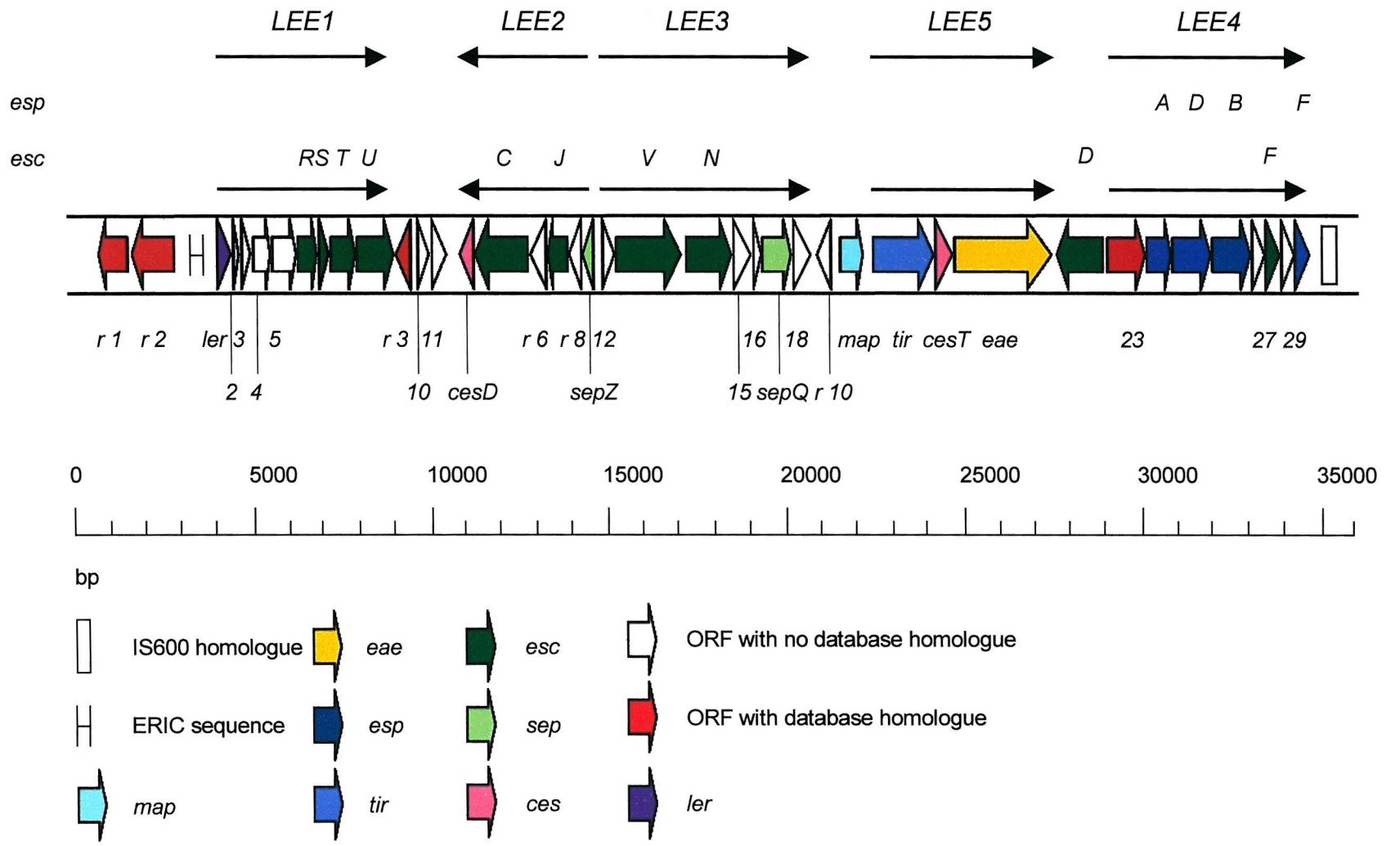


Figure 1.4. The LEE of EPEC E2348/69. The thin arrows indicate operons, whereas the thicker arrows indicate the sizes of the open reading frames, which are colour coded according to the suspected function of the corresponding protein. Adapted from Elliott *et al.* (1998). (Note: the colour coding for these gene products is continued in future illustrations)

1.7 Protein secretion and type III secretion systems

Secretion of proteins by gram-negative bacteria requires the delivery of target proteins across the inner membrane, the outer membrane and the intervening periplasmic space. Bacteria employ several methods to achieve this feat. The known mechanisms include the type I pathway, the type II sec-dependent pathway and the more recently discovered type III pathway. The second stage of the EPEC pathogenesis involves the production of a type III secretion system that is used to deliver effector proteins to eukaryotic cells, involved in triggering signalling events and cytoskeletal rearrangements. Some of these proteins are delivered into the extracellular environment, while others are translocated directly into the host cell. Type III secretion systems are not unique to EPEC and are employed for the export of virulence factors by a number of bacteria, including *Yersinia*, *Shigella flexneri*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa* (Hueck, 1998). The components of the type III secretory machinery are broadly conserved between the species while the proteins secreted across the bacterial cell envelope vary. In *Yersinia* the secreted proteins are termed Yops - *Yersinia* outer-membrane proteins, (Cornelis and Wolf-Watz, 1997) and in *Salmonella* Sips and Sops - *Salmonella* inner and outer-membrane proteins respectively (Collazo and Galan, 1997).

Type III secretion systems are complex organelles, the product of 20 or more gene products, that enable gram negative bacteria to secrete and inject pathogenicity proteins into the cytosol of eukaryotic host cells. The proteins comprising the type III secretion apparatus form a channel across both the inner and outer membrane of the bacterium, and form a pore in the host cell epithelium (Donnenberg, 2000). A common feature of proteins secreted through the type III secretion apparatus is the lack of a cleavable leader peptide sequence, and the requirement for specific chaperones. Also, the initiation methionine is present on all proteins (known at present) that are secreted through the type III secretion system other than Tir (Kenny *et al.*, 1997a).

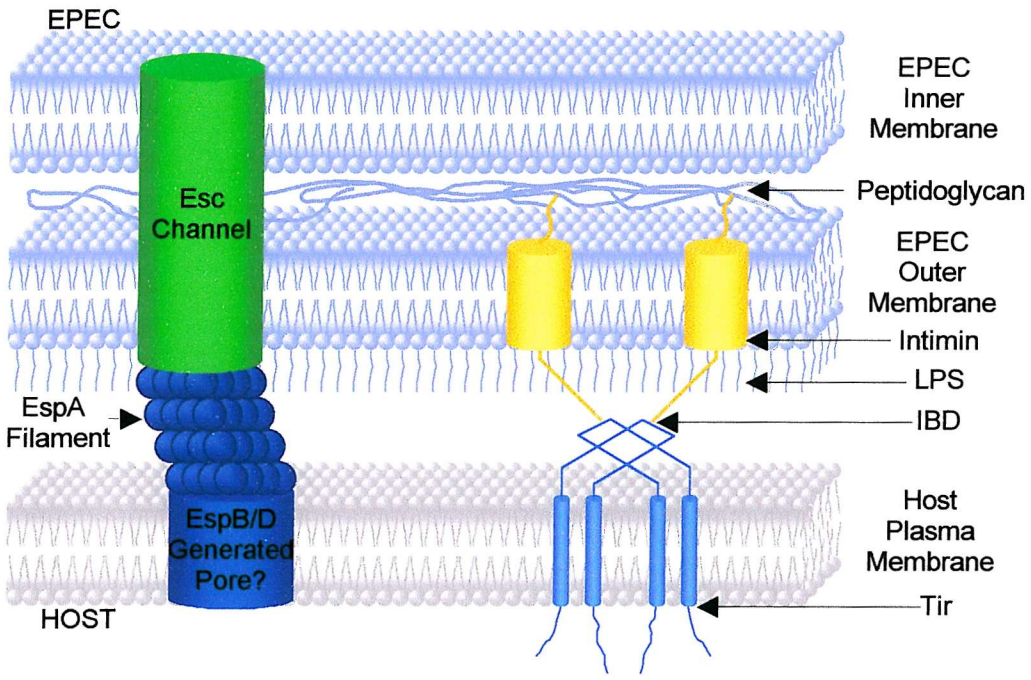


Figure 1.5. Model of the proposed translocon, consisting of pores in the bacterial envelope (EscC-generated pore) and in the host cell membrane (EspB/D-generated pore) with the pores connected by a hollow EspA filament. Energy for protein translocation may be provided by the EscN protein. Adapted from Celli *et al.* (2000).

1.7.1 EPEC-secreted proteins (Esps)

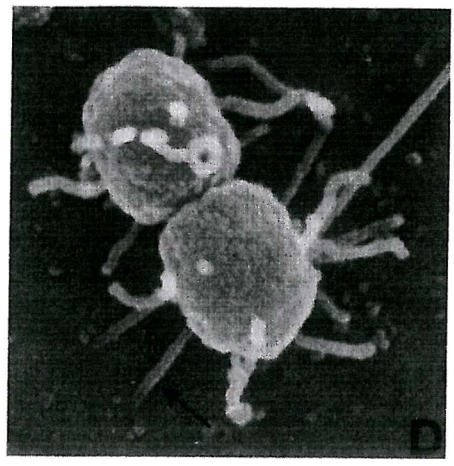
Secretion of Esps is required for signal transduction in host cells and A/E lesion formation, and is induced when bacteria are grown in a tissue culture medium that mimics the conditions of the gastrointestinal tract (Haigh *et al.*, 1995; Kenny and Finlay, 1995; Kenny *et al.*, 1997b). Proteins secreted by type III secretion systems are classified as either effector proteins, which act to subvert host cell processes, or translocases which deliver effector proteins into the eukaryotic cell (Hartland *et al.*, 2000). Although *in vitro* experiments with knockouts and human volunteer studies have indicated that Esps are necessary for A/E lesion formation, the precise role of Esps in EPEC pathogenesis is largely unknown.

1.7.2 EspA

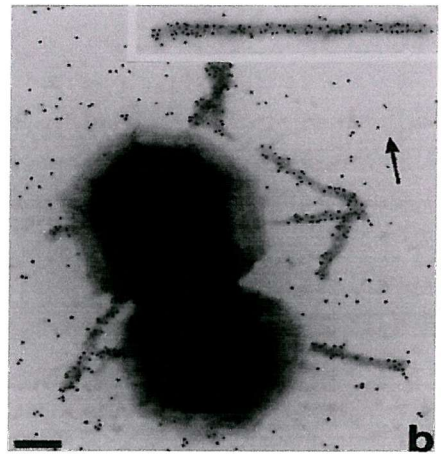
EspA (molecular weight 25 kDa) is secreted by the type III secretion system and becomes a major component of a bacterial surface organelle, forming a direct link between the bacterium and the host cell during A/E lesion formation (Knutton *et al.*, 1998). Current understanding suggests that the EspA filament bridges a pore from the bacterial cell produced by EscC, and a pore in the host cell produced by EspB and EspD, allowing the translocation of bacterial proteins such as EspB into the host cell and Tir to the host cell membrane (Kenny *et al.*, 1997a; Knutton *et al.*, 1998; Neves *et al.*, 1998). It is postulated that a molecular syringe could form, powered by the type III secretion ATPase EscN, permitting the passage of proteins from the bacterium to the host (Hartland *et al.*, 2000).

The carboxyl-terminus of EspA has an α-helical region, predicted to contain six heptad repeats, indicating a propensity for coiled-coil interactions leading to filament assembly (Lupas, 1996; Pallen *et al.*, 1997; Delahay *et al.*, 1999). Mutation of the coiled-coil regions of EspA does not affect expression or secretion of EspA, but has consequences on formation of EspA filaments, and subversion of host cell signal transduction pathways (Delahay *et al.*, 1999). EspA filaments are probably constructed from bundles of smaller fibrils resembling pili. A number of 7 nm diameter fimbrial structures are believed to come together to form a 50 nm diameter, 2 μm long hollow cylindrical filament (Figure 1.6.) (Knutton *et al.*, 1998). The hollow centre of the filament is postulated to form a channel through which proteins are delivered to the host cell (Knutton *et al.*, 1998; Frankel *et al.*, 1998). However, studies to date have been unable to show co-immunoprecipitation of EspD or Tir within EspA filaments. This could suggest that they are not located in the filament prior to host cell attachment (Hartland *et al.*, 2000). Neither EspB nor EspD is required for EspA multimerization (Delahay *et al.*, 1999), although EspD is important in assembly and elaboration of the filament on the bacterial cell surface (Knutton *et al.*, 1998). EPEC *espA* mutants do not induce phosphorylation of Tir, and do not activate epithelial cell signal transduction or cause cytoskeletal rearrangements

(Kenny *et al.*, 1996). Since the EspA filaments disappear after A/E lesion formation there is proposed to be a sensing or regulatory mechanism controlling the expression or delivery of EspA (Knutton *et al.*, 1998).



A



B

Figure 1.6. (A) Scanning electron micrograph showing EspA filaments on the bacterial surface of strain JPN15, forming a bridge between bacteria before A/E lesion formation. x 30 000 (B) Electron micrograph showing E2348/69 immunogold labelled with EspA antiserum (scale bar 200 nm). Both figures reprinted by permission from Oxford University Press (Knutton *et al.*, 1998. *EMBO J.* **17,** 2166-2176) copyright (1998) Oxford University Press.

1.7.3 EspB

EspB, formerly EaeB (Donnenberg *et al.*, 1993a), is a 37 kDa protein that is produced and secreted upon contact with HeLa cells (DeVinney *et al.*, 1999a). It is translocated shortly after bacterial contact with host cells, suggesting its secretion is cell contact dependent (Frankel *et al.*, 1998; Wolff *et al.*, 1998). EspB is not a component of the EspA filament, although, as mentioned above, these filaments are required for the translocation of EspB to the host cell surface (Knutton *et al.*, 1998). At the host cell surface, EspB becomes distributed in both the membrane and cytosol (Frankel *et al.*, 1998; Wolff *et al.*, 1998). EspB requires other proteins for its efficient translocation into host cytoplasm. Moreover, secretion of EspB into the medium

occurs in addition to its being translocated into the host cell (Taylor *et al.*, 1998). EspB co-immunoprecipitates with EspA filaments, and EspB co-localizes closely with EspA during EPEC infection of HEp-2 cells. The exact function of EspB is unknown. However; it has sequence homology with YopD of *Yersinia* and based on this homology, EspB and EspD have been suggested to form a channel in the host cell epithelium to permit the translocation of EPEC effector proteins into the host cell. In the presence of host cells, EspB is protease resistant (Kenny and Finlay, 1995) implying it doesn't have a surface location, although interaction with other Esps may prevent its degradation (Hartland *et al.*, 2000). EPEC *espB*$^-$ mutants are still able to produce EspA filaments (Knutton *et al.*, 1998), and EspA filaments interact directly with the host cell surface, even in *espB*$^-$ mutants. Thus it is proposed that even if EspB and EspD are involved in forming a pore in the host cell, this structure is not the initial receptor for EspA filament interaction with the host (Hartland *et al.*, 2000).

As with EspA, EspB is also predicted to contain a large number of serine and threonine residues near the amino terminus (Donnenberg *et al.*, 1993a; Kenny *et al.*, 1996). The predicted EspB sequence also reveals a motif common to enzymes that bind pyridoxal phosphate (Donnenberg *et al.*, 1993a). However, recent results do not support a role for pyridoxal phosphate in EspB function (Taylor *et al.*, 2001). EspB is required for activation of signal transduction cascades within the host leading to tyrosine phosphorylation of Tir, and the accumulation of actin beneath the adherent bacteria (Donnenberg *et al.*, 1993b; Foubister *et al.*, 1994a). Moreover, EspB is a virulence factor for EPEC. Tacket *et al.* (2000) demonstrated that diarrhoea developed in 9/10 people given wild-type EPEC, but only in 1/10 volunteers given an *espB*$^-$ mutant strain. In addition to its putative structural role in the formation of the translocon, EspB could also be an effector protein once transmitted into host cells. When expressed in transfected cells EspB causes a reduction in actin stress fibres and cellular morphology changes. It does not co-localise with actin, but is distributed throughout the cytoplasm (Taylor *et al.*, 1999; Tacket *et al.*, 2000).

1.7.4 EspD

EspD, the product of the *espD* gene, is a 39 kDa-secreted protein with an as yet unknown function (Lai *et al.*, 1997; Wachter *et al.*, 1999). Like EspB, EspD secretion is triggered by contact with HeLa cells (Wachter *et al.*, 1999). EspD has two predicted coiled-coil domains, possibly involved in mediating structural interactions with EspA and/or other proteins. The interactions might explain how the EspA filament is ultimately anchored to the host cell surface (Pallen *et al.*, 1997; Delahay *et al.*, 1999). It has recently been shown that EspD becomes integrated into the host cell membrane in a surface exposed location, at sites of bacterial contact, where it is postulated to become part of a translocation system delivering bacterial effector proteins into the host cell (Wachter *et al.*, 1999). EspD mutants exhibit a reduced level of secretion of EspA and EspB, and are unable to induce accumulations of actin or tyrosine phosphorylated protein beneath attached bacteria (Lai *et al.*, 1997).

1.7.5 EspF

The function of EspF in EPEC pathogenesis is currently unknown as *espF* mutants can still form A/E lesions. EspF is a proline rich protein and contains 2 proline-rich repeats similar to Src homology 3 (SH3) domains. These domains mediate protein-protein interactions in many signalling pathways in eukaryotic cells. Therefore it is proposed that EspF could be an effector protein (McNamara and Donnenberg, 1998).

1.7.6 Mitochondrial associated protein – MAP

The gene upstream of *tir*, *orf19*, has recently been shown to encode a type III secreted effector protein. The protein product of *orf19*, MAP, has homology to the EPEC TrcA/TrcP proteins and to the *Shigella* IpgB protein, and is delivered into host cells via a mechanism independent of endocytosis, but dependent on EspB (Kenny and Jepson, 2000). Within the host MAP is targeted to the host mitochondria, probably

via its putative N-terminal signal sequence, where it disrupts the membrane potential of the mitochondria underlying the site of infection, resulting in a loss of mitochondrial integrity (Kenny and Jepson, 2000). Interestingly, jejunal biopsy samples from infants infected with EPEC exhibit deformed mitochondria in damaged enterocytes (Rothbaum *et al.*, 1982).

1.7.7 Translocated intimin receptor (Tir)

Because Tir was originally observed as a tyrosine phosphorylated protein of 90 kDa in the membranes of infected host cells it was originally referred to as host protein 90 kDa (Hp 90) (Rosenshine *et al.*, 1992). A similar protein termed EspE has been described in Shiga toxin *E. coli* strains (Deibel *et al.*, 1998). Tir is a bacterial protein secreted through the type III secretory apparatus and translocated into the host cell, facilitated by the type III secreted products EspA and EspB. Following translocation it becomes tyrosine phosphorylated and assembles into the plasma membrane, where it functions as the receptor for the bacterial outer membrane protein intimin (Kenny *et al.*, 1997a). Proteins secreted by the type III secretion system retain their first methionine. However, Tir lacks the initial methinoine, which possibly suggests that it may not directly use the type III secretion system, although EspA, EspB and the type III secretion system are required for its efficient transfer to host cells (Kenny *et al.*, 1997a). Tir is predicted to encode a 56.8 kDa protein, although the protein migrates at around 78 kDa on SDS-PAGE, possibly reflecting its high glycine content and/or post-translational modifications (Kenny *et al.*, 1997a; Deibel *et al.*, 1998). Upon contact with the host cell, EPEC translocates Tir as a 78 kDa protein through the type III secretion system into the host cell membrane, concomitant with post-translational modifications in the host cell. These modifications include phosphorylation on serine and/or threonine residues associated with an increase in molecular weight to 90 kDa, possibly reflecting a conformational change. These events are accompanied by tyrosine phosphorylation of Tir, which does not alter the mass of Tir, but is essential for actin nucleating activity (Kenny *et al.*, 1997a; Kenny, 1999). The translocation of Tir into the host cell membrane increases with the duration of infection (Gauthier *et*

al., 2000). Modified Tir then interacts with the bacterial outer membrane protein intimin, which leads to intimate adherence of EPEC to the surface of the host cell, concomitant pedestal formation and host signalling events such as activation of phospholipase C γ-1 (Rosenshine *et al.*, 1992; Kenny and Finlay; 1997; Kenny, 1999; de Grado *et al.*, 1999; Gauthier *et al.*, 2000). Recently it has been shown that Tir does not encode sufficient information to facilitate its complete modification when introduced into host cells in an EPEC independent manner, implying that Tir is co-expressed with other EPEC factors to enable its full modification within cells (Kenny and Warawa, 2001).

A hairpin model has been proposed for Tir topology in the host cell membrane, in which Tir uses two transmembrane domains to span the host cell membrane, with its N- and C-terminal regions located within the host cell, and an 109 amino acid spanning region acting as an extracellular loop for the interaction with intimin (Kenny and Finlay, 1997; Kenny *et al.*, 1997a; Kenny, 1999; de Grado *et al.*, 1999; Hartland *et al.*, 1999). The region of Tir involved in interacting with intimin is named differently depending on the author, and hence is variously termed TIBA (Tir-intimin binding area) (Kenny, 1999) and IBD (intimin binding domain) (de Grado *et al.*, 1999). The intimin-binding site of Tir has been localised to a central 107-amino-acid region (Tir-M) located between two predicted transmembrane domains (Hartland *et al.*, 1999; Kenny, 1999; de Grado *et al.* 1999). The Tir-intimin interaction leads to intimate binding of the bacterium to the host cell. Tir anchors the bacterium to the host cell cytoskeleton via an interaction between the amino-terminal end of Tir, and a host protein α-actinin, in a phosphotyrosine-independent manner, (Goosney *et al.*, 2000). Currently it is not known if Tir assembles into the plasma membrane directly after injection into the cytosol of host cells or if it first localises to an intermediate sub-cellular compartment. However, the rapidity of insertion suggests the former possibility.

The C-terminal domain of Tir that becomes inserted into the host cytoplasm contains six tyrosines that are potential targets for tyrosine phosphorylation (Kenny *et al.*,

1997a). Phosphorylation of tyrosine 474 appears to be critical for the actin nucleation activity of EPEC Tir (Kenny, 1999). If Tir is substituted at Y474F, Tir is delivered and focused in the host cell but tyrosine phosphorylation and pedestal formation do not occur (Goosney *et al.*, 2000). Surprisingly EHEC does not cause tyrosine phosphorylation of Tir (tyrosine 474 of EHEC strain O157:H7 is a serine) suggesting an alternative method for pedestal formation (Ismaili *et al.*, 1995; Deibel *et al.*, 1998; Kenny, 1999).

Tir is predicted to have at least three functions (Kenny *et al.*, 1997a; Goosney *et al.*, 2000). First, it acts as a receptor on epithelial cells, for the bacterial outer membrane protein intimin. Second it nucleates actin, following intimin binding, forming a stable interaction with α-actinin through its N-terminus in a phosphotyrosine-independent manner. Third, after Tir-intimin binding, it recruits additional cytoskeletal proteins at the carboxyl terminus in a phospho-tyrosine-dependent manner.

1.8 The requirement for chaperones by type III secreted proteins

Some type III secreted proteins require small cytoplasmic proteins with chaperone functions. Studies of type III chaperones from *Yersinia* and *Shigella* indicate that they are generally specific for one or two proteins. In common with other chaperonins, they are involved in preventing cytoplasmic proteolysis and the premature folding or aggregation of secreted proteins in the bacterial cytoplasm, and may also couple translation to secretion (Hueck, 1998; Karlinsey *et al.*, 2000b)

1.8.1 CesD – A secretion chaperone?

CesD is proposed to be a secretion chaperone in EPEC, owing to its sequence similarity to chaperones from other type III secretion pathways (Wainwright and Kaper, 1998). CesD is detectable in the bacterial cytosol and inner membrane and is required for the secretion of EspD and to a lesser extent EspB (but not EspA) into culture supernatants (Wainwright and Kaper, 1998). CesD mutant strains are

negative for the FAS test, and for tyrosine phosphorylation of Tir (Wainwright and Kaper, 1998). These findings are consistent with EspD and EspB forming part of a molecular syringe through which Tir passes to the host.

1.8.2 CesT - Chaperone for *E. coli* secretion of Tir

CesT is the product of the previously named OrfU, and its gene is located between the *eae* and *tir* genes on the LEE (Elliott *et al.*, 1998). Named CesT for the chaperone for *E. coli* secretion of Tir (Abe *et al.*, 1999; Elliott *et al.*, 1999), CesT is to stabilise Tir levels in the cytoplasm by protecting Tir from proteolysis (Abe *et al.*, 1999). CesT is predicted to bind post-transcriptionally with Tir in a multimeric complex, possibly a hexamer, and to act as an anti-degradation factor (Abe *et al.*, 1999; Elliott *et al.*, 1999). CesT is located in the cytoplasm (Elliott *et al.*, 1999), and is not found in EPEC culture supernatants. This suggests that it dissociates from Tir once Tir is secreted and translocated (Abe *et al.*, 1999). Mutation of *cesT* affects the ability of EPEC to induce cytoskeletal rearrangements. In accordance with this effect, there is reduced Tir secretion into culture supernatants and decreased amounts of Tir in the bacterial cytoplasm. However, it has no effect on the secretion of other type III secreted proteins (Abe *et al.*, 1999; Elliott *et al.*, 1999). The mature form of CesT lacks the first methionine, like Tir. Thus, both proteins are accessible to an N-terminal methionine-specific amino-peptidase (Abe *et al.*, 1999).

1.9 EspC – A protein whose secretion is co-regulated with the Esps

EspC is a highly immunogenic 110 kDa protein whose secretion is co-regulated with the Esp proteins, but is secreted by an alternative mechanism to the type III secretion system (Kenny and Finlay, 1995). Unlike the other proteins secreted as part of virulence causing process, EspC is not encoded on the LEE. Instead, *espC* is located within a second EPEC pathogenicity island (Mellies *et al.*, 2001). The predicted size of EspC is 1,306 amino acids; however, the first 53 predicted amino acids (which have characteristics of signal peptides involved in mediating sec-dependent export

across the cytoplasmic membrane) are absent from the secreted 110 kDa protein (Stein *et al.*, 1996a). EspC has homology to a family of proteins distantly related to the immunoglobulin A (IgA) protease family, which are widespread among pathogenic bacteria. These include the Tsh protein of the avian-pathogenic *E. coli* strain, SepA protein from *Shigella flexneri* and PssA from Shiga-toxin producing *E. coli* (Stein *et al.*, 1996a; Djafari *et al.*, 1997). These proteins do not encode IgA protease activity, but use a method for secretion that is similar to that of the IgA protease from *Neisseria gonorrhaeae* and *H. influenzae* (Stein *et al.*, 1996a). This subfamily of autotransporters has been termed SPATE, for serine protease autotransporters of Enterobacteriaceae (Mellies *et al.*, 2001). The secretion pathway of IgA protease-like proteins involves a two-step extracellular secretion. The family of proteins possess an N-terminal signal peptide and use the sec-dependent pathway to transverse the cytoplasmic membrane. They then auto-transport by generating a β-barrel pore in the outer membrane through which the N-terminal passenger domain is believed to be translocated (Klauser *et al.*, 1993; Mellies *et al.*, 2001). The putative serine endopeptidase site of EspC is not required for cleavage of the protease domain form the C-terminal helper domain, which implies that an additional protease may be responsible for the release of EspC into the extracellular space (Stein *et al.*, 1996a).

EspC is secreted by EAF minus strains, as well as by EAF plasmid containing strains. Studies show that EspC is not necessary for mediating EPEC induced signal transduction in HeLa cells, nor is it involved in the adherence to, or invasion of cultured epithelial cells (Stein *et al.*, 1996a). EPEC have traditionally been considered not to produce secretory enterotoxins or cytotoxins (Knutton *et al.*, 1987a), and instead exert their pathogenic effects by adhering to the host cell and generating the A/E lesion. However, EspC is tentatively proposed to be an enterotoxin, and has recently been shown to cause a rise in short circuit current and potential difference in rat jejunal tissue (Mellies *et al.*, 2001). EspC has homology with the Pet protein of enteroaggregative *E. coli*. Pre-incubation of jejunal tissue with antiserum against Pet, was found to eliminate the EspC enterotoxin activity (Mellies *et al.*, 2001).

1.10 The bacterial outer membrane protein Intimin

Intimin is the product of the *eae* gene, a 94 kDa bacterial outer membrane protein required for intimate attachment of bacteria to host cells (Jerse *et al.*, 1990; Jerse and Kaper, 1991; Donnenberg and Kaper, 1991). Intimin contains a cleavable N-terminal signal sequence, and is targeted to the outer membrane independently of the type III secretion system, where it serves as an adhesin (Jerse and Kaper, 1991; Gomez-Duarte and Kaper, 1995). Intimin exists as at least five antigenically distinct subtypes, which may contribute to tissue tropism. These subtypes are: intimin α, intimin β, intimin γ, intimin δ and intimin ε (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000). The different intimin types are postulated to possess different receptor-targeting specificities in EPEC pathogenesis (Hartland *et al.*, 1999). Intimin α is expressed mainly in EPEC (e.g. strain E2348/69) and intimin γ mainly in EHEC. This could contribute to the specificity of small intestine and Peyer's patch adhesion for intimin α expressing E2348/69 and adhesion to Peyer's patch follicle-associated epithelium for intimin γ expressing strains (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000; Phillips and Frankel, 2000). Interestingly, intimin shows regions of sequence homology with invasins. The amino acid sequences of intimin and invasin are homologous in the amino terminal 500 amino acids, but more divergent in the C-terminal binding domain (Frankel *et al.*, 1994; Adu-Bobie *et al.*, 1998; Batchelor *et al.*, 2000). Two stretches of amino acids are conserved in all intimin subtypes, and two cysteine residues are conserved at the C-termini among all the intimin family members, to form part of a binding domain (Adu-Bobie *et al.*, 1998).

Intimin is immunogenic in volunteers challenged with EPEC, and is recognised by serum from patients after infection with E2348/69 (Jerse and Kaper, 1991). Intimin is a virulence factor and required for full virulence as demonstrated by human volunteer studies, in which 11/11 volunteers given E2348/69 developed diarrhoea, while diarrhoea developed in 4/11 who were given an *eae*$^-$ mutant (Donnenberg *et al.*, 1993b). Microcolonies of *eae*$^-$ mutant EPEC strains form on cells, but there is no intimate attachment to the epithelial cell membrane, or accumulation of filamentous

material beneath the adherent bacteria. However, epithelial signals are still transduced to the cell and have a faint shadow in the FAS test suggesting some ability to change the host cytoskeleton structure (Jerse *et al.*, 1990; Rosenshine *et al.*, 1992; Donnenberg and Kaper, 1991; Rosenshine *et al.*, 1996b).

The expression of intimin by E2348/69 strains is culture media dependent. Intimin expression is induced when E2348/69 are grown to mid-log phase in DMEM at 37°C, when intimin can be found distributed over the surface of the bacteria (Gomez-Duarte and Kaper, 1995; Knutton *et al.*, 1997; Adu-Bobie *et al.*, 1998). In stationary phase broth cultures, only a small fraction of E2348/69 expresses intimin (Knutton *et al.*, 1997; Adu-Bobie *et al.*, 1998). Immunogold labelling of E2348/69 with HEp-2 cells, reveals a uniform distribution of intimin over the bacterial surface except at the site of bacterial attachment to the host cell (Knutton *et al.*, 1997). Expression of intimin is higher in E2348/69 than its plasmid cured strain JPN15 when grown in DMEM (Jerse and Kaper, 1991; Gomez-Duarte and Kaper, 1995). However, JPN15 exhibits a culture independent intimin expression phenotype, and this strain is reported to have higher levels of intimin expression than E2348/69 when grown in LB (Knutton *et al.*, 1997).

During A/E lesion formation intimin interacts with Tir to trigger the focused accumulation of actin and tyrosine phosphorylated Tir beneath the adherent bacteria (Rosenshine *et al.*, 1996b; Kenny and Finlay, 1997; Kenny, 1999). Intimin is an absolute requirement, along with modified Tir, for the formation of pedestals (Rosenshine *et al.*, 1992; Kenny and Finlay, 1997). The C-terminal domain of intimin is required for intimin-mediated attachment and invasion, and has been localised to the last 280 amino acids (Frankel *et al.*, 1994; Kenny, 1999). However, intimin can bind independently to host cells in the absence of Tir, which requires an intact lectin-like molecule at the C-terminal end of the intimin polypeptide (Hartland *et al.*, 1999). Moreover, the C-terminal 280 residues of intimin can bind to uninfected host cells through the same domain that can bind to Tir (Frankel, 1994; Rosenshine *et al.*, 1996b; Kenny *et al.*, 1997a; Adu-Bobie *et al.*, 1998). The Tir binding region of

intimin has been localised to the C-terminal 190 amino acids Int190 (Batchelor *et al.*, 2000), although other regions may be involved in interacting with Tir. In addition, Cys-937 is essential for intimin mediated cell binding (Frankel *et al.*, 1995). Inactive forms of Int280 in which Cys-937 is substituted for Ser or Ala, bind Tir, but do not bind to cells in the absence of Tir (Hartland *et al.*, 1999). The carboxyl terminal 280 amino acids of EPEC intimin in the presence of divalent ions have been reported to bind to β_1 integrins (Frankel *et al.*, 1996). Interestingly, enterocytes do not possess β_1 integrins on their apical surface (Beaulieu, 1992), although they are expressed on their basolateral membranes and on both the apical and basolateral membranes of M cells (Clark *et al.*, 1998). The possible role of intimin-integrin interactions is unclear, although it implies that intimin can bind to more than one receptor (Kenny, 1999). Recent studies suggest that intimin from *C. rodentium* and EPEC can induce mucosal hyperplasia and a T helper cell type 1 response in mice (Higgins *et al.*, 1999). This suggests that the binding of intimin to a host cell receptor may modify the immune response. Mucosal T lymphocytes express β_1 integrins. The induced mucosal proliferation and increased thickening would result in an increased surface area for bacterial colonization that may in turn promote infection (Higgins *et al.*, 1999).

The C-terminal 280 amino acid residues of intimin mediate intimate bacterial host-cell interaction. NMR studies of this region indicate that Int280 is built from three globular domains, two immunoglobulin-like domains D1 and D2 and a C-type lectin-like module D3 (Kelly *et al.*, 1999). The topology of D3 is reminiscent of C-type lectins despite the lack of significant sequence homology, and suggests that carbohydrate recognition may be important in intimin-mediated adhesion (Kelly *et al.*, 1999; Batchelor *et al.*, 2000). However, if intimin binds to carbohydrate, it must do so in a unique manner, as it lacks a cluster of calcium binding acidic residues, common to other carbohydrate binding molecules (Weis and Drickamer, 1996; Luo *et al.*, 2000). The topology of D1 and D2 resembles that of the immunoglobulin superfamily, which are involved in molecular recognition. It is proposed that D1 and D2 may form a linker extending from the bacterial surface, allowing D3 to make interactions (Kelly *et al.*, 1999). Subsequent studies have identified a further Ig-like

domain within the extracellular part of intimin (Batchelor *et al.*, 2000; Luo *et al.*, 2000). The nomenclature used to describe these domains differs between these two groups. Batchelor *et al.* (2000) renamed the domains D1, 2, 3 and 4 in which D4 now becomes the lectin-like domain, while Luo *et al.* (2000) maintain the original nomenclature, and name the new domain D0. Both studies suggest that the extracellular part of intimin comprises a rod of immunoglobulin domains extending from the bacterium surface, presenting an adhesive tip to the target cell (Batchelor *et al.*, 2000; Luo *et al.*, 2000). The Tir-binding fragment of intimin spans the immunoglobulin superfamily-like domain, D3(D2), and the lectin-like domain, D4(D3). The binding site of Tir is localised to a patch of residues at the tip of D4, in which the number of residues Tir is in contact with could be as low as 5 or up to 14 (Batchelor *et al.*, 2000). One proposed model of Tir-intimin binding is shown in Figure 1.7.

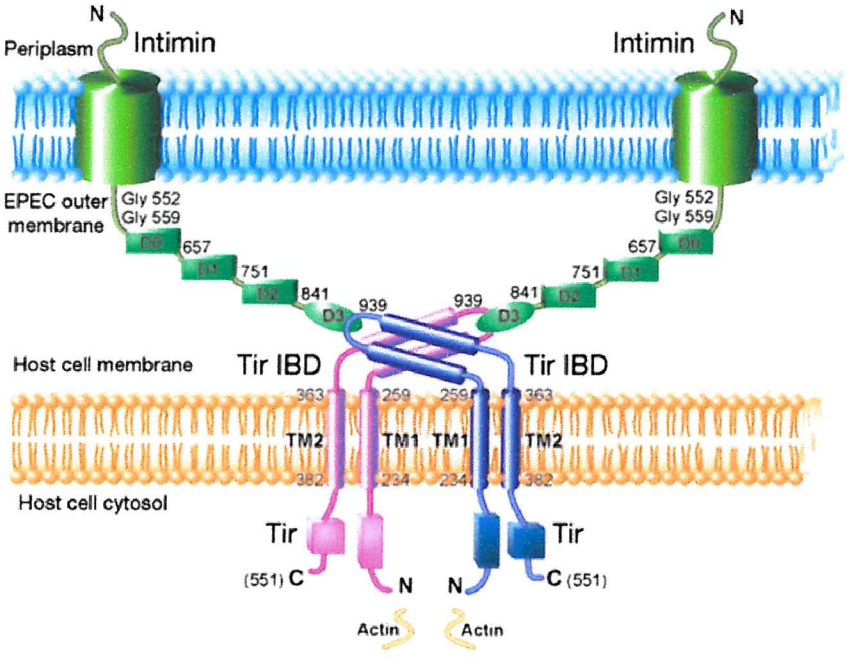


Figure 1.7. A proposed model of Tir-intimin binding based on structural data of the complex of the C-terminal fragment of intimin (domain D1, D2 and D3) and the extracellular Tir IBD. The Ig-like domains D0, D1 and D2 are shown as rectangles, and the lectin-like domain D3, which binds to the Tir IBD, as an oval. Reprinted by permission from Nature (Luo *et al.*, 2000. *Nature*. **405**, 1073-1077) copyright (2000) Macmillan Magazines Ltd.

1.11 Regulation of the LEE

While studies have concentrated on the discovery and importance of LEE encoded genes, it is only recently that the regulatory mechanisms behind the control of virulence gene expression on the LEE have started to be elucidated. A complex multi-factorial cascade system including the involvement of a number of transcriptional activators and repressors; Per, Ler, IHF, H-NS, along with quorum sensing are involved in positively and negatively regulating LEE gene expression.

1.11.1 Plasmid encoded regulator (Per)

A number of genes on the EPEC LEE are regulated by the plasmid-encoded regulator Per, encoded as a cluster of three ORFs believed to be transcribed as a single polycistronic message on the EAF plasmid. The three ORFs are termed *perA*, *perB* and *perC* and are also referred to as *bfpTVW*, (Gomez-Duarte and Kaper, 1995). Per has recently been shown to activate operons *LEE1*, *LEE2*, *LEE3* and, to some extent, *LEE4* (Mellies *et al.*, 1999). As described in section 1.11.3 activation is via the Ler protein, a LEE encoded regulator that is specified by the first gene of the *LEE1* operon. Ler acts directly or indirectly on promoter regions for *LEE2*, *LEE3* and *LEE4* in a cascade fashion (Mellies *et al.*, 1999; Elliott *et al.*, 2000). Recent studies by Bustamante *et al.* (2001) suggest that the level of transcription of the *LEE2* and *LEE3* promoters is similar in wild-type and EAF plasmid minus EPEC, suggesting that Per is not directly involved in *LEE2* and *LEE3* expression. However, PerC is indirectly required for efficient *LEE2* and *LEE3* transcription when the EAF plasmid is present, possibly to overcome the negative effect of an unknown factor that is either encoded by, or activated in the presence of EAF (Bustamante *et al.*, 2001)

1.11.2 Involvement of Integration Host Factor in the expression of genes on the LEE

The expression of the LEE genes is also dependent on the global regulator IHF, a DNA-binding protein that binds to specific consensus sites and bends the DNA to form nucleoprotein complexes (Friedberg *et al.*, 1999; Giladi *et al.*, 1998). The crystal structure of IHF bound to DNA indicates the DNA to be wrapped around the protein and bent by over 160° (Giladi *et al.*, 1998; Engelhorn and Geiselmann, 1998). IHF is involved in site-specific recombination and DNA replication, as well as in the positive and negative regulation of gene expression (Friedman, 1988). IHF is a heterodimer, composed of IHFα and IHFβ, and is encoded by the *ihfA* and *ihfB* genes, respectively (Friedberg *et al.*, 1999).

The LEE contains a near perfect IHF consensus sequence ATAtagtAACTAA on the antisense strand, 35 nucleotides upstream of the –35 region of *LEE1*. This suggests that IHF interacts directly with the transcription initiation complex (Friedberg *et al.*, 1999). Studies show that IHF activates a small number of LEE genes directly, including the *ler* gene (by binding upstream of the *LEE1* promoter region). *LEE2*, *LEE3*, *LEE4*, *tir* and *eae* are then activated as a consequence of Ler activation (Friedberg *et al.*, 1999). EPEC strains with a mutation in the *ihfA* gene do not elicit A/E associated actin rearrangements (Friedberg *et al.*, 1999).

1.11.3 LEE encoded regulator – Ler

The product of the first gene in *LEE1* - Ler - has been shown to be an important regulator of virulence genes. All the genes important for A/E lesion formation, including those coding for the type III secretion system, the secreted proteins, Tir and intimin, are regulated by *ler*, or in the case of the *LEE1* operon, co-regulated with *ler* (Mellies *et al.*, 1999; Elliott *et al.*, 2000). As well as regulating the expression of genes on the LEE involved in A/E lesion formation, Ler also regulates the expression of a number of genes on the LEE that are not required for A/E lesion formation

(Elliott *et al.*, 2000). Moreover, Ler regulates the expression of genes outside of the LEE in EPEC and EHEC that are also not essential for A/E lesion formation. These include EspC in EPEC and TagA in EHEC (Elliott *et al.*, 2000). Mutation of *ler* in EPEC and EHEC strains leads to an altered adherence of the strains to epithelial cells and the expression of novel fimbriae (Elliott *et al.*, 2000). Although Ler negatively regulates the formation of fimbriae, it is not involved in the regulation of BFP expression (Bustamante *et al.*, 2001). Elliott *et al.* (2000) suggest a mechanism whereby low levels of Ler may be associated with the initial stages of colonization, by mediating the production of fimbriae, while at higher levels of Ler expression, the genes involved in A/E lesion formation are up regulated.

Ler shares homology with the H-NS/StpA family of DNA binding proteisn, with 24% sequence identity and 44% similarity to H-NS of *Salmonella* in the C-terminal region (Elliott *et al.*, 1998; Mellies *et al.*, 1999; Elliott *et al.*, 2000). H-NS is one of the most abundant DNA binding proteins in enterobacteria and exists essentially as a homodimer with affinity for all types of nucleic acids (Atlung and Ingmer, 1997; Bertin *et al.*, 1999). H-NS binds preferentially to intrinsically curved DNA by binding to target sites that are AT rich (thus normally bent), or by binding non-specifically over DNA regions that it can cover completely (Atlung and Ingmer, 1997; Laurent-Winter *et al.*, 1997). The protein contains two structural domains, a C-terminal domain involved in DNA-protein interactions and an N-terminal dimerization region containing a coiled-coil domain involved in protein-protein interactions (Lupas, 1996; Atlung and Ingmer, 1997; Williams and Rimsky, 1997; Bertin *et al.*, 1999). H-NS can affect the overall structure of DNA by compacting it as well as by altering DNA superhelicity and inducing DNA bending (Williams and Rimsky, 1997).

Mellies *et al.* (1999) first proposed that Ler was likely to have a different mechanism of action to H-NS, (demonstrated by Elliott *et al.* 2000), as H-NS generally negatively regulates transcription, while Ler activates transcription (Atlung and Ingmer, 1997; Mellies *et al.*, 1999). Recent studies show that Ler protects a region of 121 bp

between the divergently transcribed *LEE2* and *LEE3* regions (Sperandio *et al.*, 2000). This implies that Ler may bind in a multimerized form to the *LEE2* regulatory region in order to activate transcription (Sperandio *et al.*, 2000). Ler possesses a conserved DNA binding motif of the H-NS proteins and the amino-terminal segment of Ler contains a stretch of residues predicted to adopt a coiled-coil conformation, previously shown to be important for dimerization between subunits (Lupas, 1996; Elliott *et al.*, 2000; Sperandio *et al.*, 2000). The importance of the amino-terminal domain in Ler is highlighted by the fact that purified Ler, mutated in the coiled-coil region, is unable to activate transcription and bind to the *LEE2* regulatory region (Sperandio *et al.*, 2000). Previously it has been speculated that the binding of Ler upstream of *LEE2* could activate transcription by changing the DNA conformation or dislodging a repressor protein such as H-NS, allowing transcription of the operons to proceed (Sperandio *et al.*, 2000). Recent studies indicate that Ler acts as an antirepressor protein, overcoming the H-NS mediated silencing on the *LEE2/LEE3* divergent promoter region caused by the formation of a repressing H-NS nucleoprotein complex (Bustamante *et al.*, 2001).

The expression of the *LEE2* and *LEE3* operons is negatively regulated by flanking common upstream and downstream silencing regulatory sequences, SRS1 and SRS2, which act negatively for both promoters (Bustamante *et al.*, 2001). The SRS regions are involved in negative regulation in the presence of ammonium in the culture medium, or at temperatures above 37°C (Bustamante *et al.*, 2001). In the absence of SRS1 or SRS2, or in the absence of H-NS, the expression of the *LEE2* and *LEE3* operons from the two divergent overlapping promoters is independent of Ler, implying these operons are constitutively expressed in the absence of H-NS (Bustamante *et al.*, 2001). Also contained within the *LEE2/LEE3* promoter region is an attenuating regulatory sequence (ARS) involved in transcriptional attenuation of the *LEE3* operon (Bustamante *et al.*, 2001). While it is not yet known how Ler acts as an antirepressor of H-NS, it is postulated that Ler may compete with H-NS for its binding sites and/or alter the local DNA architecture to hinder H-NS binding. Thus, interfering with the formation of a repressing nucleoprotein complex would allow

RNA polymerase to interact with the promoters (Bustamante *et al.*, 2001). This seems plausible as previous DNA-protein binding studies have shown that Ler can bind to and protect a region between *LEE2* and *LEE3*, which contain SRS and ARS sequences (see Figure 1.8.). The other method of anti-repression suggested by Bustamante *et al.* (2001), proposes Ler binding to and sequestering H-NS, thereby preventing it from interacting with SRS1 and SRS2.

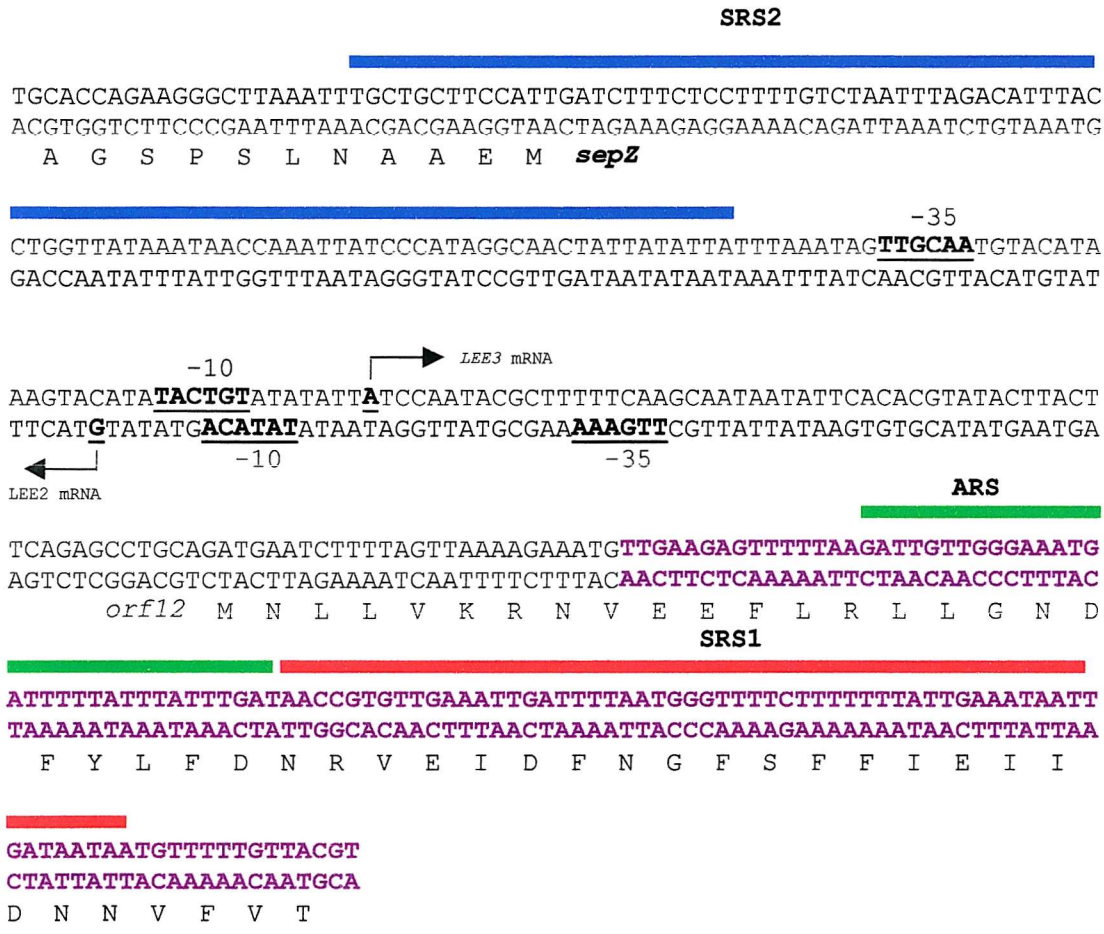


Figure 1.8. Nucleotide sequence of the divergent promoter region of the *LEE2* and *LEE3* operons. The transcriptional start sites are shown in bold underlined letters and by broken arrows. The putative –35 and –10 promoter sequences are shown in bold underlined letters. The regions containing the SRS sequence are shown in red and blue for SRS1 and SRS2, respectively. The region containing the putative ARS is shown in green. While the region protected by the Ler footprint (Sperandio *et al.*, 2000) is shown in bold purple letters. (Figure adapted from Bustmante *et al.*, 2001).

1.11.4 Quorum sensing influences expression of the type III secretion gene transcription

Quorum sensing is a mechanism of cell-to-cell signalling via the production of autoinducers that allow a bacterium to sense its own population as well as the population of other bacteria in a given environment (Sperandio *et al.*, 1999). Quorum sensing regulates the majority of the genes encoded on the LEE. The *LEE1* and *LEE2* operons are directly regulated by quorum sensing, while the *LEE3*, *LEE4* and *LEE5* operons are indirectly activated by quorum sensing by the product of the first gene in the *LEE1* operon (Sperandio *et al.*, 1999). Quorum sensing also regulates *per* in an indirect fashion through an unknown factor that appears to be present in a K-12 background (Sperandio *et al.*, 1999). Maximal induction of *LEE1* expression occurs when bacteria are grown in DMEM preconditioned by the growth of bacteria able to secrete the autoinducer AI-2 (Sperandio *et al.*, 1999). Several groups have speculated that the low infectious dose of EHEC may be attributable to AI-2 production from bacteria within the large intestine, thereby activating the type III secretion system and permitting intestinal colonization to occur (Sperandio *et al.*, 1999; Elliott *et al.*, 2000). The secretion of effector molecules is a more efficient process in EPEC than in EHEC, probably due to the fact that EPEC contains Per. Per may respond to environmental signals and control BFP expression, leading to microcolony formation in the small intestine, where the levels of other bacteria that could produce AI-2 are lower than those in the large intestine (Mellies *et al.*, 1999; Sperandio *et al.*, 1999; Elliott *et al.*, 2000).

In summary, the regulation of virulence genes on the LEE required for the development of the LA phenotype and A/E lesion formation is a complex phenomenon. The status of the regulatory mechanisms at the time of writing is summarised in Figure 1.9. While there may be more positive or negative regulatory conditions or elements modulating LEE expression, current understanding suggests that LEE gene expression is regulated by environmental conditions, and that the product of the first *LEE* operon - Ler acts as an antirepressor of H-NS, that occupies

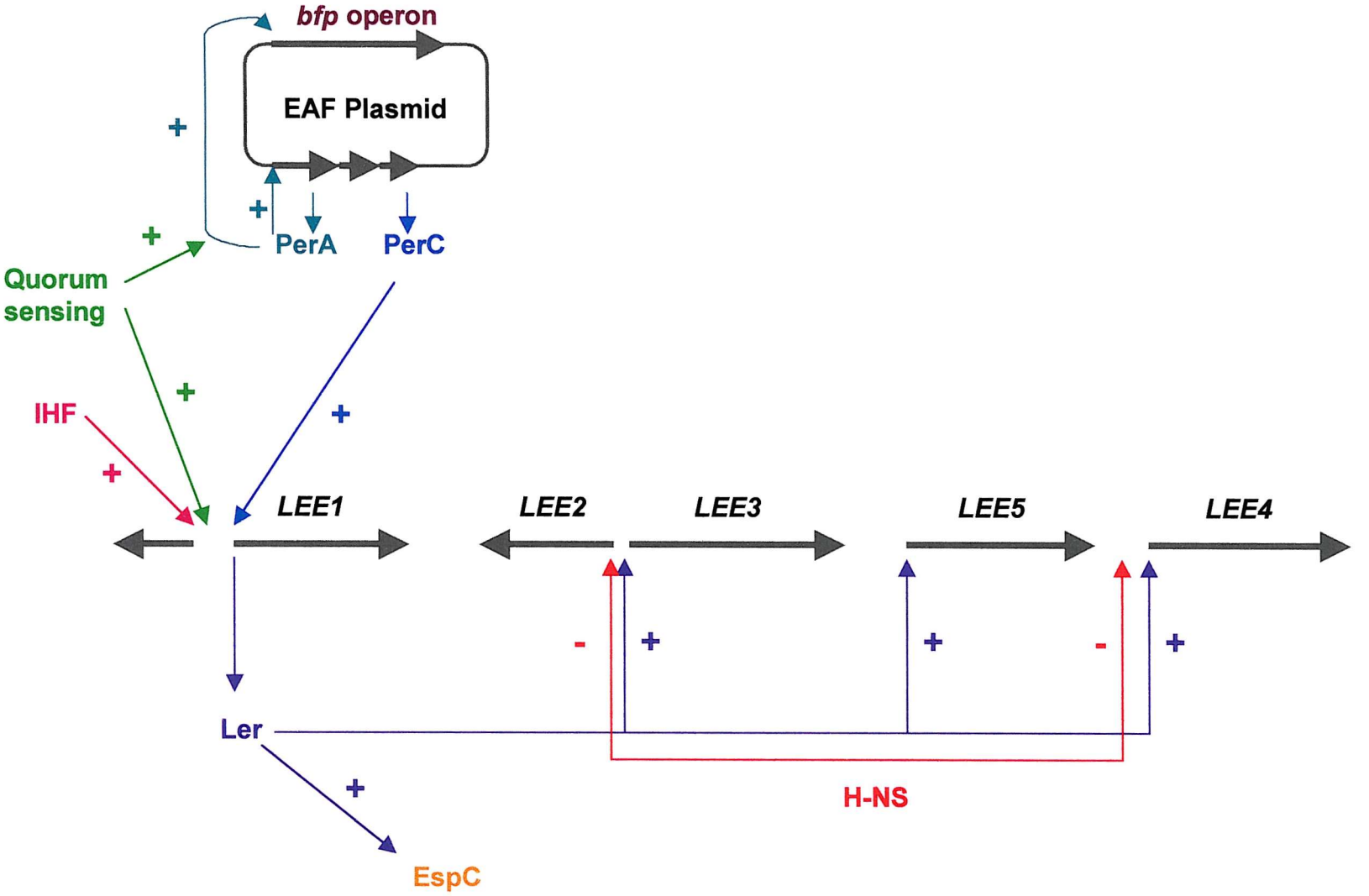


Figure 1.9. Current model of the regulatory cascade of LEE gene expression

sites on the *LEE* of an otherwise constitutively expressed operon. While Ler may be central to regulating the cascade, Ler is itself regulated by Per (Mellies *et al.*, 1999), IHF (Friedberg *et al.*, 1999) and by quorum sensing (Sperandio *et al.*, 1999).

1.12 Structure of the A/E lesion

The reorganisation of cytoskeletal actin in infected epithelial cells of the gut that results from EPEC signalling events, leads to the effacement of microvilli and the formation of actin rich structures known as pedestals. Although not formally proven, the disappearance of microvilli connected with the formation of actin rich pedestals could be triggered by bacteria. It is presumed that the depolymerised actin is subsequently re-polymerized to form the pedestal structures. However, it is proposed that this is not just the rebuilding of microvilli, and that the presence of some cytoskeletal components may be present as a consequence of the high concentration of F-actin (Celli *et al.*, 2000).

Pedestals have been observed after EPEC infection of animals, human biopsies and cultured epithelial cells. EPEC adhere to and become partially surrounded by the cup-like projections, with the bacteria remaining 10 to 12 nm above the host membrane in these pedestal structures (Knutton *et al.*, 1987a).These pedestals have a propensity to bend and undulate and can change their length of projection, extending up to 10 μm from the host cell surface (Nataro and Kaper, 1998). The precise role of pedestals is unclear. Undoubtedly they provide a strong attachment of the bacterium to the host cell, and hence probably prevent their dislodgement during diarrhoea. It has also been postulated that the pedestal may serve as a strategy to remain extracellular (Goosney *et al.*, 1999).

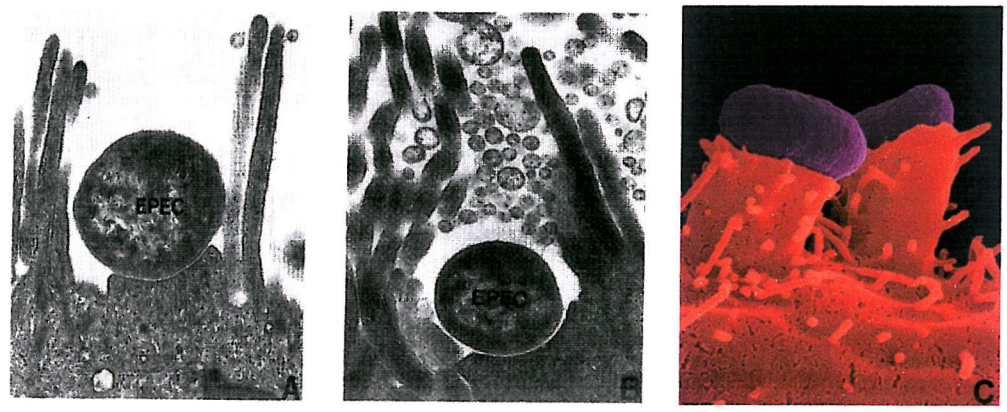


Figure 1.10. (A) Electron micrograph (x 45000) indicating intimate bacterial attachment and pedestal formation. Reprinted by permission from American Society for Microbiology (Knutton *et al.*, 1987a. *Infect. Immun.* 55, 69-77) copyright (1987) American Society for Microbiology. (B) Electron micrograph (x 45000) showing localised destruction of microvilli by cytoskeletal breakdown. Reprinted by permission from American Society for Microbiology (Knutton *et al.*, 1987a. *Infect. Immun.* 55, 69-77) copyright (1987) American Society for Microbiology. (C) SEM image of wild type EPEC bound to HeLa cells (EPEC are artificially coloured purple, and HeLa orange). Reprinted by permission from Oxford University Press (Rosenshine *et al.*, 1996b. *EMBO J.* 15, 2613-2624) copyright (1996) Oxford University Press.

Given the location of Tir at the tip of the pedestal, it is predicted to govern the linking of EPEC to the host cytoskeleton through the membrane. It is thought to accomplish this by interacting with cytoskeletal regulators to direct actin accumulation and pedestal formation, possibly exploiting regulators of actin dynamics to initiate actin polymerisation. Neither RhoA, Rac1 nor Cdc-42 activity is required for actin polymerisation and pedestal formation (Ben-Ami *et al.*, 1998). Instead pedestal formation depends on the localised recruitment and activation of the heptameric Arp2/3 complex (Arp2/3c) an actin nucleating factor and members of the Wiskatt-Aldrich syndrome (WAS) family of proteins (WASP and N-WASP), which bind to and activate Arp2/3c (Kalman *et al.*, 1999; Machesky and Insall, 1999). Arp2/3c

recruitment depends on WASP, and WASP recruitment depends on its GTPase-binding domain. WASP colocalises to the tip of EPEC induced pedestals in HeLa cells as well as to Arp2/3. The C-terminus of WASP associates with and potentiates the nucleating activity of Arp2/3c, while the acidic domain directly binds a subunit of Arp2/3c. The recruitment of Arp2/3c to the WASP-C-terminus and subsequent activation stimulates actin nucleation and polymerisation; this in turn drives pedestal formation (Kalman *et al.*, 1999).

Immunofluorescence studies of EPEC induced pedestals have revealed that in addition to membrane bound Tir and WASP, which are found at the tip of the pedestal, several host cytoskeletal components are present. F-actin is distributed at the tip and along the length of the pedestal, while other cytoskeletal components involved in cross-linking actin filaments, such as α-actinin, villin, talin and ezrin are found along the length of the pedestal (Finlay *et al.*, 1992; Knutton *et al.*, 1989). (Figure 1.11.) Molecules involved in actin-mediated movement such as tropomyosin and non-muscle myosin II have also been shown to accumulate at the pedestal base (Finlay *et al.*, 1992; Vallance and Finlay, 2000). Infection of HeLa cells with EPEC results in the accumulation of cortactin under the adherent bacteria, which is able to bind other cytoskeletal proteins and crosslinks F-actin (Cantarelli *et al.*, 2000).

1.13 Signal transduction and A/E lesion formation

Many signal transduction pathways are stimulated within epithelial cells following EPEC infection. However, the precise molecular mechanisms involved in the rearrangements of host cell epithelium are still unclear, with recent findings contradicting previous results.

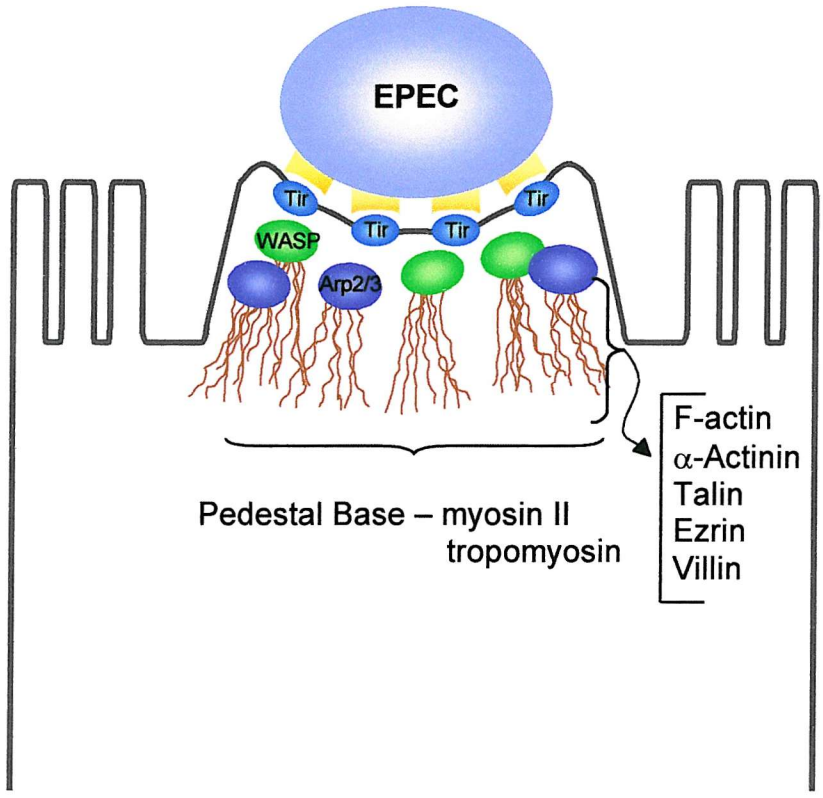


Figure 1.11. The structure of the EPEC pedestal. EPEC adheres to the host cell through an interaction of intimin (yellow) with its translocated receptor (blue). WASP and Arp2/3 are recruited to the tip of the pedestal, while α-actinin, villin, talin

Previously it was thought that the breakdown of the host cytoskeleton and the effacement of microvilli were due to changes in calcium levels within the host cell. It was postulated that the release of calcium from intracellular stores cause the activation of calcium-dependent actin remodelling proteins such as villin (Bretscher and Weber, 1980), thereby breaking the actin in the microvilli into short filaments, which would result in the breakdown of the microvillous core into vesicles (Knutton *et al.*, 1987a; Baldwin *et al.*, 1991; Baldwin, 1998). Elevated levels of intracellular calcium in the epithelial cell post-infection have been proposed (Baldwin *et al.*, 1993), and this increase in intracellular calcium was shown to be dependent on a functional type III secretion system and intimate attachment (Baldwin *et al.*, 1991; Dytoc *et al.*, 1994). Buffering the intracellular calcium with BAPTA (AM bis-

(aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid) a calcium chelator was shown to inhibit pedestal formation suggesting a role for calcium in EPEC induced actin rearrangements (Baldwin *et al.*, 1991).

Elevated levels of the second messenger inositol 1,4,5-triphosphate (IP_3) were reported upon EPEC infection of cultured epithelial cells (Baldwin *et al.*, 1991; Foubister *et al.*, 1994b; Dytoc *et al.*, 1994), which was postulated to mediate the release of calcium from IP_3 sensitive stores (Berridge, 1993). Consistent with these findings, Kenny and Finlay, (1997) report that EPEC infection triggers the tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1). PLC-γ1 is a 150 kDa cytoplasmic protein able to mediate the production of IP_3 and hence bring about the release of calcium from IP_3 sensitive intracellular stores (related to the endoplasmic reticulum). Following tyrosine phosphorylation, PLC-γ1 can interact with and cleave the phosphatidylinositol diphosphate (PIP_2) lipids in the membrane, resulting in the release of the second messengers IP_3 and DAG, which are both implicated in the release of intracellular calcium and activation of protein kinase C (PKC). The latter is an enzyme involved in the control of normal ion fluxes across mammalian cell membranes (Figure 1.13.) (Baldwin *et al.*, 1991; Berridge, 1993; Kenny and Finlay 1997; Baldwin, 1998). However, the effacement seen with EPEC infection is localised at the site of the adherent bacteria. Thus the widespread changes in calcium signalling within the epithelial cell possibly do not account for the localised rearrangements seen in the host cell epithelium. These localised changes in host cell structure prompted Bain *et al.* (1998) to look for localised signalling events induced by EPEC.

Bain *et al.* (1998) used calcium-imaging fluorescence microscopy to permit temporal and spatial measurements of intracellular calcium within live cells. They concluded that there was no significant increase in intracellular calcium levels at the site of A/E EPEC adhesion. Moreover, they propose that there are no significant changes in intracellular calcium in EPEC infected cells compared to uninfected cells (Bain *et al.*, 1998). Further results from this study showed that the buffering of intracellular

calcium with BAPTA did not prevent A/E lesion formation (Bain *et al.*, 1998). As yet the reasons for the apparent conflicting results are still not clear although Bain *et al.* (1998) propose that the calcium changes reported by others may reflect cytotoxic effects of EPEC on cells, rather than specific calcium signalling events associated with A/E lesion formation. It should also be noted, that the time-scales used by Bain *et al.* (1998) were different from those used in previous studies. Kenny (1999) has suggested that the translocated EspB, Tir or as yet unidentified effector proteins may be responsible for inducing the localised cytoskeletal rearrangements involved in A/E lesion formation. Thus further studies are required to clarify the apparent contradictory evidence.

1.13.1 Activation of host protein kinases

During EPEC infection, at least two protein kinases are activated in host tissues, and the stimulation of protein kinase activity appears to be an important aspect of A/E lesion formation. The production of the second messenger diacylglycerol (DAG), in response to phospholipase C action on PIP_2, activates the phospholipid-dependent protein kinase C (Figure 1.12.). Additionally there is the calcium-dependent activation of myosin light chain kinase (MLCK) (Baldwin, 1998).

1.13.2 Protein kinase C (PKC)

A role for PKC in the formation of A/E lesions was first suggested by Baldwin *et al.* (1990), when they showed that treatment of HEp-2 cells with PLC and phosphatidic acid (components of the signal transduction pathway by which PKC is activated) resulted in phosphorylation patterns identical to those seen during EPEC infection of HEp-2 cells. Crane and Oh (1997) have since demonstrated PKC stimulation as a result of EPEC adherence to human cell lines. After activation, PKC undergoes a conformational change resulting in its movement from the cytosol to the membrane. This results in the phosphorylation of several proteins. However, the importance of activated PKC in the development of diarrhoea associated with EPEC is still not

clear. PKC activation occurs early in EPEC infection and may promote a secretory response by phosphorylation of chloride transport proteins in microvilli (Baldwin, 1998). PKC is able to activate the main chloride channel in the brush border of enterocytes, i.e. the CFTR, along with another plasma-membrane-associated chloride channel separate from the CFTR (Crane and Oh, 1997). Indeed, the activation of PKC in rat small intestine elicits a secretory response that is mediated by chloride ion secretion (Fondacaro and Henderson, 1985). The activation of PKC may also affect the architecture of the tight junctions, affecting cellular permeability. Incubation of polarised cell monolayers with EPEC results in decreased transepithelial electrical resistance (TEER), and increased permeability to sodium and mannitol (Crane and Oh, 1997).

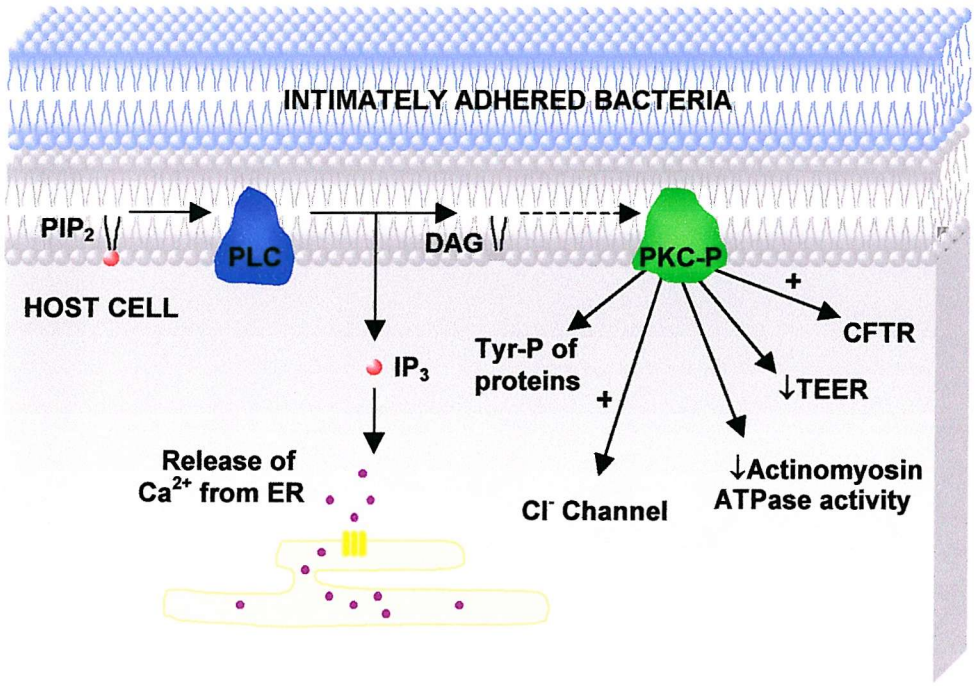


Figure 1.12. The production of the second messenger DAG in response to the action of PLC on PIP_2, and the subsequent activation of PKC. Adapted from Lodish *et al.*, (1995).

1.13.3 Myosin Light Chain Kinase (MLCK)

Infection of epithelial cells with EPEC induces the phosphorylation of the 20 kDa protein, Myosin Light Chain, by Myosin Light Chain Kinase, which leads to the loss of tight junction integrity (Manjarrez-Hernandez *et al.*, 1991; Manjarrez-Hernandez *et al.*, 1992; Yuhan *et al.*, 1997). The phosphorylation state of MLC may be important in the effacement of the brush border and formation of pedestals, since phosphorylated MLC is involved in the organisation of actin.

EPEC infection of host cells activates an increase in intracellular calcium, which binds to the calcium binding protein, calmodulin, which in turn binds to and activates myosin kinase. MLCK phosphorylates MLC and shifts the distribution of MLC from the cytoplasm to the cytoskeleton, where phosphorylated MLC associates with actin (Adelstein and Eisenberg, 1980). Subsequently, myosin ATPase becomes activated resulting in cytoskeletal contraction and an increase in paracellular permeability. In contrast, dephosphorylation of MLC results in the loss of actin microfilament bundles (Yuhan *et al.*, 1997; Frankel *et al.*, 1998). MLC can be phosphorylated by either MLCK or PKC, but only phosphorylation by MLCK increases myosin ATPase activity and contraction, whereas phosphorylation of MLC by PKC decreases actomyosin ATPase activity (Yuhan *et al.*, 1997).

It is postulated that during the early stages of bacterial adherence MLC is phosphorylated by PKC, causing MLC to dissociate from the polymerised actin and move into the soluble cell fraction. The destabilisation of the actin structure in the microvillous core and the activation of villin could lead to calcium-mediated core breakdown and loss of microvilli (Baldwin, 1998). During the subsequent stages of lesion development, local actin polymerisation beneath the sites of bacterial attachment would be stabilised by actin association with MLCK phosphorylated MLC, leading to the development of pedestal structures.

1.13.4 Activation of cytokines during EPEC infection

EPEC infection affects cytokine release from host tissues, EPEC infection of an intestinal epithelial monolayer model using T84 cells has shown that EPEC infection triggers the activation of Nuclear Factor kappa B (NF-κB) (Savkovic *et al.*, 1996; Savkovic *et al.*, 1997). NF-κB is involved in the regulation of genes activated during inflammatory, immune and acute phase responses and is present in the cytoplasm of a variety of cell types in an inactive form that is complexed with IκB (Pahl and Baeuerle, 1997; Akira and Kishimoto, 1997; Mercurio and Manning, 1999). The intermediate events linking EPEC induced signalling with NF-κB stimulation are still to be resolved. However, the stimulation of cells with pro-inflammatory cytokines, antigens, UV, γ-irradiation, and bacterial and viral infections, rapidly induces the activity of NF-κB (Pahl and Baeuerle, 1997). Activation of NF-κB is caused by the accumulation of proteins in the ER membrane, a process referred to as ER overload (Pahl and Baeuerle, 1997). The release of calcium from the ER is accompanied by an increase in the intracellular concentration of reactive oxygen intermediates (ROIs),. This in turn leads to the phosphorylation, and subsequent proteolytic degradation of IκB, resulting in the dissociation of the IκB-NF-κB complex. The resulting exposure of the nuclear localization signal of NF-κB, frees the transcription factor to interact with the nuclear import machinery and translocate to the nucleus where it binds to cognate DNA binding sites and activates transcription (Figure 1.13.) (Pahl and Baeuerle, 1997; Mercurio and Manning, 1999).

The activation of NF-κB within host cells during EPEC infection has been shown to require EspB but not intimate adherence, and results in increased interleukin (IL)-8 production and the transmigration of polymorphonuclearleukocytes (PMNs) to the intestinal lumen (Savkovic *et al.*, 1996). These PMNs migrate from the basolateral side of the epithelial cell to the apical or luminal side, where they release 5' adenosine monophosphate. This compound gets converted to adenosine, which can in turn bind to adenosine receptors and stimulate Cl^- secretion (Yuhan *et al.*, 1997; Goosney *et al.*,

1999). IL-8 acts as a neutrophil chemokine and can also stimulate responses such as the respiratory burst and degranulation of neutrophils.

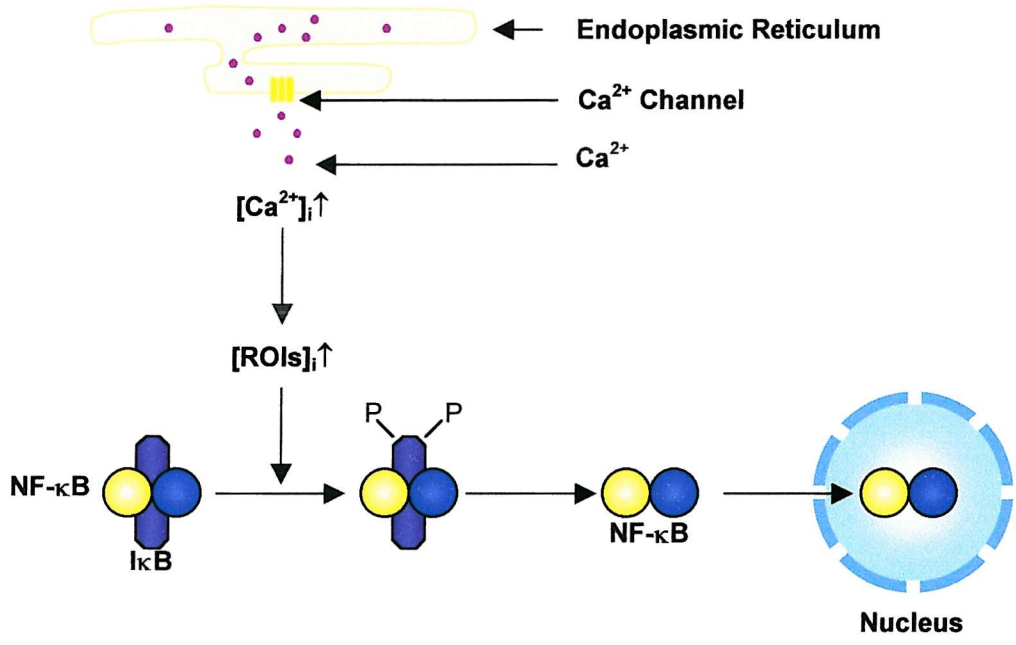


Figure 1.13. Activation of NF-κB. (Adapted from Pahl and Baeuerle, 1997).

Lysates of EPEC directly inhibit cytokine production by lymphoid cells (Klapproth *et al.*, 1995; Klapproth *et al.*, 1996; Malstrom and James, 1998). The cytokine inhibition is mediated by the protein product of *lifA*, lymphostatin, which specifically inhibits lymphocyte production, IL-2, IL-4 and gamma interferon (γIFN) production (Klapproth *et al.*, 2000). The disruption of *lifA* does not affect EPEC pathogenesis; moreover, lymphostatin is postulated to suppress the immune response to the bacteria, allowing for prolonged infection (Klapproth *et al.*, 2000).

1.14 Mechanisms of EPEC-mediated diarrhoea

The mechanisms whereby EPEC cause diarrhoea are not understood, although it is postulated that EPEC induced secretory processes are likely to be important in early infection (Spitz *et al.*, 1995), while the loss of epithelial microvilli could lead to

decreased absorption and may account for the persistent diarrhoea. Clinical findings indicate that EPEC mediated diarrhoea contains high levels of sodium, characteristic of a secretory type of diarrhoea (Stein *et al.*, 1996b). Although an EPEC-induced increase in intracellular calcium is still controversial, raised calcium levels could inhibit sodium and chloride ion absorption and stimulate chloride secretion in enterocytes contributing to the diarrhoea (Nataro and Kaper, 1998). The active chloride secretion of Cl^- ions due to activation of CFTR by PKC, PMN transmigration, and adenosine mediated stimulation of chloride secretion, have all been suggested to be responsible for the initial onset of diarrhoea (Donnenberg *et al.*, 1997; Goosney *et al.*, 1999). As well as interacting with the host intestinal epthelium, bacteria that produce A/E lesions must also encounter and circumvent innate host defences. PMNs are known to participate in the elimination of pathogens via phagocytosis and ROI production, in addition to their action on induction of water and electrolyte movements in epithelial cells. The transmigration of PMNs (transmigrated PMNs have a greater capacity to phagocytose *E. coli*), suggests that inflammatory pathways may be involved in diarrhoea (Donnenberg *et al.*, 1997; Goosney *et al.*, 1999; Hofman *et al.*, 2000).

The phosphorylation of MLC has been suggested to lead to changes in the integrity of tight junctions, leading to increased intestinal permeability and the loss of transepithelial electrical resistance (TEER). Adherence of EPEC to polarised epithelial monolayers of Caco-2 or MDCK causes a decrease in TEER (Canil *et al.*, 1993). Canil *et al.* (1993), suggested that the drop in resistance was attributable to a disruption of the intracellular pathway rather than by disrupting intracellular tight junctions. Moreover, it now appears that alterations in tight junctions, possibly through the phosphorylation of MLC (which can lead to increased permeability of tight junctions), may be the reason for the decrease in TEER upon EPEC infection. Further studies in another cell line have shown that EPEC adherence to T84 cell monolayers diminishes barrier function, as demonstrated by a decrease in TEER (Spitz *et al.*, 1995). Blocking the increase in intracellular calcium prevents the EPEC

induced drop in TEER, indicating the involvement of calcium in this event (Donnenberg *et al.*, 1997).

Collington *et al.* (1998a) demonstrated that EPEC induce alterations in host cell electrolyte transport after initial adhesion, a process that requires EPEC signal transduction. The transepithelial electrical parameters of Caco-2 monolayers were measured by exposing the monolayer to short circuit conditions (Collington *et al.*, 1998a; Collington *et al.*, 1998b). It was observed that EPEC stimulate a rapid three-fold increase in short circuit current (*I*sc), coinciding with A/E lesion formation and dependent on a type III secretion system and intimate attachment (Collington *et al.*, 1998b). The increase in *I*sc peaked after 10 minutes and was shown not to be associated with a decrease in TEER (although this decreased later). Moreover, a third of the *I*sc was purported to be predominantly the result of chloride secretion. (Collington *et al.*, 1998a; Collington *et al.*, 1998b). Since the chloride secretion was transient, the possible contributory effect on diarrhoea is unclear. However, recent studies suggest that EPEC does not stimulate active ion secretion, and that changes in *Isc* caused by EPEC infection are not associated with chloride secretion, but may be attributable to altered HCO_3^- secretion (Hecht and Koutsouris, 1999). As yet the reasons for the apparent conflicting results are not clear although different models, cell lines and methods may account for the observed differences.

EPEC infection of T84 induces a decrease in TEER, the formation of A/E lesions and cytokine production. EPEC induces the tyrosine phosphorylation of several proteins in T84 including p46 and p52 Shc isoforms, also, ERK1/2, p38 and c-Jun N-terminal kinase (JNK) MAP kinases are activated (Czerucka *et al.*, 2001). Both adhesion and signal transduction by the type III secretion system are necessary to induce tyrosine phosphorylation in T84 cells. ERK1/2 and p38 MAP kinase are implicated in IL-8 expression but do not participate in A/E lesion formation or TEER modification (Czerucka *et al.*, 2001). *Saccharomyces boulardii* a non pathogenic yeast used in the treatment of infectious diarrhoea, abrogates the alterations induced by EPEC on

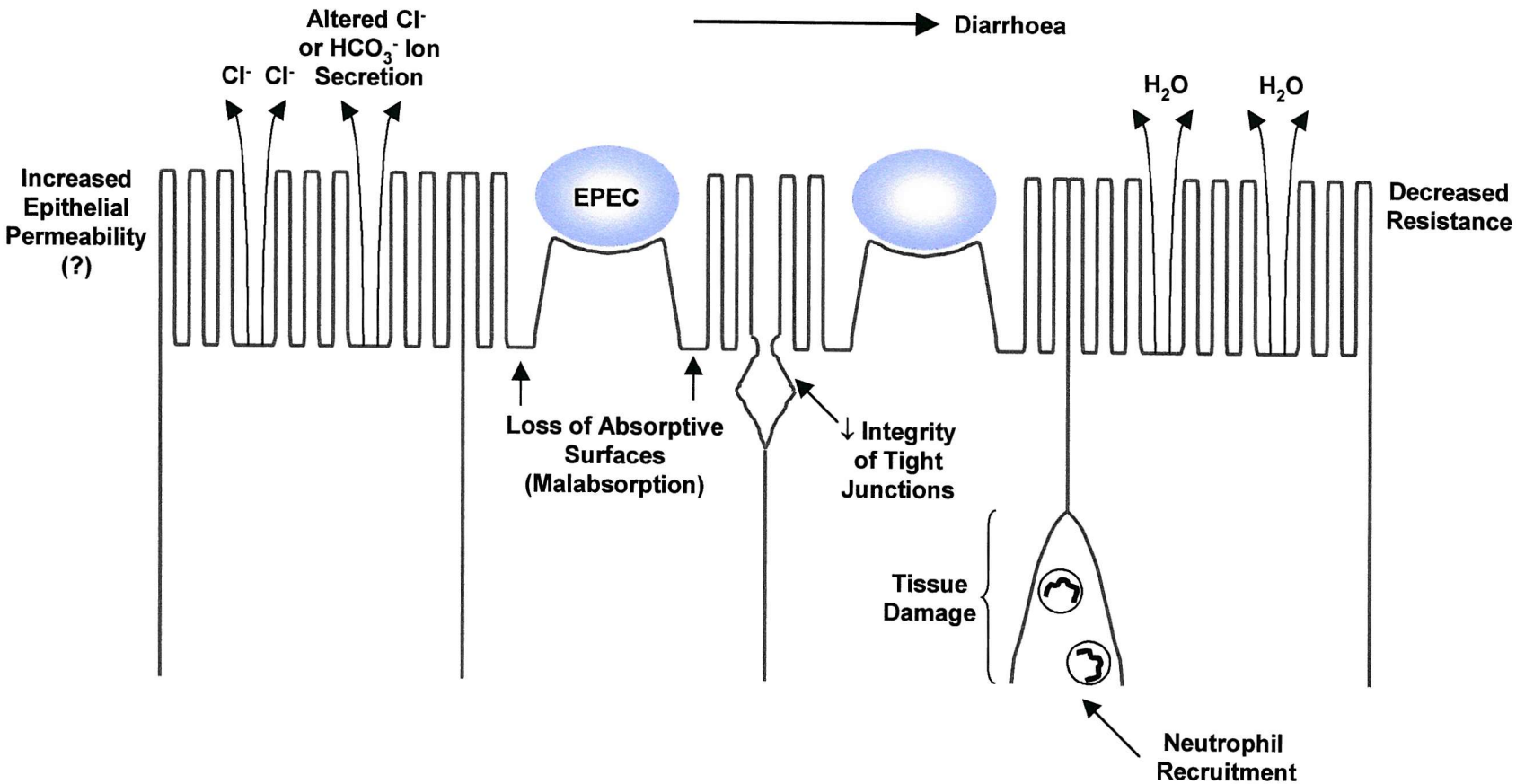


Figure 1.14. EPEC interaction with host cells induces a number of putative mechanisms of diarrhoea. These include an increase in epithelial cell permeability, alterations in Cl^- and HCO_3^- ion secretion, loss of absorptive surfaces, reduced tight junction permeability and tissue damage. (Figure adapted from Vallance and Finlay, 2000)

TEER, and the distribution of the tight junction-associated protein ZO-1 in T84 cells (Czerucka *et al.*, 2000). In addition, the tyrosine phosphorylation of p46 and p52 Shc isoforms and ERK1/2 MAP kinase in T84 infected cells is diminished in the presence of *Saccharomyces boulardii*. Thus *Saccharomyces boulardii* modulates signalling pathways induced by bacterial infection, eliciting a protective effect on the epithelial cell (Czerucka *et al.*, 2000).

1.15 Rationale for the project

Phagocytic cells are a critical component of the body's defense against pathogenic micro-organisms (Fields *et al.*, 1989). In particular PMNs have been implicated in EPEC pathogenesis and have a phagocytic action that generally allows them to engulf the bacteria they encounter. The importance of PMNs in EPEC pathogenesis is at present not clear. Traditionally, EPEC strains have been considered non-invasive. However, intracellular EPEC have been observed in animal models (Moon *et al.*, 1983), in biopsies from infected humans (Ulshen and Rollo, 1980), and in *in vitro* studies with HEp-2, HeLa and Caco2 cell lines (Donnenberg *et al.*, 1989; Rosenshine *et al.*, 1992). Although there is no experimental evidence on the fate of EPEC, theoretically the death of invading bacteria may be achieved by oxygen dependent and oxygen independent processes (Elsbach and Weiss, 1988; Heffron *et al.*, 1989). The oxidative process is characterised by the 'respiratory burst' in which an NADPH oxidase within the PMN becomes activated after bacteria are bound to their cell surfaces. Associated with this activation of NADPH is a sharp increase in oxygen uptake and metabolism within the PMN (the respiratory burst), and the generation of oxygen-derived radicals such as superoxide and hydrogen peroxide that are effective bactericidal agents (Odell and Segal, 1988). Non-oxygen dependent antimicrobial factors include the acidification of the phagosome and cytotoxic agents such as defensins, cathepsin G, azurocidin, lysozyme and bactericidal/permeability-increasing protein (Elsbach and Weiss, 1988; Heffron *et al.*, 1989). The last of these is of particular interest as it accounts for the majority of the antibacterial activity in PMN lysates against gram-negative bacteria such as EPEC.

1.16 Bactericidal/permeability increasing protein (BPI)

BPI is a cationic (pI > 9.6) boomerang-shaped protein of 57 kDa (Elsbach and Weiss, 1988; Elsbach and Weiss, 1993; Beamer *et al.*, 1997). The protein has approximate dimensions of 135 by 35 by 35 Å, and consists of two domains (NH_2- and COOH-terminals) of similar size connected by a proline rich linker (Beamer *et al.*, 1997). The amino terminal half of BPI is rich in lysine residues and hence is cationic, whereas the carboxyl terminal half is less charged and hydrophobic (Elsbach and Weiss, 1993). BPI has no activity towards gram-positive bacteria, fungi or mammalian cells but is highly cytotoxic towards most gram-negative bacteria, due to the attraction of the cationic BPI for the negatively charged LPS of the outer membrane of gram-negative bacteria (Mannion *et al.*, 1989). The N-terminal domain of BPI retains the bactericidal, LPS binding and LPS-neutralisation activities of the intact protein (Beamer *et al.*, 1999). Moreover, the cationic amino terminal domain of BPI retains activity when expressed as a recombinant protein in *E. coli* (Qi *et al.*, 1994). Indeed, it has also been established that peptides including residues 90-99 of BPI retain significant anti-bacterial activity (Little *et al.*, 1994; Gray and Haseman, 1994). For example, a peptide termed P2, including residues 86-104 of BPI, was shown to retain potent anti-bacterial activity against a range of gram-negative organisms including *E. coli.* (Barker, 1997).

The outer membrane of gram-negative bacteria constitutes a major barrier to antibiotics and to hydrophobic substances. Phospholipids make up the inner leaflet, and the lipid portion of lipopolysaccharides (LPS) constitutes the outer leaflet. A hydrophilic barrier thus protects the gram-negative bacteria against hydrophobic substances (Elsbach and Weiss, 1988). This barrier is stabilised in *E. coli* by divalent cations such as calcium and magnesium, which cross-link the LPS molecules (Elsbach and Weiss, 1988). BPI competes for the divalent cation sites and upon binding displaces the Ca^{2+} or Mg^{2+}, causing a breakdown in the LPS cross-linked structure (Elsbach and Weiss, 1988; Elsbach and Weiss, 1993). This results in a permeabilization of the outer membrane of the bacteria, and activation of an

endogenous phospholipase A_2, as well as of peptidoglycan degrading enzymes (Weiss *et al.*, 1983; Mannion *et al.*, 1989). Recent studies by Barker *et al.* (2000) suggest that the primary lesion caused by the BPI derived peptide P2, is not gross permeabilization of the bacterial cytoplasmic membrane, but disruption of the respiratory chain. Therefore the region of BPI that corresponds to P2 may function as a 'warhead' interfering with the respiratory chain, while the rest of the holoprotein delivers this region to the cytoplasmic membrane (Barker *et al.*, 2000).

1.17 Discovery and characterisation of BipA

Given the defensive role of BPI it might be expected that microbial pathogens have evolved counter-measures to deal with exposure to it. In this respect, 2-D gel analysis of protein profiles of BPI–treated and untreated *Salmonella* Typhimurium cultures highlighted a number of induced and repressed proteins. Comparisons of these gels identified a protein of around 72 kDa that was strongly induced (7 fold) in response to challenge with BPI (Qi *et al.*, 1995). Microsequencing of this protein and database searches revealed that the first 10 residues matched the amino terminal region of a previously hypothetical protein *in E. coli* K-12, specified by open reading frame 591, located at 88 min next to *glnA* (Plunkett *et al.*, 1993; Qi *et al.*, 1995). 2-D gel analysis indicated the protein to be a 72 kDa protein with a pI of 5.22, present at approximately 1000 copies per cell. Translation of *o591* predicted a protein of molecular weight 65.44 and a pI of 5.10 (Qi *et al.*, 1995). The protein was termed BPI inducible protein A (BipA) due to its strong induction in response to BPI in *Salmonella* Typhimurium (Qi *et al.*, 1995). Parallel studies by Freestone *et al.* (1995) using an EPEC strain identified a major phosphoprotein that apparently copurified with RNA polymerase, which they termed TypA for Tyrosine phosphorylated protein A (Freestone *et al.*, 1998). Sequencing of this protein confirmed it to correspond to the product of the hypothetical *o591* gene in *E. coli* K-12; indeed TypA/BipA are the same protein. Further inspection of the BipA sequence strongly suggested it belonged to the GTPase superfamily, raising the question of its mechanism of action and role in responses to human BPI.

1.18 GTPases

GTPases are conserved molecular switches that are activated by the binding of GTP and de-activated by the hydrolysis of GTP to GDP. The GTPase superfamilies are found in all domains of life, have a wide versatility and are involved in a number of processes. These include the sorting and amplification of transmembrane signals, directing the synthesis and translocation of proteins, and control of proliferation and differentiation (Bourne *et al.*, 1991; Kaziro *et al.*, 1991). The GTPase cycle includes three conformational states of the protein illustrated in Figure 1.15. Briefly, release of bound GDP converts the inactive form of the protein into a transient empty state. Under ambient conditions in the cytoplasm GTP is more likely than GDP to enter the empty guanine nucleotide binding site, and upon binding GTP, the protein adopts an active conformation. Guanine nucleotide release proteins (GNRPs) catalyse the release of bound GDP, promoting its replacement by GTP, while GTPase activating proteins (GAPs) speed up GTP hydrolysis (Bourne *et al.*, 1991; Kjeldgaard *et al.*, 1996).

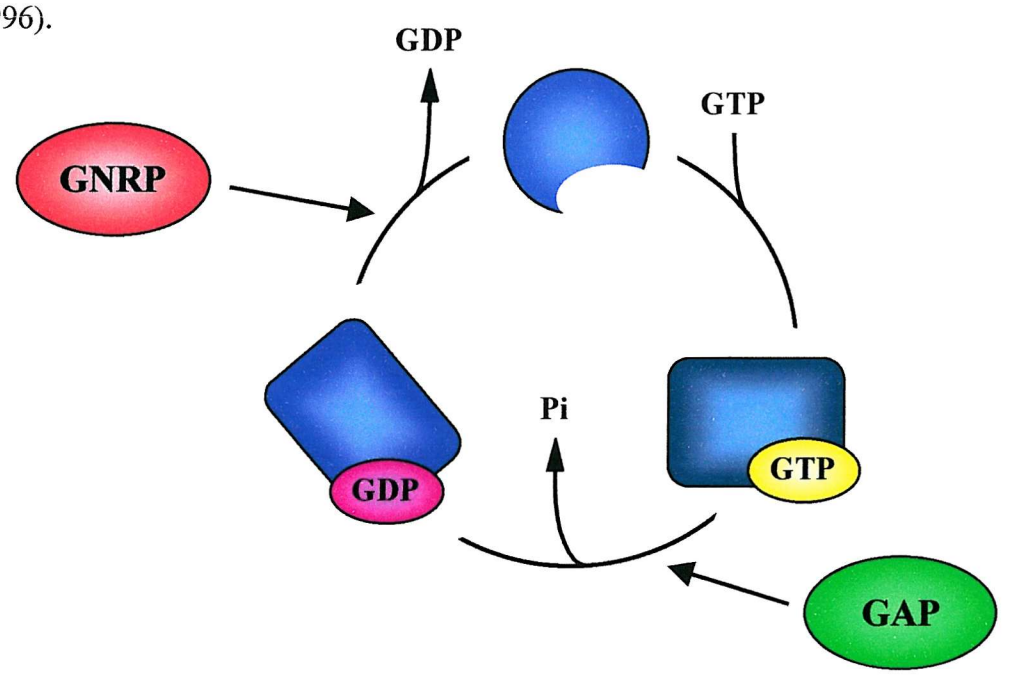


Figure 1.15. GTP cycle, indicating the three conformational states of the protein and action of the GTPase activating proteins (GAP) and guanine nucleotide release proteins (GNRPs). Figure adapted from Bourne *et al.* (1991).

1.19 BipA is a member of the GTPase superfamily and displays sequence similarity to elongation factors

The deduced protein sequence shows that BipA has similarity with the first 131 residues of ribosome binding GTPases, e.g. elongation factor G (EF-G) and the tetracycline resistance TetM/TetO family (Figure 1.16.) (March and Inouye, 1985; Salyers *et al.*, 1990; Qi *et al.*, 1995). The sequence similarity is most highly conserved in the amino-terminal third of the protein that includes areas defining a guanine nucleotide-binding region. G-proteins possess a common structural core for nucleotide binding, which contains common consensus sequence elements involved in the binding of the nucleotide (Kjeldgaard *et al.*, 1996). These conserved sequence motifs include GxxxxGK(S/T) referred to as the phosphate binding loop or the glycine rich loop. The second motif DxxG is located close to the phosphate binding loop and is believed to be involved in the conformational change that takes place between the GDP and GTP forms and a third motif NKxD which determines the specificity for guanine (Kjeldgaard *et al.*, 1996). BipA contains these three motifs known to characterise a G-protein, (residues 12-20, 74-83 and 128-131) (Bourne *et al.*, 1991; Farris *et al.*, 1998), and also contains a fourth motif RGITI that is specifically found in ribosome binding GTPases (Ævarsson, 1995). There are also conserved pockets of nucleotide sequence stretching throughout the gene, including an area of sequence similarity with domain IV of EF-G that has been shown to interact with the 30S ribosomal subunit (Nissen *et al.*, 1995; Stark *et al.*, 2000). The role of GTPases in eukaryotes is well documented but comparatively little is known about such proteins in prokaryotes, although bacterial GTPases have been shown to be involved in protein transport and protein translation (Britton *et al.*, 1998).

1.20 Ribosome binding GTPases

The ribosome binding GTPases EF-G and EF-Tu are involved in the translational elongation cycle and interact with the 70S ribosome during protein biosynthesis. In the GTP form, EF-Tu binds to aminoacylated tRNAs forming a stable ternary

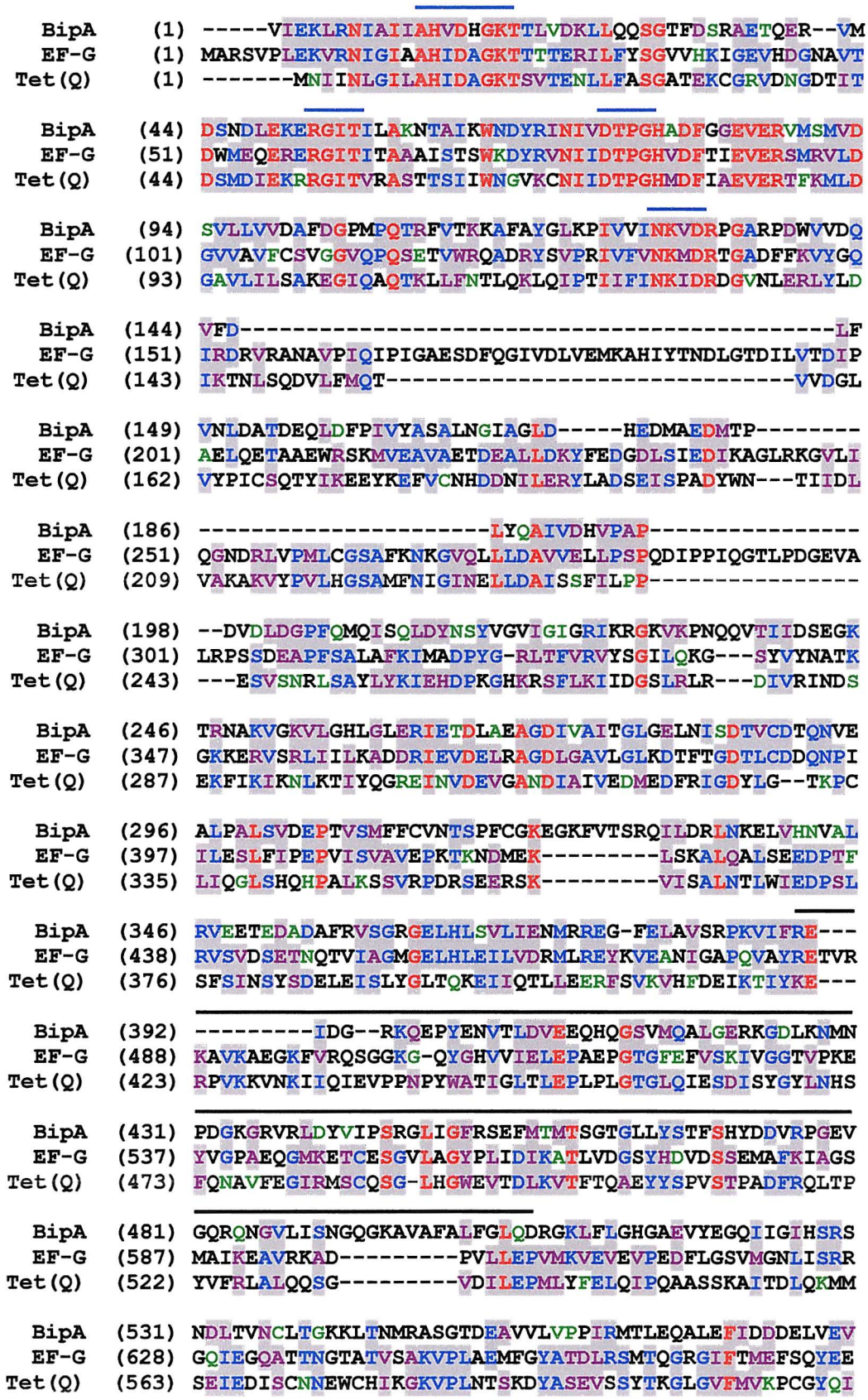

BipA (1) -----VIEKLRNIAIIAHVDHGKTTLVDKLLQQSGTFDSRAETQER--VM
EF-G (1) MARSVPLEKVRNIGIAAHIDAGKTTTTERILFYSGVVHKIGEVHDGNAVT
Tet(Q) (1) -------MNIINLGILAHIDAGKTSVTENLLFASGATEKCGRVDNGDTIT
BipA (44) DSNDLEKERGITILAKNTAIKWNDYRINIVDTPGHADFGGEVERVMSMVD
EF-G (51) DWMEQERERGITITAAAISTSWKDYRVNIIDTPGHVDFTIEVERSMRVLD
Tet(Q) (44) DSMDIEKRRGITVRASTTSIIWNGVKCNIIDTPGHMDFIAEVERTFKMLD
BipA (94) SVLLVVDAFDGPMPQTRFVTKKAFAYGLKPIVVINKVDRPGARPDWVVDQ
EF-G (101) GVVAVFCSVGGVQPQSETVWRQADRYSVPRIVFVNKMDRTGADFFKVYGQ
Tet(Q) (93) GAVLILSAKEGIQAQTKLLFNTLQKLQIPTIIFINKIDRDGVNLERLYLD
BipA (144) VFD---LF
EF-G (151) IRDRVRANAVPIQIPIGAESDFQGIVDLVEMKAHIYTNDLGTDILVTDIP
Tet(Q) (143) IKTNLSQDVLFMQT-----------------------------VVDGL
BipA (149) VNLDATDEQLDFPIVYASALNGIAGLD-----HEDMAEDMTP--------
EF-G (201) AELQETAAEWRSKMVEAVAETDEALLDKYFEDGDLSIEDIKAGLRKGVLI
Tet(Q) (162) VYPICSQTYIKEEYKEFVCNHDDNILERYLADSEISPADYWN---TIIDL
BipA (186) ----------------------LYQAIVDHVPAP----------------
EF-G (251) QGNDRLVPMLCGSAFKNKGVQLLLDAVVELLPSPQDIPPIQGTLPDGEVA
Tet(Q) (209) VAKAKVYPVLHGSAMFNIGINELLDAISSFILPP----------------
BipA (198) --DVDLDGPFQMQISQLDYNSYVGVIGIGRIKRGKVKPNQQVTIIDSEGK
EF-G (301) LRPSSDEAPFSALAFKIMADPYG-RLTFVRVYSGILQKG---SYVYNATK
Tet(Q) (243) ---ESVSNRLSAYLYKIEHDPKGHKRSFLKIIDGSLRLR---DIVRINDS
BipA (246) TRNAKVGKVLGHLGLERIETDLAEAGDIVAITGLGELNISDTVCDTQNVE
EF-G (347) GKKERVSRLIILKADDRIEVDELRAGDLGAVLGLKDTFTGDTLCDDQNPI
Tet(Q) (287) EKFIKIKNLKTIYQGREINVDEVGANDIAIVEDMEDFRIGDYLG--TKPC
BipA (296) ALPALSVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVAL
EF-G (397) ILESLFIPEPVISVAVEPKTKNDMEK---------LSKALQALSEEDPTF
Tet(Q) (335) LIQGLSHQHPALKSSVRPDRSEERSK---------VISALNTLWIEDPSL
BipA (346) RVEETEDADAFRVSGRGELHLSVLIENMRREG-FELAVSRPKVIFRE---
EF-G (438) RVSVDSETNQTVIAGMGELHLEILVDRMLREYKVEANIGAPQVAYRETVR
Tet(Q) (376) SFSINSYSDELEISLYGLTQKEIIQTLLEERFSVKVHFDEIKTIYKE---
BipA (392) ---------IDG--RKQEPYENVTLDVEEQHQGSVMQALGERKGDLKNMN
EF-G (488) KAVKAEGKFVRQSGGKG-QYGHVVIELEPAEPGTGFEFVSKIVGGTVPKE
Tet(Q) (423) RPVKKVNKIIQIEVPPNPYWATIGLTLEPLPLGTGLQIESDISYGYLNHS
BipA (431) PDGKGRVRLDYVIPSRGLIGFRSEFMTMTSGTGLLYSTFSHYDDVRPGEV
EF-G (537) YVGPAEQGMKETCESGVLAGYPLIDIKATLVDGSYHDVDSSEMAFKIAGS
Tet(Q) (473) FQNAVFEGIRMSCQSG-LHGWEVTDLKVTFTQAEYYSPVSTPADFRQLTP
BipA (481) GQRQNGVLISNGQGKAVAFALFGLQDRGKLFLGHGAEVYEGQIIGIHSRS
EF-G (587) MAIKEAVRKAD---------PVLLEPVMKVEVEVPEDFLGSVMGNLISRR
Tet(Q) (522) YVFRLALQQSG---------VDILEPMLYFELQIPQAASSKAITDLQKMM
BipA (531) NDLTVNCLTGKKLTNMRASGTDEAVVLVPPIRMTLEQALEFIDDDELVEV
EF-G (628) GQIEGQATTNGTATVSAKVPLAEMFGYATDLRSMTQGRGIFTMEFSQYEE
Tet(Q) (563) SEIEDISCNNEWCHIKGKVPLNTSKDYASEVSSYTKGLGVFMVKPCGYQI

Figure 1.16. Predicted sequence of the EPEC E2348/69 BipA protein and its alignment with elongation factor G (EF-G) and Tet(Q) the tetracycline resistance protein. EF-G and Tet(Q) sequences shown are from *Anacystis nidulans* (P18667) and *Bacteroides fragilis* (Q08425), respectively, and were chosen because they show the highest degree of sequence identity with E2348/69 BipA (AJ278218). Residues that are identical are indicated with a red foreground and grey background. A blue foreground and a grey background indicate conservative residues; a purple foreground and a grey background depict similar residues, a green foreground and a white background depict weakly similar residues, while non-homologous residues are indicated by a black foreground and white background. Domain IV of EF-G, which is believed to interact with the small subunit of the ribosome and mimics the anti-codon arm of tRNA (Nissen *et al.,* 1995; Rodnina *et al.*, 1997), is depicted by a black line. Blue lines depict motifs known to characterise a G-protein. Sequences were aligned using Vector NTI AlignX.

complex EF-Tu:GTP:aa-tRNA, which binds to the acceptor (A) site of the ribosome where the anticodon on tRNA recognises a codon on the mRNA (Kjeldgaard *et al.*, 1996; Wilson and Noller, 1998). Subsequently GTPase activity is induced, the inactive EF-Tu:GDP is released and the aminoacyl tRNA is bound to the A site, (the specialised nucleotide exchange factor EF-Ts converts the inactive form of EF-Tu to the active GTP form) (Kjeldgaard *et al.*, 1996; Wilson and Noller,1998). After peptide bond formation, the growing peptide chain is transferred to the A site tRNA, and the deacylated tRNA moves to the exit (E) site. The movement of the peptidyl tRNA from the A to the peptidyl (P) site of the ribosome is mediated by EF-G. The mechanism of translocation catalysed by EF-G and the role of GTP hydrolysis are not fully understood. It is speculated that GTP hydrolysis and/or the dissociation of inorganic phosphate induces a conformational strain in EF-G which is connected to a

structural change of the ribosome, promoting the movement of tRNA (Rodnina *et al.*, 1997; Stark *et al.*, 2000).

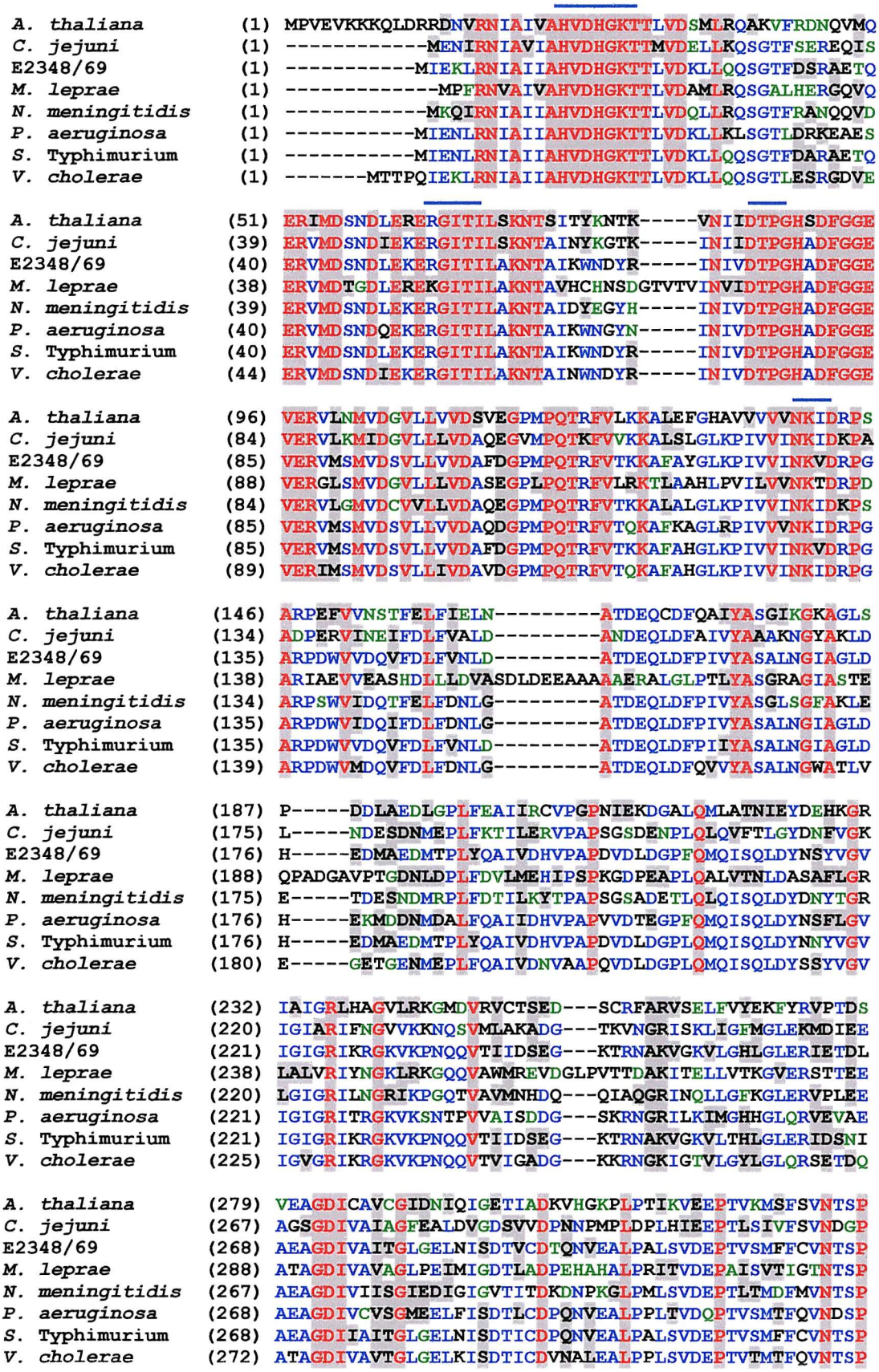
1.21 EPEC BipA is tyrosine phosphorylated

The attachment of a phosphate group to a side chain of an amino acid within a protein is used to control metabolic pathways, a means of rapidly switching the activity of proteins from one state to another. Protein phosphorylation is typically a reversible process in which a γ-phosphate group is transferred from ATP/GTP to the acceptor hydroxyl residue of the protein substrate by a kinase, and removed by a phosphatase. Amino acid side chains that are commonly phosphorylated include serine, threonine, histidine, aspartate and tyrosine.

Freestone *et al.* (1998) report that TypA from EPEC strain MAR001 is phosphorylated in protein extracts supplemented with radioactive ATP or GTP and manganese or magnesium. Farris *et al.* (1998) extended this observation by providing evidence, that BipA/TypA undergoes tyrosine phosphorylation in the same strain (unlike BipA in *E. coli* K-12 and *Salmonella* Typhimurium, which apparently does not undergo tyrosine phosphorylation). The biological importance of these findings is unclear at present and only a few bacterial proteins modified by phosphorylation on tyrosine have been identified (Bakal and Davies, 2000). Whether BipA autophosphorylates or is phosphorylated by a tyrosine kinase has yet to be determined. However, a protein tyrosine kinase, Etk, expressed specifically by a range of strains of *E. coli* has recently been identified (Ilan *et al.*, 1999). It remains to be seen whether BipA is a specific Etk substrate.

1.22 Background to the present studies

Homologues of BipA have been found in most bacteria and intriguingly, also in plants (Figure 1.17). Moreover, BipA has sequence similarity with ribosome-binding elongation factors, suggesting that it may use a novel mechanism to modulate gene


A. thaliana (1) MPVEVKKKQLDRRDNVRNIAIVAHVDHGKTTLVDSMLRQAKVFRDNQVMQ
C. jejuni (1) ----------MENIRNIAVIAHVDHGKTTMVDELLKQSGTFSEREQIS
E2348/69 (1) ----------MIEKLRNIAIIAHVDHGKTTLVDKLLQQSGTFDSRAETQ
M. leprae (1) ----------MPFRNVAIVAHVDHGKTTLVDAMLRQSGALHERGQVQ
N. meningitidis (1) ----------MKQIRNIAIIAHVDHGKTTLVDQLLRQSGTFRANQQVD
P. aeruginosa (1) ----------MIENLRNIAIIAHVDHGKTTLVDKLLKLSGTLDRKEAES
S. Typhimurium (1) ----------MIENLRNIAIIAHVDHGKTTLVDKLLQQSGTFDARAETQ
V. cholerae (1) -------MTTPQIEKLRNIAIIAHVDHGKTTLVDKLLQQSGTLESRGDVE
A. thaliana (51) ERIMDSNDLERERGITILSKNTSITYKNTK-----VNIIDTPGHSDFGGE
C. jejuni (39) ERVMDSNDIEKERGITILSKNTAINYKGTK-----INIIDTPGHADFGGE
E2348/69 (40) ERVMDSNDLEKERGITILAKNTAIKWNDYR-----INIVDTPGHADFGGE
M. leprae (38) ERVMDTGDLEREKGITILAKNTAVHCHNSDGTVTVINVIDTPGHADFGGE
N. meningitidis (39) ERVMDSNDLEKERGITILAKNTAIDYEGYH-----INIVDTPGHADFGGE
P. aeruginosa (40) ERVMDSNDQEKERGITILAKNTAIKWNGYN-----INIVDTPGHADFGGE
S. Typhimurium (40) ERVMDSNDLEKERGITILAKNTAIKWNDYR-----INIVDTPGHADFGGE
V. cholerae (44) ERVMDSNDIEKERGITILAKNTAINWNDYR-----INIVDTPGHADFGGE
A. thaliana (96) VERVLNMVDGVLLVVDSVEGPMPQTRFVLKKALEFGHAVVVVVNKIDRPS
C. jejuni (84) VERVLKMIDGVLLLVDAQEGVMPQTKFVVKKALSLGLKPIVVINKIDKPA
E2348/69 (85) VERVMSMVDSVLLVVDAFDGPMPQTRFVTKKAFAYGLKPIVVINKVDRPG
M. leprae (88) VERGLSMVDGVLLLVDASEGPLPQTRFVLRKTLAAHLPVILVVNKTDRPD
N. meningitidis (84) VERVLGMVDCVVLLVDAQEGPMPQTRFVTKKALALGLKPIVVINKIDKPS
P. aeruginosa (85) VERVMSMVDSVLLVVDAQDGPMPQTRFVTQKAFKAGLRPIVVVNKIDRPG
S. Typhimurium (85) VERVMSMVDSVLLVVDAFDGPMPQTRFVTKKAFAHGLKPIVVINKVDRPG
V. cholerae (89) VERIMSMVDSVLLIVDAVDGPMPQTRFVTQKAFAHGLKPIVVINKIDRPG
A. thaliana (146) ARPEFVVNSTFELFIELN---------ATDEQCDFQAIYASGIKGKAGLS
C. jejuni (134) ADPERVINEIFDLFVALD---------ANDEQLDFAIVYAAAKNGYAKLD
E2348/69 (135) ARPDWVVDQVFDLFVNLD---------ATDEQLDFPIVYASALNGIAGLD
M. leprae (138) ARIAEVVEASHDLLLDVASDLDEEAAAAAERALGLPTLYASGRAGIASTE
N. meningitidis (134) ARPSWVIDQTFELFDNLG---------ATDEQLDFPIVYASGLSGFAKLE
P. aeruginosa (135) ARPDWVIDQIFDLFDNLG---------ATDEQLDFPIVYASALNGIAGLD
S. Typhimurium (135) ARPDWVVDQVFDLFVNLD---------ATDEQLDFPIIYASALNGIAGLD
V. cholerae (139) ARPDWVMDQVFDLFDNLG---------ATDEQLDFQVVYASALNGWATLV
A. thaliana (187) P-----DDLAEDLGPLFEAIIRCVPGPNIEKDGALQMLATNIEYDEHKGR
C. jejuni (175) L-----NDESDNMEPLFKTILERVPAPSGSDENPLQLQVFTLGYDNFVGK
E2348/69 (176) H-----EDMAEDMTPLYQAIVDHVPAPDVDLDGPFQMQISQLDYNSYVGV
M. leprae (188) QPADGAVPTGDNLDPLFDVLMEHIPSPKGDPEAPLQALVTNLDASAFLGR
N. meningitidis (175) E-----TDESNDMRPLFDTILKYTPAPSGSADETLQLQISQLDYDNYTGR
P. aeruginosa (176) H-----EKMDDNMDALFQAIIDHVPAPVVDTEGPFQMQISQLDYNSFLGV
S. Typhimurium (176) H-----EDMAEDMTPLYQAIVDHVPAPDVDLDGPLQMQISQLDYNNYVGV
V. cholerae (180) E-----GETGENMEPLFQAIVDNVAAPQVDLDGPLQMQISQLDYSSYVGV
A. thaliana (232) IAIGRLHAGVLRKGMDVRVCTSED---SCRFARVSELFVYEKFYRVPTDS
C. jejuni (220) IGIARIFNGVVKKNQSVMLAKADG---TKVNGRISKLIGFMGLEKMDIEE
E2348/69 (221) IGIGRIKRGKVKPNQQVTIIDSEG---KTRNAKVGKVLGHLGLERIETDL
M. leprae (238) LALVRIYNGKLRKGQQVAWMREVDGLPVTTDAKITELLVTKGVERSTTEE
N. meningitidis (220) LGIGRILNGRIKPGQTVAVMNHDQ---QIAQGRINQLLGFKGLERVPLEE
P. aeruginosa (221) IGIGRITRGKVKSNTPVVAISDDG---SKRNGRILKIMGHHGLQRVEVAE
S. Typhimurium (221) IGIGRIKRGKVKPNQQVTIIDSEG---KTRNAKVGKVLTHLGLERIDSNI
V. cholerae (225) IGVGRIKRGKVKPNQQVTVIGADG---KKRNGKIGTVLGYLGLQRSETDQ
A. thaliana (279) VEAGDICAVCGIDNIQIGETIADKVHGKPLPTIKVEEPTVKMSFSVNTSP
C. jejuni (267) AGSGDIVAIAGFEALDVGDSVVDPNNPMPLDPLHIEEPTLSIVFSVNDGP
E2348/69 (268) AEAGDIVAITGLGELNISDTVCDTQNVEALPALSVDEPTVSMFFCVNTSP
M. leprae (288) ATAGDIVAVAGLPEIMIGDTLADPEHAHALPRITVDEPAISVTIGTNTSP
N. meningitidis (267) AEAGDIVIISGIEDIGIGVTITDKDNPKGLPMLSVDEPTLTMDFMVNTSP
P. aeruginosa (268) AEAGDIVCVSGMEELFISDTLCDPQNVEALPPLTVDQPTVSMTFQVNDSP
S. Typhimurium (268) AEAGDIIAITGLGELNISDTICDPQNVEALPALSVDEPTVSMFFCVNTSP
V. cholerae (272) ATAGDIVAVTGLGELKISDTICDVNALEALPPLSVDEPTVTMTFQVNTSP

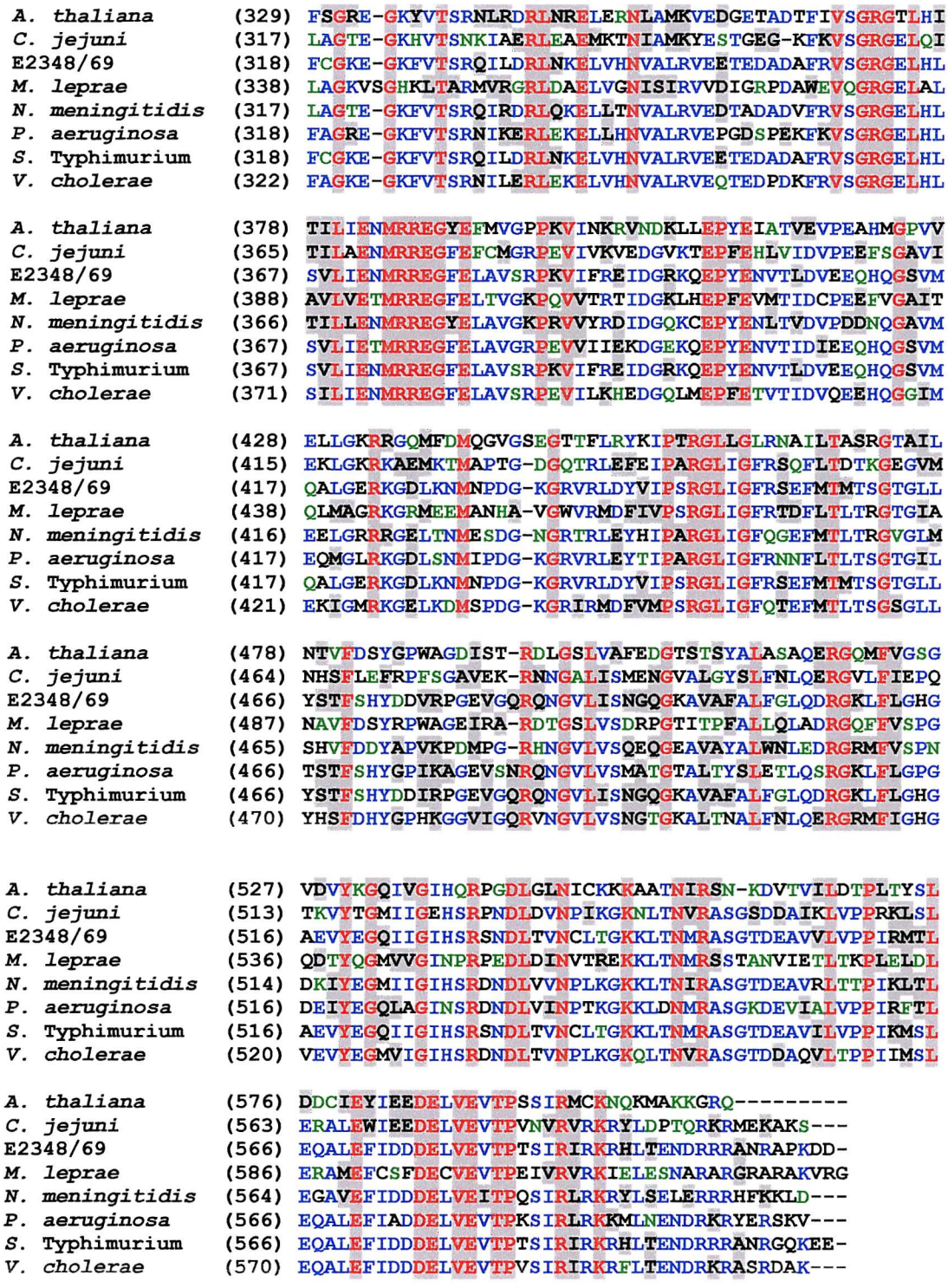


Figure 1.17. Predicted sequence of the EPEC E2348/69 BipA protein and its alignment with various BipA sequences from bacteria and plants. BipA sequences are shown from *Arabidopsis thaliana* (AB0060704), *Campylobacter jejuni* NCTC 1168 (CJ11168X1), E2348/69 (AJ278218), *Mycobacterium leprae* (MLEPRTN6),

Neisseria meningitidis MC58 (AE002467), *Pseudomonasa aeruginosa* (AE004924), *Salmonella* Typhimurium (STY276889) and *Vibrio cholerae* (AE004339). Residues that are identical are indicated with a red foreground and grey background. Similar residues are shown as a black foreground and grey background, a blue foreground and a white background indicate conservative residues; a green foreground and a white background depict weakly similar residues, while non-homologous residues are indicated by a black foreground and white background. Sequences were aligned using Vector NTI AlignX. Regions of homology are apparent throughout the alignment. Blue lines depict motifs known to characterise a G-protein. Table 1.1 indicates the percentage homology between BipA sequences compared to the E2348/69 sequence.

Alignment between BipA from	**Homology (%)**
E2348/69 and *A. thaliana*	47.9
E2348/69 and *C. jejuni*	53.0
E2348/69 and *M. leprae*	45.5
E2348/69 and *N. meningitidis*	62.9
E2348/69 and *P. aeruginosa*	72.8
E2348/69 and *S.* Typhimurium	96.4
E2348/69 and *V. cholerae*	76.9

Table 1.1. Percentage homology between predicted BipA protein sequences. (Percentage homology shown with reference to the E2348/69 sequence).

expression. Indeed, BipA from EPEC strain E2348/69 has recently been shown to interact with ribosomes (R. Owens, unpublished observations). Studies suggest that BipA has GTPase activity that is stimulated in the presence of 70S ribosomes, characteristic of other ribosome binding GTPases (Chinali and Parmeggiani, 1982).

Moreover, BipA and EF-G appear to bind to the same site, or overlapping sites on the ribosome (R. Owens, unpublished results). The current mechanism of action of BipA is undetermined although from the available evidence, it tempting to speculate, that BipA may act as an elongation factor for the translation of a subset of mRNAs. BipA regulates a number of stress- and virulence-associated properties of *E. coli*. These properties include resistance to the antibacterial effects of a host defence protein,expression of the genes for the synthesis of the K5 capsule, flagella-mediated cell motility and the ability of an enteropathogenic strain to trigger cytoskeletal rearrangements in infected host cells (Farris *et al.*, 1998; Rowe *et al.*, 2000).

To date, studies into the effect that BipA has in regulating virulence in EPEC have concentrated mainly on the strain MAR001. The lineage of MAR001 is not certain, other than that it lacks the EAF plasmid, and hence does not contain the *per* and *bfp* genes. Considering the prospective importance of BipA in virulence and its potential novel regulatory mechanism, it would be of some interest to determine if the original observations could be extended to the prototype EPEC strain E2348/69 (serotype O127:H6) that is relatively well characterised. This would facilitate future attempts to dissect the regulatory mechanism used by BipA to exert its various effects. Accordingly, a collaborative initiative (between the labs of Dr David O'Connor, University of Southampton and Professor Peter Williams, University of Leicester) was initiated to clone the *bipA* gene from the prototype EPEC strain E2348/69 (O127:H6) and to produce null mutants in order to investigate the influence of BipA in EPEC pathogenesis. BipA null mutants of JPN15 (i.e. E2348/69 cured of the EAF plasmid) were also required, since the presence of BFP on the surface of E2348/69 results in clumping of bacteria, which could affect the collection of data or interpretation of results. Previous studies have determined that JPN15 adheres to HeLa cells to a lesser extent than E2348/69 and induces fewer pseudopods (extended pedestals) per host cell, although those that form are indistinguishable from those induced by E2348/69 (Rosenshine *et al.*, 1996b).

With this in mind, the aims of the present study are:

- To examine more closely the phenotype of *bipA* null mutants, especially those of the prototypic EPEC strain E2348/69 and its derivatives;

- To determine the means by which BipA regulates expression of an A/E lesion

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

Materials

The following specialist materials were used:

Nitrocellulose transfer membrane (Schleicher and Schuell)
Sterile disposable filters 0.22 μm (Sartorius)
Phase Lock Gel™ III Light Extraction Tubes (5 Prime to 3 Prime)
Blue sensitive X-ray film (GRI)
NuPAGE™ 4–12 % Bis-Tris Gels (NOVEX)
Tissue culture dishes (Nunclon)

Reagents

The following specialist reagents were used:

DNA 1 kb ladder size marker (BRL)
Protein Broad Range Marker (New England Biolabs)
RNase Zap (Invitrogen)
SuperSignal Chemiluminescent Substrate (Pierce)
Qiaprep spin kits (Qiagen)
Dulbecco's modified Eagles's medium (Gibco BRL Life Technologies)
Radiochemicals (Amersham International plc)
Restriction endonucleases and DNA modifying enzymes (New England Biolabs, Promega, Stratagene)
GATEWAY™ Cloning Technology (Gibco BRL Life Technologies)
EZ::TN Transposase (Epicentre)

Unless stated all other reagents were acquired from New England Biolabs, Promega or Sigma.

2.1 General Techniques

2.1.1 Bacterial strains, plasmids, tissue culture and media

Bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2, respectively. All strains were stored at –70°C in 66% LB/33% glycerol (v/v). Bacteria were typically cultivated in Luria-Bertani medium at 37°C. However, for motility plates, tryptone medium was used (Silverman and Simon, 1974), and for expression studies of virulence proteins Dulbecco's modified Eagle medium (DMEM) with Glutamax-1, sodium pyruvate, glucose (0.45% v/v), and pyridoxine (Gibco BRL Life Technologies) was used. Other applications required specific growth conditions, and these are given where necessary. When testing for a cold-sensitive growth phenotype, cells were incubated for 14 hours at 27°C. An overnight culture typically consisted of a single colony being used to inoculate a 10 ml culture that was grown with shaking at 37°C.

Luria-Bertani medium, containing per litre: 10 g tryptone, 5 g yeast extract and 5 g NaCl, was autoclaved at 121°C (15psi) for 20 minutes. Supplementing LB media with 1.5% agar produced LB plates. TB sucrose plates contained per litre: 10 g tryptone, 5 g yeast extract, 5% sucrose and 1.2% agar. M9 minimal media contained per litre: 1 mM $MgSO_4$, 100 μM $CaCl_2$, 1 mM thiamine and 0.2% glucose, supplemented when required with 1.5% agar. Motility agar plates contained per litre: 10 g tryptone, 5 g NaCl, 0.1 g thymine and 0.35% agar. TB medium for L-arabinose induction assays was prepared as for motility agar plates, (excluding agar). SB medium (Mayer, 1995) contained per litre: 32 g NZ amine, 20 g yeast extract, 5 g NaCl and 0.5 ml 10M NaOH, SB plates were produced by supplementing SB media with 1.5% agar. MacConkey plates were produced by autoclaving 40 g of MacConkey agar base (Difco) in a litre of water. DMEM plates were produced by diluting pre-warmed DMEM to two-thirds strength with autoclaved sterile distilled water and agar and to give a final concentration of 1.5% agar. MOPS medium

Table 2.1. Bacterial strains used in this study

Strain	Genotype or Characteristics	Source/Reference
E2348/69	Prototype O127:H6 EPEC strain	M. Donnenberg
E2348/69Δβ-gal Sm^r	Streptomycin resistant *lacZ*⁻ E2348/69	R. Haigh
JPN15	Plasmid cured strain of E2348/69, lacking EAF plasmid	Jerse A.E.
DH5α	F^- ϕ80d*lacZ ΔM15 Δ*(*lacZYA-argF*)U169 *recA*1 *end*A1 *hsdR*17(rk^-mk^+) *sup*E44 λ^- *thi*-1 *gyr*A *rel*A	Hanahan *et al.*, 1991
DH5αλ*pir*	DH5α (λ*pir*)	P. Alefounder
SM10 λ*pir*	Permissive strain for replication of plasmid pCVD442. *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pir*	M. Donnenberg
S17-1(λ*pir*)	Permissive strain for replication of plasmid pCVD442. *recA thi pro hsdRM*.RP4:2-Tc:Mu:Km Tn7 Sm^r λ*pir*	ATCC
MC4100	F^- *araD139 Δ*(*argF-lac*)*U169 rpsL150* (Str^r) *relA1 flbB5301 deoC1 ptsF25 rbsR*	ATCC
JM109	F^- *traD36 lacI*^{q} *Δ*(*lacZ*)*M15 proA*$^+$*B*$^+$/*e*14-(McrA$^-$) *Δ*(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* ($r_k^-m_k^+$) *relA1 supE44 recA1*	I. Clarke
MG1655	F^-	CGSC
MG1655Δ*bipA*	MG1655 with Δ*bipA* inserted into chromosomally located *bipA*	Arigoni *et al.*, 1998
MAR001	EPEC clinical isolate, plasmid cured	V. Norris
MAR001*bipA::cat*	EPEC isolate MAR001*bipA::cat*	Farris *et al.*, 1998
EE347 LB5000	Spontaneous Str^r derivative of LB5000	C. Lee
AG1	E2348/69 with Δ*bipA::cat* inserted into chromosomally located *bipA*, Cm^r	This study
AG2	E2348/69 with Δ*bipA::cat* inserted into chromosomally located *bipA*, Cm^r	This study
AG3	Spontaneous rifampicin resistant mutant of E2348/69 Rif^r	This study

AG4	JPN15 with Δ*bipA::cat* inserted into chromosomally located *bipA*, Cm^r	This study
AG5	JPN15 with Δ*bipA::cat* inserted into chromosomally located *bipA*, Cm^r	This study
AG6	JPN15 with Δ*bipA::cat* inserted into chromosomally located *bipA*, Cm^r	This study
AG7	E2348/69 with Δ*bipA* inserted into chromosomally located *bipA* Rif^r	This study
AG8	E2348/69 with Δ*lacZ* inserted into chromosomally located *lacZ*	This study
AG14	AG2 with Δ*lacZ* inserted into chromosomally located *lacZ*	This study
AG21	MC4100 (λ*pir*)	This Study
AG22	AG8 with *espD::lacZ::Km*^{r} inserted into chromosomally located *espD*, Km^r	This study
AG25	AG14 with *espD::lacZ::Kn*^{r} inserted into chromosomally located *espD* Cm^r , Km^r	This study

Table 2.2. Plasmids used in this study

Plasmid	Genotype or Characteristics	Source/Reference
pMPM123	Low-copy number vector containing an L-arabinose-regulated expression cassette Km^r, Sp^r	Mayer, 1995
pT7T318U	Multifunctional cloning vector Ap^r	Pharmacia
pBR322	Cloning vector Ap^r, Tc^r	Bolivar *et al.*, 1977
pCVD442	π-dependent, *sacB*-containing positive-selection suicide vector Ap^r	Donnenberg and Kaper, 1991
pACYC184	Low-copy number cloning vector encoding Cm^r, Tc^r	New England Biolabs
pDOC70	Derivative of pKK233-2 with deletion of *Eco*RI-*Sal*I fragment of this expression vector and insertion of 1.45 kb *Pst*I fragment from pRME1, carrying Km^r gene of Tn903 and flanked by polylinker	C. D. O'Connor
pTrcHisB	Expression vector Ap^r	Invitrogen
pMOD<MCS>	High copy number transposon construction vector containing a 63 bp multiple cloning site flanked at each end by the hyperactive 19 bp Mosaic End EZ::TN Transposase recognition sequence Ap^r	Epicentre
pHP45Ω	Vector containing Sm^r/Spc^r segment flanked by inverted repeats carrying the T4 transcription-termination signals, and translational stop signals Ap^r	Prentki and Krisch, 1984
pRTP1::Tn5B50	Cloning vector pRTP1 containing the transposon Tn5B50 with *npt* promoter Ap^r, Tc^r	Lee *et al.*, 1992
pT7T318U/MAR*bipA*	*Eco*RI-*Hin*dIII fragment containing MAR001 *bipA* gene and intragenic region cloned in pT7T318U derivative	Farris *et al.*, 1998
pTrc99A	Expression vector Ap^r	Invitrogen
pTrcHisB*bipA*	*Bam*HI-*Hin*dIII fragment containing MAR001 *bipA* gene and intragenic region cloned in pTrcHisB derivative	Farris *et al.*, 1998

pTrcHisB*bipA*(G77V)	*Bam*HI-*Hin*dIII fragment containing MAR001 *bipA* gene and intragenic region cloned in pTrcHisB derivative	C.D.O'Connor
pPA3065	pCVD442 with *attP1-ccdB-Cm^R-attP2* replacing the region between the *Xba*I sites of pCVD442	P. Alefounder
pCVD450	pACYC184 containing the *per* regulatory genes	Gomez-Duarte and Kaper, 1995
pII30	*Eco*RI-*Hin*dIII fragment containing E2348/69 *slyA* gene cloned into pTrc99A, to give IPTG-controlled expression of SlyA Ap^r	S. Swift
pAJG0a	1.2 kb *Xho*I-*Pme*I Δ*bipA* fragment cloned into *Sal*I-*Sma*I digested suicide vector pCVD442 Ap^r	This Study
pAJG0b	*Xmn*I-*Hin*cII fragment containing *cat* gene from pACYC184 cloned into *Swa*I digested pAJG0a Ap^r, Cm^r	This Study
pAJG0c	*Not*I PCR fragment containing *cat* gene from pACYC184 cloned into *Not*I digested pAJG0a Ap^r, Cm^r	This Study
pAJG1	*Sph*I-*Xho*I fragment containing incorrect variant truncated *bipA* gene sequence from E2348/69 cloned into pBR322, Ap^r	This Study
pAJG2	*Sph*I-*Xho*I fragment containing incorrect variant truncated *bipA* gene sequence from E2348/69 cloned into pBR322, Ap^r	This Study
pAJG3	*Sph*I-*Xho*I fragment containing incorrect variant truncated *bipA* gene sequence from E2348/69 cloned into pBR322, Ap^r	This Study
pAJG4	*Age*I-*Sma*I fragment from pT7T318U/MAR*bipA* replaced with an *Age*I-*Sma*I fragment from pAJG1 Ap^r	This study
pAJG9	PCR product of E2348/69 *bipA* gene used to replace an *Age*I-*Hin*dIII fragment from pAJG4 Ap^r	This study
pAJG10	PCR product of E2348/69 *ler* gene with c-Myc tag and 283 bp of upstream sequence cloned as an *Eco*RI-*Swa*I fragment into *Eco*RI-*Xmn*I digested pACYC184 Tc^r	This Study

pAJG11	PCR product of E2348/69 *bipA* gene used to replace an *Age*I-*Hin*dIII fragment from pTrcHisB*bipA* Ap^r	This study
pAJG13	*Age*I-*Hin*dIII fragment from pTrcHisB*bipA*(G77V) replaced with an *Age*I-*Hin*dIII fragment from pAJG11 Ap^r	This study
pAJG14	2.1 kb *Eco*RI fragment containing the Omega interposon from pHP45Ω cloned into *Eco*RI site of pAJG10 Tc^r, Sm^r, Spc^r	This Study
pAJG19	1.5 kb *Sac*I fragment containing a Km^r cassette from pDOC70 cloned into similarly digested pCVD442 Ap^r, Km^r	This Study
pAJG21	*Sal*I-*Sma*I Δ*lacZ* fragment PCR amplified from E2348/69Δβ-gal Sm^r cloned into similarly digested pAJG19 Ap^r	This Study
pAJG27	2.1 kb *Eco*RI fragment containing the Omega interposon from pHP45Ω cloned into *Eco*RI site of pMOD<MCS> Ap^r, Sm^r, Spc^r	This Study
pAJG28	1.3 kb *Sac*I-*Xba*I fragment from pMPM123 containing *araC* and P_{BAD} inserted into pAJG27 Ap^r, Sm^r, Spc^r	This Study
pAJG31	TnΩP_{BAD} insertion into pAJG9 Ap^r, Sm^r, Spc^r	This Study
pAJG32	TnΩP_{BAD} insertion into pAJG9 Ap^r, Sm^r, Spc^r	This Study
pAJG33	TnΩP_{BAD} insertion into pAJG9 Ap^r, Sm^r, Spc^r	This Study
pAJG34	PCR product of *sacB* and 300 bp of upstream sequence cloned as a *Bgl*II fragment into *Bam*HI digested pRTP1::Tn5B50	This Study
pAJG35	PCR product of *espD* with internal linker cloned into pPA3065 using GATEWAY technology Ap^r	This Study
pAJG36	PCR product of MG1655 *lacZ* gene was cloned as a *Not*I-*Asc*I fragment into similarly digested pAJG35 Ap^r	This Study
pAJG38	L-arabinose-inducible *bipA* generated by removal of a 1.7 kb *Sal*I-*Pst*I fragment from pAJG31, and replacement with a 1.6 kb *Sal*I-*Pst*I fragment PCR amplified from E2348/69 chromosomal DNA Ap^r	This Study

pAJG39	PCR amplified Km^r cassette from pDOC70 cloned as an *Asc*I-*Pme*I fragment into similarly digested pAJG36 Ap^r, Km^r	This Study

contained per litre: 40 mM Potassium morpholinopropane sulphonate (MOPS), 4 mM N-Tris(hydroxymethyl)-methyl glycine (Tricine), 10 μM $FeSO_4$, 95 mM NH_4Cl, 0.276 mM K_2SO_4, 0.5 μM $CaCl_2$, 0.528 mM $MgCl_2$, 50 mM NaCl, 3.0 nM $(NH_4)_6(MO_7)_{24}$, 0.4 μM H_3BO_3, 0.01 μM $CuSO_4$, 0.08 μM $MnCl_2$, 0.01 μM $ZnSO_4$, 1.32 mM K_2HPO_4, 0.05% casamino acids, supplemented with 0.4% glucose (high glucose), 0.02% glucose (low glucose). A stock solution of 40 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide was used to prepare a working concentration of 40 μg/ml in X-gal containing agar plates.

Pre-conditioned medium for Quorum sensing experiments was prepared by growing *E. coli* MG1655 in DMEM to an OD_{600} of 1.2. Bacteria were removed by centrifugation at 5000 rpm in a Beckman JA-20 for 15 minutes, and the supernatant was passed through a 0.2 μm filter to further remove any residual bacteria. The filtrate was termed pre-conditioned medium. For studies into quorum sensing, the pre-conditioned medium was diluted 50:50 with fresh pre-warmed DMEM.

A rifampicin resistant derivative of E2348/69 (AG3) was produced, by harvesting bacteria from a 10 ml overnight culture of E2348/69, resuspending in 200 μl LB and plating on a Rifampicin plate.

HeLa cells were grown as a monolayer under 5% CO_2 at 37°C in 35 mm chambers (Nunclon), to approximately 75% confluence in DMEM supplemented with 10% foetal calf serum (Gibco BRL), 0.5 mg/ml fungizone, 1% L-glutamine and 0.55 mg/ml gentamycin. Prior to infection with bacteria, HeLa cells were washed three times with supplement-free DMEM to remove all traces of antibiotics and foetal calf serum.

2.1.2 Antibiotics

Antibiotics where necessary, were added at the indicated concentrations. If required, antibiotic supplements were added to autoclaved medium once it had cooled to below

45°C. Stock solutions were made up in filter sterilised analytical grade water except where indicated. All stock solutions were stored at –20°C.

Ampicillin	-	100 μg/ml	
Chloramphenicol	-	7 μg/ml	(made up in 100 % ethanol)
Kanamycin	-	25 μg/ml	
Rifampicin	-	100 μg/ml	(made up in methanol)
Spectinomycin	-	50 μg/ml	
Streptomycin	-	100 μg/ml	
Tetracycline	-	12.5 μg/ml	(50% ethanol, 50% water)

2.1.3 SlyA induction studies

A 10 ml overnight culture grown in LB was spun at 7000 rpm in a Beckman JA-20 rotor at 24°C for 7 minutes to pellet bacteria. The supernatant was removed and the pellet resuspended in 10 ml of fresh pre-warmed LB. A 1:20 dilution of this culture was used to inoculate 10 ml of LB supplemented where necessary with antibiotic and IPTG. Cultures were grown at 37°C shaking for 3 hours to an OD_{600} of 1.5; bacterial whole cell extracts were then prepared.

2.1.4 Replica plating

Replica plating of agar plates was performed using velveteen squares and a support, as per manufacturers instructions (obtained from Sigma).

2.1.5 Biolog ES MicroPlates

Biolog ES MicroPlates were used, with a slightly modified procedure, from that supplied by the manufacturer. A 10 ml overnight culture was spun at 4000 rpm in a Beckman JA-20 rotor for 10 minutes to pellet bacteria. Supernatant was removed and the pellet resuspended in 0.85% NaCl. 150 μl of suspension was then added to each

of the 96 wells, the plates were incubated at 37°C for 16 hours, followed by reading of the MicroPlate at a wavelength of 590 nm using a microplate reader.

2.2 Techniques Involving DNA

2.2.1 Genomic DNA preparation

A 10 ml overnight culture was spun at 4000 rpm in a Beckman JA-20 rotor for 10 minutes to pellet the bacteria. The supernatant was removed and the pellet resuspended in TE buffer pH 8.0 (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, containing 1 mg/ml proteinase K). The contents were shaken and then incubated standing at 70°C for 30 minutes. SDS (1% final concentration) was then added and the solution left at 70°C for a further 60 minutes. Phenol/chloroform (2 ml) was added to the sample in a phaselock tube (5 prime to 3 prime). The solution was gently agitated and spun at 4000 rpm in a Megafuge 1.0 Heraus 7570E. The supernatant was decanted to a fresh phaselock tube, and the phenol extraction repeated twice more. The supernatant was transferred to a Falcon tube containing room temperature absolute ethanol (3 ml), and the tube inverted until the precipitated DNA appeared fluffy. DNA was removed with a hook fashioned from a pasteur pipette and swirled in -20°C, 75% ethanol. The residual liquid was allowed to drip from the DNA, before the pellet was left to air dry for 30 minutes. The pellet was resuspended in TE buffer and stored at -20°C.

2.2.2 Plasmid DNA preparation

Bulk DNA preparations were obtained using the Qiagen Maxi kit, following the manufacturers instructions. Qiagen plasmid purification protocols were based upon a modified alkaline lysis procedure. Following binding of the DNA from a cleared lysate to an anion-exchange resin under low salt conditions, the DNA was eluted from the column in a high-salt buffer then concentrated and desalted by isopropanol precipitation. DNA was resuspended in sterile analytical grade water.

Small-scale plasmid DNA preparations were obtained from overnight cultures using the Qiagen Mini prep spin kit, following the manufacturers instructions. The DNA was eluted from the silica with sterile analytical grade water.

2.2.3 Phenol, chloroform, ether extraction

This was carried out essentially as per Sambrook *et al.* (1989). Purification of DNA was achieved by the addition of an equal volume of phenol to the sample, which was vortexed thoroughly, followed by centrifugation in a micro-centrifuge to separate the layers. The aqueous layer was removed, and the phenol extraction repeated twice more. This was followed by three extractions in chloroform and, finally, three extractions in ether. After the final ether extraction the sample was left to air dry for 5 minutes, allowing residual ether to evaporate. DNA was resuspended in TE or sterile analytical grade water.

2.2.4 Ethanol precipitation

DNA was recovered, by the addition of 0.1 volumes of 3 M NaAc (pH 5.6), and 2.5 volumes of absolute ethanol. The sample was incubated in a methanol dry-ice bath for 20 minutes, after which time the sample was briefly thawed then spun for 10 minutes at 15000 rpm in a micro-centrifuge to pellet the DNA. Ethanol was removed and the pellet washed in 300 µl -20°C, 75% ethanol to remove salts. The pellet was left to air dry for 5 minutes before being resuspended in sterile analytical grade water.

2.2.5 Estimation of DNA quantity and purity

DNA concentrations were measured by UV spectroscopy taking absorbance readings in quartz cuvettes at 260 nm against a suitable blank. Protein contamination within the sample was estimated by taking an absorbance reading of the sample at 280 nm. If the ratio of absorbance at 260 nm to 280 nm was below 1.75, the DNA was refined by sequential extraction with phenol, chloroform, and ether.

2.2.6 DNA modifications

Routine DNA modifications including: Restriction endonuclease digestion of DNA, modifications of DNA and ligations were carried out as per Sambrook *et al.* (1989). Ligations were carried out using T4 DNA ligase (Promega), and were performed in a Techne thermal cycler using the following conditions: 4 hours at 22°C, followed by 4 hours at 20°C, 4 hours at 18°C, 4 hours at 16°C and 4 hours at 4°C, followed by ethanol precipitation. Unless otherwise stated, all restriction enzymes, modifying enzymes and DNA polymerases were purchased from Promega and New England Bioloabs.

2.2.7 Agarose gel electrophoresis

DNA fragments were resolved by the use of horizontal agarose gels prepared as described by Sharp *et al.* (1973) and McDonnell *et al.* (1977). Agarose was made up in TAE buffer (0.04 M Tris-acetate 0.001 M EDTA) containing 0.6 μg/ml of ethidium bromide. DNA samples were mixed with 0.2 volumes of gel loading buffer (30% v/v glycerol, 0.3% w/v bromophenol blue, made up in TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA)), and loaded into a gel that had been set for 30 minutes. Either side of the samples to be run, 5 μl of a 1 kb DNA ladder (Gibco BRL) (Table 2.3) was used as a size marker. Electrophoresis was carried out at 100 V for 40 minutes in TAE buffer containing 0.6 μg/ml of ethidium bromide. DNA within the gel was viewed by the use of a transilluminator (UVP) with 300 nm UV light.

2.2.8 Extraction of DNA from agarose

DNA fragments to be purified from agarose gels were excised using a clean scalpel blade. The DNA from within these agarose segments was then extracted using the Qiagen Gel-Extraction kit, following the manufacturers instructions. The silica-membrane columns absorb DNA in the presence of high salt, while contaminants pass through the column; DNA was eluted into sterile analytical grade water.

DNA band (BRL 1kb)	Size (kbases)
1	12.21
2	11.19
3	10.18
4	9.16
5	8.14
6	7.12
7	6.01
8	5.09
9	4.07
10	3.05
11	2.03
12	1.63
13	1.01
14	0.51
15	0.50
16	0.39
17	0.34
18	0.29
19	0.22
20	0.20
21	0.15
22	0.13
23	0.04

Table 2.3. Gibco BRL 1 kb DNA marker

2.2.9 PCR amplification of DNA

Polymerase chain reactions (PCR) were carried out essentially as described by Sambrook *et al.* (1989). All PCR reactions were performed in 25 µl reaction volumes in 0.5 ml eppendorf tubes in a Techne thermal cycler, using a 50:50 mixture of *Taq* polymerase (Promega) and high fidelity *Pfu* DNA polymerase (Stratagene), unless otherwise stated. PCR buffer consisted of a final concentration of 1 x Promega Buffer, 2 mM $MgCl_2$, 0.2 nM each dNTP, 20 pmols of each primer and 2.5 U of Polymerase mixture. After addition of template DNA (typically around 200 ng), the PCR mixture was denatured at 94°C for 1 minute before the addition of Polymerase mixture. Thermal cycler conditions typically consisted of: 94°C 1 minute, 55°C 1 minute, 72°C 2 minutes, all experiments used 30 cycles and a final 10 minute hold step. The oligonucleotides used for amplification by PCR, for sequencing and for primer extension were synthesised by Oswel (except MARBIPR2, MARBIPF2, MARBIPF3, MARBIPR4, MARBIPR7, MARBIPF8, MARBIPF9, MARBIPR11 and pBR322F1, which MWG Biotech synthesised, and AJG111 and AJG114 which were synthesised by Life Technologies). Table 2.4 lists the oligonucleotides used in this study.

2.2.10 Crossover PCR deletion

The crossover PCR deletion process of Link *et al.* (1997) was used to create in-frame deletions and was principally carried out in two stages:

(i) Two different asymmetric PCRs were used to generate fragements to the left and right of the sequence to be deleted.

(ii) The left and right fragments were annealed at the overlapping region (incorporated into the inner primers), and amplified by PCR as a single fragment using the outer primers.

The procedure is shown schematically in Figure 2.1 (section 2.2.19).

Table 2.4. Oligonucleotides used in this study

Primer	Primer sequence (5'→3')
MARBIPR2	CGCACGTAGACCATGGTAA
MARBIPF2	GAGACGTACGCGGCCTTT
MARBIPF3	GATAATCTGACCTTCGTAA
MARBIPR4	GAAGGTCAGATTATCGG
MARBIPR7	ACGAGCTGGTAGAAGTGAC
MARBIPF8	ACAGACAGGTGCAGTTCGCC
MARBIPF9	AATCCCACGCTCTTTCT
MARBIPR11	AAAAACATGCCAGACGGT
pBR322F1	AAGTGCGGCGACGATA
LER2	GCTGAATGTATGGACTTGTTGTATGTG
AJG001	AAAAAATCAGCATGCGGACATACTTTAACTCTCCT
AJG014	AAAAAAAACTCGAGATCGAAAAATTGCGTAATATC
AJG015	TACGTATTTAAATGCGGCCGCAGAAGCGTAAACGATCGGGAA
AJG016	GCGGCCGCATTTAAATACGTACGTGAAGGTTTCGAACTGGCG
AJG017	GGGGGGGGGTTTAAACAGAGCTTGTTCCAGAGTCAT
AJG020	GGGGGGGGCTCGAGTCAGTGACGTTAACGAATACG
AJG024	GGGGGGGGGTCGACTCAGTGACGTTAACGAATACG
AJG025	GGGGGGGGCTCGAGTCGGTGCGCGGTTGGCGCGGC
AJG026	GGGGGGGGCTCGAGGATCGGGAAGTCCAGCTGCTC
AJG027	CCCCCCCCGCGGCCGCTGTGACGGAAGATCACTTCGCA
AJG028	TTTTTTTTGCGGCCGCCATAGCTGTTCCTCTAGTCAGTTACGCCCCGCCCTGCCACTC
AJG029	GACCTGACTGTAAACTGC
AJG040	TTCGACTCTCGTGCCGAA
AJG041	CAGATCGAATACCTGATC
AJG044	AAAAAAAATGGATCCACTGAGTCCTTCCTGATAAGGAT
AJG045	GGGGGGGGGCATGCATTTAAATGCGGCCGCTTAGTGGTGGTGGTGGTGGTGAATATTTTTCAGCGGTAT
AJG049	GAAATTGAAACTTCGGAAAATCC
AJG050	TAAGGGAGATACTCGCATTGGTG
AJG051	AAAAAAAAGAATTCTGACTAGCTAAGTCGTTTGTTAACGAGAT

AJG052	GGGGGGGGCAGCTGATTTAAATGCGGCCGCTTACAGATCCTCTTCTGAGATGAGTT TTTGTTCAATATTTTTCAGCGGTAT
AJG053	AAAAAAAAACCGGTAAGAAACTGACCAACATGCGT
AJG054	GGGGGGGGAAGCTTTAATCGTCTTTCGGTGCGCG
AJG055	AAAAAAAACTCGAGGTGAAACCAGTAACGTTATAC
AJG069	TTCTTTCGGTCATTGGCATGTTCA
AJG080	GGGGGGGGCCCGGGTTAAGCGACTTCATTCACCTG
AJG087	GCTGTGGAAGCTGCCTGC
AJG088	CTTCACCGCCTGGCCCTG
AJG089	GAATTCATCGATGATGGT
AJG090	ATGCAAGCTTCAGGGTTG
AJG095	AAAAAAAAGTCGACTAAATAGATGAGCGGCCGCGAAGGAGGGATCCGTGATCGAAA AATTGCGT
AJG096	CCCCCCCCCTGCAGACCGAACAGCGCGAACGC
AJG098	GGATGTGCTGCAAGGCGA
AJG099	CCATCCTATGGAACTGCC
AJG106	GCGGGTCTGGACCACGAA
AJG107	GCGCTGCCGGCACTCTCC
AJG108	TCACCACCGAAGTCGGCG
AJG109	AAAAAAAAATCGATGCGGCCGCACAGGAAACAGCTATGACCATGATTACGGATTCA
AJG110	GGGGGGGGGGTCCCGTTTAAACTTAGAAAAACTCATCGAGCAT
AJG111	GGGGACCACTTTGTACAAGAAAGCTGGGTATGCTTAATGTAAATAACGAT
AJG112	CATACCGGTCTCCTTCGTTTAAACGGCGCGCCAAGCTTGCGGCCGCAACAGCCCAG AGTGCCGGGTT
AJG113	GCGGCCGCAAGCTTGGCGCGCCGTTTAAACGAAGGAGACCGGTATGGTTGCTATTA GTGCAACAGCA
AJG114	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTAAACTCGACCGCTGACAAT
AJG119	CAGAGCGGGTAAACTGGC
AJG120	GGGGGGGGGGTACCGGCGCGCCTTATTTTTGACACCAGACCAA
AJG121	AAAAAAAAGAATTCGGCGCGCCTAGCGCTGAGGTCTGCCTCGT
AJG127	AAAAAAAAAGATCTTAGGCCCGTAGTCTGCAA
AJG128	CCCCCCCCAGATCTTTATTTGTTAACTGTTAA
AJG129	CGCAGAATCAGACATCAG
AJG135	ACGAGTGCACGTTCTGAT
AJG136	CTGGTGCTGTCAGTCGTG
AJG139	TGGCACTGCAAACTGCAG

AJG141	CAATTTGTCGAGTTAAAT
AJG142	ACGTGCTACAATTCGTAC
AJG143	GGGCAGTGAGCGCAACGC
AJG145	TAAGCACATAATTGCTCA

2.2.11 DNA sequencing

Oswel, performed the sequencing reactions using an ABI-100 sequencer unless otherwise stated. Where indicated sequencing reactions were analysed using a Li-Cor 4000 automated sequencer. DNA was sequenced by the cycle sequencing method using an Amersham Thermosequenase Sequencing Kit and 5' IRD-800 labelled primers (MWG Biotech), as per manufacturer's instructions. Reactions were stopped by the addition of Stop Buffer (0.01 M EDTA (pH 9.5), 10 mM NaOH, 10 mg Blue Dextran in 10 ml deionised formamide) and stored at -20°C in the dark.

2.2.12 Transformations

Electroporation

Cells for transformation by electroporation were grown to mid-exponential phase, (an absorbance of 0.4 – 0.5 at 600 nm was used). Cultures were chilled on ice for 15 minutes after which time bacteria were pelleted at 6000 rpm at 4°C for 15 minutes in a Beckman JLA 10,500 rotor. Culture supernatants were removed and the pellet resuspended in a suitable volume of ice-cold sterile analytical grade water and re-spun; this was repeated. The pellet was resuspended in 25 ml of sterile ice cold 10% glycerol, prior to a further spin at 6000 rpm 4°C for 15 minutes in a Beckman JA-20 rotor. Supernatant was removed and the bacteria resuspended in the residual glycerol, before being aliquoted into 40 µl volumes and stored at –70°C.

Electroporation reactions were performed essentially as described by Dower *et al.* (1988). Desalted DNA (10-100 ng) was added to the thawed 40 µl aliquot of bacteria, and incubated on ice for 1 minute. The sample was transferred to an electroporation cuvette (1 mm gap) and the bacteria shocked with a voltage of 1.25 mV for 4.5 – 5 msec. Bacteria were resuscitated immediately by the addition of 1 ml of sterile SOC medium (10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, 20 g Bacto-tryptone and 5 g Bacto-yeast extract in 1 litre) to the

cuvette. After recovery for one hour at 37°C with shaking, cells were serially diluted and plated.

Calcium competent cells

Cells were made competent by $CaCl_2$ treatment described by Sambrook *et al.* (1989). Essentially bacteria were grown to mid-exponential phase, (an absorbance of 0.4 – 0.5 at 600 nm was used). Cultures were chilled on ice for 15 minutes after which time bacteria were pelleted at 6000 rpm at 4°C for 15 minutes in a Beckman JLA 10,500 rotor. Culture supernatants were removed and the pellet resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and re-spun; this was repeated. The pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ before being aliquoted into 200 µl volumes and stored at –70°C. DNA was added to the competent bacteria, mixed by swirling and incubated on ice for 30 minutes, after which time the mixture was heat shocked at 42°C for 90 seconds followed by incubation on ice for 2 minutes. Bacteria were resuscitated immediately by the addition of 800 µl of sterile SOC medium, after recovery for one hour at 37°C with shaking, cells were serially diluted and plated.

2.2.13 Southern Blotting

The method used for Southern blotting was a modification of the method described by Sambrook *et al.* (1989).

Transfer of DNA

The DNA to be probed was digested with the appropriate restriction enzyme and loaded onto a 0.8% agarose gel, which was run at 120 V for 4 hours. The gel was washed in 0.1 M HCl for depurination, and the DNA denatured by immersing the gel for 30 minutes in several volumes of an alkali-denaturing solution (1.5 M NaCl, 0.5 M NaOH), with constant gentle shaking. The gel was neutralised by immersing for 30 minutes in several volumes of neutralisation solution (1 M Tris-HCl pH 7.4, 1.5 M

NaCl). Capillary action transferred the DNA onto nitrocellulose membrane (Schleicher and Schuell). Nucleic acids were fixed to the membrane after transfer, by placing the membrane in an envelope of Whatman 3MM paper and baking in an 80°C oven for two hours. Membranes were stored wrapped in cling film and foil prior to being probed.

Preparation of probe

The probe fragment was produced from a PCR product and radiolabelled via random hexamers using the Prime-a-Gene Kit (Promega). The labelling reaction mix contained, in the following order, a final concentration of 1 x labelling buffer, 20 μM each of the unlabelled dNTPS (dCTP, dGTP, dTTP), 500 ng/ml of DNA template (previously denatured by heating to 95°C for 2 minutes followed by transfer to ice), 400 μg/ml nuclease-free BSA, 1mCi/ml [α ^{32}P] dATP (3000Ci/mmol) (Amersham Corporation) and 100 u/ml DNA polymerase Klenow fragment. The reaction-mix volume was made up to 50 μl with the addition of nuclease-free water. The reaction was incubated at 37°C for 60 minutes, and then heated to 95°C for 2 minutes followed by the addition of EDTA to a final concentration of 20 mM and incubation on ice to stop the reaction.

Hybridisation of probe

The nitrocellulose membrane was washed in 2 x SSC (0.2 M NaCl, 0.03 M trisodium citrate (pH 7.0)) rolled gently and placed in a hybridisation bottle. The membrane was pre-hybridised in 10 ml of pre-warmed hybridisation solution (5 x SSC, 0.5% SDS, 5 x Denhardt's solution (0.1% Ficoll, 0.1% Polyvinylpyrolidine 0.1% Bovine Serum Albumin), 0.2 mg/ml Salmon Sperm DNA, made up in analytical grade sterile distilled water) at 65°C for 1 hour. The pre-hybridisation solution was then replaced with 10 ml of hybridisation solution (as pre-hybridisation solution, with the addition of the 50 μl labelled probe). Hybridisation was performed at 65°C overnight, followed by washing of the membrane in 50 ml of Wash Buffer 1 (2 x SSC and 0.5%

SDS), pre-warmed to 65°C. After incubation for 1 minute, the Wash Buffer was removed, and the 65°C wash repeated. Two further washes were carried out with Wash Buffer 1 at 65°C for 15 minutes. Finally two washes in Wash Buffer 2 (1 x SSC and 0.1% SDS) were carried out for 15 minutes at 25°C. The membrane was blotted dry and wrapped in cling film, before being placed on a Phosphor screen for 2 hours and developed using a Molecular Dynamics Storm Imager and Image Quant analysis.

2.2.14 Cloning and sequence analysis of EPEC *bipA*

Standard genetic techniques were employed to clone the EPEC *bipA* gene from E2348/69 (Sambrook *et al.*, 1989). Following amplification of *bipA* and its intragenic region by PCR from E2348/69 chromosomal DNA, the *bipA* gene was cloned into plasmid pBR322, generating pAJG1. Further constructs were produced in a similar manner, however, sequencing highlighted PCR artefacts within the *bipA* sequences. Thus to create a cloned copy of E2348/69 *bipA* with no errors, a partial gene replacement was carried out using pAJG1 (this study) and pT7T318U/MAR*bipA* (Farris *et al.*, 1998) generating pAJG4. The EPEC *bipA* gene was sequenced on both strands using an ABI-100 sequencer and custom synthesised primers (Table 2.4) to confirm its structure.

2.2.15 Sequence analysis

Software package Omiga 1.1.3 (Oxford Molecular Ltd.) was used for sequence analysis. Software package Vector NTI AlignX (Informax Inc) was used for homology alignments and software package Vector NTI (Informax Inc) was used for drawing plasmid maps.

2.2.16 MG1655 *bipA* sequence (ECAE462) used to guide the design of 3' *bipA* specific primers contains a deletion in the 3' end

Recent homology alignments of the EPEC E2348/69 and K-12 *bipA* sequences with *Salmonella* and a number of other bacterial species highlighted an extended 3' region in all strains but K-12 and E2348/69 (R. Haigh, unpublished observations). In the K-12 and E2348/69 strains there was a potential frameshift in the last codon, prematurely terminating the BipA protein. The removal of the frameshift by the addition of a further 'c' at nucleotide position 1774 extended the protein by a further 16 amino acids in keeping with the *Salmonella* sequence. Primers were designed to PCR amplify a region of the MG1655 and E2348/69 chromosome in order to confirm whether there was a frameshift in the K-12 and E2348/69 *bipA* sequence. Sequence analysis of the MG1655 and E2348/69 *bipA* gene highlighted the presence of an additional 'c' at position 1774 extending the open reading frame of *bipA* to 1824 nucleotides, in keeping with other *bipA* gene sequences. In view of the frameshift, pAJG4 was modified. The resulting construct, designated pAJG9, contained a 2227 bp fragment in which full length *bipA* was expressed from its native promoter(s).

2.2.17 Nucleotide sequence accession number

The nucleotide sequence of the *bipA* gene from EPEC E2348/69 was deposited in the EMBL Nucleotide Sequence Database with accession number AJ278218.

2.2.18 Generation of full-length hexahistidine-tagged E2348/69 *bipA* derivatives

The production of a hexahistidine-tagged and full-length version of the BipA protein was performed as described for pAJG9. The entire *bipA* gene in the resulting construct, pAJG11 was sequenced using custom synthesised primers to confirm its integrity. A full length E2348/69 pTrcHisB*bipA*(G77V) substituted mutant was also produced by gene replacement generating the plasmid pAJG13.

2.2.19 Construction of *bipA* deletion mutants in EPEC E2348/69

Standard genetic techniques (Sambrook *et al.*, 1989), as well as the modification of a crossover PCR method used to create in-frame deletion constructs (Link *et al.*, 1997), were employed to construct a *bipA* null mutant of EPEC strain E2348/69. The pCVD442Δ*bipA* construct, which was used for the production of *bipA* null mutants, was produced by separately cloning out the 5' and 3' regions of the *bipA* gene using PCR amplification. The resulting fragment contained a deletion of around 600 bp in the middle region of the *bipA* gene, and was cloned into the suicide vector pCVD442 generating the construct pAJG0a. Targeted disruption was achieved by inserting the structural gene for cat from pACYC184. (Described in detail in Chapter 3 section 3.3 and shown in Figure 2.1).

Transfer of the constructs to E2348/69 and selection for allelic exchange with the chromosomal *bipA* gene was performed using sucrose-TB medium, as described previously (Donnenberg and Kaper, 1991), and a modification of a conjugation reaction described by Chikami *et al.* (1985). Briefly 200 µl of overnight culture of the E2348/69 recipient strain was mixed with 100 µl of Donor strain containing the disrupted *bipA* gene on a suicide plasmid, and pipetted onto a sterile 0.22 µm nitrocellulose filter (Millipore) on an LB plate without spreading. After four hours incubation at 37°C, the filter was removed and bacteria were resuspended in 1 ml of Phosphate Buffered Saline (PBS) (8 mM Na_2HPO_4, 137 mM NaCl, 0.5 mM $MgCl_2$, 1.6 mM KH_2PO_4, 2.7 mM KCl). Recipients carrying the plasmid integrated into their chromosome due to a single cross-over event were selected on M9 Minimal Media plates containing suitable antibiotics. Constructs having undergone successful transfer were then plated onto sucrose-TB medium containing suitable antibiotics to select for a second cross-over bringing about allelic exchange with the chromosomally located *bipA* gene. (The process of allelic exchange is shown diagrammatically in Figure 2.2). The resultant mutants with a chromosomally located Δ*bipA* were confirmed by PCR using primers to DNA regions outside of the *bipA*

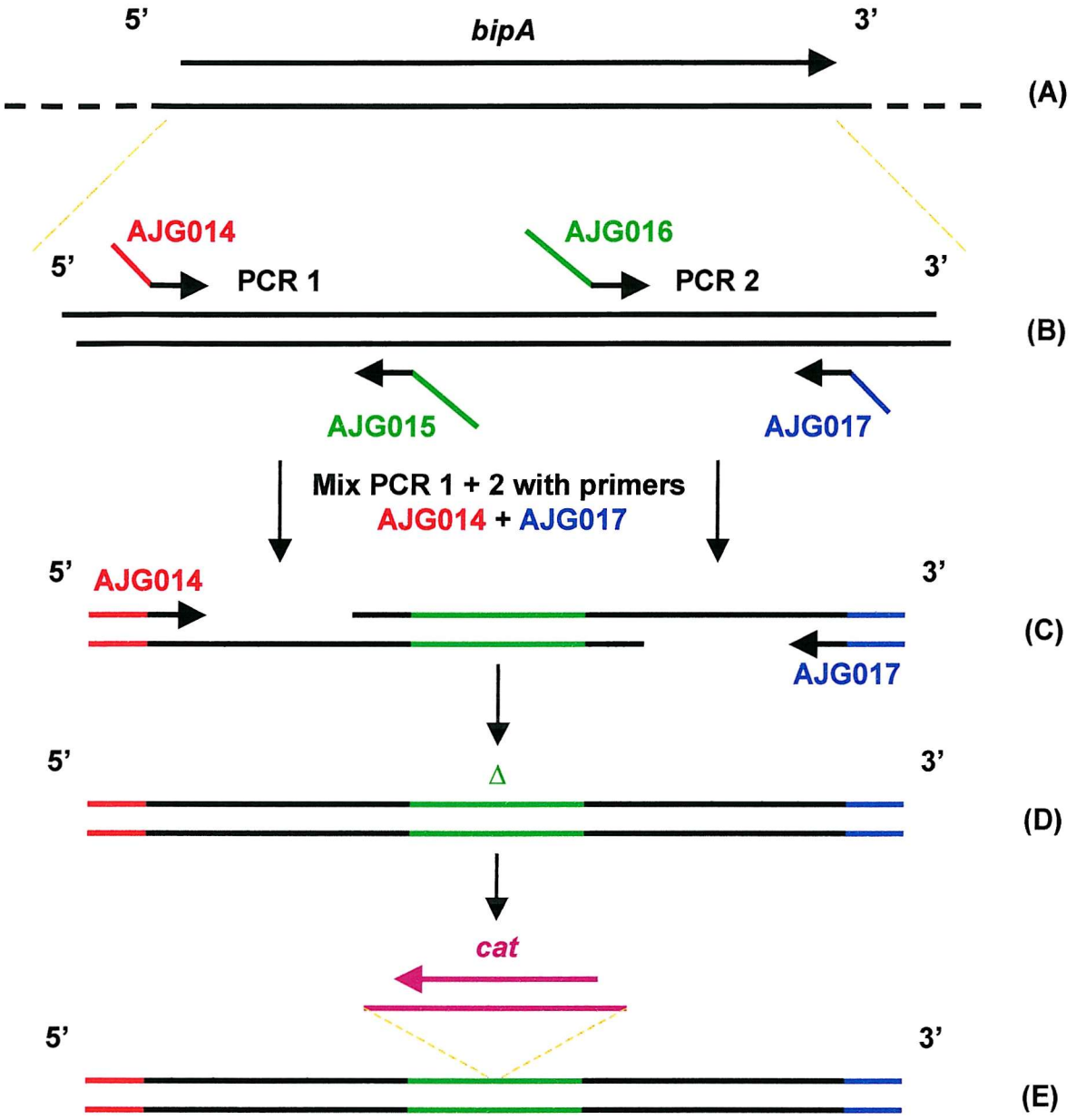


Figure 2.1. Targeted disruption of the *bipA* gene of EPEC E2348/69, adapted from Link *et al.* (1997).

(A) Region of the chromosome containing the *bipA* gene

(B) Shows the binding regions of the primers, indicating the two PCR reactions that will produce Δ*bipA* when fused

(C) The fused molecule is amplified by PCR, using AJG014 and AJG017

(D) Amplified product generates Δ*bipA*

(E) Insertion of *cat* cassette into engineered sites in complementary region

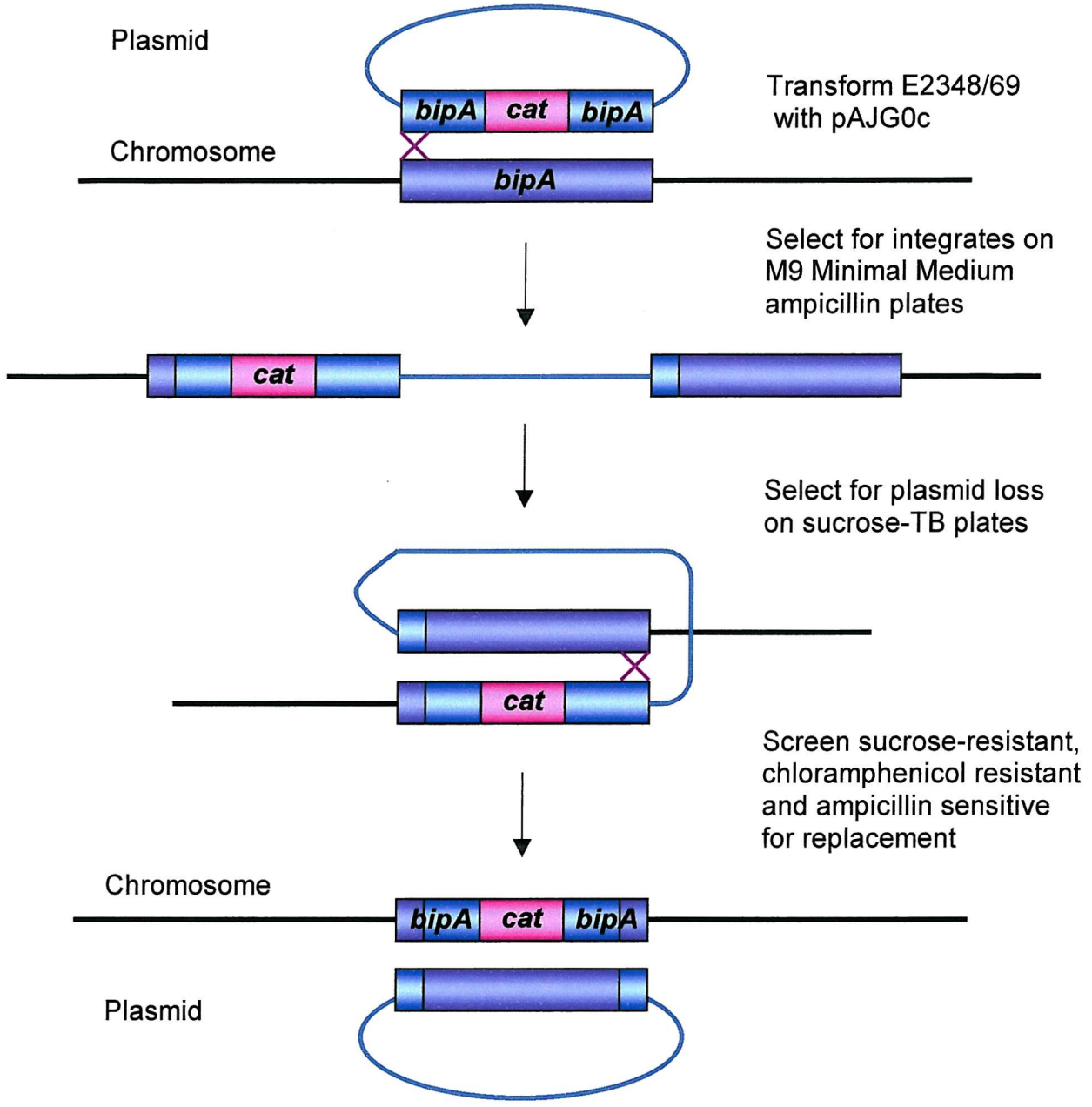


Figure 2.2. Protocol used for replacing the wild-type *bipA* sequence on the chromosome with an *in vitro* altered sequence. Diagram adapted from Link *et al.* (1997). The construct containing the *in vitro* altered sequence is transformed into EPEC, an integration event allows replication of plasmid sequences by the chromosomal origin. The counter-selectable *sacB* marker is used to select for a second recombination event, resulting in loss of the plasmid.

gene (AJG001 and AJG024; Table 2.4). Additionally, candidates were verified by Southern hybridisation. Western Blotting also confirmed that mutants failed to cross-react with anti-BipA antibodies.

2.2.20 Production of an *in vitro* transposon system for highly regulated gene expression

The plasmid pMOD<MCS> (Epicentre Technologies Inc.), which contains a multiple cloning site flanked by 19 bp inverted repeats that are recognised by a hyperactive form of Tn5 transposase (Goryshin *et al.*, 2000; Goryshin and Reznikoff, 1998), was used to construct the transposable promoter (described in detail in Chapter 8 section 8.2). The *in vitro* transposon (TnΩP_{BAD}) thus made could be conveniently excised from pAJG28 as a 3.5 kb fragment using *Pvu*II

2.2.21 *In vitro* transposition

A 3.5 kb *Pvu*II fragment carrying the *in vitro* transposable element (TnΩP_{BAD}) was excised form pAJG28, gel purified and desalted using a QIAquick gel extraction kit. The *in vitro* transposon insertion reaction mix (9 µl) was composed of 50 mM Tris-acetate, pH 7.5, 0.15 M potassium acetate, 10 mM magnesium acetate, 4 mM spermidine, containing 0.2 µg of pAJG9 target DNA and the molar equivalent of TnΩP_{BAD}. After the addition of 1 µl of hyperactive Tn5 transposase (Epicentre Technologies), the reaction was incubated for 2 hours at 37°C, and was stopped by the addition of 1 µl of EZ::TN 10x Stop Solution (Epicentre Technologies), followed by heating to 70°C for 10 minutes. 1 µl was used for electroporation into DH5α; transformants bearing the transposable promoter were selected on plates containing ampicillin, spectinomycin and streptomycin.

2.2.22 Production of a *bipA* derivative with arabinose-inducible expression

An L-arabinose-dependent construct was produced by deleting the region between P_{BAD} and the *bipA* open reading frame of pAJG31, generating the plasmid pAJG38 (described in detail in Chapter 8 section 8.3).

2.2.23 Production of a c-Myc tagged *ler*

The *ler* gene along with its upstream promoter region and encoding a c-Myc tag at the 3' end was PCR amplified from E2348/69 and cloned into pACYC184 generating the plasmid pAJG10, which was electrotransformed into DH5α, confirmed by restriction analysis and sequenced using an ABI-100 sequencer and custom made primers.

2.2.24 Production of a c-Myc tagged *ler* preceded by the Omega fragment

The Omega interposon bearing a streptomycin/spectinomycin resistance marker flanked by strong transcriptional and translational terminators (Prentki and Krisch, 1984) was inserted as a 2.1 kb *Eco*RI fragment from the plasmid pHP45Ω (Prentki and Krisch, 1984), into the *Eco*RI site of pAJG10 generating the plasmid pAJG14.

2.2.25 Construction of a kanamycin resistant derivative of pCVD442

Restriction sites in the multiple cloning site of plasmid pCVD442 were separated by the introduction of a kanamycin resistance cassette. This not only tagged the construct with a further selectable marker but aided restriction digestion with certain enzymes. The kanamycin resistance marker, originally from Tn903 (Oka *et al.*, 1981), was transferred from pDOC70 to pCVD442 as a *Sac*I fragment and the resulting plasmid was designated pAJG19.

2.2.26 Construction of EPEC delta *lacZ* strains in the E2348/69 wild-type and *bipA* null mutant strains

E2348/69 wild-type and the *bipA*⁻ mutant Δ*lacZ* mutants of the strains were produced by the allelic exchange of chromosomally located *lacZ* with inactivated Δ*lacZ* on the plasmid pAJG21. The plasmid pAJG21 containing a DNA fragment carrying the deleted *lacZ* gene was produced by the PCR amplification of the *lacI* Δ*lacZ lacY* region from chromosomal DNA prepared from an E2348/69 Δβ-galactosidase Sm^r strain (R. Haigh, personal gift). pAJG21 was electrotransformed into DH5α(λ*pir*) and confirmed by the analysis of restriction digests. The structure of representative isolates with chromosomally located Δ*lacZ* genes were confirmed by Southern blotting.

2.2.27 Production of MC4100(λ*pir*) strain

To aid the cloning of a functional *lacZ* gene into the pCVD442-derived plasmid pAJG35, it was desirable to use a *lacZ* null mutant that also expressed the *pir* gene to allow plasmid replication (Donnenberg and Kaper, 1991). A derivative of the Δ*lacZ* strain MC4100 containing λ*pir* was prepared essentially as previously detailed by Silhavy *et al.* (1984). Samples of 1.5 ml of an overnight MC4100 culture were spun at 13000 rpm in a microcentrifuge. Following resuspension of the pellets in 1 ml of 10 mM $MgSO_4$, 100 µl of the cells was mixed with 2.5 ml of molten TB top agar and poured over TB plates. Following serial dilution of a lysate of λ*pir* in λ diluent, 200 µl samples of the 10^{-1} to 10^{-4} dilutions were spotted onto the plates. After drying, the plates were incubated at 37°C overnight. A scraping from a resulting area of lysis was restreaked onto a fresh LB plate, which was incubated overnight at 37°C. Colonies from this plate were restreaked, generating the strain AG21.

2.2.28 Production of an *espD::lacZ* transcriptional fusion in EPEC

The plasmid pAJG39, harbouring the cloned *espD::lacZ::km*^{r} fusion, was created in a multi stage cloning procedure utilising the recent GATEWAY cloning technology (Life Technologies), a PCR method adapted from Link *et al.* (1997), and standard cloning techniques (Sambrook *et al.*, 1989), (described in detail in Chapter 7 section 7.2).

GATEWAY™ Cloning Technology replaces the requirement for restriction enzymes and ligase with a mechanism based on the site-specific recombination of bacteriophage λ, whereby lambda can integrate site specifically into a gene by lysogenisation mediated by Int and IHF. The *espD* gene from E2348/69 was cloned into the suicide vector pPA3065 the 'Donor Vector' (P. Alefounder) by an *att*B + *att*P reaction mediated by BP CLONASE Enzyme Mix containing Int and IHF. pPA3065 contains *att*P sites, for recombination, and *ccdB*, encoding a toxic protein that interferes with the rejoining step of DNA gyrase, causing the chromosome to be digested. Plasmids containing the *ccdB* gene require propagation in a gyrase mutant (*gyrA462*) strain, thus *gyr*$^+$ cells harbouring a Donor Vector that has not undergone recombination will be killed.

The BP Cloning Reaction was composed on ice as: BP Reaction Buffer (4 µl), PCR product (400 ng), Donor Vector (300 ng) and TE to 16 µl. BP CLONASE was thawed on ice for 2 minutes, vortexed twice for 2 seconds, and 4 µl added to the reaction mix. The reaction was vortexed and incubated at 25°C for 16 hours in a Techne thermal cycler. After incubation, 2 µl of Proteinase K solution was added to the reaction, which was then incubated at 37°C for 10 minutes. Following recombination, 1 µl of the BP reaction was transformed into calcium competent DH5α(λ*pir*). Cells in which the Δ*espD* gene was present in pPA3065 were selected by growth on the appropriate media, followed by restriction analysis of plasmid DNA. A suitable construct designated pAJG35 was sequenced using primers AJG111, AJG112, AJG113 and AJG114.

The *lacZ* gene was PCR amplified from MG1655 chromosomal DNA and cloned into pPA3065 generating the plasmid pAJG36 which was transformed by electroporation into MC4100(λ*pir*) and selected for by plating on media containing the appropriate antibiotic and X-gal. The plasmid was further confirmed by PCR and restriction analysis of plasmid DNA. The kanamycin resistance cassette was amplified by PCR from pDOC70 and cloned into pAJG36, generating the plasmid pAJG39. Chromosomally located *espD::lacZ* fusions were produced by the allelic exchange of chromosomally located *espD* with the *espD::lacZ::km*^{r} fragment on the plasmid pAJG39. A representative AG8*espD::lacZ::km*^{r} construct was named AG22, and a representative AG14*espD::lacZ::km*^{r} construct was named AG25 (Figure 7.3).

2.2.29 Production of pAJG34 – a derivative of pRTP1::Tn5B50 that contains *sacB*

The *sacB* gene from pCVD442 was amplified by PCR and inserted into pRTP1::Tn5B50, the resulting construct was designated pAJG34. To validate the use of pAJG34 as a transposon delivery system, it was electrotransformed into AG2. Serial dilutions were then plated on tetracycline plates, followed by replica plating onto sucrose-TB + tetracycline plates. Colonies that grew on these plates were further subjected to replica plating onto tetracycline plates and ampicillin + tetracycline plates. From 1400 colonies replica plated at this last stage around 7% were found to still be ampicillin resistant. This figure is significantly higher than the background 0.2% reported by Lee *et al.* (1992), but was considered a manageable number for future transposition selection events.

2.3 Techniques Involving RNA

2.3.1 RNA extraction

Total cell RNA was isolated using a modification of the acid phenol procedure described by Aiba *et al.* (1981).

A 40 ml culture was grown to OD_{600} of 0.5 and bacteria were harvested at 4000 rpm in a Beckman JA-20 rotor at 4°C for 15 minutes. The supernatant was removed and the pellet resuspended in 0.8 ml of lysis solution (0.15 M sucrose, 0.01 M NaAc (pH 4.5) 1% SDS) and incubated at 60°C for 5 minutes. 0.5 ml of acid phenol pre-heated to 60°C was added to the solution and incubated at 60°C for 5 minutes with occasional shaking. The mixture was incubated on ice for 10 minutes and spun at 13000 rpm in a Beckman JA-20 rotor at 4°C for 5 minutes. The aqueous phase was removed and the phenol extraction repeated twice more. 1 µl of RQ1 DNase (DNase I, RNase free, Boehringer) was added to the collected aqueous layer and incubated at room temperature for 30 minutes, followed by the extraction of the aqueous phase with phenol/chloroform/isoamyl alcohol (24:24:1). RNA was precipitated with 2.5 volumes of ice-cold absolute alcohol at –70°C for 60 minutes. The precipitate was collected by centrifugation at 13000 rpm in a micro-centrifuge at 4°C for 30 minutes, the ethanol was removed and the pellet washed with ice-cold 70% ethanol. The precipitate was collected by centrifugation at 13000 rpm in a micro-centrifuge at 4°C for 15 minutes, the pellet was left to air dry for 10 minutes before being resuspended in 50 µl of DEPC treated water. RNA was stored at –70°C to aid dissolving and to prevent degradation.

2.3.2 Estimation of RNA quantity and purity

RNA concentrations were calculated by UV spectroscopy, performing absorbance readings in quartz cuvettes at 260 nm against a suitable blank. RNA purity was estimated by comparing the ratio of absorbance at 260 nm and 280 nm. Further conformation of the quality of the RNA, was tested, by using agarose gel electrophoresis, in which the gel and buffer were prepared using DEPC treated water and the apparatus treated with RNase Zap (Invitrogen).

2.3.3 5' end labelling of primers

The labelling reaction mix contained, in the following order, a final concentration of: 100 pmoles of primer, 25 μCi [γ ^{32}P] dATP (Amersham Corporation), DEPC treated water to a final volume of 28.5 μl, 3 μl of 10 x kinase buffer (pH 7.6) (70 mM Tris-HCl, 7 mM $MgCl_2$, 5 mM DTT) and 15 units of T4 Polynucleotide kinase. The reaction was incubated at 37°C for 30 minutes and then quenched by the addition of 470 μl of analytical grade water and incubation on ice, or storage at –20°C.

2.3.4 Primer extension reaction

The mix contained in the following order: 5 μg of RNA, 0.5 pmol of labelled primer, 1.5 μl of 5 x hybridisation buffer (250 mM HEPES (pH 7.0), 500 mM KCl in DEPC treated water). The reaction-mix volume was raised to 7.5 μl with DEPC treated water. The reaction was incubated at 90°C for 3 minutes then cooled slowly to 45°C, followed by incubation on ice. The following were then added to the mix: 2.5 μl of 5 x avian myeloblastosis virus reverse transcriptase (AMV RT) buffer (Boehringer), 2 mM of each dNTP, 20 units of RNase Inhibitor (Boehringer) and 25 units of AMV RT (Boehringer). The reaction was incubated at 42°C for 60 minutes before being stopped by the addition of 10 μl of sequencing stop buffer (95% formamide, 20 mM EDTA (pH 8.0), 0.05% Bromophenol blue, 0.05% xylene cyanol), and stored at -20°C until use.

2.4 Protein Techniques

2.4.1 Estimation of protein concentration

Protein concentrations were calculated by taking absorbance readings in quartz cuvettes at 280 nm against a suitable blank. (It was assumed that an absorbance of 1 was equal to 1 mg/ml).

Protein concentrations were also measured spectrophotometrically by the bicinchoninic (BCA) assay (Pierce), performed according to the manufacturers instructions. Bovine serum albumin (BSA) standards were diluted from a stock solution with sample buffer. Duplicate standards in the range of 200 µg/ml to 1200 µg/ml were plated along with an appropriate blank and the unknown samples in 10 µl volumes into the appropriate microtitre plate wells. 200 µl of BCA solution (50A:1B) was added to each well and the samples were mixed for 30 seconds on a plate shaker. The plate was covered, and incubated at 37°C for 30 minutes. Absorbance readings were obtained at 570 nm using a microtitre plate reader. A standard curve was plotted from the absorbance readings of the standards and the protein concentration of the unknown samples determined.

2.4.2 Preparation of bacterial whole cell extracts

Unless otherwise stated, bacteria were harvested from 1.5 ml of culture by centrifugation in a micro-centrifuge at 13000 rpm, 4°C for 2 minutes. The supernatant was removed by aspiration, and the bacterial pellet resuspended in 350 µl of 2 x Final Sample Buffer (FSB) (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-Mercaptoethanol, 0.05% bromophenol blue). Samples were boiled for 6 minutes, and lysates cleared by centrifugation at 13000 rpm 4°C for 5 minutes, protein samples were stored at –20°C.

2.4.3 SDS polyacrylamide gel electrophoresis of proteins

Unless otherwise stated, separation of proteins by size was performed using 12% SDS gels as previously detailed by Laemmli, (1970) using the Bio-Rad mini-gel electrophoresis apparatus as per manufacturer's instructions. NuPAGE™ 4-12% Bis-Tris gels from Novex were used for separation of proteins, where indicated. Gels were run as per manufacturer's instructions, using NuPAGE™ MES SDS running buffer with the addition of NuPAGE™ antioxidant to the inner chamber. Protein bands were visualised in the gel by staining with Coomassie Blue (0.25% (w/v)

Coomassie Brilliant Blue R-250, dissolved in 9% (v/v) glacial acetic acid and 45% (v/v) methanol). Gels were destained in 5% (v/v) glacial acetic acid, 50% (v/v) methanol. Broad Range Marker (New England Biolabs, Table 2.5) was used for size approximation.

2.4.4 Preparation of EPEC secreted proteins

Analysis of culture supernatants from EPEC secreted proteins was performed using trichloroacetic acid (TCA) precipitations as detailed previously by Kenny and Finlay, (1995), with modifications. Briefly, bacteria were removed by centrifugation at 13000 rpm in a micro-centrifuge for 10 minutes, and supernatant proteins were precipitated by the addition of TCA to 10% (wt/vol). Samples were incubated on ice for 60 minutes, and proteins were collected by centrifugation at 13000 rpm in a micro-centrifuge for 30 minutes at 4°C. Supernatants were removed by aspiration and the pellet was resuspended in a suitable volume of 2 x FSB, and neutralised by the addition of NaOH. Lysates were boiled for 6 minutes and cleared by centrifugation at 13000 rpm in a micro-centrifuge for 5 minutes, prior to loading on SDS-polyacrylamide gels.

2.4.5 Preparation of EPEC secreted proteins to detect EspC

The preparation of proteins to detect EspC from culture supernatants was carried out essentially as 2.4.5 with the following modifications. Bacteria were grown in DMEM to an OD_{600} of 0.6 and removed from 40 ml of culture by centrifugation at 8000 rpm, in Beckman JA-20. 8.75 μg of BSA was added to the supernatant as an internal standard, and supernatant proteins were precipitated by the addition of TCA to 10% (wt/vol). Samples were incubated on ice for 60 minutes, and proteins were collected by centrifugation at 13000 in a Beckman JA-20 for 30 minutes at 4°C. Supernatants were removed by aspiration and the pellet washed twice with acetone before being resuspended in a suitable volume of 2 x FSB. Lysates were boiled for 6 minutes and

cleared by centrifugation at 13000 rpm in a micro-centrifuge for 5 minutes, prior to loading on SDS-polyacrylamide gels.

2.5 Immunological Techniques

2.5.1 Western blotting

Proteins to be immunoblotted were separated on a 12% SDS-polyacrylamide gel or 4-12% Bis-Tris NuPAGE™ gels as stated. A Mini-Trans Blot Electrophoresis Transfer Cell (BioRad) was used for transfer as per manufacturer's instructions. The gel and nitrocellulose (Schleicher and Schuell) were equilibrated in ice-cold transfer buffer(25 mM Tris, 192 mM glycine, 20% (v/v) methanol) prior to transfer. Each transfer was run at 100 V for sufficient time to permit the current to rise to 350 mA. Following transfer, the gel was stained was coomassie blue and de-stained to ensure transfer had occurred. The nitrocellulose membrane was washed in 150 ml of room temperature Blocking Buffer (10 mM Tris (pH 7.5), 1.975 mM Na_2EDTA pH 8.0, 133 mM NaCl, 0.5% BSA (w/v), 0.5% Triton X-100 (v/v)) for 2 hours. After 2 hours the blocking buffer was removed and the primary antibody, diluted in the same buffer, was added and incubated with the filter at 4°C with gentle agitation overnight. After incubation with the primary antibody, the membrane was washed three times in blocking buffer then incubated with the secondary horseradish peroxidase (HRP) labelled antibody diluted in blocking buffer, for 2 hours at room temperature with gentle agitation. The membrane was washed three times in Tris-saline buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl), prior to detection of the bound antibodies using SuperSignal chemiluminescence substrate (Pierce); the emitted light was detected using X-ray film. After detection, the nitrocellulose membrane was stained with Amido Black (1 mg/ml Amido Black, 45% (v/v) Methanol, 10% (v/v) Acetic Acid) to reveal the extent to which the protein bands had been transferred.

Protein Broad Range Molecular Marker (NEB)	**Molecular Mass (kDa)**
Myosin	212 000
MBP-β-galactosidase	158 194
β-galactosidase	116 351
Phosphorylase b	97 184
Serum albumin	**66 409**
Glutamate dehydrogenase	55 561
Maltose-binding protein 2	42 710
Lactose dehydrogenase	36 487
Triose phosphate isomerase	**26 625**
Trypsin inhibitor	(20 040 – 20 167)
Lysozyme	14 313
Aprotinin	6 517
Insulin A, B chain	(2 340 – 3 400)

Table 2.5. New England Biolabs Broad Range Molecular Weight Marker

2.5.2 Affinity purification of antibodies

Peptide (1 mg) was resuspended in 20 mM HEPES pH 7.5 (1 ml) and incubated with 1 ml of Affigel (Biorad) at 4°C overnight on a 10 rpm rotating wheel. Unbound sites on the resin were blocked with 1 M ethanolamine (pH 8.0) for 1 hour at 4°C, before the Affigel was packed into a 10 ml column and washed with 50 ml of column buffer A (100 mM K_2HPO_4/KH_2PO_4, (pH7.5), 100 mM NaCl) at a flow rate of 1 ml/min. Antibody solution was passed through the column 3 times, followed by 50 ml column buffer A. Non-specifically bound proteins were removed by washing the column with 30 ml of column buffer B (as for buffer A but with 500 mM NaCl). Antibodies were eluted from the resin with 20 ml of 100 mM glycine-HCl (pH 2.7-2.8). Samples were collected into 200 µl 1 M Tris-HCl (pH 8.0)/ml. Absorbance readings of the fractions at 280 nm were taken, and the highest absorbances pooled. Samples were dialysed against 1 l of column buffer A containing 35% glycerol (v/v) for 16 hours at 4°C.

2.5.3 Primary antibodies

Antibody	Dilution	Source
Anti BipA polyclonal	1:400	M. Farris
Anti EspA polyclonal	1:2000	B. Kenny
Anti EspB polyclonal	1:2000	B. Kenny
Anti EspD polyclonal	1:5	F. Ebel
Anti H-NS monoclonal	1:400	J. Hinton
Anti IHF	1:2000	R. Johnson
Anti Intimin polyclonal	1:750	G. Frankel
Anti Myc monoclonal	1:5000	Invitrogen
Anti Tir polyclonal	1:400	M. Farris

2.5.4 Secondary antibodies

Antibody	Dilution	Source
Goat anti-mouse HRP	1:3000	Autogen
Goat anti-rabbit HRP	1:3000	Autogen

2.5.5 Scanning of gels and densitometry analysis

Gels and immunoblots were scanned using an EPSON GT-8000, with EPSON SCAN II32 ver 1.12E software. Densitometry analysis of immunoblots was performed using Phoretix2D_v5.01

2.6 Tissue Culture

2.6.1 Preparation of secreted proteins and bacterial whole cell extracts from bacteria incubated in the presence of HeLa cells

An overnight bacterial culture grown in LB at 37°C shaking was diluted 1:50 into DMEM, and grown for 3 hours at 37°C, in 5% CO_2, without shaking. When the absorbance at 600 nm reached 0.6, (around 3 hours) bacteria were pelleted and resuspended in 0.5 ml of DMEM. Three samples were prepared for each bacterial strain, one to be incubated in the presence of HeLa cells, one in the absence of HeLa, and the bacteria from the third sample were pelleted (7000 rpm for 1 min at 4°C), and resuspended in 100 µl 2 x FSB. The proteins in the supernatant were also pelleted by addition of TCA as previously described. HeLa cells were washed 3 times with 2.5 ml of supplement-free DMEM. Bacteria were incubated standing in the presence and absence of HeLa for 90 minutes at 37°C, 5% CO_2. After 90 minutes DMEM and unattached bacteria were removed from the well, and the remaining bacteria were pelleted as before, and a TCA precipitation was performed on the supernatant to pellet secreted proteins, (this yields secreted proteins not associated with the host cells). HeLa cells were washed three times with 5 ml of cold PBS, pH 8.0 and

scraped into 1.5 ml of cold PBS pH 8.0, and spun for 1 minute at 7000 rpm, 4°C. Pellet was lysed in 0.1 ml of lysis solution (1% Triton X-100, 50 mM Tris-HCl pH 7.6, 0.4 mM Na_3VO_4, 1 mg/ml PMSF, NaF, 10 μg/ml leupeptin), and the lysate spun at 13000 rpm for 1 minute, 100 μl of 2 x FSB was added to the supernatant (Triton X-soluble) fraction, the Triton X-100 insoluble pellet was resuspended in 100 μl 2 x FSB. All lysates were boiled for 6 minutes, and then fractions cleared by centrifuging at 13000 rpm for 5 minutes.

2.6.2 Scanning electron microscopy

These procedures were performed essentially as described by Farris *et al.* (1998). Bacteria were grown overnight without shaking, collected by centrifugation and resuspended in supplement free DMEM medium. Bacteria (0.1 OD_{600} units) were then added to semi-confluent HeLa cells, grown under 5% CO_2 at 37°C in 35 mm chambers (Nunclon). After incubation for 6 hours, samples were fixed for 30 minutes with 4% (w/v) paraformaldehyde in PBS and washed three times with PBS. A Hitachi S-800 scanning electron microscope fitted with a field emission source was used for sample visualisation.

2.6.3 Confocal microscopy

For fluorescence microscopy, HeLa cells infected with GFP-tagged bacteria were washed three times with cold PBS (pH 8.0), fixed for 30 minutes with 4% paraformaldehyde in PBS, a further wash in PBS was performed, followed by an incubation with 0.1% Triton X-100 in PBS. Cells were incubated with 5 μg/ml rhodamine-conjugated phalloidin for 60 minutes at 37°C in the dark, followed by a wash with PBS. Samples were mounted in 10% (v/v) glycerol in PBS and examined using a confocal laser-scanning microscope (Bio-Rad MRC-600).

2.7 Biological Assays

2.7.1 Cold shock phenotype

A bacterial colony was streaked using a platinum wire, first onto a plate to be placed at 27°C and then onto another plate to be placed at 37°C. Plates were typically removed after 14 hours unless otherwise stated, and growth phenotypes compared.

2.7.2 Motility plate assays

Motility assays were performed as described by Silverman and Simon, (1973). Briefly, a platinum wire was dipped into stationary phase cultures and used to stab the motility agar plate. The plates were incubated at 37°C for 16 hours unless otherwise stated, after which time the diameter of each colony was measured.

2.7.3 Growth rate studies

1 ml of an overnight culture was added to 50 ml of pre-warmed media in a 250 ml flask, the absorbance of culture was checked against a suitable blank at an absorbance of 600 nm at time 0 and at 30 minute intervals thereafter, for a period of 10 hours.

2.7.4 H-NS protein expression levels in high and low glucose MOPS

Two 2 ml samples from a 10 ml overnight culture were spun at 6000 rpm in a micro-centrifuge for 2 minutes to pellet the bacteria. The pellet from each sample was resuspended in 1 ml of ice cold sterile PBS, the wash step was repeated twice more. One pellet was resuspended in 1 ml high-glucose MOPS and the other in 1 ml of low-glucose MOPS. Samples were used to inoculate 100 ml of high-glucose MOPS and low-glucose MOPS respectively. Bacteria were grown for 4 hours at 37°C with shaking, after which time the absorbance of the culture was checked against a suitable blank at 600 nm. Bacteria were centrifuged at 6000 rpm, 4°C for 15 minutes in a

Beckman JA-20 rotor. The pellet was resuspended in 2 ml of ice cold sterile PBS, bacteria were pelleted and the wash step repeated twice more. The bacterial pellet was resuspended in an appropriate volume of 2 x Final Sample Buffer then boiled for 5 minutes, before being resolved on a 12% SDS-polyacrylamide gel to verify equal protein loadings. H-NS protein samples were run on a 4-12% NuPAGE SDS-polyacrylamide gel and transferred to nitrocellulose as described. Protein expression levels within the samples were detected immunologically by western blotting using a monoclonal antibody against H-NS.

2.7.5 Effect on killing of cells by BPI-derived peptides

E2348/69 or its *bipA*⁻ derivative were inoculated into 10 ml of tryptic soy broth (TSB) and grown overnight at 37°C with shaking. Cells were harvested by centrifugation at 8000 rpm in Beckman JA-20, washed with an equal volume of 20 mM sodium phosphate buffer (0.01 M $NaH_2PO_4.2H_20$, 0.0116 M NaOH) and resuspended in 1 ml of the same buffer. Samples were diluted with PBS to obtain an OD_{600} of 0.5. 100 µl samples were dispensed into sterile micro-centrifuge tubes, and peptide P2 (dissolved in 20 mM sodium phosphate buffer) or sodium phosphate as a control was added. (Bactericidal peptide P2 (Barker *et al.*, 2000) was obtained from Dr N. Kinsella). The volume was made up to 500 µl with 20 mM sodium phosphate and bacteria were incubated standing at 37°C. Samples were taken at time points 0 and 30 min, serially diluted in PBS to 10^{-6} and 100 µl aliquots plated in duplicate on LB agar plates. Plates were incubated at 37°C overnight, colonies counted and colony forming units/ml calculated.

2.7.6 β-galactosidase assays

β-galactosidase assays were performed essentially as described by Miller, (1977). Briefly, bacteria were grown in DMEM, 5% CO_2 at 37°C standing, to an OD_{600} of 0.6. 100 µl aliquots were removed, and bacteria pelleted by centrifugation in a micro-centrifuge at 13000 rpm, 4°C for 2 minutes. Bacterial pellets were resuspended in 1

ml of Z buffer (pH 7.0) (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, 0.05 M β-mercaptoethanol). 2 drops of chloroform and 1 drop of 0.1% SDS were then added to the cell culture, followed by 10 seconds of vortexing, and a 5 minute equilibration at 28°C. Resuspended cell pellets were assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. 0.2 ml of ONPG in Z buffer was added to the sample, after 10 minutes the reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3. Units of β-galactosidase activity (units/mg protein) were calculated using the following equation: $1000 \times (A_{420} - (1.75 \times A_{550})) / (t \times 0.1 \times A_{600})$ where t = time in minutes

CHAPTER 3

CLONING AND SEQUENCE ANALYSIS OF EPEC HOMOLOGUE OF BipA AND CONSTRUCTION OF EPEC *bipA* NULL MUTANTS

CHAPTER 3: CLONING AND SEQUENCE ANALYSIS OF EPEC HOMOLOGUE OF *bipA* AND CONSTRUCTION OF EPEC *bipA* NULL MUTANTS

3.1 Introduction

Sequence analysis reveals the BipA protein to have sequence similarity with ribosome-binding GTPases. Moreover, Farris *et al.* (1998) report that the BipA protein from MAR001 has GTPase activity, and recent findings indicate that the equivalent protein from E2348/69 interacts directly with ribosomes (R. Owens, unpublished observations). Evidence is therefore mounting to suggest that BipA uses a novel regulatory mechanism to regulate the expression of target proteins. Farris *et al.* (1998) have reported that, unlike *E. coli* K-12 and *Salmonella*, EPEC strains such as MAR001 phosphorylate BipA on tyrosine. Both the processes of GTPase hydrolysis and tyrosine phosphorylation are closely involved in the transmission of intracellular signals. Therefore it is not unreasonable to assume that BipA may be involved in controlling or influencing a number of signal transduction processes, possibly by a novel regulatory mechanism.

The gene for BipA is present in a wide range of bacteria and plants. Previous studies have shown that BipA regulates a number of stress- and virulence-associated properties of *E. coli*. These properties include resistance to the antibacterial effects of a host defence protein, expression of the genes for synthesis of the K5 capsule, flagella mediated cell motility and the ability of an enteropathogenic strain to trigger cytoskeletal rearrangements in infected host cells (Farris *et al.*, 1998; Rowe *et al.*, 2000). BipA therefore appears to have a role in the regulation of virulence. At present, many of theses observations are restricted to a strain of EPEC that is poorly characterised. It would therefore be of interest to determine if BipA also controls such processes in other strains.

As a prerequisite, this chapter details the cloning and sequence analysis of the *bipA* gene of EPEC E2348/69, and the construction of *bipA* null mutants in EPEC strains E2348/69 and JPN15.

3.2 Cloning of the EPEC E2348/69 *bipA* gene

It has previously been shown that *bipA* shares the promoter region of *glnA* the gene for glutamine synthetase in *S.* Typhimurium, *E. coli* K-12 and EPEC MAR001 but that it is transcribed divergently (Plunkett *et al.*, 1993; Qi *et al.*, 1995; Farris *et al.*, 1998). PCR analysis of EPEC E2348/69 confirmed the *bipA* gene to be divergently transcribed from *glnA*. To clone the EPEC E2348/69 *bipA* gene, Primer AJG001 was designed to the very 5' end of *glnA*, and Primer AJG020 to the very 3' end of E2348/69 *bipA* sequence (Stuart Clarke, personal correspondence), thereby permitting the PCR amplification of the *bipA* gene and its intragenic region. Following amplification of *bipA* and its intragenic region by PCR from E2348/69 chromosomal DNA using Primers AJG001 and AJG020 (Table 2.4), the *bipA* gene was cloned as an *Sph*I-*Xho*I fragment into *Sph*I-*Sal*I digested plasmid pBR322, generating the plasmid pAJG1. The EPEC *bipA* gene was sequenced using a Li-Cor 4000 automated sequencer and custom-synthesised dye-labelled primers (Table 2.4). A further clone was produced in the same way, and designated pAJG2. A subsequent clone was produced from an independent PCR reaction using primers AJG001 and AJG025 (Table 2.4) generating the plasmid pAJG3. All constructs were shown to contain PCR artefacts in the *bipA* gene. Thus, to create a cloned copy of E2348/69 *bipA* with no errors, a partial gene replacement was carried out using pAJG1 (this study) and pT7T318U/MAR*bipA* (Farris *et al.*, 1998). This was achieved by replacing a 1388 bp *Age*I-*Sma*I fragment from pT7T318U/MAR*bipA* by the corresponding *Age*I-*Sma*I *bipA* fragment from pAJG1. The resulting plasmid was designated pAJG4. The EPEC *bipA* gene was sequenced on both strands using an ABI-100 sequencer and custom synthesised primers (Table 2.4) to confirm its structure.

Multiple alignment of the *bipA* sequences from different organisms suggested that the *E. coli* K-12 homologue might contain a frameshift. Therefore, primers were designed to amplify a region of the MG1655 and E2348/69 chromosome, to confirm whether there was a frameshift in the published *bipA* sequence. A stretch of residues around 90 bp downstream of the new predicted *bipA* stop codon was used to design a

3' primer complementary to the to MG1655 sequence; the primer was designated AJG050. Some preliminary sequence data 3' to *bipA* from E2348/69 was obtained from R. Haigh (personal correspondence) and was used to design a primer around 100 bp downstream of the new predicted *bipA* stop codon. Primers AJG016 and AJG050 were used with MG1655 chromosomal DNA as the template, thereby permitting the PCR amplification of the 3' end of *bipA* and its downstream sequence. Similarly the corresponding region of E2348/69 was amplified using primers AJG016 and AJG049, using E2348/69 chromosomal DNA as the template. The resulting PCR products were sequenced using the 5' and 3' primers used for their construction. Sequencing identified the presence of an additional nucleotide 'c' at position 1774, which extended the *bipA* sequence by a further 48 nucleotides, in keeping with other *bipA* sequences (Figure 3.1.). Thus, the BipA protein expressed from pAJG4 lacks the last 16 amino acids; however, it is biologically active and complements EPEC *bipA* null mutant strain in all phenotypes tested (Chapters 4, 5 and 6).

In view of the frameshift, pAJG4 was modified. To construct a plasmid expressing a full-length version of the BipA protein, a portion of the *bipA* gene was amplified from E2348/69 chromosomal DNA using primers AJG053 and AJG054 and used to replace an *Age*I-*Hin*dIII fragment from pAJG4. The entire *bipA* gene in the resulting construct, pAJG9, was sequenced using custom-synthesized primers to confirm its integrity. pAJG9, contained a 2227 bp fragment in which *bipA* was expressed from its native promoter(s). The production of a cloned copy of E2348/69 *bipA* is summarised in Figure 3.2.

Previous studies have suggested that the *bipA* gene specifies a 65 kDa polypeptide although the protein runs anomalously in SDS PAGE around 72 kDa (Farris *et al.*, 1998). These studies have identified that the *bipA* gene previously cloned is truncated, calculation of the predicted molecular weight for EPEC truncated BipA using ExPASy molecular weight computing tool (www.expasy.ch/tools/pi_tool.html) is 67.8 kDa, while full-length BipA is 69.7 kDa. This new predicted molecular weight is in closer agreement with the estimated size of BipA; the observed

(A)

```
     1760          1770
     |             |
cgt att cgt aaa cgt cac tga
Arg Ile Arg Phe Arg His Stop
```

(B)

```
     1760          1770          1780          1790          1800
     |             |             |             |             |
cgt att cgt aaa cgt cac ctg acg gaa aac gat cgt cgt cgc gcc aac cgc
Arg Ile Arg Phe Arg His Leu Thr Glu Asn Asp Arg Arg Arg Ala Asn Arg

    1810          1820
    |             |
gca ccg aaa gac gat taa
Ala Pro Lys Asp Asp Stop
```

Figure 3.1. Sequence analysis identifies that K-12 *bipA* has a frameshift next to the proposed stop codon, extending the *bipA* gene. (A) Plasmids containing a cloned copy of the *bipA* gene MAR001 (Farris *et al.*, 1998) and E2348/69 this study, contained a *bipA* gene PCR amplified from the appropriate chromosomal DNA using primers designed to MG1655 *bipA* sequence (ECAE462). These constructs contained a stop codon at position 1776 as illustrated. (B) Sequencing of MG1655 and E2348/69 chromosomal DNA highlighted an additional base (**c**) at position 1774, which results in a frameshift, and extends *bipA* by a further 48 bases, resulting in an additional 16 amino acids.

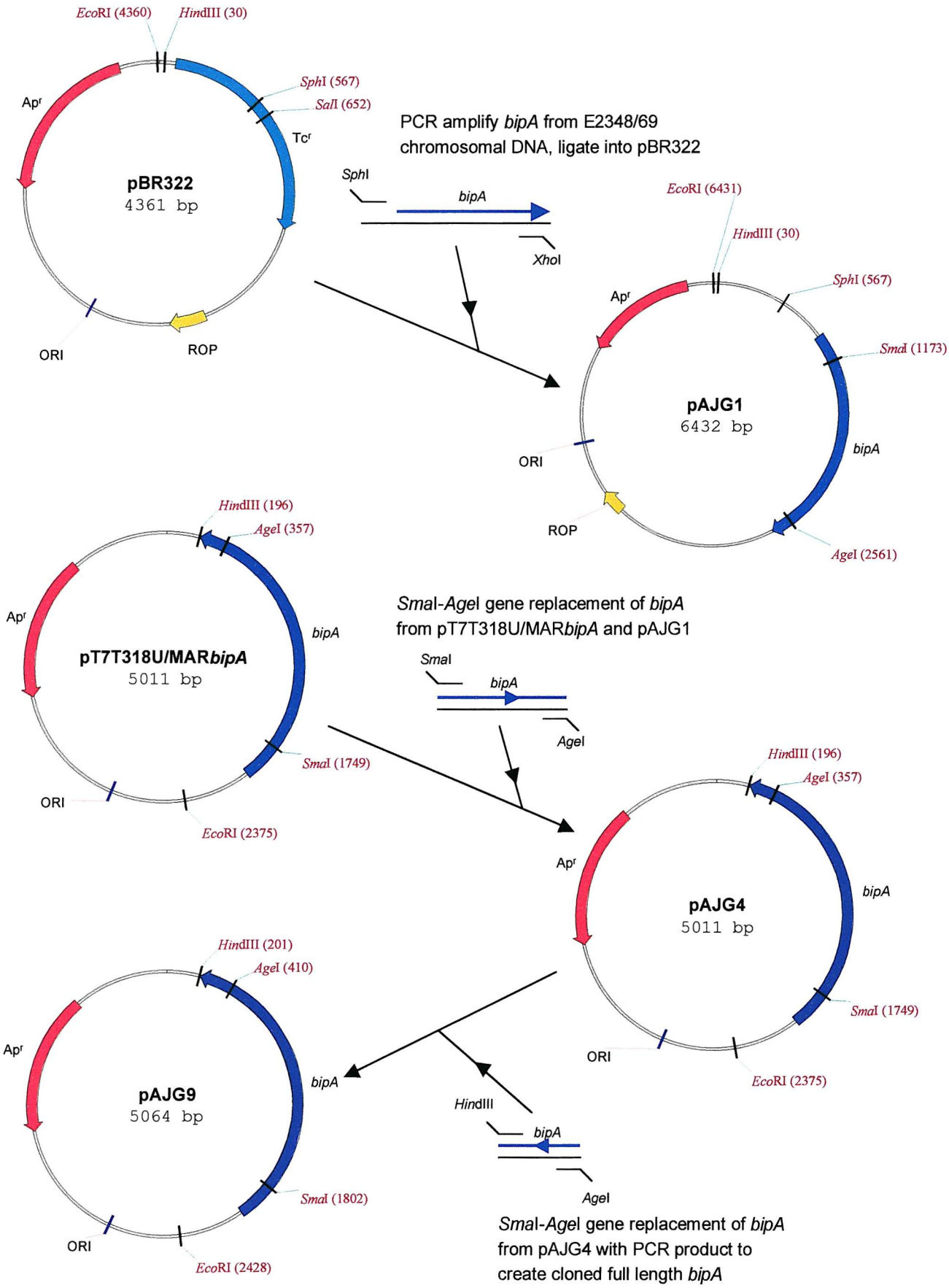


Figure 3.2. Schematic indicating stages in the cloning of the wild-type *bipA* gene from E2348/69. Restriction enzyme sites important for cloning are shown.

difference may be due to post-translational modifications, or anomalous migration through SDS-PAGE.

Further constructs previously produced were also incorrect. These include pTrcHisB*bipA*, a derivative of the *lacI*^{q} - containing expression vector pTrcHisB (Invitrogen), which contains the *bipA* gene from the EPEC strain MAR001 (Farris *et al.*, 1998), and a mutated version of this plasmid which contained a substitution G77V in the putative nucleotide binding region (DTPG) resulting in a functionally inactive protein. To construct a plasmid expressing a hexahistidine-tagged and full-length version of the E2348/69 BipA protein, a gene replacement (as previously detailed for construct pAJG9) was carried out using pTrcHisB*bipA* and a PCR amplified region of the E2348/69 chromosome generating the plasmid pAJG11. To create a plasmid expressing a hexahistidine-tagged and full length version of the BipA protein with the substitution G77V, a partial gene replacement was carried out using pTrcHisB*bipA*(G77V) (C.D. O'Connor) and pAJG11 (this study). An *Age*I–*Hin*dIII fragment from pTrcHisB*bipA*(G77V) was recovered and replaced with the corresponding fragment from pAJG11. The entire *bipA* gene in the resulting construct, pAJG13 was sequenced to confirm its integrity.

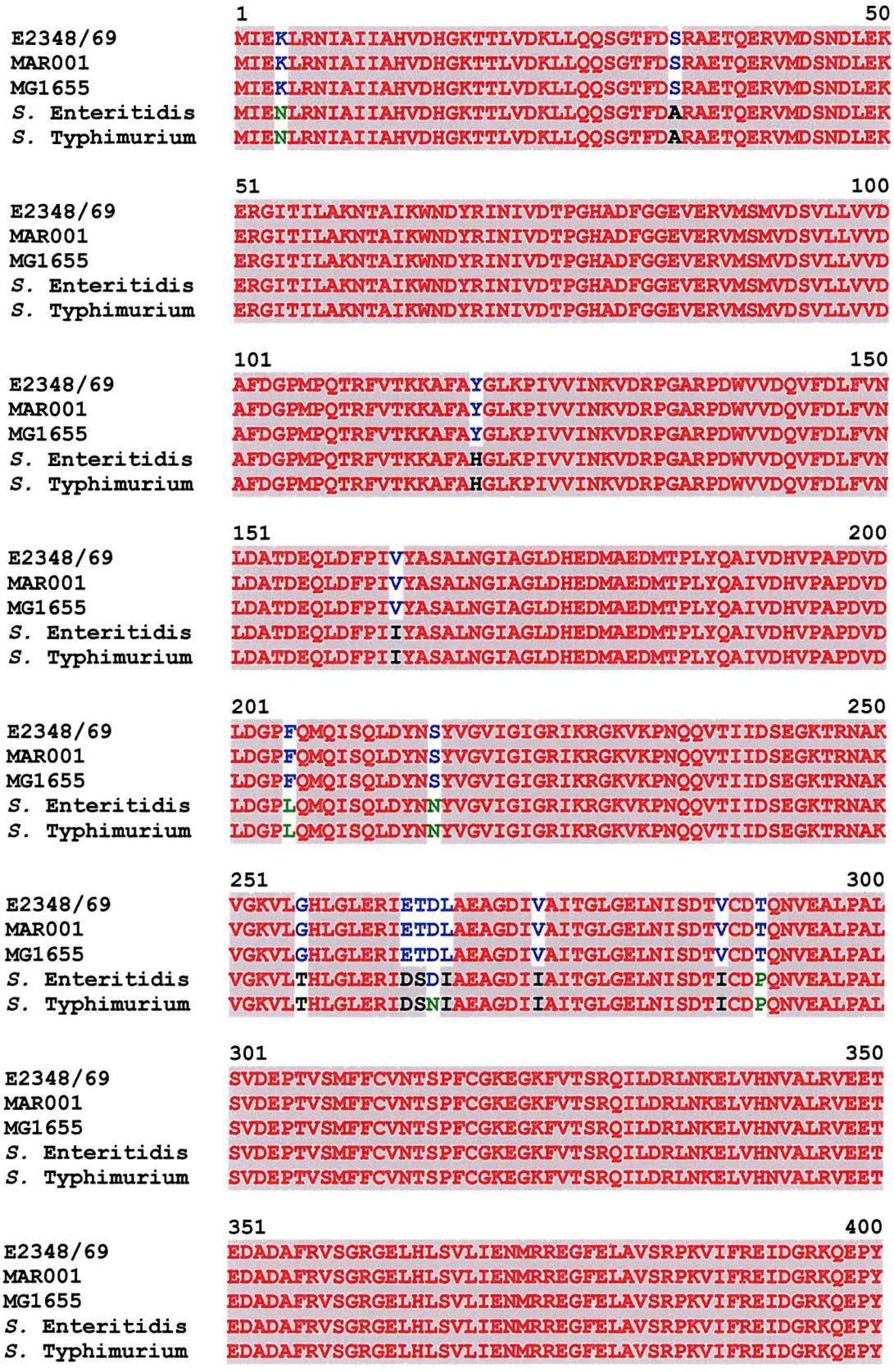
3.2.1 Sequence analysis of the EPEC *bipA* gene and protein product

The sequence of the *bipA* gene of E2348/69 was aligned with that of MG1655, MAR001, *Salmonella* Typhimurium and *Salmonella* Enteritidis using Vector NTI AlignX (data not shown). The degrees of homology are shown in Table 3.1

Alignment between	Homology (%)
E2348/69 and MAR001	99.83
E2348/69 and MG1655	99.21
E2348/69 and *S.* Typhimurium	90.64
E2348/69 and *S.* Enteritidis	90.86
MAR001 and MG1655	98.93
MAR001 and *S.* Typhimurium	90.47
MAR001 and *S.* Enteritidis	90.58
MG1655 and *S.* Typhimurium	90.75
MG1655 and *S.* Enteritidis	90.81
S. Typhimurium and *S.* Enteritidis	99.38

Table 3.1. Percentage homology between *bipA* gene sequences from the translational start to nucleotide 1773, prior to truncated stop codon.

Between the full-length *bipA* genes from E2348/69 and *S.* Typhimurium there was 90.35% homology, between E2348/69 and *S.* Enteritidis there was 90.57% homology and between *S.* Typhimurium and *S.* Enteritidis there was 99.38% homology. There was a greater degree of conservation seen when the *bipA* nucleotide sequences were translated to their corresponding protein sequences. Figure 3.3 shows the alignment of BipA protein sequences, while Table 3.2 indicates the percentage homology between the aligned BipA sequences.


1 50
E2348/69 MIEKLRNIAIIAHVDHGKTTLVDKLLQQSGTFDSRAETQERVMDSNDLEK
MAR001 MIEKLRNIAIIAHVDHGKTTLVDKLLQQSGTFDSRAETQERVMDSNDLEK
MG1655 MIEKLRNIAIIAHVDHGKTTLVDKLLQQSGTFDSRAETQERVMDSNDLEK
S. Enteritidis MIENLRNIAIIAHVDHGKTTLVDKLLQQSGTFDARAETQERVMDSNDLEK
S. Typhimurium MIENLRNIAIIAHVDHGKTTLVDKLLQQSGTFDARAETQERVMDSNDLEK
51 100
E2348/69 ERGITILAKNTAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVD
MAR001 ERGITILAKNTAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVD
MG1655 ERGITILAKNTAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVD
S. Enteritidis ERGITILAKNTAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVD
S. Typhimurium ERGITILAKNTAIKWNDYRINIVDTPGHADFGGEVERVMSMVDSVLLVVD
101 150
E2348/69 AFDGPMPQTRFVTKKAFAYGLKPIVVINKVDRPGARPDWVVDQVFDLFVN
MAR001 AFDGPMPQTRFVTKKAFAYGLKPIVVINKVDRPGARPDWVVDQVFDLFVN
MG1655 AFDGPMPQTRFVTKKAFAYGLKPIVVINKVDRPGARPDWVVDQVFDLFVN
S. Enteritidis AFDGPMPQTRFVTKKAFAHGLKPIVVINKVDRPGARPDWVVDQVFDLFVN
S. Typhimurium AFDGPMPQTRFVTKKAFAHGLKPIVVINKVDRPGARPDWVVDQVFDLFVN
151 200
E2348/69 LDATDEQLDFPIVYASALNGIAGLDHEDMAEDMTPLYQAIVDHVPAPDVD
MAR001 LDATDEQLDFPIVYASALNGIAGLDHEDMAEDMTPLYQAIVDHVPAPDVD
MG1655 LDATDEQLDFPIVYASALNGIAGLDHEDMAEDMTPLYQAIVDHVPAPDVD
S. Enteritidis LDATDEQLDFPIIYASALNGIAGLDHEDMAEDMTPLYQAIVDHVPAPDVD
S. Typhimurium LDATDEQLDFPIIYASALNGIAGLDHEDMAEDMTPLYQAIVDHVPAPDVD
201 250
E2348/69 LDGPFQMQISQLDYNSYVGVIGIGRIKRGKVKPNQQVTIIDSEGKTRNAK
MAR001 LDGPFQMQISQLDYNSYVGVIGIGRIKRGKVKPNQQVTIIDSEGKTRNAK
MG1655 LDGPFQMQISQLDYNSYVGVIGIGRIKRGKVKPNQQVTIIDSEGKTRNAK
S. Enteritidis LDGPLQMQISQLDYNNYVGVIGIGRIKRGKVKPNQQVTIIDSEGKTRNAK
S. Typhimurium LDGPLQMQISQLDYNNYVGVIGIGRIKRGKVKPNQQVTIIDSEGKTRNAK
251 300
E2348/69 VGKVLGHLGLERIETDLAEAGDIVAITGLGELNISDTVCDTQNVEALPAL
MAR001 VGKVLGHLGLERIETDLAEAGDIVAITGLGELNISDTVCDTQNVEALPAL
MG1655 VGKVLGHLGLERIETDLAEAGDIVAITGLGELNISDTVCDTQNVEALPAL
S. Enteritidis VGKVLTHLGLERIDSDIAEAGDIIAITGLGELNISDTICDPQNVEALPAL
S. Typhimurium VGKVLTHLGLERIDSNIAEAGDIIAITGLGELNISDTICDPQNVEALPAL
301 350
E2348/69 SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEET
MAR001 SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEET
MG1655 SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEET
S. Enteritidis SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEET
S. Typhimurium SVDEPTVSMFFCVNTSPFCGKEGKFVTSRQILDRLNKELVHNVALRVEET
351 400
E2348/69 EDADAFRVSGRGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPY
MAR001 EDADAFRVSGRGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPY
MG1655 EDADAFRVSGRGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPY
S. Enteritidis EDADAFRVSGRGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPY
S. Typhimurium EDADAFRVSGRGELHLSVLIENMRREGFELAVSRPKVIFREIDGRKQEPY

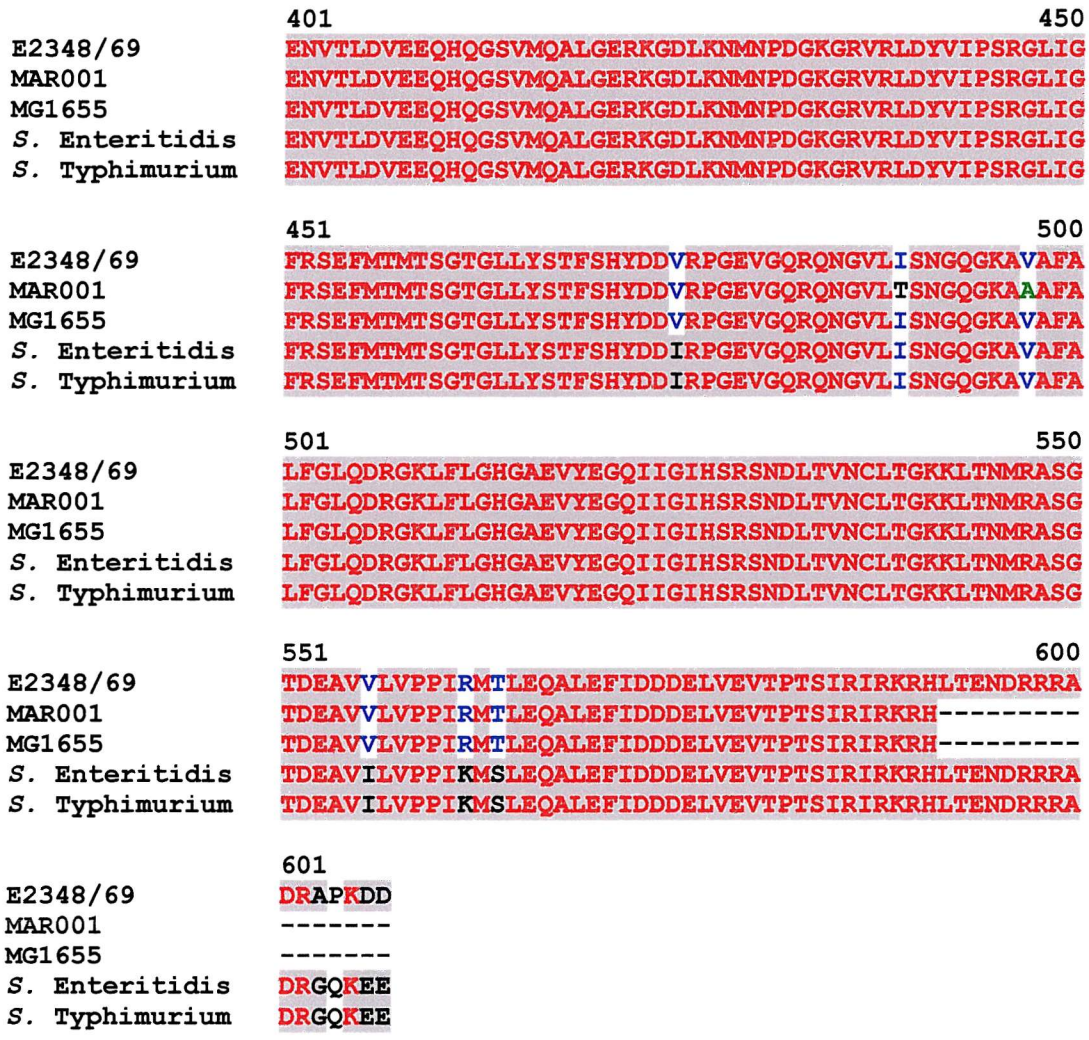


Figure 3.3. Predicted sequence of EPEC E2348/69 BipA protein (Accession number AJ278218), and its alignment with predicted BipA sequences for MAR001 (ECAJ4871), MG1655 (ECAE462), *Salmonella* Enteritidis (SEN277943) and *Salmonella* Typhimurium (STY276889). Residues that are identical are indicated with a red foreground and grey background. Similar residues are shown as a black foreground and grey background, a blue foreground and a white background indicate conservative residues; a green foreground and a white background depict weakly similar residues, while non-homologous residues are indicated by a black foreground and white background. Sequences were aligned using Vector NTI AlignX.

Alignment between BipA from	Homology (%)
E2348/69 and MAR001	99.66
E2348/69 and MG1655	100
E2348/69 and *S.* Typhimurium	96.95
E2348/69 and *S.* Enteritidis	97.12
MAR001 and MG1655	99.66
MAR001 and *S.* Typhimurium	96.62
MAR001 and *S.* Enteritidis	96.79
MG1655 and *S.* Typhimurium	96.95
MG1655 and *S.* Enteritidis	97.12
S. Typhimurium and *S.* Enteritidis	99.83

Table 3.2. Percentage homology between predicted BipA protein sequences from the start codon, to codon 591, which is just prior to the truncated stop codon.

Between the full-length BipA protein sequences there was a high degree of homology, between E2348/69 and *S.* Typhimurium (96.38% homology), between E2348/69 and *S.* Enteritidis there was 96.54% homology and between *S.* Typhimurium and *S.* Enteritidis there was 99.84% homology.

Previous studies by Farris *et al.* (1998) identified that the MAR001 BipA sequence differed from the predicted gene product of the K-12 sequence at two sites, Ile → Thr and Val → Ala at positions 489 and 497 respectively. These substitutions were not observed in EPEC E2348/69 and appear to be specific to MAR001.

3.2.2 Sequence analysis of *bipA* promoter region

In *E. coli* K-12, the *bipA* gene (*o591*) is divergently transcribed to *glnA*, the gene for glutamine synthetase (Plunkett *et al.*, 1993). The *bipA* gene is also divergently transcribed from *glnA* in *Salmonella* Typhimurium, *Salmonella* Enteritidis, and EPEC strains E2348/69 and MAR001. The intragenic region between *bipA* and *glnA* is complex, and contains transcriptional start sites for *glnA*, along with binding sites for NtrC/NR1 (nitrogen response element 1) and CAP (catabolite activator protein) which have previously been shown to control the expression of *glnA* (Covarrubias and Bastarrachea, 1983; Ueno-Nishio *et al.*, 1984; Reitzer and Magasanik, 1985). The untranslated region also contains three putative transcriptional start sites for *bipA*. Homology alignments of the intragenic region between *bipA* and *glnA* for EPEC, K-12 and *Salmonella* Typhimurium strains exhibit a high degree of homology, the sequence is conserved in the three putative –35 and –10 control regions for *bipA*, although there are small insertions and deletions in other regions of the *Salmonella* sequence (data not shown). The significance of three promoter sites for *bipA* is currently under investigation, as is the possibility of control by NtrC or CAP. Homology searches indicate that in a number of bacterial species such as *Helicobacter pylori*, *Campylobacter jejuni*, *Pseudomonas aeruginosa* and *Neisseria meningitidis*, *bipA* is not divergently transcribed from *glnA*, and possesses upstream promoter sequences different to those in *E. coli* K-12.

3.3 Production of pCVD442Δ*bipA*, a construct suitable for targeted disruption of the *bipA* gene

The pCVD442Δ*bipA* construct, which was used for the production of *bipA* null mutants, was produced by separately cloning out the 5' and 3' regions of the *bipA* gene using PCR amplification. Briefly, two asymmetric PCR reactions were used to generate fragments to the left and right of the sequence of *bipA* to be deleted. The 5' region of the *bipA* gene was amplified from E2348/69 chromosomal DNA by PCR using the primers AJG014 and AJG015 (Table 2.4), while the 3' region of the *bipA*

gene was amplified using primers AJG016 and AJG017 (Table 2.4). Primers AJG015 and AJG016 were complementary over 21 nucleotides, so that when the two PCR products were mixed, the complementary regions annealed and primed at the 3' overlapping region for a 3' extension of the complementary strand (Figure 2.1). The annealed structure was amplified by PCR using the outer primers AJG014 and AJG017; the resulting Δ*bipA* fragment was cloned as an *Xho*I-*Pme*I fragment into *Sal*I-*Sma*I digested pCVD442 to create the plasmid pAJG0a.

A fragment carrying the *cat* gene was inserted into the Δ*bipA* gene in pAJG0a to facilitate the selection of null mutants. The structural gene for *cat* from pACYC184 was isolated as an *Xmn*I-*Hin*cII fragment (containing the *cat* gene and its promoter, along with some extraneous pACYC184 sequence), and cloned into *Swa*I - digested pAJG0a, generating the plasmid pAJG0b. A further construct was produced using a *cat* gene with its promoter region (Alton and Vapnek, 1979), which was PCR amplified from pACYC184 using primers AJG027 and AJG028 (Table 2.4). The amplified product was digested with *Not*I and cloned into the *Not*I site engineered into the 21 nucleotide sequence inserted within the Δ*bipA* gene. In this construct the 3' region of *bipA* was preceded by translational stop codons in all three reading frames, a consensus ribosome binding site and a translational start codon, (which had been engineered into the *cat* primer) to permit translation of the remaining 3' portion of the mutated gene. Restriction enzyme analysis was used to confirm a construct in which the *cat* gene had been inserted in the same direction as *bipA*. The resulting plasmid was designated pAJG0c and was transformed into S17-1(λ*pir*) by electroporation.

3.4 Replacing the wild-type *bipA* gene on the EPEC chromosome

To determine the role of BipA in EPEC, null mutants were generated by allelic exchange of the wild-type *bipA* gene on the chromosome with an inactivated *bipA* gene cloned into the positive-selection suicide vector pCVD442 (Donnenberg and Kaper, 1991). Transfer of the construct to E2348/69 and selection for allelic

exchange with the chromosomal *bipA* gene was performed as described in section 2.2.19 of Chapter 2. Suicide vector pCVD442 requires the product of the *pir* gene, the π protein, for initiation of DNA replication at *ori*R6K (Inzuka and Helsinki, 1978). The gene encoding this protein is absent from the suicide vector but is supplied by a chromosomal copy from S17-1(λ*pir*) in the donor strain, thus pCVD442*bipA*⁻ mutant derivatives were unable to replicate outside the donor strain due to the requirement of the protein being supplied *in trans*. The chromosome of the donor strain also bears a conjugative plasmid – RP-4 that facilitates transfer of plasmid DNA from donor to recipient. To aid the selection of double cross-over events, the suicide vector contains *sacB* which encodes levansucrase, an enzyme that degrades sucrose to produce toxic metabolites resulting in cell death (Gay *et al.*, 1985). Therefore growth of candidates in the presence of sucrose selects for colonies that do not contain a copy of *sacB*, e.g. due to recombinational loss of the plasmid.

Double cross-over events were confirmed by PCR using primers designed to chromosomal regions upstream and downstream of the cloned region of *bipA* (Figures 3.4 and 3.7). Candidate *bipA*⁻ strains were further confirmed by immunoblotting, probing whole cell lysates of E2348/69 and null mutants with anti-BipA antibodies (Figures 3.5 and 3.8), and by Southern hybridisation analysis (Figures 3.6 and 3.9). Southern hybridisation was performed using chromosomal DNA digested with *Bgl*II, *Sal*I or *Ssp*I and probed with a 5' ^{32}P labelled PCR product of *bipA*. Restriction enzymes *Bgl*II and *Sal*I cut neither in *bipA* nor *cat*, as expected the *bipA* probe bound to a *Bgl*II/*Sal*I restriction fragment in AG2 that was approximately 200 bp larger than the corresponding band in the parent. It also bound to a restriction fragment in AG1 that was approximately 1 kb larger than the corresponding band in the parent and a fragment in AG7 that was around 600 bp smaller than the corresponding band in the parent. Restriction enzyme *Ssp*I cuts within the *cat* gene. As expected the *bipA* probe bound to a *Ssp*I restriction fragment in AG2 that was approximately 1.6 kb smaller than the corresponding band in the parent, while it bound to a restriction fragment in AG1 that was approximately 1.3 kb smaller than the corresponding band

in the parent. It also bound to a fragment in AG7 that was around 600 bp smaller than the corresponding band in the parent.

pAJG0c was transformed into SM10(λ*pir*) by electroporation and transferred to the wild-type JPN15 strain by mobilisation. Transfer and allelic exchange were performed as for AG1. Three different isolates were chosen for further study, AG4, AG5 and AG6. The structure of a representative isolate with chromosomally located *bipA::cat* fusion was confirmed as previously detailed.

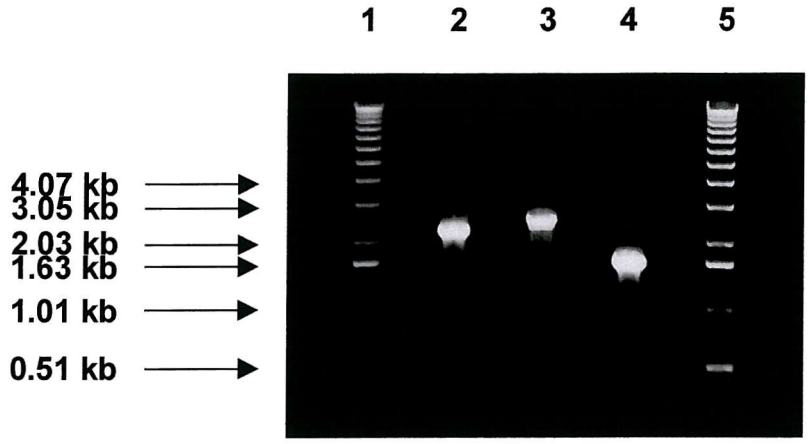


Figure 3.4. Agarose gel of PCR analysis of chromosomal DNA from E2348/69 wild-type and BipA null mutants using primers AJG001 and AJG025. PCR analysis of the clones shows that the amplified band from AG2 was approximately 200 bp greater than that of wild-type E2348/69, while the amplified band from AG7 was approximately 600 bp smaller as expected. Lane 1, 1 kb DNA ladder (BRL); lane 2, E2348/69; lane 3, AG2; lane 4, AG7; lane 5, 1 kb DNA ladder (BRL).

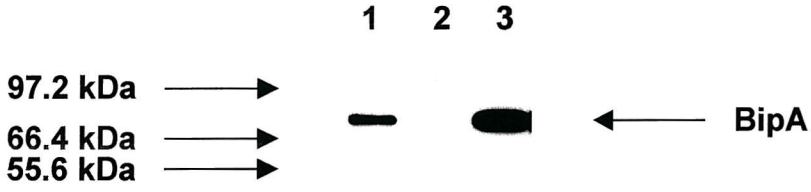


Figure 3.5. Immunoblot of whole cell extracts from E2348/69 wild-type (lane 1), BipA null mutant (lane 2) and transcomplemented strains (lane 3). Note the absence of a positive signal with the *bipA*⁻ strain, while the null mutant strain containing a cloned copy of the *bipA* gene over-expresses the BipA protein. Bacteria were grown in LB to an OD_{600} of 0.6-0.7; whole cell extracts were prepared as previously detailed. Protein samples were separated by polyacrylamide gel electrophoresis on 4-12% Bis-Tris NuPAGE gels, transferred onto nitrocellulose and probed with antisera specific to BipA.

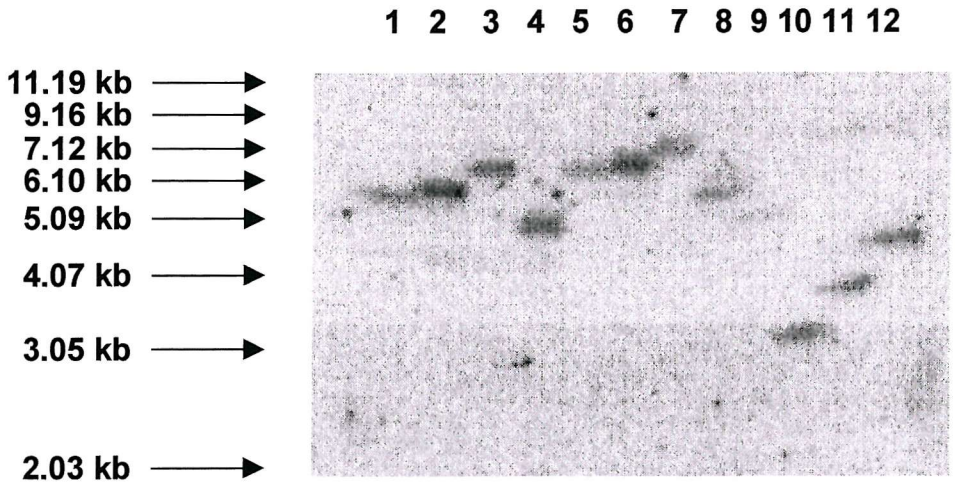


Figure 3.6. Southern hybridisation analysis using E2348/69 wild-type and BipA null mutants. Chromosomal DNA was digested with *Bgl*II, *Sal*I or *Ssp*I and probed with a 5' ^{32}P labelled PCR product of *bipA*. Lane 1, E2348/69 *Bgl*II digested; lane 2, AG2 *Bgl*II digested; lane 3, AG1 *Bgl*II digested; lane 4, AG7 *Bgl*II digested; lane 5, E2348/69 *Sal*I digested; lane 6, AG2 *Sal*I digested; lane 7, AG1 *Sal*I digested; lane 8, AG7 *Sal*I digested; lane 9, E2348/69 *Ssp*I digested; lane 10, AG2 *Ssp*I digested; lane 11, AG1 *Ssp*I digested; lane 12, AG7 *Ssp*I digested.

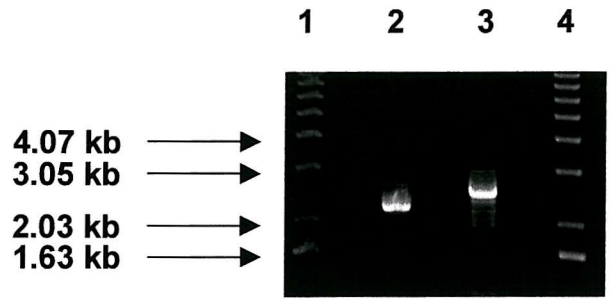


Figure 3.7. Agarose gel of PCR analysis of chromosomal DNA from JPN15 wild-type and *bipA*⁻ strains using primers AJG001 and AJG025. PCR analysis of the clones shows that the amplified band from AG4 was approximately 200 bp greater than that of wild-type JPN15. Lane 1, 1 kb DNA ladder (BRL); lane 2, JPN15; lane 3, AG4; lane 4, 1kb DNA ladder (BRL).

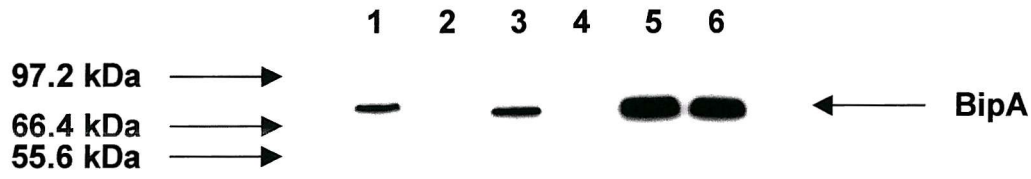


Figure 3.8. Immunoblot of whole cell extracts from JPN15 wild-type, *bipA*⁻ mutant and transcomplemented strains. Note the absence of a positive signal with the *bipA*⁻ strain (lane 2), while the wild-type and null mutant strain containing a cloned copy of the *bipA* gene over-expresses the BipA protein (lanes 5 + 6). Bacteria were grown in LB to an OD_{600} of 0.6-0.7; whole cell lysates were prepared as previously detailed. Protein samples were separated by polyacrylamide gel electrophoresis on 4-12% Bis-Tris NuPAGE gels, transferred onto nitrocellulose and probed with antisera specific to BipA. Lane 1, JPN15; lane 2, AG4; lane 3, JPN15(pT7T318U); lane 4, AG4(pT7T318U); lane 5, JPN15(pAJG4); lane 6, AG4(pAJG4).

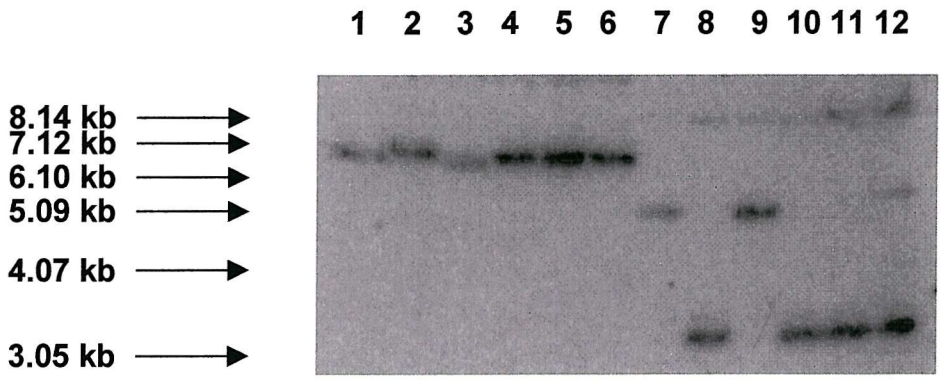


Figure 3.9. Southern hybridisation analysis using JPN15 wild-type and BipA null mutants. Chromosomal DNA was digested with *Bgl*II, *Sal*I or *Ssp*I and probed with a 5' ^{32}P labelled PCR product of *bipA*. Lane 1, JPN15 *Bgl*II digested; lane 2, AG4 *Bgl*II digested; lane 3, AG5 *Bgl*II digested; lane 4, AG6 *Bgl*II digested; lane 5, JPN15 *Sal*I digested; lane 6, AG4 *Sal*I digested; lane 7, AG5 *Sal*I digested; lane 8, AG6 *Sal*I digested; lane 9, JPN15 *Ssp*I digested; lane 10, AG4 *Ssp*I digested; lane 11, AG5 *Ssp*I digested; lane 12, AG6 *Ssp*I digested.

3.5 Discussion

Prior to this study the *bipA* gene of E2348/69 sequence was suggested to display a number of base changes from the *E. coli* K-12 nucleotide sequence, which once translated to protein sequence, resulted in six amino acid substitutions (Freestone *et al.*, 1998). As a result of producing a number of independent clones all of which were sequenced on more than one occasion with primers specific for both DNA strands (this study), we conclude that there is considerable sequence identity between the E2348/69 and *E. coli* K-12 *bipA* nucleotide sequence (99.21%) and that, once translated, the homology is in 100% accordance up to codon 591 which precedes the stop codon as suggested by the K-12 sequence (ECAE462). Further sequencing of MG1655 and E2348/69 chromosomal DNA has suggested a sequence that differs from the K-12 *bipA* sequence in the database (ECAE462), in which an additional 'c' residue is found at position 1774, which extends the nucleotide sequence by an additional 48 residues. The E2348/69 sequence from this study was submitted to the EMBL database, Accession number AJ278218. The extended BipA sequence is in close agreement with the BipA sequence from a number of other bacteria (R. Haigh unpublished observations). It is intriguing that a cloned copy of the truncated E2348/69 *bipA* gene is functionally active, and complements the *bipA*$^{-}$ mutation in a number of phenotypic studies (Chapters herein).

BipA shows sequence similarity with ribosome binding GTPases such as elongation factor G (EF-G). This sequence similarity is more highly conserved in the amino-terminal third of the protein, that includes areas that define a guanine nucleotide-binding region (residues 12-20, 74-83 and 128-131) (Figure 1.16). There are also conserved pockets of nucleotide sequence stretching throughout the gene, including an area of sequence similarity with domain IV of EF-G. The sequence of the protein from strain MAR001 differs from the *E. coli* K-12 sequence by two residues, located within domain IV (Ile to Thr and Val to Ala at positions 489 and 497 respectively) (Farris *et al.*, 1998). Sequence analysis of the EPEC E2348/69 BipA sequence (this

study), has confirmed it to have 100% homology to *E. coli* K-12 BipA sequence, thus it does not have the residue changes that EPEC MAR001 has in domain IV.

Domain IV has structural homology with the anti-codon arm of tRNA in the ternary complex of elongation factor Tu, and is thought to interact with the small ribosomal subunit (Nissen *et al.*, 1995; Rodnina *et al.*, 1997). Farris *et al.*, (1998) propose that the conformational change within Domain IV caused by the differences in the two amino acid residues within this region in *E. coli* K-12 and EPEC MAR001, may account for the observed differences between the two proteins in their ability to undergo tyrosine phosphorylation. Freestone *et al.* (1998) report tyrosine phosphorylation of TypA (the product of the *o591* reading frame – alternative name for BipA), in EPEC strains MAR001 and E2348/69. The two amino acid substitutions seen between MAR001 and *E. coli* K-12 are not changed in EPEC E2348/69 sequence. This suggests that another function accounts for the differences in BipA to undergo tyrosine phosphorylation in different strains.

For the characterisation of the biological role of BipA in EPEC it was necessary to produce chromosomal *bipA* null mutants in E2348/69 and JPN15. Null mutants were successfully produced, and confirmed by PCR amplification, using primers to chromosomal regions of DNA upstream and downstream of *bipA*, and by Southern hybridisation using a range of restriction endonucleases. The absence of a positive signal with the BipA antibody was also confirmed for these mutants by Western blotting.

The availability of these mutants now permits a detailed analysis of the role of BipA in the genesis of an attaching and effacing lesion. These studies are described in the next chapter.

CHAPTER 4

PRELIMINARY SURVEY OF PROPERTIES OF THE BipA NULL MUTANTS

CHAPTER 4: PRELIMINARY SURVEY OF PROPERTIES OF THE BipA NULL MUTANTS

4.1 Introduction

The co-ordinated regulation of bacterial virulence factors is believed to be critical for successful colonization of host organisms. Bacteria also have a requirement to control the expression of virulence factors to prevent unnecessary wastage of energy on the synthesis of these factors when they are not needed. Bacteria possess thermosensing devices to adjust to changes in temperature, which for enteric bacteria may reflect transfer from the environment to a host (Hurme and Rhen, 1998). Temperature-mediated regulation occurs at the levels of transcription and translation and events involved in thermoregulation include supercoiling, changes in mRNA conformation, and changes in protein conformation (Hurme and Rhen, 1998).

One protein in particular, H-NS, is implicated in thermoregulation, and transcription of the *hns* gene is markedly induced upon a shift from 37°C to 10°C (La Teana *et al.*, 1991). Mutations in *hns* alter the expression of genes regulated by environmental factors such as osmolarity, pH, oxygen availability, and temperature (Laurent-Winter *et al.*, 1997; Hommais *et al.*, 2001).

Bacteria have to constantly monitor environmental conditions and respond to changes in key parameters, such as nutrients, pH, osmolarity and temperature. The ability of bacteria to move in a directed manner allows bacteria to move towards attractants to increase the efficiency of nutrient acquisition and also to access optimal colonisation sites and to move away from repellents, such as host defence components (Ottemann and Miller, 1997). In bacteria such as *E. coli*, motility is dependent on the presence of surface filamentous appendages, flagella, which serve as propellers. These multicomponent structures require the expression of around 50 genes, and their synthesis requires approximately 2% of the cells total energy.

4.1.1 Rotation of the flagellar filament

The rotating part of the flagellum that propels the cell is termed the filament. In *E. coli*, flagellar filaments are thin helical appendages found at random locations on the surface of a bacterium, at a density of 5 to 10 appendages per cell (Bertin *et al.*, 1994; Macnab, 1996). Flagellar convert trans-membrane proton potential energy into mechanical work. Protons pumped out of the cell by the respiratory chain, pass back into the cell, powering the flagellar motor. The torque generated produces rotation, which is transmitted from the motor to the flagellar filament (Macnab, 1992). The filament is a left-handed helix, rotation of the flagellar filament counter clockwise (CCW) causes the flagellar filaments to wrap around one another and form a bundle promoting a forward movement, swimming. In contrast, bacteria with clockwise (CW) rotation have a poorly defined orientation, known as tumbling, (Iino, 1977; Macnab, 1996; Macnab, 1999). The modulation of motility permits bacteria to respond to environmental signals such as chemical stimuli.

4.1.2 Structure of the *E. coli* flagellum

The flagellum is composed of three sub-structures; (i) the basal body, located in the cell membrane (ii) an extracellular hook which connects the flagellar filament to the cell and acts as a flexible linker allowing for flexible movement of the filament (iii) the filament. (Iino, 1977; Macnab, 1992; Hughes *et al.*, 1993 Minamino *et al.*, 1999). (The general view of a flagellum is shown in Figure 4.1). The assembly of the flagellum in the extracellular environment involves the transport of components through a pore in the basal body structure. Thus the flagellum is not just the organelle of propulsion for the bacterium, but also functions as a type III export apparatus. Indeed, recent studies with *Yersinia* show that certain virulence factors can also exit through the flagellar apparatus (Young *et al.*, 1999). In *E. coli*, twelve proteins are known to be exported through the type III flagellar pathway and, as with other type III systems, none of the proteins secreted undergo signal peptide cleavage during export (Minamino and Macnab, 2000).

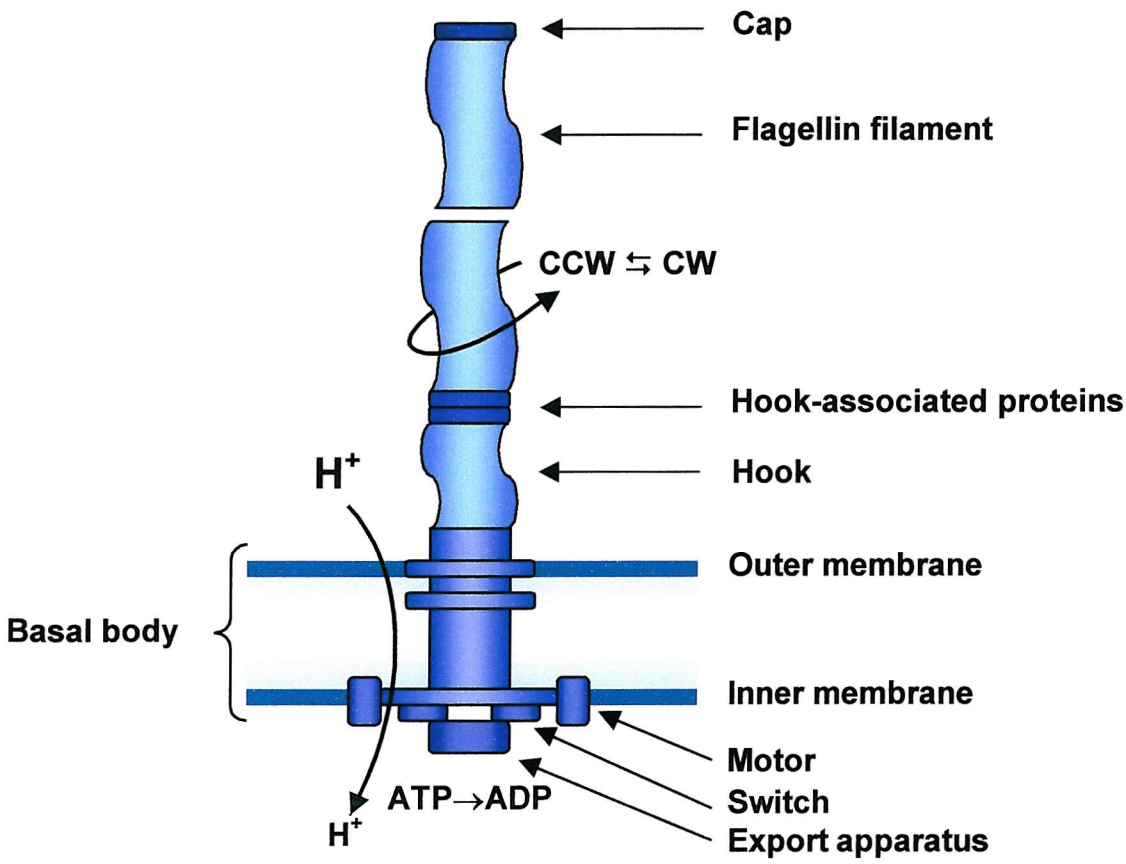


Figure 4.1. Biosynthesis of the flagellar structure. Adapted from Hughes *et al.* (1993) and Macnab, (1999)

4.1.3 Regulation of flagellar biosynthesis

Flagellar operons form a co-ordinated system of expression, a regulon, whereby the transcription of specific gene sets are only initiated after the transcription of a gene or number of genes higher up in the cascade has been completed. (Bertin *et al.*, 1994; Prüß and Wolfe, 1994). There are three classes of genes: early, middle and late. The early class of genes includes two master regulatory genes *flhC* and *flhD*, which are positive regulators of the middle class of genes. The genes of the middle class are required for the structure and assembly of the hook-basal body complex. One middle class gene is *fliA*, which encodes the alternative sigma factor, σ^{28}, required for the transcription of the late class of genes. *flgM* is both a middle and late class of gene and encodes an anti sigma factor σ^{28} that negatively regulates the late gene operon (Kutsukake *et al.*, 1990; Macnab, 1992; Hughes *et al.*, 1993; Chilcott and Hughes, 1998). FlgM inhibits the expression of the late flagellar genes until the hook-basal

body structure is assembled and ready for the export of flagellar late proteins, FlgM is then exported from the cytoplasm through the flagellar filament. This permits σ^{28} to bind to the core RNA polymerase and initiate transcription of the late promoters, (Chilcott and Hughes, 1998; Karlinsey *et al.*, 2000a; Karlinsey *et al.*, 2000b).

The expression of *flhDC* is affected by many global regulatory factors. The cyclic AMP-catabolite activator protein (cAMP-CAP) complex positively regulates the expression of the master operon (Soutourina *et al.*, 1999). H-NS also acts as a positive regulator of genes involved in the biogenesis of flagella, independent of the cAMP-CAP pathway (Bertin *et al*, 1994). *crp* and *hns* mutants have been shown to be non-motile due to reduced expression of *fliA* and *fliC* which encode the specific flagellar sigma factor and flagellin respectively (Soutourina *et al.*, 1999). The positive control of CAP is due to a direct interaction between the cAMP-CAP complex and RNA polymerase (Soutourina *et al.*, 1999), activation of *flhC* and *flhD* transcription by H-NS is mediated through the negative regulator HdfR (a LysR family protein) (Ko and Park, 2000). H-NS negatively modulates the expression of *hdfR*, thus when H-NS is inactive, the expression of the *hdfR* gene is increased leading to an increase in the production of HdfR. This in turn reduces the transcription of *flhC* and *flhD* by binding to the upstream control region (Ko and Park, 2000). Recent studies suggest that the global regulator CsrA is also required for motility and flagellar biosynthesis, through the post-transcriptional activation of *flhDC* expression (Wei *et al.*, 2001).

Recent studies with an ill-characterised EPEC strain (MAR001) suggest that BipA null mutants have a pleiotropic phenotype and, in particular, show defects in cell motility and resistance to the host defence protein BPI (Farris *et al.*, 1998). Having constructed equivalent mutations in the *bipA* gene of the classical EPEC strain E2348/69 and its plasmid-cured derivative JPN15, it was therefore of interest to survey these strains for general defects. In this chapter, the growth phenotypes of *bipA*$^{-}$ mutants of E2348/69 are measured in complex and minimal media, along with response to cold shock, cell motility and the levels of H-NS protein expression.

4.2 Growth characteristics of E2348/69 *bipA*$^+$ and *bipA*$^-$ strains are the same at 37°C

As a prerequisite to more detailed studies, the growth characteristics of the E2348/69 wild-type, *bipA*$^-$ mutant and complemented strains were compared in nutrient rich broth (LB) and nutrient deprived broth (M9 minimal media). Bacteria from an overnight culture were used to inoculate a fresh culture grown at 37°C, and absorbance readings at 600 nm were taken every 30 minutes thereafter for a period of 10 hours (Figure 4.2). The BipA null mutant showed no significant difference in growth with respect to wild-type E2348/69 at all phases of growth in either nutrient rich broth (LB) or nutrient deprived broth (M9 minimal media).

4.3 EPEC *bipA*$^-$ strains exhibit a reduced growth phenotype below 30°C

Although there was no difference in the growth rates of wild-type and *bipA*$^-$ EPEC strains at 37°C to stationary phase, further studies showed that the BipA protein is required for the growth of EPEC at temperatures below 30°C. E2348/69 wild-type cells grew equally well on an LB plate incubated at 27°C or at 37°C, (although colonies were slightly smaller on the plate incubated at 37°C). However, the E2348/69 *bipA*$^-$ mutant strain showed substantially reduced growth on an LB plate incubated at 27°C, with the absence of defined isolated colonies (Figure 4.3). The observed phenotype was due to mutation of the *bipA* gene, as growth of the *bipA*$^-$ strain was restored on the addition of pAJG4, which contains a cloned copy of the *bipA* gene. Complementation was as a result of adding *bipA* as AG2, containing just the cloning vector pT7T318U exhibited a phenotype that was the same to that of AG2.

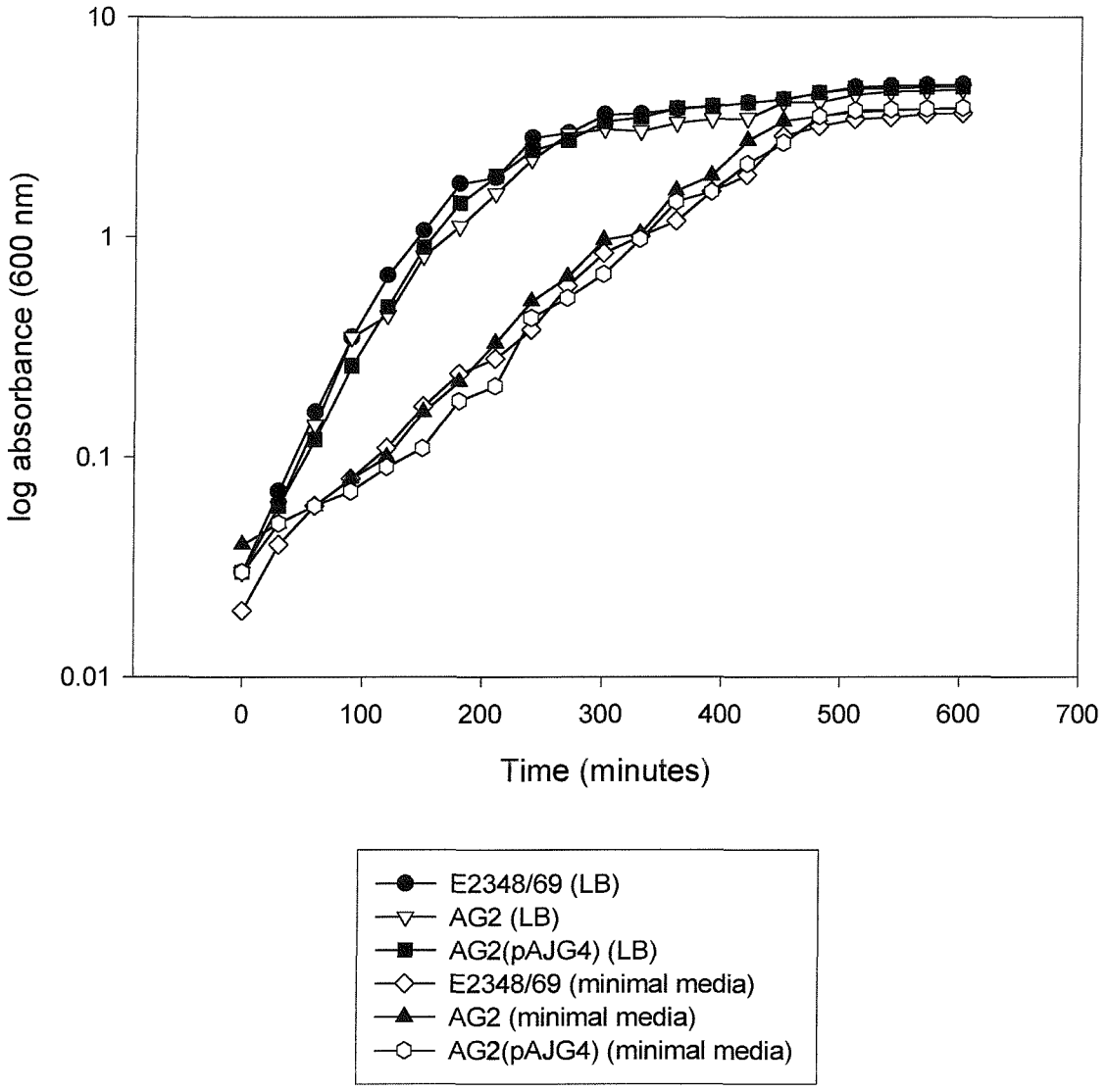


Figure 4.2. Comparison of growth rates of E2348/69 wild-type, *bipA*$^{-}$ and transcomplemented strains grown in nutrient rich media (LB), and nutrient deprived media (M9 minimal media) to stationary phase at 37°C. No significant differences in growth rate were observed between the *bipA*$^{+}$ and *bipA*$^{-}$ E2348/69 strains in either media.

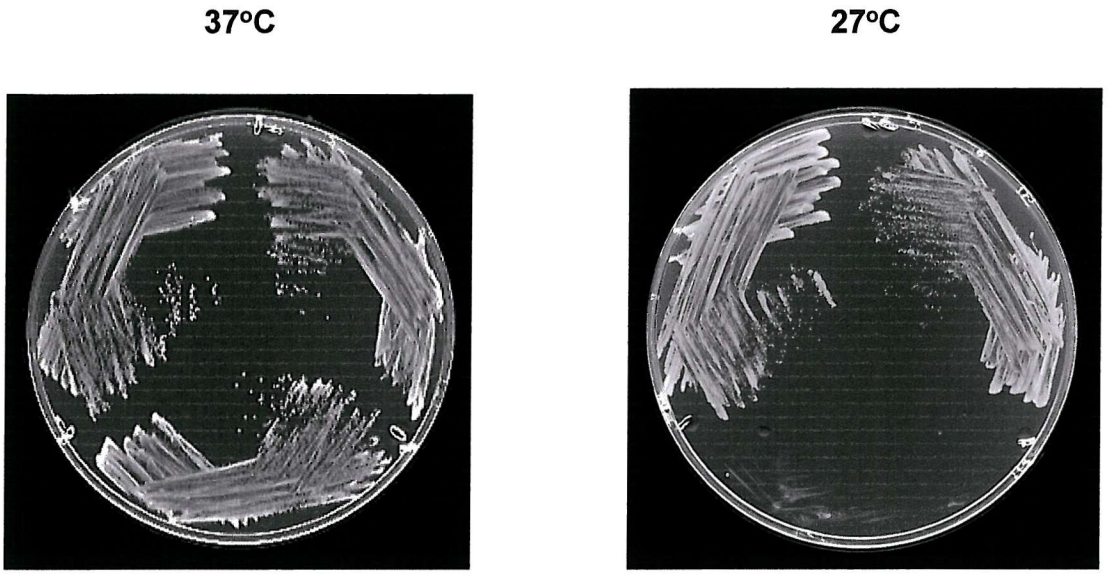


Figure 4.3. BipA is required for growth at low temperatures. E2348/69, AG2 and AG2(pAJG4) were plated onto two LB agar plates and incubated at 27°C and 37°C for 14 hours. The top right segment of each plate corresponds to E2348/69 whereas the top left and bottom segments correspond to AG2(pAJG4) and AG2 respectively. The photo demonstrates the absence of well-defined isolated colonies of the E2348/69 *bipA*$^{-}$ mutant strain grown at 27°C.

4.4 Biolog ES MicroPlates confirm *E. coli* K-12 Δ*bipA* is in MG1655

To determine if absence of a functional *bipA* gene caused a general defect in the ability of *E. coli* K-12 to grow on defined carbon or nitrogen sources Biolog ES Microplates were used. The results showed that the phenotype of MG1655 *bipA*$^-$ cells was indistinguishable from the corresponding wild-type cells. Thus this system not only confirmed the phenotype of MG1655 but shows that BipA does not compromise the ability of this strain to utilise a wide range of carbon and nitrogen sources.

4.5 Other EPEC and *E. coli* K-12, but not *Salmonella bipA*$^-$ strains exhibit a reduced growth phenotype below 30°C

The reduced growth of *bipA*$^-$ mutants at temperatures below 30°C was extended to the EPEC *bipA*$^-$ strains of JPN15 and MAR001, and also to the *E. coli* K-12 *bipA*$^-$ strain of MG1655. Complementation of this phenotype with plasmids containing a cloned copy of the *bipA* gene established that the truncated E2348/69 BipA protein encoded by pAJG4, full length E2348/69 BipA expressed from pAJG9, full-length E2348/69 His tagged BipA and *Salmonella* Typhimurium BipA expressed from pYL98-2, complement the *bipA*$^-$ mutants. His-tagged BipA(G77V) did not complement any strain tested. Moreover, truncated MAR001 BipA expressed from pT7T318UMAR*bipA* failed to complement the defect in any strain tested. As expected BipA null mutant strains transformed with the cloning vectors pT7T318U or pTrcHisB exhibited phenotypes that were identical to the BipA null mutant strains. This data is summarised in Table 4.1. The BipA protein is thus required for growth of EPEC and *E. coli* K-12 strains at temperatures below 30°C. Interestingly *bipA*$^-$ mutant strains of *Salmonella* Typhimurium and *Salmonella* Enteritidis do not show a reduced growth phenotype at temperatures below 30°C (data not shown).

Plasmid / Strain	pAJG4	pAJG9	pAJG11	pAJG13	pMAR*bipA*	pYL98-2	pT7T318U	pTrcHisB
AG2	✓	✓	✓	✗	✗	✓	✗	✗
AG4	✓	✓	✓	✗	✗	✓	✗	✗
MAR001 *bipA::cat*	✓	✓	✓	✗	✗	✓	✗	✗
MG1655Δ*bipA*	✓	✓	✓	✗	✗	✓	✗	✗

Table 4.1. Comparison of the ability of the protein product of cloned versions of the *bipA* gene from different bacterial strains to complement the growth of EPEC and *E. coli* K-12 *bipA*$^-$ mutants at temperatures below 30°C. Complementation is indicated by (✓), while the inability of the plasmid to complement the defect is indicated by (✗). Truncated E2348/69 BipA (pAJG4), full length (pAJG9) and His-tagged (pAJG11) constructs complement growth in all strains tested, as does BipA from *Salmonella* Typhimurium (pYL98-2). The His-tagged mutant BipA(G77V) - pAJG13, pT7T318UMAR*bipA* and the cloning vectors pT7T318U and pTrcHisB did not complement the defect.

4.6 E2348/69 *bipA*⁻ mutant hyper-secretes flagellin

Previous studies by Farris *et al.* (1998) indicate the increased export of a 60 kDa protein identified as flagellin in the spent culture medium of MAR001 *bipA*⁻ mutant grown in LB. Accordingly, proteins secreted by E2348/69 *bipA*⁺ and *bipA*⁻ strains were precipitated from spent LB culture medium using TCA precipitation, and resolved by SDS PAGE (Figure 4.4). As observed with the EPEC MAR001 strain, elevated levels of a 60 kDa protein were detected in the spent culture medium from the E2348/69 *bipA*⁻ mutant. This band migrated around the size expected for flagellin (FliC), which has a molecular weight of 60.5 kDa. In view of the results of Farris *et al.* (1998) and further results from this study, attempts to confirm the protein as flagellin were not performed.

4.7 BipA negatively regulates flagella-mediated cell motility in EPEC strains

These studies and those of Farris *et al.* (1998) have demonstrated that EPEC BipA negatively regulates the expression or export of the bacterial flagellum. Moreover, MAR001 *bipA*⁻ strains were found to be markedly more motile than either the parent or the transcomplemented strain (Farris *et al.*, 1998). In view of the effects observed with the poorly characterised EPEC strain MAR001, it was of interest to determine whether EPEC E2348/69 and JPN15 *bipA*⁻ mutant strains exhibited the same phenotype, thus the motilities of the strains was compared. Bacteria from stationary phase cultures were stabbed into motility agar plates and left at 37°C before scoring the diameters of the colonies. As observed with the MAR001 *bipA* null mutant (Farris *et al.*, 1998), the E2348/69 and JPN15 *bipA* null mutants formed significantly larger colonies than the parent strain on motility agar plates (Figures 4.5 – 4.7). Colonies of E2348/69 wild-type grown on motility agar were 53% smaller than those of the *bipA*⁻ strain, while colonies of JPN15 wild-type were 44% smaller than those of the *bipA*⁻ strain. The phenotype was in part restored when the mutant strains were transformed with the plasmids; pAJG4, pAJG9, pAJG11, pT7T318UMAR*bipA* and pYL98-2 which encode a cloned copy of the *bipA* gene. In contrast, the (G77V) His-

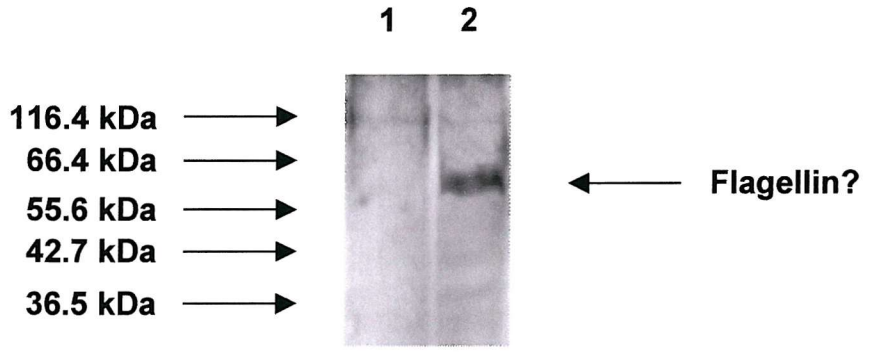


Figure 4.4. Comparison of secreted protein profiles from supernatants of wild-type and the *bipA*⁻ mutant E2348/69 strains. Bacteria were grown in LB to an OD_{600} of 0.8. After removal of bacteria by centrifugation, 1.5 ml of supernatant was extracted, and supernatant proteins were precipitated by the addition of 10% trichloroacetic acid. Proteins were separated by polyacrylamide gel electrophoresis, and the gel stained by coomassie blue staining. Lane 1, E2348/69; lane 2, AG2.

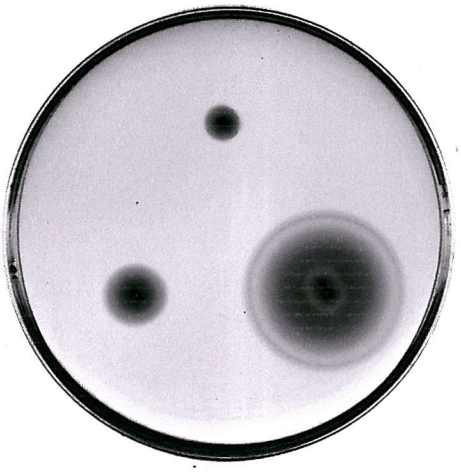


Figure 4.5. Picture of a motility agar plate showing the difference in colony diameter of E2348/69 wild-type, *bipA*⁻ mutant and transcomplemented strains. Bacteria were stabbed into motility agar plates and left at 37°C for 22 hours, before scoring the diameters of the colonies. The top segment of the plate corresponds to E2348/69 whereas the bottom left and bottom right segments correspond to AG2(pAJG4) and AG2, respectively.

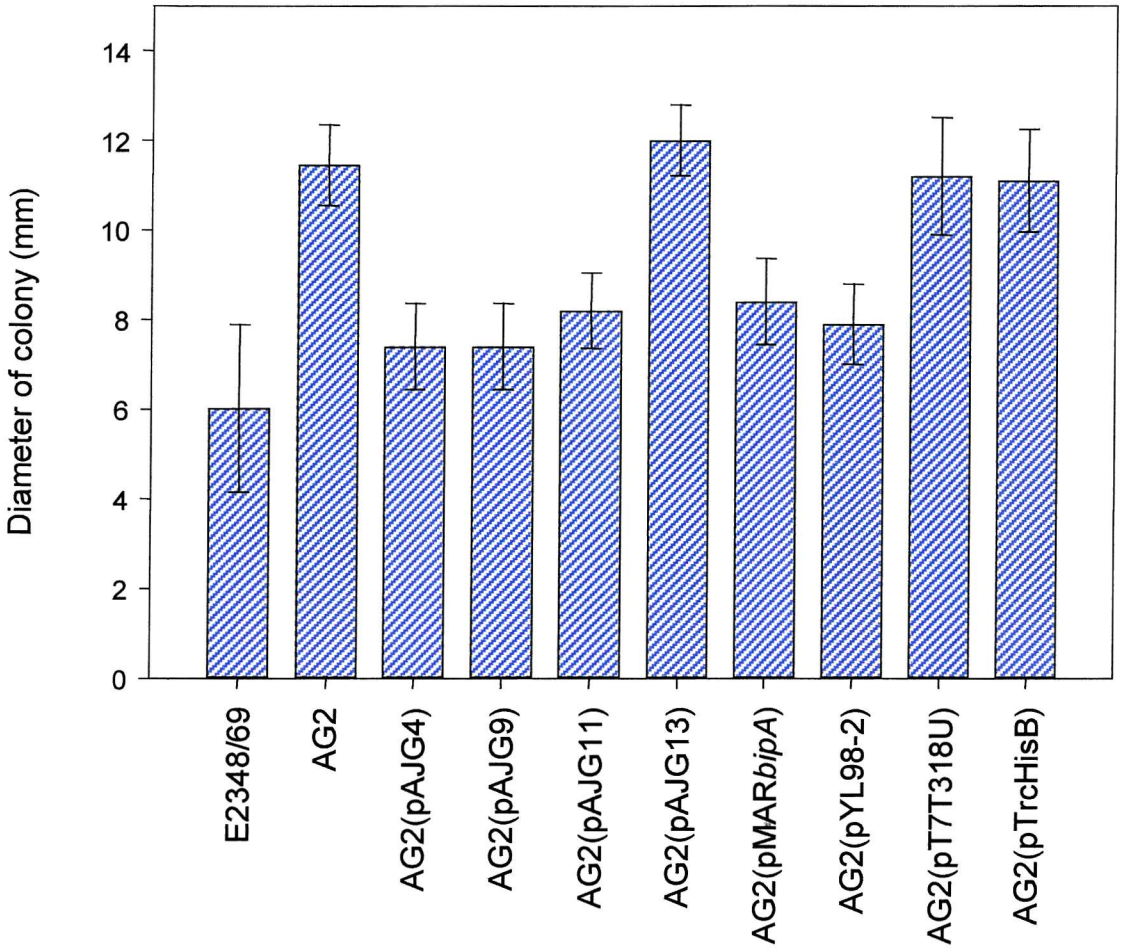


Figure 4.6. Motility of E2348/69 or its derivatives. The graph shows the diameter of colonies of E2348/69 wild-type, *bipA*⁻ mutant and null mutant strains transformed with various *bipA* expressing plasmids and cloning vectors. Wild-type, *bipA*⁻ mutant and a *bipA*⁻ mutant strain transformed with a particular plasmid were stabbed into motility agar plates and left at 37°C for 20 hours, before scoring the diameters of the colonies. The graph shows the mean averages of 5 experiments for each of the plasmid-containing strains, and the mean averages of 40 experiments for the wild-type and *bipA*⁻ mutant strains. Error bars are plotted as standard deviation. The graph shows that E2348/69 *bipA*⁻ mutants were more motile than the wild-type strain. *bipA*⁻ mutant strains expressing truncated, full length and His-tagged E2348/69 BipA, along with BipA from *Salmonella* Typhimurium or MAR001, complemented the *bipA*⁻ defect in part, while the His-tagged mutant (G77V) did not complement the *bipA*⁻ defect. The complementation was as a result of adding *bipA in trans* as AG2 containing the cloning vectors pT7T318U or pTrcHisB exhibited a phenotype that was identical to AG2.

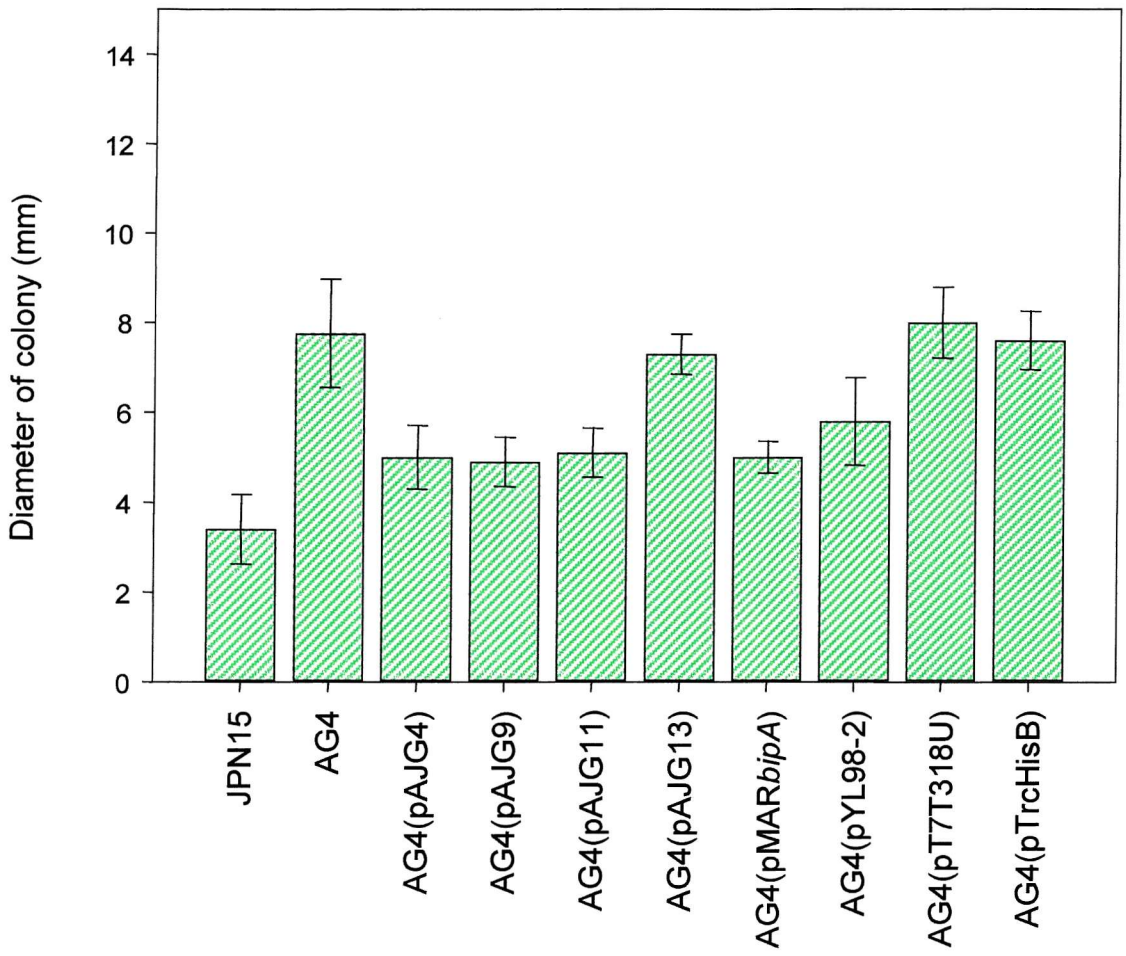


Figure 4.7. Motility of JPN15 or its derivatives. The graph shows the diameter of colonies of JPN15 wild-type, *bipA*⁻ mutant and null mutant strains transformed with various *bipA* expressing plasmids and cloning vectors. Wild-type, *bipA*⁻ mutant and a *bipA*⁻ mutant strain transformed with a particular plasmid were stabbed into motility agar plates and left at 37°C for 20 hours, before scoring the diameters of the colonies. The graph shows the mean averages of 5 experiments for each of the plasmid-containing strains, and the mean averages of 40 experiments for the wild-type and *bipA*⁻ mutant strains. Error bars are plotted as standard deviation. The graph shows that JPN15 *bipA*⁻ mutants were more motile than the wild-type strain. *bipA*⁻ mutant strains expressing truncated, full length and His-tagged E2348/69 BipA, along with BipA from *Salmonella* Typhimurium or MAR001, complemented the *bipA*⁻ defect in part, while the His-tagged mutant (G77V) did not complement the *bipA*⁻ defect. The complementation was as a result of adding *bipA in trans* as AG4 containing the cloning vectors pT7T318U or pTrcHisB exhibited a phenotype that was identical to AG4.

tagged mutant did not complement the BipA defect. The complementation was as a result of adding a cloned copy of *bipA* as AG2 and AG4 containing the cloning vectors pT7T318U or pTrcHisB, exhibited a phenotype that was identical to the plasmid free mutant strains.

4.8 BipA positively regulates flagella-mediated cell motility in *E. coli* K12 strains

Results from this study and those of Farris *et al.* (1998) indicate that BipA negatively regulates cell motility and/or chemotaxis in EPEC. However, the phenotype is reversed in *E. coli* K12. The *bipA*$^-$ mutant strain of MG1655 was found to be less motile than the corresponding wild-type strain (Figure 4.8). Colonies of MG1655 wild-type grown on motility agar were 48% larger than those of the *bipA*$^-$ strain. This observation is in accordance with the findings that *bipA*$^-$ *Salmonella* Typhimurium and *Salmonella* Enteritidis mutants are less motile than *bipA*$^+$ strains (A. White and N. Kinsella unpublished observations). Interestingly the presence of a cloned copy of *bipA* in MG1655 *bipA*$^-$ does not complement the defect, a result echoed in the *Salmonella* Enteritidis strains (A. White, unpublished observations). Indeed, the presence of a cloned copy of *bipA* on the plasmids pAJG4, pAJG9 and pT7T318UMAR*bipA* seems to augment the motility defect further.

4.9 BipA does not control the expression of H-NS in EPEC E2348/69

In view of the effect of BipA on cell motility and other pleiotropic effects in the BipA mutants. The levels of H-NS in E2348/69, its *bipA*$^-$ mutant, and a transcomplemented strain grown in high and low glucose MOPS, were measured by western blotting. H-NS was chosen for study as it has previously been shown to regulate genes involved in both thermoregulation and motility. The E2348/69 *bipA*$^-$ mutant strain exhibited no significant difference in H-NS expression levels with respect to the wild-type E2348/69 strain grown in either high glucose or low glucose MOPS (Figure 4.9).

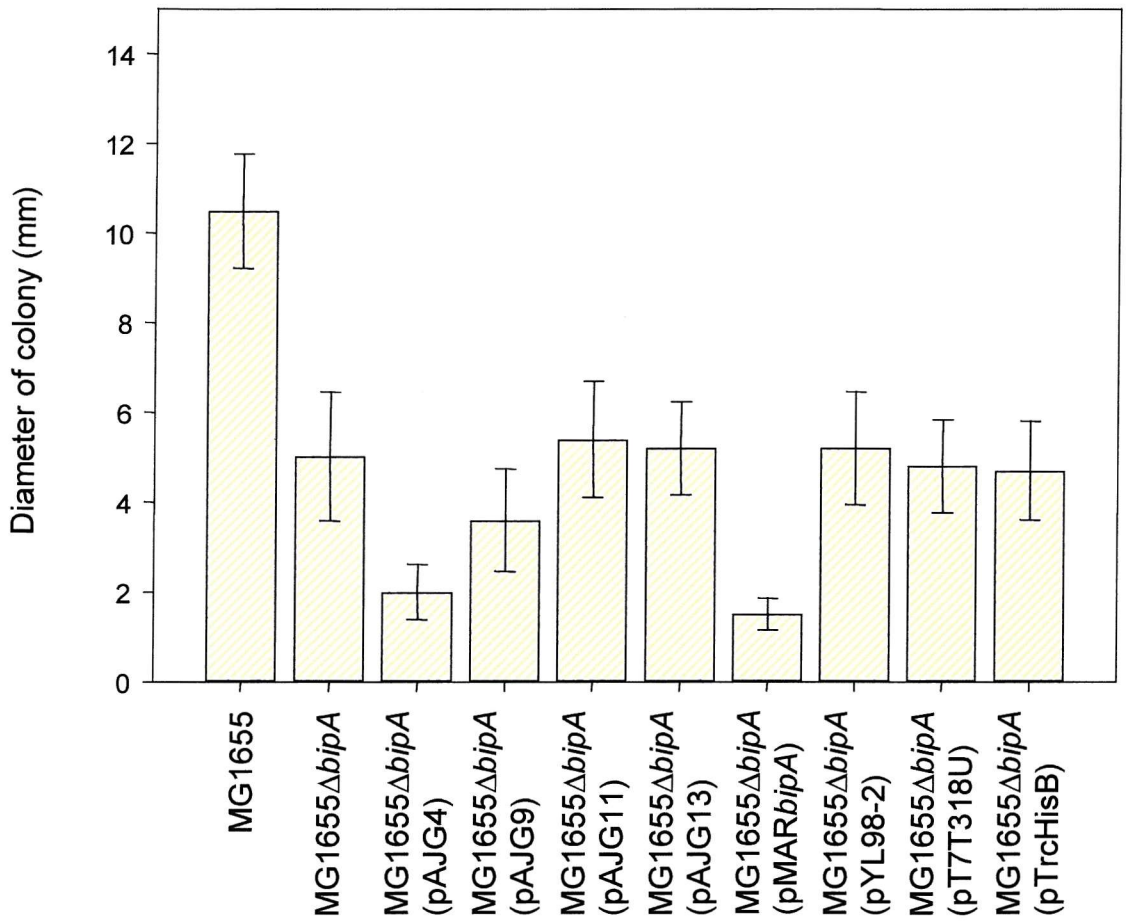


Figure 4.8. Motility of MG1655 or its derivatives. The graph shows the diameter of colonies of MG1655 wild-type, *bipA*⁻ mutant and null mutant strains transformed with various *bipA* expressing plasmids or cloning vectors. Wild-type, *bipA*⁻ mutant and a *bipA*⁻ mutant strain transformed with a particular plasmid were stabbed into motility agar plates and left at 37°C for 20 hours, before scoring the diameters of the colonies. The graph shows the mean averages of 5 experiments for each of the plasmid-containing strains, and the mean averages of 40 experiments for the wild-type and *bipA*⁻ mutant strains. Error bars are plotted as standard deviation. The graph shows that the *bipA*⁻ derivative of MG1655 was less motile than the parent strain. Interestingly, this is a reversal of the phenotype observed with EPEC strains. *bipA*⁻ mutant strains expressing truncated and full length E2348/69 BipA, along with BipA from MAR001 appear to have a further reduced motility while the remaining *bipA* expressing plasmids did not complement the defect, or cause a further reduction in motility. The MG1655 *bipA*⁻ mutant transformed with the cloning vectors exhibited a phenotype that was identical to that of the *bipA*⁻ mutant.

Figure 4.9. Comparison of H-NS levels in whole cell extracts from E2348/69 wild-type, *bipA*⁻ mutant and transcomplemented strains grown in high and low glucose MOPS. Growth of the bacteria in high and low glucose MOPS was achieved as detailed in section 2.7.4 of Chapter 2. Whole cell extracts were prepared as previously detailed and proteins were separated by polyacrylamide gel electrophoresis, using 4-12% BisTris NuPAGE gels. Proteins were transferred onto nitrocellulose and probed with anti H-NS antibodies. Immunoreactive bands were then visualised using SuperSignal chemiluminescence substrate. The samples for lanes 1, 3 and 5 were grown in low glucose MOPS whereas the samples for lanes 2, 4 and 6 were grown in high glucose MOPS. Lanes 1 + 2, E2348/69; lanes 3 + 4, AG2; lanes 5 + 6, AG2(pAJG4).

4.10 Sensitivity to BPI-derived peptide P2

BipA was initially discovered when *Salmonella* Typhimurium cells were exposed to sub-lethal concentrations of Bactericidal/permeability-inducing protein (BPI), a cationic host defence component produced by human granulocytes. Such treatments induced BipA expression by more than sevenfold (Qi *et al.*, 1995). Studies by Little *et al.* (1994) and Qi *et al.* (1994) indicate that certain peptides derived from BPI retain anti-bacterial activity. In particular a peptide termed P2, which includes residues 86 – 104, was shown to retain considerable anti-bacterial activity against a range of bacteria including *E. coli* (Barker, 1997). The MAR001 *bipA*$^-$ mutant was found to be more than two log orders more sensitive to P2 than the parent strain (Farris *et al.*, 1998) suggesting that BipA mediates resistance to P2. The sensitivity of E2348/69 wild-type and *bipA*$^-$ mutant strains to P2 was investigated by measuring cell viability after exposing bacteria to a range of P2 concentrations. No significant difference in sensitivity to P2 was seen between the *bipA*$^+$ and *bipA*$^-$ E2348/69 strains (Figure 4.10). Thus although BipA is important for mediating resistance to the peptide P2 in MAR001, this phenotype appears not to be exhibited by the prototype EPEC strain E2348/69.

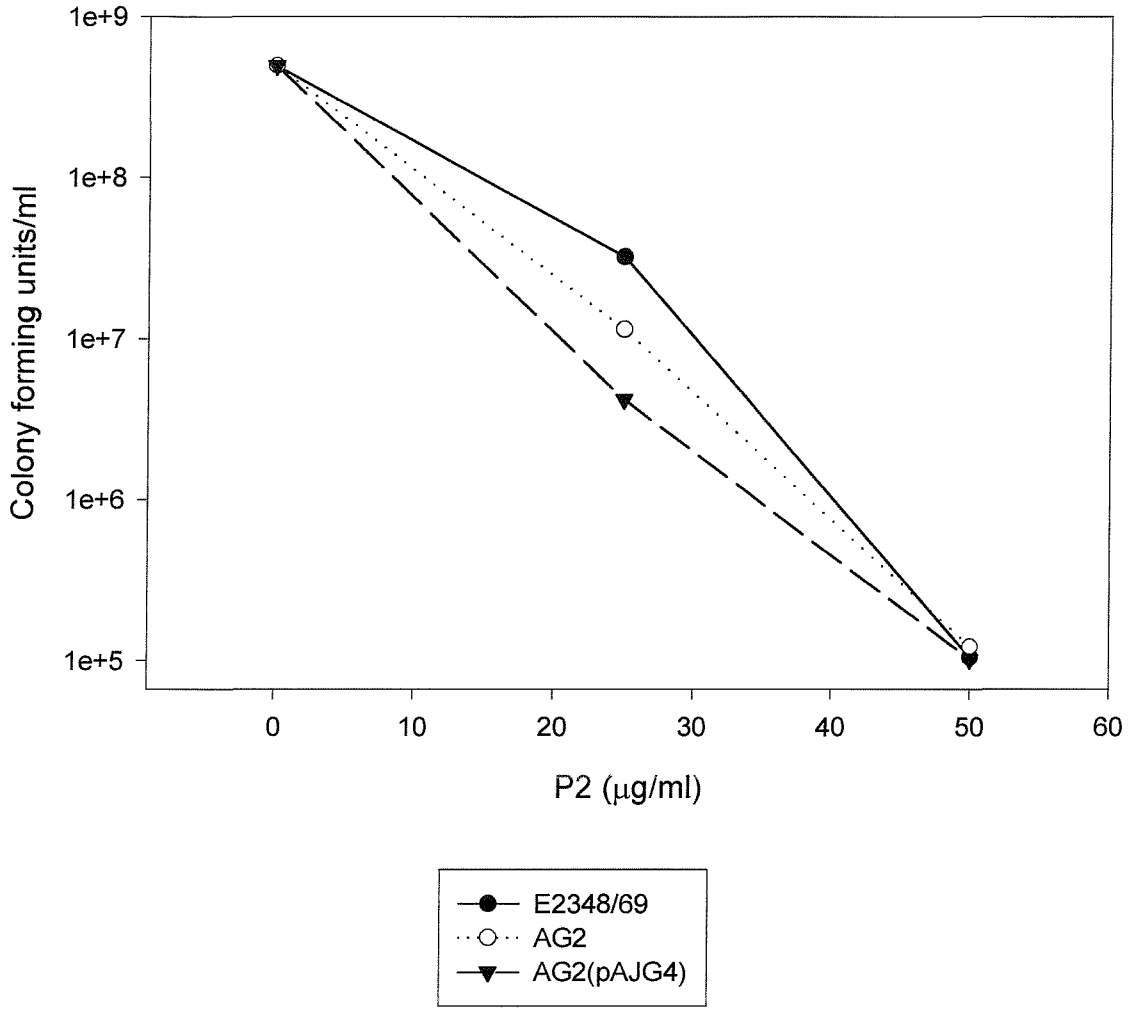


Figure 4.10. Graph showing the susceptibility of E2348/69 and BipA mutants to the bactericidal peptide P2. Bacteria were grown to mid-exponential phase in tryptic soy broth. Bacteria were pelleted by centrifugation, washed and resuspended in 20 mM sodium phosphate buffer. Bacteria were incubated at 37°C for 30 minutes with the bactericidal peptide P2 (SKISGKWKAQKRFLKMNSGNFGC) before being serially diluted and plated on LB agar plates. Survivors were scored after overnight incubation at 37°C. There was no significant difference in the susceptibility of the E2348/69 *bipA*⁻ mutant strain to killing by P2 with respect to the wild-type.

4.11 Discussion

Recent studies indicate that *bipA*⁻ mutants are pleiotropic, suggesting that the GTPase has a regulatory function. The results described in this chapter both support and extend this conclusion.

4.11.1 Growth characteristics of the *bipA*⁻ mutants

For characterisation of the biological role of BipA in the prototype EPEC strain, a null mutant of E2348/69 was produced by the non-polar insertion of a chloramphenicol acetyltransferase (*cat*) cassette into the *bipA* gene (Chapter 3, section 3.3). Having produced the null mutant, growth of the strain relative to the wild-type was measured. It was observed that growth of AG2 in nutrient rich broth (LB) or nutrient depleted medium (M9 Minimal Medium) at 37°C to stationary phase was not significantly different from the wild-type E2348/69. Additionally supplying BipA *in trans* in AG2 made no difference to the overall growth rate of the strain. These results suggest that EPEC BipA is not required for viability at 37°C, and indicate that the *bipA*⁻ mutants are not 'sickly'.

However, BipA is required for growth of EPEC and *E. coli* K-12 strains at low temperatures as demonstrated by the reduction of growth of MAR001, E2348/69, JPN15 and MG1655 *bipA*⁻ mutants relative to respective wild-type cells at 27°C. Normal growth could be restored by supplying *bipA in trans* on the high copy number vectors; pAJG4, pAJG9, pAJG11 and pYL98-2. Interestingly, the complementation studies suggest that the C-terminally truncated E2348/69 BipA, expressed from pAJG4, was functionally active. Also, the addition of a His-tag did not perturb the functional activity of E2348/69 BipA, while substitution of G77V, which maps to one of the proposed nucleotide binding domains, rendered the protein inactive. At present, it is unclear if loss of activity is due to a conformational change. However, it is tempting to speculate that this observation adds further credence to the suggestion that it is the GTPase function of BipA rather that its tyrosine phosphorylation that is

important for activity. Interestingly, normal growth was not restored, by supplying MAR001 *bipA in trans* on the high copy number vector, pT7T318U. The ability of this construct to complement *bipA*⁻ mutants, has previously been reported (Farris *et al.*, 1998) which suggests that, although C-terminally truncated, a functionally active form of *bipA* was cloned. These studies have indicated that EPEC and *E. coli* K-12 BipA is essential for growth at low temperature. However, this is not the case for *bipA*⁻ mutants of *Salmonella* Typhimurium or *Salmonella* Enteritidis, which do not show a reduced growth phenotype at 27°C.

At present, the molecular basis for the cold-sensitive phenotype of EPEC *bipA*⁻ mutants is not known. However, recent studies with other proteins that confer a similar phenotype when lacking, e.g. the major cold shock-inducible protein of *E. coli*, CspA, suggests that CspA functions as an RNA chaperone, preventing the formation of secondary structures in RNA molecules at low temperatures (Herschlag, 1995; Jiang *et al.*, 1997). Cold shock also induces a ribosomal associated protein CsdA, a protein with helix destabilising activity important for ribosomal function, to increase translational efficiency of mRNAs by unwinding stable RNA secondary structures found at low temperatures (Jones *et al.*, 1996). It is proposed that the function of CspA and CsdA may be coupled, CspA co-operatively binding to mRNAs unwound by CsdA to prevent mRNAs from annealing (Jones *et al.*, 1996; Jiang *et al.*, 1997). In this respect, it is intriguing that Caldon *et al.* (1999) has recently suggested that a critical characteristic of bacterial GTPases is their ability to interact with RNA.

The *era* (*E. coli ras*-like) gene encodes an essential 34 kDa GTP binding protein involved in cell cycle progression and essential for *E. coli* viability (Inada *et al.*, 1989; Lin *et al.*, 1994; Shimamoto and Inouye, 1996; Britton *et al.*, 1998). Interestingly, a mutant of *era* has been shown to be cold-sensitive, a phenotype that can be suppressed by the over-expression of *ksgA*, the gene for 16S rRNA methyltransferase (Lu and Inouye, 1998). Further studies have indicated that Era binds to 16S rRNA and the 30S ribosomal subunit (Sayed *et al.*, 1999; Johnstone *et al.*, 1999; Meier *et al.*, 1999; Meier *et al.*, 2000). The expression of the *era* gene is

translationally coupled with the expression of the *rnc* gene (encoding the double-strand-dependent endonuclease RNase III), expression of both genes is negatively controlled by RNase III itself (Chen *et al.*, 1990). RNase III and RNase E (two endonucleases) along with PNPase and RNase II (two exonucleases) and poly(A)polymerase form part of a multi-protein complex called the degradosome (Manago, 1999; Lopez *et al.*, 1999). The expression of polynucleotide phosphorylase, PNPase, is post-transcriptionally autoregulated at the level of translation, and stability of the RNase III-processed mRNA. It is proposed that PNPase binds to RNA determinant(s) located at the 5' end of the RNase III-processed transcript, thereby inhibiting translation and inducing RNA degradation (Zangrossi *et al.*, 2000). Mutants in *pnp* are viable. However, they are unable to grow below 30°C (Manago, 1999). It is therefore suggested that PNPase is important at low temperatures in forming a cold-adapted degradosome (Beran and Simons, 2001).

It is therefore possible that the BipA GTPase interacts with RNA (either mRNA or rRNA) and has the ability to act as an RNA chaperone and promote its unfolding. This might occur only when the protein associates with ribosomes and hence may be difficult to test, but it is an idea that merits further investigation in view of the phenotype observed here. In view of the ribosome binding capacity of BipA, it is intriguing that ribosomes have been suggested to act as sensors of heat and cold shock (VanBogelen and Neidhardt, 1990). The reason for a cold-sensitive phenotype in EPEC and *E. coli* K-12 *bipA*$^-$ strains, but not in *Salmonella* Typhimurium or *Salmonella* Enteritidis strains is unclear due to the uncertainty of the involvement of BipA. However, if EPEC BipA operates as an RNA chaperone, it could be that the unwanted secondary structures are not formed in the *Salmonella* strains, or that the function of BipA is substituted by an alternate chaperone or helicase.

4.11.2 BipA does not affect H-NS expression

The DNA binding protein H-NS affects the expression of many different genes and is proposed to play a major role in signal transduction pathways involving; virulence,

cold shock, pH and osmotic regulation. These studies suggest that H-NS levels are not altered in E2348/69 *bipA*⁻ strains grown in high or low glucose MOPS, or in DMEM (Chapter 7, section 7.5). These findings conflict with recent data published by Freestone *et al.* (1998) who demonstrated that *E. coli* K-12 TypA/BipA mutants were defective in the expression of H-NS. Specifically, they report that TypA/BipA mutants of *E. coli* K-12 show a substantial increase in the level of H-NS in growth-arrest conditions, and a modest increase in H-NS synthesis during exponential growth. The discrepancy might be due to the differences in experimental conditions. Equally, it is possible that there are differences in the regulatory targets of BipA between strains of *E. coli*. Whatever the reason, the results obtained here suggest that the pleiotropic phenotype of BipA and null mutants of E2348/69 cannot be explained by changes in H-NS levels.

4.11.3 BipA does not protect E2348/69 against the antibacterial peptide P2

BipA was first detected as a polypeptide that was strongly induced on expression of *Salmonella* Typhimurium cells to sub-lethal amounts of BPI, an antibacterial human defence protein produced by granulocytes (Qi *et al.*, 1995). Further to this, Farris *et al.*, (1998) showed that MAR001 BipA is important for resistance to antibacterial proteins such as BPI and its derivatives. MAR001 *bipA*⁻ was found to be over 2 log orders more sensitive to a recombinant form of BPI, BPI_{153}, (Qi *et al.*, 1994) or to peptide P2, relative to the parent strain (Farris *et al.*, 1998). Results from this study suggest that BipA of E2348/69 is not important for resistance to bactericidal peptide P2. Barker *et al.* (2000), have indicated that lysis of the cytoplasmic membrane mediated by peptide P2 is dependent on incubation time and the growth phase of bacteria. However, the bactericidal assays in this study were performed as previously described by Farris *et al.* (1998). The observed differences are likely to reflect differences in the physiology of the bacteria. In this respect, it is interesting to note that, E2348/69 cells appear more sensitive than MAR001 cells to peptide P2.

4.11.4 BipA controls flagellar mediated cell motility

As observed with the *bipA*⁻ mutant of MAR001 (Farris *et al.*, 1998), *bipA*⁻ mutants of EPEC E2348/69 and its plasmid cured derivative JPN15 were more motile than the respective parent strains on motility agar plates. In addition, a band migrating at the size expected for flagellin was upregulated in the spent culture medium of the E2348/69 *bipA*⁻ mutant, which is consistent with the findings of Farris *et al.* (1998). Farris *et al.* (1998) also conclude that in MAR001, BipA negatively regulates the biosynthesis of flagellin as well as its secretion from the cell. Further to this, *bipA*⁻ mutants were hyper-flagellated. Although not tested, it is likely that EPEC E2348/69 and JPN15 *bipA*⁻ mutants would confer the same phenotype. However, it should be noted that the colonies produced on motility agar plates were, smaller than those produced by the corresponding MAR001 derivatives for reasons that are unclear. Since E2348/69 and the plasmid cured strain JPN15, both have reduced colony size on motility plates with respect to MAR001, the retardation in motility is not mediated by a factor encoded by the EAF plasmid.

It is not yet known which steps in EPEC flagellar biosynthesis that BipA affects. Although *bipA*⁻ mutants hyper-secrete flagellin, the bacteria still synthesise functional flagellar, so it is unlikely to be control of the Cap proteins. Control could be due to the upregulation of σ^{28}, decreased expression of FlgM, or control exerted at the early class of flagellar genes, *flhC* and *flhD*. It is interesting to note that recent studies suggest that *flhDC* expression is regulated post-transcriptionally as well as at the level of transcription.

In EPEC, BipA negatively regulates flagellin and flagellar mediated cell motility. However, in *E. coli* K-12, *Salmonella* Typhimurium and *Salmonella* Enteritidis BipA positively regulates flagellar mediated cell motility (A. Grant and A. White, unpublished observations). This is of interest as; hitherto, flagellar synthesis in *E. coli* and *Salmonella* has been thought to be very similar. The differences are unlikely to be attributable to differences in BipA sequence as the *E. coli* K-12 and E2348/69

sequences are identical. Interestingly, EPEC BipA, unlike its counterparts in *E. coli* K-12 and *Salmonella* undergoes detectable tyrosine phosphorylation, although the importance of this is unclear. It is possible that BipA has different targets in different bacterial strains. Attempts to complement the motility defect in *E. coli* K-12 (this study) and in *Salmonella* Typhimurium and *Salmonella* Enteritidis (A. White and N. Kinsella, unpublished observations) failed, suggesting that the level of BipA must be tightly regulated for controlling flagella mediated cell motility.

Motility is an important virulence attribute for many gastrointestinal pathogens, because it allows access to optimal colonization sites (Ottemann and Miller, 1997). The negative regulation of flagellar mediated cell motility in EPEC *bipA*$^-$ mutants underscores the involvement of BipA in virulence-related processes. This role is explored more fully in later chapters.

CHAPTER 5

IDENTIFICATION OF COMPONENTS OF THE LEE THAT ARE REGULATED BY BipA

CHAPTER 5: IDENTIFICATION OF COMPONENTS OF THE LEE THAT ARE REGULATED BY BipA

5.1 Introduction

The capacity for EPEC to cause disease is linked to their ability to adhere tightly to the epithelium of the small intestine inducing A/E lesions, in which microvilli are replaced by microfilamentous structures protruding from the cell surface. Producing a pedestal to which the bacteria become intimately attached (Moon *et al.*, 1983; Knutton *et al.*, 1987a). All genes specifically necessary for the formation of the A/E lesion are encoded on the 35 kb chromosomal pathogenicity island named the LEE, for locus of enterocyte effacement (McDaniel *et al.*, 1995; McDaniel and Kaper 1997) (See also Chapter 1).

Localised adherence of EPEC to host cell epithelium is followed by a number of signal transduction events both within the bacteria and the host cell. A type III secretion system, also encoded on the LEE, translocates a number of LEE encoded proteins including EspA, EspB and EspD from the bacterial cytoplasm into the external environment. Mutation of the secreted proteins EspA, EspB, EspD and Tir prevents A/E lesion formation in epithelial cell culture models (Nataro and Kaper, 1998). Several LEE encoded proteins are required for full virulence of EPEC in human volunteer studies, including the secreted protein EspB (Tacket *et al.*, 2000). EspA is involved in forming a filamentous organelle (Knutton *et al.*, 1998), that is transiently expressed on the surface of the bacteria and that probably forms a translocon through which other secreted proteins such as EspB may be translocated into the host. EspB is translocated to the host in an EspA dependent manner, where it adopts a location in the host cell membrane. Knutton *et al.* (1998) and Wolff *et al.* (1998), propose that EspB and EspD form a pore in the host cell through which other proteins may be translocated. In support of this, EspB and EspD have homology to pore forming molecules of *Yersinia*, YopD and YopB respectively (Wachter *et al.*,

1999), and EPEC exhibit a type III secretion-dependent contact-mediated haemolytic activity (Warawa *et al.*, 1999).

Following signal transduction, intimate attachment of the bacteria to the host cell, is mediated by an interaction between the bacterial outer membrane protein intimin and the bacterially produced and secreted receptor Tir (Kenny *et al.*, 1997a; Kenny, 1999). The latter is translocated to the host, where it undergoes a number of tyrosine and probably serine and threonine phosphorylation events before assembly into the plasma membrane to serve as the receptor for intimate attachment (Kenny *et al.*, 1997a).

The known properties of BipA, which include its phosphorylation on tyrosine and its GTPase activity, are consistent with a role in signal transduction. We therefore sought to further determine its mode of action in EPEC pathogenesis using the better-characterised prototypical strain E2348/69 and its derivatives, rather than the ill-defined MAR001 strain. This chapter describes initial experiments to investigate the involvement of BipA on EPEC directed pathogenesis, and specifically in the production of the characteristic A/E lesion.

5.2 BipA is necessary for EPEC-directed cytoskeletal rearrangements in host cells

To determine the role of the protein encoded by *bipA* in A/E lesion formation in E2348/69 and JPN15, null mutants were produced by allelic exchange as previously described in section 2.2.19 of Chapter 2. For complementation studies the *bipA* gene of E2348/69 was amplified by PCR and inserted into the cloning vector pT7T318U creating constructs pAJG4 (3' truncated *bipA*) and pAJG9 (full length *bipA*), in which *bipA* was expressed from its native promoter(s). We examined the effect of BipA depletion and over-expression in E2348/69 on the formation of actin rich pedestals in host cells. HeLa cells were infected for 6 hours with E2348/69, AG2 or AG2(pAJG4) and then examined by scanning electron and fluorescence microscopy. The parental strain triggered the destruction of microvilli and induced the accumulation of actin beneath adherent bacteria as expected. Under identical conditions, AG2 failed to trigger cytoskeletal rearrangements although some bacteria adhered to the host cells. Infection of HeLa cells with the *bipA* null mutant bacteria over-expressing BipA as a result of the presence of *bipA* on a high copy number plasmid, restored actin accumulation, but also aided the production of numerous EPEC associated pedestals (Figures 5.1 and 5.2). These results extend those of Farris *et al.* (1998), to the prototype EPEC strain E2348/69, and indicate that BipA is necessary for the EPEC directed reorganization of the cytoskeleton in host cells.

5.3 BipA positively regulates the expression of EspA, EspB, and EspD

Having identified that *bipA*$^-$ mutants adhere to cultured epithelial cells but fail to trigger the characteristic cytoskeletal rearrangements found in cells infected with wild-type EPEC, we sought to further elucidate the level of control causing this phenotype. Therefore we next examined the ability of *bipA*$^-$ strains to secrete the effector proteins EspA, EspB and EspD.

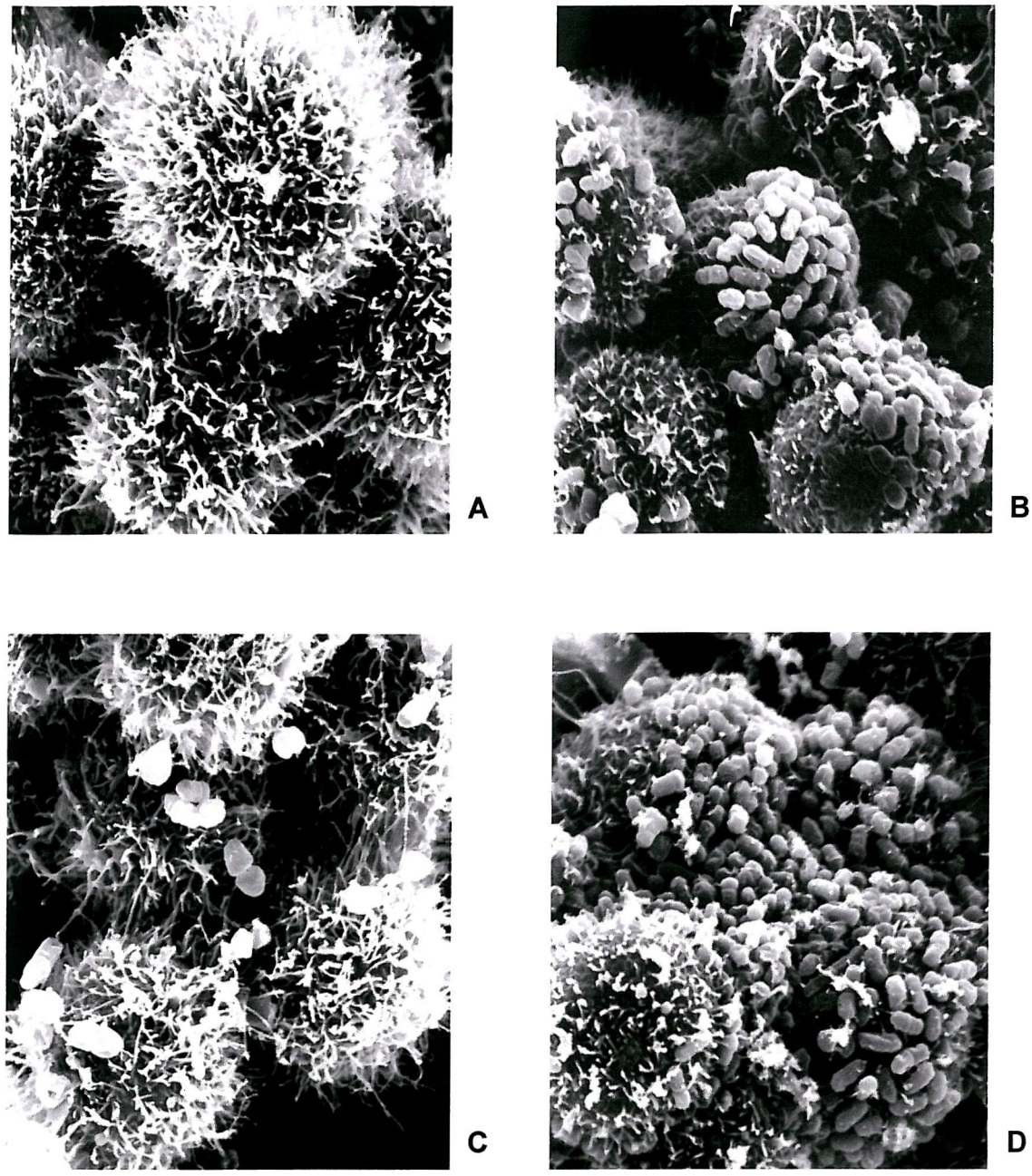


Figure 5.1. An E2348/69 *bipA*$^{-}$ strain fails to trigger host cell cytoskeletal rearrangements characteristic of wild-type EPEC. Infected cells were fixed and examined by scanning electron microscopy. The figure shows uninfected HeLa cells (A), HeLa cells infected with E2348/69 (B), AG2 (C) and AG2(pAJG4) (D). Experiments and photos kindly produced by Dr Michele Farris.

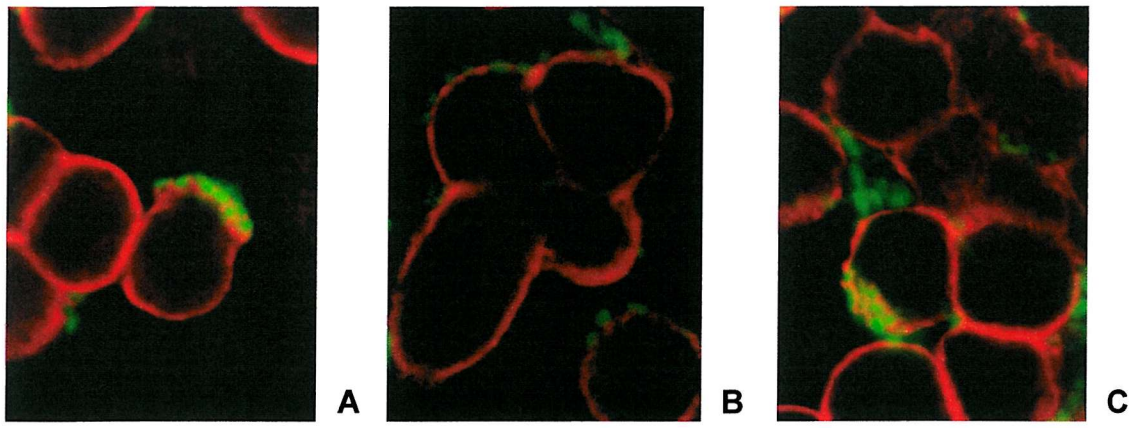


Figure 5.2. An E2348/69 *bipA*$^{-}$ strain fails to trigger host cell cytoskeletal rearrangements characteristic of wild-type EPEC. Infected cells were labelled with rhodamine-conjugated phalloidin before analysis by confocal microscopy. EPEC cells were first tagged with Green fluorescent protein (GFP) by the introduction of an appropriate GFP-expressing plasmid. The figure shows HeLa cells infected with E2348/69 (A), AG2 (B) and AG2(pAJG4) (C). Experiments and photos kindly produced by Dr Michele Farris.

Proteins secreted into the supernatant from bacteria grown in the presence of HeLa cells were harvested as previously detailed. The proteins were separated by PAGE, transferred to nitrocellulose and probed with anti-EspA, anti-EspB and anti-EspD antibodies. Control samples from DH5α grown in the presence of HeLa and from the supernatant of uninfected HeLa cells were also probed (Figure 5.3). The results indicate that a mutation in the *bipA* gene almost completely abolishes secretion of EspA, EspB and EspD into the culture supernatant. The observed defect was due to mutation of the *bipA* gene as the wild-type protein profile was restored when the *bipA* strain was transformed with pAJG4, which encodes a cloned copy of the *bipA* gene. Average densitometry data of immunoblots reacted with EspA, EspB and EspD (n=3 for each experiment) showed a 64, 70 and 63% complementation in secretion, in the *bipA*$^-$ strain containing pAJG4. Complementation was as a result of adding *bipA* as AG2, containing just the cloning vector pT7T318U, exhibited a phenotype that was the same as AG2 (data not shown). The antisera against EspA, EspB and EspD did not cross react with anything in the control samples, as expected. Although it was difficult to detect the proteins in samples from JPN15 and its derivatives, it was clear that their expression was reduced in AG4 compared to the wild-type, prolonged exposure was needed to detect any EspB in the JPN15 strains (Figure 5.4). The band migrating around 30 kDa in the wild-type and complemented strains may be a breakdown product of EspB, its level is greatly reduced in the *bipA*$^-$ strain. Collectively these results suggest that BipA is necessary for the secretion of effector proteins EspA, EspB, and EspD.

5.3.1 BipA positively regulates the intracellular expression of EspA

A possible explanation for the observed decrease in the level of the effector proteins secreted by the *bipA*$^-$ strains was down regulation of the type III secretion system encoded by the LEE or, alternatively, a reduction in their biosynthesis. In the absence of antisera specific to components of the type III secretion system, we sought to address this question by measuring the intracellular expression levels of the Esp proteins. Unadhered mid-log phase bacteria grown in the presence of HeLa cells for

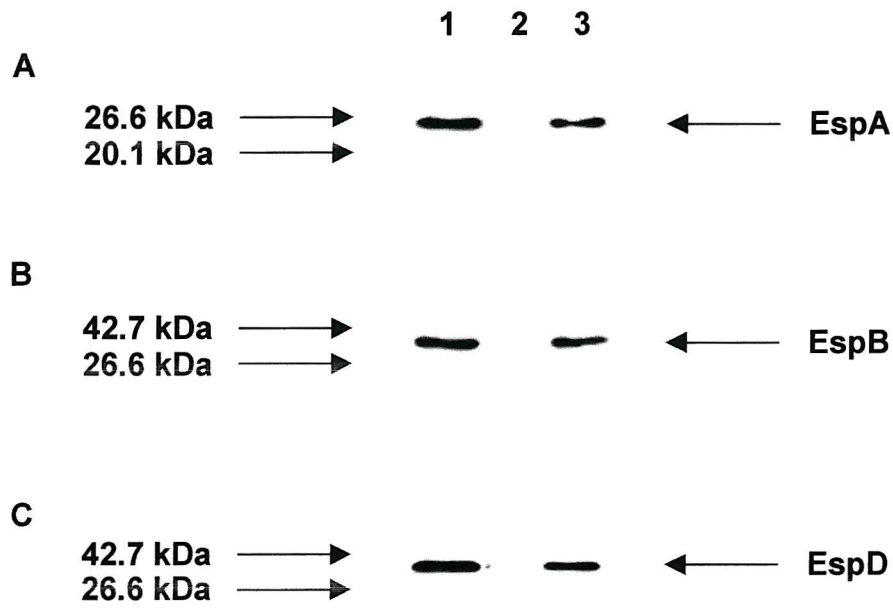


Figure 5.3. Role of EPEC BipA in Esp protein secretion. Comparison of secreted protein profiles from supernatants of wild-type, *bipA*⁻ null mutant and complemented E2348/69 strains. Bacteria were grown in DMEM to an OD_{600} of 0.3-0.4, then grown in DMEM in the presence of HeLa cell monolayers for a further 1 hour. After removal of bacteria by centrifugation, supernatant proteins were precipitated with 10% trichloroacetic acid, separated by PAGE, transferred onto nitrocellulose and probed with antisera specific to either EspA (A), EspB (B) or EspD (C). Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG4).

90 minutes were pelleted and whole cell extracts prepared as previously detailed. Protein samples from E2348/69 and JPN15 and their BipA mutants along with the control DH5α cells, grown in DMEM in the presence of HeLa cells, were separated by PAGE, transferred to nitrocellulose and then probed with anti-EspA antibodies. Although the background due to non-specific antibody binding was higher, the results indicate that the intracellular level of EspA is dramatically decreased in the BipA null mutant relative to the wild type E2348/69 strain. This decrease was complemented when BipA was expressed in *trans* (Figure 5.5). Similarly, the antisera detected EspA-specific bands in JPN15 and complemented null mutant but not in the null mutant. However, the level of EspA detected was lower than that found in the E2348/69 strain even though equal amounts of protein were loaded. These results suggest that it is the biosynthesis of EspA, rather than its secretion, that requires the BipA GTPase. Although, these studies do not preclude the possibility that BipA may be involved in regulating the intracellular degradation of EspA, either at the mRNA or protein level.

5.4 BipA positively regulates Tir expression

The importance of the translocated intimin receptor (Tir) in mediating intimate adherence of the bacterium to the host cell is well documented (Kenny *et al.*, 1997a; Kenny, 1999). Since Tir is secreted through the type III secretion system and also encoded on the LEE, by a gene in a different operon to those for the Esps, it was of interest to determine if the levels of this protein were also decreased in the *bipA*$^-$ background. EPEC E2348/69, JPN15 and the BipA mutants were grown in DMEM in the presence of HeLa cells. Whole cell extracts from unadhered bacteria were separated by PAGE, transferred to nitrocellulose and then probed with anti-Tir antibodies. Immunoblots identified a significant decrease in the biosynthesis of Tir in both AG2 and AG4, levels of Tir expression being much lower in the JPN15 strain compared to the E2348/69 strain. As expected the expression of Tir was restored to near wild-type levels when the *bipA*$^-$ strain was transformed with pAJG4, which contains a cloned copy of the *bipA* gene (Figure 5.6). These observations suggest that

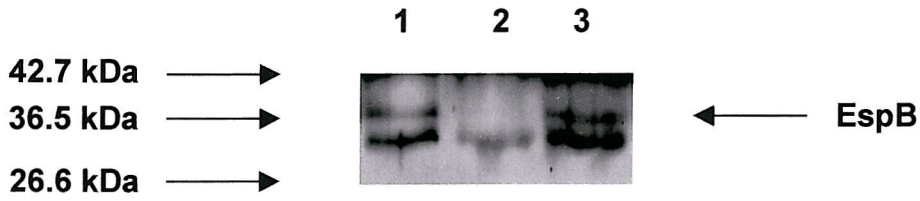


Figure 5.4. Role of BipA in Esp secretion in JPN15 strains. Overexposure of part of a blot from Figure 5.3 (B) showing comparison of secreted protein profiles from supernatants of wild-type, *bipA*⁻ mutant and transcomplemented JPN15 strains. Lane 1, JPN15; lane 2, AG4; lane 3, AG4(pAJG4).

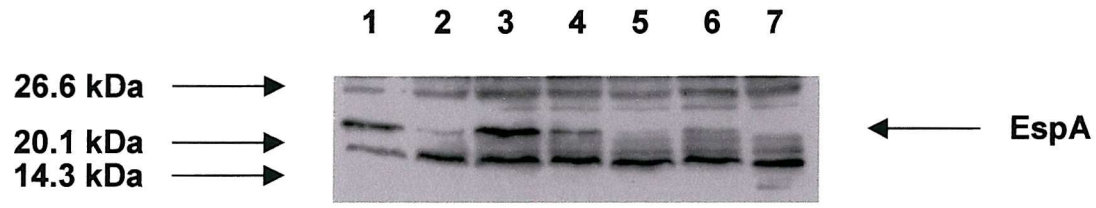


Figure 5.5. Effect of BipA on EPEC biosynthesis of EspA. Immunoblot of bacterial whole cell extracts of wild-type, *bipA*⁻ null mutant and complemented E2348/69 and JPN15 strains. Bacteria were grown in DMEM to an OD_{600} of 0.3-0.4, then grown in DMEM in the presence of HeLa cell monolayers for a further 1 hour. After which time unadhered bacteria were removed, pelletted by centrifugation and whole cell extracts prepared. Protein samples were separated by PAGE, transferred onto nitrocellulose, and probed with antisera specific to EspA. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG4); lane 4, JPN15; lane 5, AG4; lane 6, AG4(pAJG4), lane 7, DH5α.

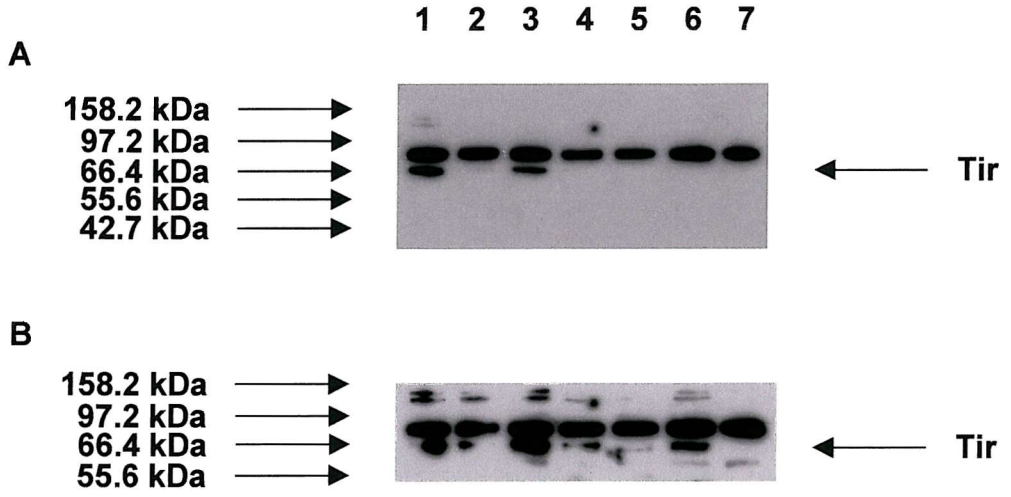


Figure 5.6. Immunoblot detection of Tir in whole cell extracts of mid-log phase bacteria. HeLa cell monolayers were infected with wild-type, *bipA*⁻ null mutant and complemented E2348/69 and JPN15 strains. Bacteria were grown in DMEM to an OD_{600} of 0.3-0.4, then grown in DMEM in the presence of HeLa cell monolayers for a further 1 hour. After incubation, unadhered bacteria were removed, pelletted by centrifugation and whole cell extracts prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to Tir. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Figure (A) shows an immunoblot of bacterial whole cell extracts of wild-type, *bipA*⁻ null mutant and complemented E2348/69 and JPN15 strains probed with antisera specific to Tir. An overexposed blot to show Tir expression profiles in JPN15 and the mutant strains is shown in (B). Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG4); lane 4, JPN15; lane 5, AG4; lane 6, AG4(pAJG4), lane 7, DH5α.

BipA is necessary for the biosynthesis of the bacterial translocated intimin receptor (Tir).

5.5 BipA positively regulates intimin expression

The bacterial outer membrane protein intimin, which is not secreted through the type III secretion system but targeted to the bacterial cell surface, is crucial for AE lesion formation (Gomez-Duarte and Kaper, 1995). Since BipA was required for the expression of LEE secreted effector proteins, it was also of interest to determine whether BipA was involved in the expression of other components of the LEE. Tir and intimin can be transcribed from the same operon. Therefore, depending on the regulatory mechanism of BipA it may be expected that intimin levels would be reduced in the *bipA*⁻ mutant strain in keeping with the Tir results; we sought to confirm this. EPEC E2348/69 and the BipA mutants were grown in DMEM in the absence of HeLa cells, bacteria were harvested and whole cell extracts prepared as previously detailed. Bacterial whole cell extracts were separated by PAGE, transferred to nitrocellulose and probed with anti-intimin antibodies. Immunoblotting of bacterial whole cell extracts demonstrated that intimin levels are markedly reduced in the absence of BipA in E2348/69 (Figure 5.7). Additionally, the level of intimin was partially restored in the transcomplemented strain but not quite to the levels found in the wild-type strain. Average densitometry data of immunoblots reacted with intimin (n=3 for each experiment) showed that the *bipA*⁻ strain expressed intimin at 13% that of the wild-type, while the introduction of pAJG4 into the *bipA*⁻ strain complemented expression to 90% that of the wild-type. These results extend the observation that BipA is necessary for the expression of a number of LEE encoded virulence factors.

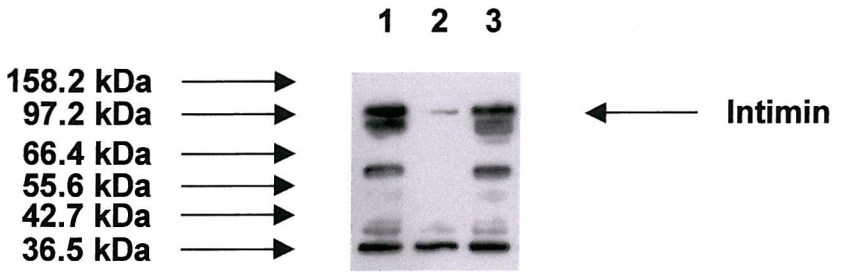


Figure 5.7. Role of BipA in the expression of the bacterial outer membrane protein intimin. Immunoblot of bacterial whole cell extracts of wild-type, *bipA*⁻ null mutant and complemented E2348/69 strains. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7; bacteria were pelletted by centrifugation and whole cell extracts prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to intimin. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG4).

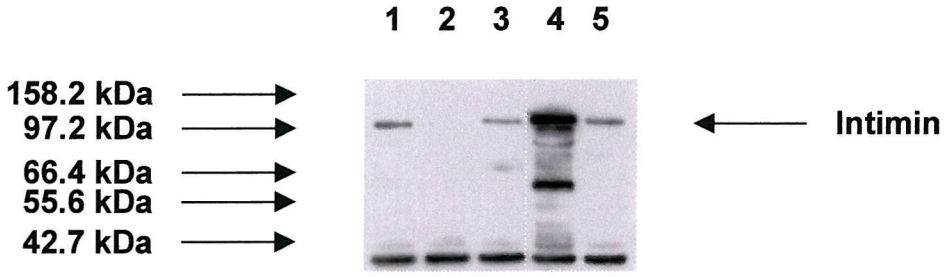


Figure 5.8. Role of BipA in the expression of intimin in the plasmid cured EPEC strain JPN15. Immunoblot of bacterial whole cell extracts of wild-type, *bipA*⁻ null mutant and complemented E2348/69 and JPN15 strains. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7; bacteria were pelletted by centrifugation and whole cell extracts prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to intimin. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, JPN15; lane 2, AG4; lane 3, AG4(pAJG4); lane 4, E2348/69; lane 5, AG2.

5.5.1 BipA positively regulates the expression of intimin in an EAF cured strain

Previous work has shown that the expression of the *eae* gene (encoding intimin) is under the control of the Per regulon (possibly with an additional factor) on the EAF plasmid. Therefore, the effect of BipA on the expression of intimin in JPN15, which lacks the EAF plasmid, was investigated to ascertain if BipA operates by interacting with an EAF-specific component. Wild-type JPN15 and the BipA mutant derivatives were grown in DMEM in the absence of HeLa cells. Whole cell extracts were prepared, separated by PAGE, transferred to nitrocellulose and probed with anti-intimin antibodies. Immunoblots showed decreased expression of intimin in the BipA null mutant with respect to the wild-type strain. Expressing *bipA in trans* complemented this decrease in the *bipA*$^-$ mutant, but not to wild-type levels (Figure 5.8). Collectively these results, in addition to those for the expression of Tir and the Esps, imply that BipA mediated control of the LEE is not through Per, since JPN15 lacks the EAF plasmid.

5.5.2 BipA positively regulates the expression of intimin in sub-optimal conditions

The expression of intimin is under the control of the *per* locus, and is regulated by growth phase and environmental conditions. Maximal expression occurs in conditions similar to those in the intestine, during growth in DMEM to late-exponential/early-stationary phase at 37°C, 5% CO_2, (Gomez-Duarte and Kaper, 1995; Knutton *et al.*, 1997; Adu-Bobie *et al.*, 1998). Previous studies have shown that the Esps are not secreted when EPEC are grown in the nutrient rich medium LB (Kenny and Finlay, 1995). Moreover, the bacterial outer membrane protein intimin is expressed when EPEC are grown in LB, but at reduced levels to those seen in optimal growth conditions (Knutton *et al.*, 1997). We therefore compared the difference in intimin expression between *bipA*$^+$ and *bipA*$^-$ strains in sub-optimal conditions for expression. Bacteria were grown in broth-culture (LB) as well as tissue culture

medium (DMEM). Figure 5.9, highlights decreased intimin expression in the E2348/69 *bipA*⁻ mutant with respect to the wild-type, in strains grown in LB medium. The observed decrease in intimin expression in the *bipA*⁻ strains grown in optimal conditions was extended to strains grown in LB, indicating that the effect of BipA is not culture dependent. Interestingly the immunoblots do not show a significant decrease in expression of intimin in the wild-type strain grown in LB compared to the same strain grown in DMEM.

5.5.3 Truncated intimin polypeptides are reduced in a BipA null mutant

Knutton *et al.* (1997), demonstrated a uniform distribution of intimin over the surface of an E2348/69 bacterium in contact with HEp-2 cells for 3 hours, except at the site of bacterial attachment (probably due to inaccessibility of the site for staining). In contrast, after 6 hours incubation, intimin is absent from these cells, while JPN15 still expresses intimin over its surface. Interestingly the down regulation of intimin was shown not to occur in DMEM in the absence of host cells, or following adhesion to fixed cells and is proposed to require host cell factors (Knutton *et al.*, 1997).

Since intimin is an outer membrane protein, the decrease in expression could be as a result of its degradation. Inspection of immunoblots probed with antisera specific for intimin showed a reduced number of putative breakdown products of intimin in samples from the BipA null mutant (Figure 5.10, also Figures 5.7, 5.8 and 5.9). These bands, in particular the band around 57 kDa, were apparently absent from JPN15 derivatives, and from the E2348/69 strains grown in LB medium (Figures 5.8 and 5.9). Also, a band of around 38 kDa appeared to be growth culture regulated, and was expressed more optimally in nutrient rich media (Figure 5.9). Intimin appears not to be degraded in conditions that do not favour maximal expression of intimin or other virulence associated proteins (compare lanes 1 and 3 in Figure 5.9). While we cannot rule out the possibility that this difference in truncated intimin polypeptides is due to differences in the expression levels of intimin, a further explanation may be that BipA positively regulates the degradation of intimin, either by controlling the

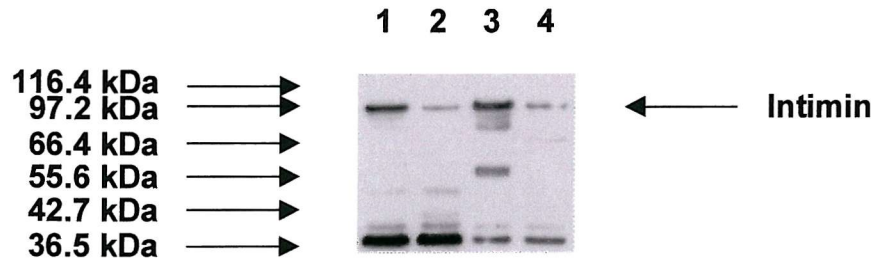


Figure 5.9. Effect of BipA on intimin expression under sub-optimal growth conditions. Immunoblot of bacterial whole cell extracts of wild-type and *bipA*⁻ null mutant E2348/69 strains. Bacteria were grown in LB (lanes 1 + 2), and DMEM (lanes 3 + 4) to an OD_{600} of 0.6-0.7; bacteria were pelletted by centrifugation and whole cell extracts prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to intimin. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, E2348/69; lane 4, AG2.

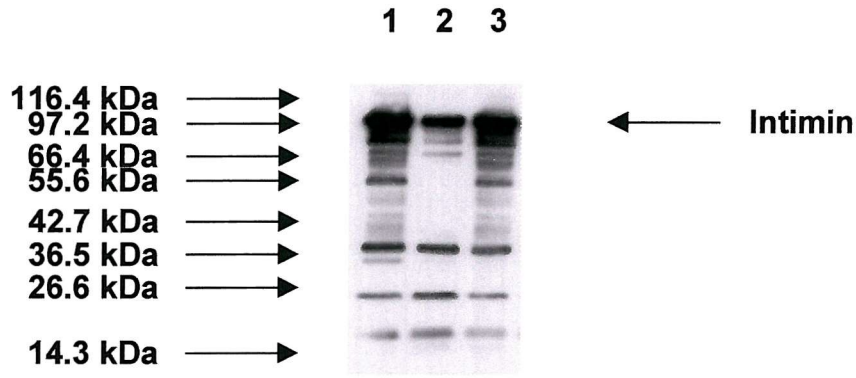


Figure 5.10. Role of EPEC BipA in the degradation of intimin. Immunoblot of bacterial whole cell extracts of wild-type and *bipA*⁻ null mutant E2348/69 strains. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7; bacteria were pelletted by centrifugation and whole cell extracts prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to intimin. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG4).

expression of a protease or by regulating the destruction of the mRNA for intimin. Since the truncated intimin polypeptides are absent in strains grown in LB compared to the same strains grown in DMEM it is tempting to speculate that if intimin is degraded by a protease, this protease may be growth- or culture-medium dependent.

5.6 BipA positively controls the expression of EspC

EPEC secretes at least seven proteins into the extracellular environment when grown in tissue culture media under optimal conditions (Kenny and Finlay, 1995; McNamara and Donnenberg, 1998; Kenny and Jepson, 2000). Secretion of one of these proteins, EspC, is co-regulated with the Esp proteins even though it uses an auto-transporter mechanism for export rather than the LEE secretion apparatus (Kenny and Finlay, 1995). EspC belongs to the IgA protease family (Stein *et al.*, 1996a), and its secretion is culture dependent. EspC has been shown not to be necessary for mediating EPEC induced signal transduction in HeLa cells, or involved in the adherence to tissue culture cells. In view of the effect of BipA on the secretion of EspA, EspB and EspD, we sought to determine whether BipA was also involved in the secretion of EspC, an EPEC secreted protein not encoded on the LEE. Recent results tentatively suggest the involvement of EspC in EPEC pathogenesis. In view of its likely proteolytic properties and cell surface location, we speculate that it may be involved in proteolytic degradation of intimin. To establish whether BipA affected the expression of EspC, proteins secreted into the supernatant from E2348/69 and AG2 grown in DMEM were harvested as previously detailed, the supernatant proteins were precipitated with trichloroacetic acid, separated by PAGE and visualised by silver staining. Coomassie stained SDS gels of secreted proteins prepared from wild-type and AG2 indicate the absence of a band migrating around 110 kDa, the molecular weight of EspC. N-terminal sequencing of the 110 kDa species confirmed the identity of the protein as EspC (Figure 5.11). These observations suggest that BipA is necessary for the expression of virulence factors not encoded on the LEE, and do not preclude the possibility that EspC may be involved in the proteolytic degradation of intimin in a BipA dependent manner.

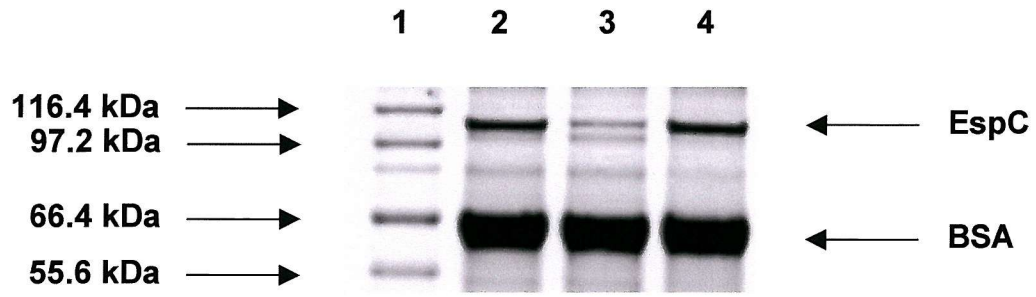


Figure 5.11. Role of EPEC BipA in EspC protein secretion. Comparison of secreted protein profiles from supernatants of wild-type, *bipA*⁻ null mutant and complemented E2348/69 strains. The *bipA*⁻ mutant does not secrete a band migrating at 110 kDa the molecular weight of EspC. N-terminal sequencing of the 110 kDa species (AQLNIDNVW) confirmed the identity of the protein as EspC. Bacteria were grown in DMEM to an OD_{600} of 0.6, after removal of bacteria by centrifugation, supernatant proteins were precipitated with 10% trichloroacetic acid, separated by PAGE on a 4-12% BisTris NuPAGE gel and visualised by coomassie blue staining as previously detailed. Lane 1, NEB Broad Range Molecular Weight Marker; lane 2, E2348/69; lane 3, AG2; lane 4, AG2(pAJG9).

5.7 Discussion

The studies described in this chapter indicate that BipA is required for the LEE-directed rearrangement of actin in host cells infected with EPEC – strain E2348/69. Further investigation showed that BipA positively regulates the secreted effector proteins EspA, EspB, EspD and Tir as well as a protein EspC encoded outside of the LEE. Finally, the studies suggest that BipA also regulates intimin and possibly its degradation.

5.7.1 BipA and actin rearrangements in EPEC-infected host cells

Bacteria producing A/E lesions are identified by the FAS test (Knutton *et al.*, 1989), in which FITC-conjugated phalloidin is used to identify the filamentous actin that accumulates beneath the adherent bacteria. Previous FAS studies by Farris *et al.* (1998), identified that EPEC MAR001 *bipA*⁻ mutants adhere to cultured epithelial cells, but fail to trigger the characteristic cytoskeletal rearrangements found in cells infected with wild-type EPEC. Moreover, increased expression of BipA enhanced actin remodelling, resulting in the hyperformation of pseudopods. Confocal microscopy studies of HeLa cells infected with EPEC E2348/69 and the BipA mutants further extends the observation of the inability of E2348/69 *bipA*⁻ mutants to trigger the actin rearrangements of host cells observed with wild-type infection. It is not yet possible to confirm the observed pseudopods seen with the overexpression of BipA in the MAR001 *bipA*⁻ mutant strains, in the complemented E2348/69 mutants. Moreover, introduction of multiple copies of *bipA* (on plasmid pAJG4) into the null mutant strain did not lead to zones of condensed actin that were larger than the zones seen in host cells infected with the wild-type strain. Thus, hyperformation of EPEC-induced pseudopods and increased actin condensation may be a phenomenon specific to MAR001 strains.

5.7.2 Positive regulation of secreted effector proteins by BipA

The co-ordinated and controlled expression and secretion of the Esps is important for mediating signal transduction in host cells and A/E lesion formation. The secretion of these Esps is induced when bacteria are grown in tissue culture medium under conditions that mimic the conditions of the gastrointestinal tract (Kenny and Finlay, 1995; Kenny *et al.*, 1997c). Studies with *esp*$^-$ mutant strains underscore the importance of the Esps in activating epithelial cell signal transduction and causing cytoskeletal rearrangements (Donnenberg *et al.*, 1993a; Foubister *et al.*, 1994a; Lai *et al.*, 1997). Therefore the inability to express/secrete Esps is associated with the inability to form A/E lesions, a phenotype characteristic of the EPEC *bipA*$^-$ mutant strains. Analysis of secreted proteins from EPEC *bipA*$^-$ mutant strains showed that BipA positively regulates the expression of EspA, EspB and EspD.

The secreted effector proteins EspA, EspB and EspD are encoded by genes that are transcriptionally coupled on the operon *LEE4*, and transcribed as a single polycistronic mRNA (Figure 5.12) (Mellies *et al.*, 1999).

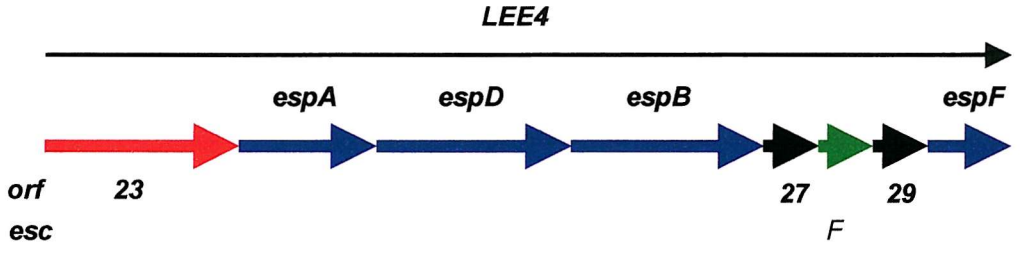


Figure 5.12. Organisation of *espA*, *espB* and *espD* in the *LEE4* operon of the LEE. The arrows indicate the direction of transcription of each open reading frame. Red indicates an open reading frame with database homologue, while blue indicates Esps. Black and green indicate open reading frames with no database homologue and Esc respectively.

In view of the operon structure of *LEE4*, it is possible that BipA controls the genes up- and down-stream of *espA*, *espD* and *espB*.

Even though the *esp*s are transcriptionally coupled on *LEE4*, the effect of *bipA* depletion was monitored for EspA, EspB and EspD due to the availability of antisera for these proteins, and the unknown level of regulation exerted by BipA. It was not known whether BipA might operate at the level of translation on individual components of the LEE. Throughout the course of these studies, some experiments were conducted in the presence of HeLa cells. Previous studies have highlighted that there is increased secretion of effector proteins such as EspB into culture medium in the presence of eukaryotic cells (Wolff *et al.*, 1998). It should be noted that studies were also conducted in the absence of eukaryotic cells, and the phenotypes were comparable.

The translocation and secretion of Tir and Esps require a type III protein secretion system. While it remains to be determined if BipA regulates the secretory apparatus, intracellular production of EspA is reduced in the BipA null mutant (see Figure 5.5). This suggests that it is the synthesis and not the secretion of EspA that is mediated by BipA. However, these studies do not exclude the possibility that BipA negatively regulates a factor involved in the degradation of Esp mRNA or protein. Since EspB and EspD are transcribed with EspA as a single polycistronic mRNA, intracellular levels of these proteins would also be expected to be decreased in the BipA null mutant, along with EspA. Thus BipA positively controls the expression and not the secretion of the Esps.

Results from this study also indicate that BipA positively regulates the expression of Tir and intimin. Recent studies suggest that *tir*, *cesT* and *eae* are on the same mRNA transcript, and form the operon *LEE5* (Mellies *et al.*, 1999; Abe *et al.*, 1999; Elliott *et al.*, 1999). However, a transcription start site for *eae* has been identified 130 bp upstream from the ATG start codon of *eae* by Gomez-Duarte and Kaper, (1995). Additionally, a potential transcription start site for *cesT* has been located 97 nucleotides upstream of the ATG start codon for *cesT* (Elliott *et al.*, 1999). It is proposed that the independent promoters for *cesT* and *eae* may be activated under certain environmental conditions, thus offering the possibility of a differential

regulation (Mellies *et al.*, 1999; Elliott *et al.*, 1999). Owing to the operon structure of *LEE5*, BipA may be required for the expression of the Tir chaperone CesT, however, because of the potential for fine control of expression from *LEE5*, this may not be the case. Owing to the availability of antisera specific to both Tir and Intimin, the expression of both proteins was monitored in the *bipA*$^{+}$ and *bipA*$^{-}$ backgrounds to facilitate the detection of any possible individual transcriptional regulation.

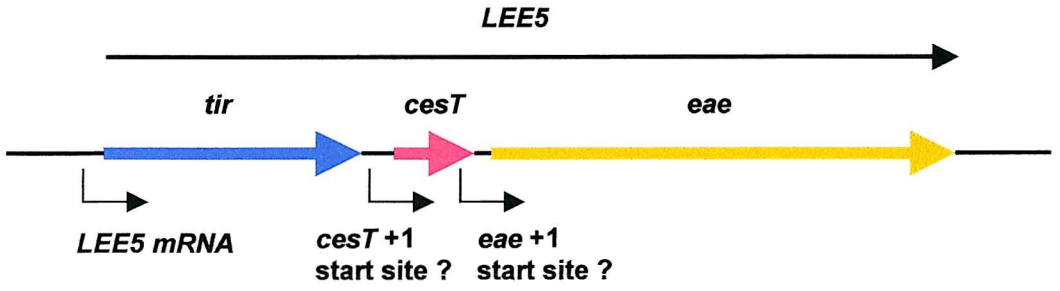


Figure 5.13. Organisation of *tir*, *cesT* and *eae* in the *LEE5* operon of the LEE. The coloured arrows indicate the direction of transcription of each open reading frame. The transcriptional start sites are shown by broken arrows underneath the arrows corresponding to the open reading frames.

5.7.3 BipA regulation of intimin expression

Knutton *et al.* (1997) have shown that the expression of intimin by EPEC is regulated by growth phase and environmental conditions. E2348/69 intimin expression is low in stationary phase cultures but induced during log phase growth, and is increased when bacteria are grown in DMEM tissue culture medium instead of LB (Knutton *et al.*, 1997; Adu-Bobie *et al.*, 1998). The positive regulation of intimin expression by BipA observed for E2348/69 in DMEM is also evident in cells grown in LB. Results from the present study indicate a modest increase in intimin expression from EPEC grown in DMEM compared to growth in LB. However, intimin breakdown increases when cells are grown in DMEM rather than in LB.

In view of the results obtained with EspA, EspB, EspD and Tir, where no significant proteolytic degradation was obtained in the presence or absence of BipA, it is likely that the protein also regulates intimin at the level of *eae* transcription. However, BipA could also affect the stability of intimin, possibly by an effect on a protease or by regulating the degradation of the mRNA transcript(s) for intimin. In this respect, there is a striking absence of truncated forms of intimin in the E2348/69 *bipA*⁻ mutant strains relative to the wild-type and transcomplemented strains (see Figures 5.7, 5.8, 5.9 and 5.10). An attractive explanation for this finding is that BipA positively regulates the EspC protease. In keeping with this hypothesis, analysis of the protein profile of culture supernatants from E2348/69 and AG2 samples showed that the latter had secreted abrogated amounts of EspC. EspC has been shown not to be necessary for mediating EPEC-induced signal transduction in HeLa epithelial cells and not to play a role in adherence or invasion of tissue culture cell (Stein *et al.*, 1996a). However, cell surface proteases are important in determining the location of other cell surface molecules involved in interactions with host cells. For example, in *Shigella* the SopA/IcsP protease restricts the localisation of proteins such as IcsA, that are involved in actin 'comet' formation to a pole of the bacterium (Egile *et al.*, 1997; Shere *et al.*, 1997). EspC may be doing something similar in EPEC, so that intimin or Tir is expressed only in certain regions of the cell surface. However, Knutton *et al.* (1997) showed that immuno-labelling with antisera against intimin revealed a uniform distribution of intimin over the bacterial surface except at the site of intimate bacterial attachment where there was no intimin labelling. They did however report a down regulation of intimin expression following A/E lesion formation, although this down regulation was shown not to occur in DMEM in the absence of cells. It was concluded that the down regulation seen following A/E lesion formation was in response to host cell factors (Knutton *et al.*, 1997). These observations do not support the involvement of BipA in regulating this process, however further investigation may be of some merit.

Interestingly truncated intimin polypeptides were not observed when E2348/69 were grown in LB, a trend that is also observable on inspection of immunoblots from

Knutton *et al.* (1997). Thus the degradation of intimin appears to be culture dependent. The secretion of EspC is also culture dependent, and it is not secreted into LB (Kenny and Finlay, 1995). Whilst truncated intimin polypeptides were not seen in the JPN15 strains, it has previously been shown that this strain secretes EspC (Stein *et al.*, 1996a).

5.7.4 BipA regulation in the absence of the Per regulator

Studies have shown that the expression of intimin is higher in E2348/69 than the plasmid cured strain JPN15 (Jerse and Kaper, 1991; Gomez-Duarte and Kaper, 1995; this study). Results from this study support the observation of decreased intimin expression in the plasmid cured strain, and also show that BipA positively regulates the expression of intimin in the EPEC plasmid cured strain JPN15. In favour of intimin being degraded by a protease, an attractive proposal could be that intimin reaches a maximum level and is then degraded. Therefore, JPN15, which expresses a lower level of intimin, may not be expected to show truncated intimin polypeptides.

Previous studies have indicated that the plasmid-encoded regulator (Per) activates the expression of the *bfp* operon located on the EAF plasmid, and operons *LEE1*, *LEE2* and *LEE3*, and modestly increases the expression of *LEE4* (Mellies *et al.*, 1999). This activation was mediated through a direct effect on the first gene transcribed from *LEE1*, known as *ler* for LEE encoded regulator (previously known as *orf1*). To exclude the possibility that the BipA regulation of Tir, Intimin and the Esps A, B and D was not mediated through Per, a *bipA* deletion was constructed in the EAF plasmid cured EPEC strain (JPN15). It was reasoned that if the JPN15 *bipA*$^-$ cells showed the same phenotypes as the E2348/69 *bipA*$^-$ cells, then BipA must be able to regulate the LEE independently of Per. The results show that BipA affects the expression of intimin, EspA, EspB, and Tir in JPN15 as well as E2348/69. Thus, BipA is unlikely to exert its effects through Per.

Since BipA affects genes in the LEE that belong to different operons, it may operate via some regulatory protein that is already known to control a number of operons on the LEE. The results described here appear to rule out Per, however, recent studies show that as well as growth conditions, and quorum sensing, Ler, IHF and H-NS are also involved in regulating LEE gene expression. The next chapter describes attempts to establish whereabouts in the regulation cascade BipA mediates its control.

CHAPTER 6

DEFINING THE REGULATORY MECHANISM USED BY BipA TO REGULATE THE LEE

CHAPTER 6: DEFINING THE REGULATORY MECHANISM USED BY BipA TO REGULATE THE LEE

6.1 Introduction

It is only in recent years that the regulatory mechanisms behind the control of virulence gene expression in EPEC, have begun to be elucidated. All of the genes necessary for the formation of the A/E lesion are encoded on the LEE, which is now believed to contain five polycistronic operons (see Figure 1.4). The protein product of the first gene in operon LEE1, Ler, positively regulates the expression of many other genes encoded in the LEE (Mellies *et al.*, 1999; Elliott *et al.*, 2000). Moreover, other studies have indicated that the DNA binding protein IHF is required for the activation of *ler* (Friedberg *et al.*, 1999), while further positive control is mediated through the presence of Per (specifically PerC) specified by the EAF plasmid (Mellies *et al.*, 1999; Bustmante *et al.*, 2001). Per is itself regulated by environmental growth conditions, and the growth phase. A quorum sensing system is also involved in the regulation of virulence gene expression (Sperandio *et al.*, 1999). However, its effect is marginal, in the conditions studied, and its mechanism of action is currently poorly defined.

The fact that at least two of the known regulators of the LEE act via Ler suggests that the latter is a key control point. Recent studies by Bustmante *et al.* (2001), suggest that Ler may mediate its effects on the control of EPEC virulence by either displacing the global regulator H-NS from binding sites in the *LEE* promoter regions, thereby relieving repression, or by sequestering H-NS and thus preventing it from negatively regulating *LEE* gene expression. Indeed, it is now proposed that the *LEE* is constitutively expressed in the absence of H-NS in a Ler independent manner. Thus Ler may not act as a positive regulator of LEE gene expression, but may operate as an antirepressor factor, preventing the negative regulatory effects of H-NS on an otherwise constitutively expressed operon system.

The present studies have demonstrated that BipA is necessary for the EPEC directed reorganization of the cytoskeleton in host cells. As described in the last chapter, BipA null mutants adhere to host cells in a reduced manner, but fail to induce the A/E lesion formation. This is probably a direct result of an inability to express the effector proteins EspA, EspB, EspD, Tir and intimin in the E2348/69 *bipA*$^-$ strain. As indicated above, and in more detail in Chapter 1, there are a number of multifactorial processes controlling the regulation of virulence gene expression in EPEC. This chapter details studies to elucidate the possible mechanism of control exerted by BipA on virulence gene expression.

6.2 A cloned copy of *per* increases the expression of LEE encoded proteins in wild-type and BipA null mutant E2348/69 and JPN15 strains

Although the regulatory mechanism of BipA is unlikely to be mediated through Per, as BipA-specific control is maintained in JPN15 which lacks the EAF plasmid, it was of interest to determine whether the introduction of pCVD450 which contains a cloned copy of the *per* operon (Gomez-Duarte and Kaper, 1995) compensates for the *bipA*⁻ defect. Accordingly, a plasmid bearing the *per* gene, pCVD450, was transformed into wild-type and BipA null mutant strains of E2348/69 and JPN15. The strains were then grown in DMEM to an OD_{600} of 0.6-0.7 and bacterial whole cell extracts were prepared as previously detailed. Immunoblots of bacterial whole cell extracts with antisera specific for either EspA or intimin demonstrated that EspA and intimin levels in the strain AG2 were restored to wild-type levels and beyond, whilst the levels of LEE encoded proteins in E2348/69(pCVD450) were markedly higher than the wild-type (Figure 6.1). This trend was also true for the JPN15 strains, in which a cloned copy of *per* increased the existing level of expression of EspA, Tir and intimin, but also restored the levels of these proteins in the JPN15 BipA null mutant (Figure 6.2). These observations indicate that the presence of the *per* operon on a high copy number plasmid can complement the *bipA*⁻ defect. Interestingly the levels of effector proteins in *bipA*⁻ strains carrying pCVD450 were restored to wild-type levels. Moreover, the levels of effector proteins in the wild-type strains carrying pCVD450 were exaggerated from wild-type expression levels.

JPN15(pCVD450) exhibited the characteristic intimin breakdown products previously detailed for the wild-type strain E2348/69 (see Figures 6.2 and 5.10). In contrast, the *bipA*⁻ derivative of JPN15(pCVD450) did not produce the breakdown products. The *bipA*⁻ derivative of E2348/69(pCVD450) also showed the breakdown products. However, the pattern of breakdown products in the E2348/69 wild-type strain carrying the plasmid appeared different to that of the plasmid minus strain, with a greater prominence of a band at around 80kDa, and weaker expression of the band around 57kDa in the strains expressing the plasmid (see Figures 6.1 and 5.10). It is

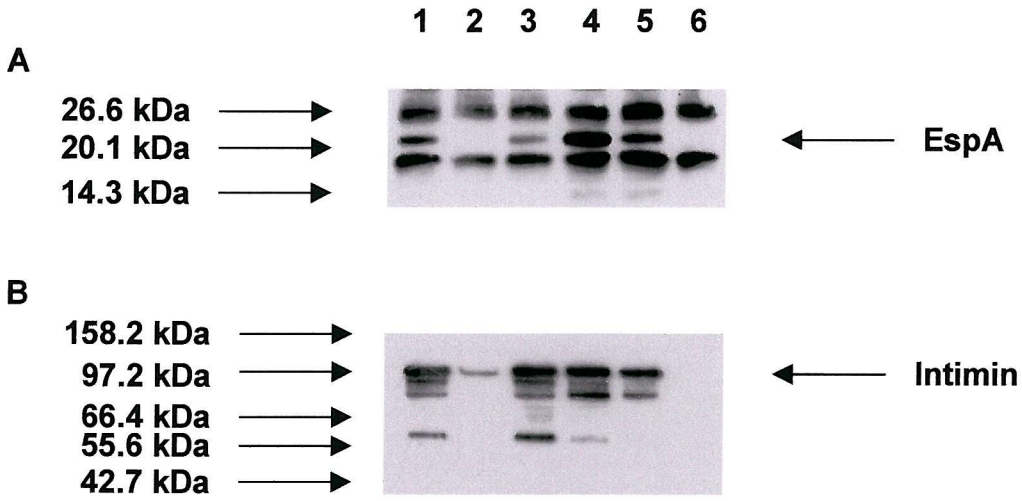


Figure 6.1. A cloned copy of *per* complements the *bipA*⁻ defect in E2348/69. Immunoblots of whole cell extracts of wild-type and *bipA*⁻ E2348/69 strains expressing pCVD450, indicating an increase in expression of both EspA and intimin in those strains expressing pCVD450. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7; whole cell extracts were prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to EspA (A) or intimin (B). Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG9); lane 4, E2348/69(pCVD450); lane 5, AG2(pCVD450); lane 6, MG1655.

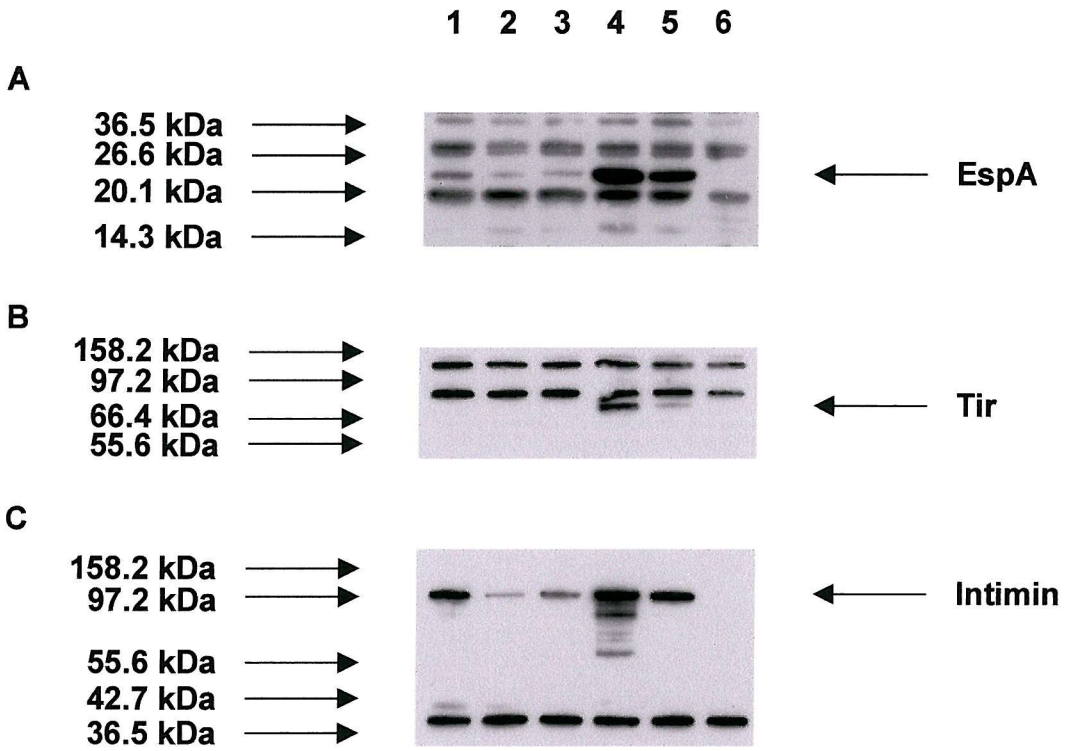


Figure 6.2. A cloned copy of *per* complements the *bipA*⁻ defect in JPN15. Immunoblots of whole cell extracts of wild-type and *bipA*⁻ JPN15 strains expressing pCVD450, indicating an increase in expression of EspA, Tir and intimin in those strains expressing pCVD450. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7; whole cell extracts were prepared, protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to EspA (A), Tir (B) or intimin (C). Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, JPN15; lane 2, AG4; lane 3, AG4(pAJG9); lane 4, JPN15(pCVD450); lane 5, AG4(pCVD450); lane 6, MG1655.

interesting to note that the level of intimin is not expressed much past wild-type levels, even when the strain expresses the plasmid pCVD450 carrying a cloned copy of *per*. A possible explanation is that once intimin levels reach a threshold level, they are degraded, possibly because of saturation of the outer membrane.

In contrast to intimin expression levels, which are not greatly increased further from wild-type expression levels in wild-type strains expressing pCVD450, the levels of EspA expressed in the E2348/69 and JPN15 expressing pCVD450 were significantly increased from wild-type expression levels.

6.3 IHF levels are the same in wild-type and BipA null mutant strains

Integration host factor (IHF) is a DNA binding protein that binds to specific sites and bends the DNA to form nucleoprotein complexes (Giladi *et al.*, 1998). The protein has been shown to be required for EPEC to elicit A/E lesions and, IHF binding at sites upstream of the *ler* promoter activates the expression of *ler*, *orf3*, *orf5* and *rorf2* directly (Nash, 1996; Rice *et al.*, 1996; Friedberg *et al.*, 1999). IHF directly activates the expression of *rorf2* and *ler*; the protein product Ler then positively regulates *LEE2*, *LEE3*, *LEE4* and *LEE5* (Friedberg *et al.*, 1999). Since present studies have demonstrated that BipA positively regulates the expression of Tir, intimin (LEE5) and Esps A, B and D (LEE4), it was of interest to determine whether the requirement for BipA was mediated through IHF.

Accordingly, E2348/69 and JPN15 wild-type, *bipA*$^-$ and transcomplemented strains were grown in the presence of HeLa cells. Bacteria that failed to adhere were harvested and whole cell extracts prepared as previously detailed. Protein samples were separated by PAGE, transferred to nitrocellulose, and then probed with anti-IHF antibodies. Immunoblotting of bacterial whole cell extracts demonstrated that IHF levels do not alter in a *bipA*$^+$ or *bipA*$^-$ background, nor do they alter between E2348/69 and the plasmid cured JPN15 strains (Figure 6.3). This result indicates that

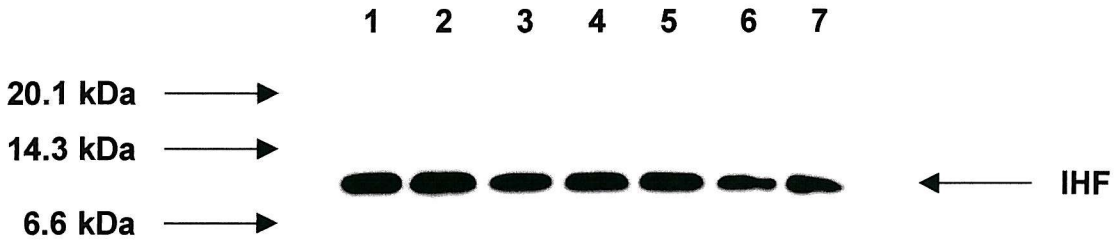


Figure 6.3. IHF protein expression levels are the same in *bipA*$^{+}$ and in *bipA*$^{-}$ EPEC. Immunoblots of whole cell extracts of wild-type and *bipA*$^{-}$ E2348/69 and JPN15 strains. Bacteria were grown in DMEM to an OD_{600} of 0.3-0.4, and then grown in DMEM in the presence of HeLa cell monolayers for a further 1 hour. After which time unadhered bacteria were removed and pelletted by centrifugation, and whole cell extracts were prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to IHF. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG4); lane 4, JPN15; lane 5, AG4; lane 6, AG4(pAJG4); lane 7, purified IHF.

BipA is not required for IHF expression, and that the necessity for BipA in virulence gene expression from the LEE is not mediated through IHF.

6.4 BipA does not appear to mediate its positive regulatory effect on LEE encoded genes through quorum sensing

The process of quorum sensing allows bacteria to sense the population they are in, via cell-to-cell signalling mediated by the production of autoinducers (Sperandio *et al.*, 1999). Recent studies suggest that *LEE1* and *LEE2* are directly regulated by quorum sensing in EPEC through the product of the *luxS* gene, while *LEE3*, *LEE4* and *LEE5* are indirectly activated by quorum sensing by Ler, the product of the first gene of LEE1 (Sperandio *et al.*, 1999).

To ascertain whether BipA acts through quorum sensing, the levels of *luxS* transcripts were measured in the E2348/69 *bipA*$^+$ and *bipA*$^-$ backgrounds, using a primer that was complementary to the start region of *luxS*. Wild-type, *bipA*$^-$, and transcomplemented strains grown overnight in LB, were diluted into prewarmed DMEM and grown to an OD_{600} 0.6-0.7. Bacteria were then harvested and RNA extracted as previously detailed. No significant differences in the transcript levels of *luxS* were seen in a *bipA*$^+$ or *bipA*$^-$ background (Figure 6.4). This result tentatively suggests that *bipA* does not control the transcription of *luxS*. However, as previously detailed, BipA is anticipated to mediate control post-transcriptionally. Thus this result does not preclude an effect on *luxS* at the post-transcriptional level.

This possibility was addressed by examining whether media that had been preconditioned by the growth of bacteria capable of producing AI-2 (maximal induction of *LEE1* expression occurs when bacteria are grown in DMEM preconditioned by the growth of bacteria able to secrete the autoinducer - AI-2 (Sperandio *et al.*, 1999)) stimulated the expression of LEE encoded factors in the E2348/69 *bipA*$^-$ strain. We tested for possible quorum sensing effects between wild-type, *bipA*$^-$ and transcomplemented strains grown in media preconditioned by

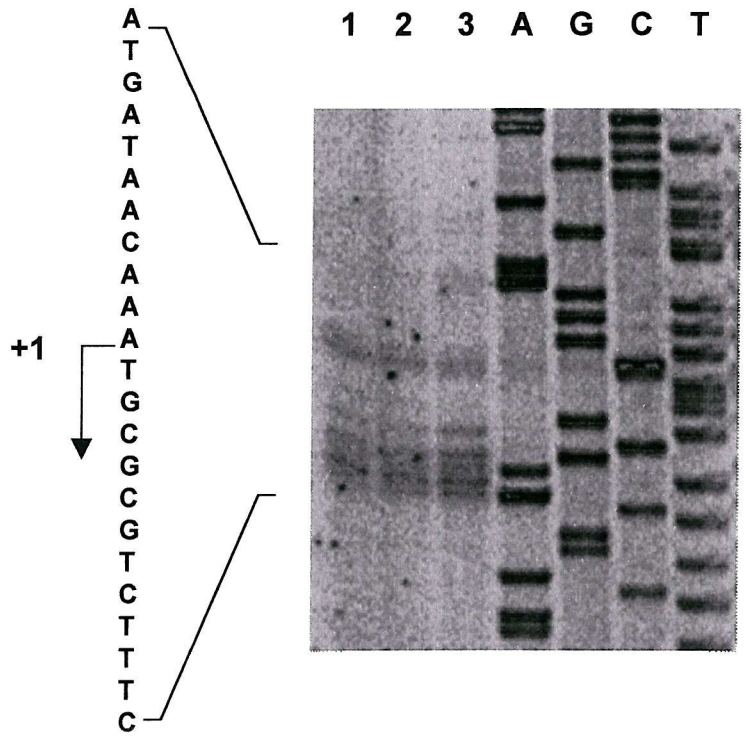


Figure 6.4. The transcript levels of *luxS* are the same in EPEC *bipA*$^+$ and *bipA*$^-$ strains. Primer extension analysis was performed with RNA extracted from E2348/69 wild-type, *bipA*$^-$ and transcomplemented strains, using a procedure as previously described, and a primer to the start region of *luxS* (AJG134). As a reference, an M13mp18 DNA sequencing ladder is shown in lanes A, G, C, T. The transcript map indicates no significant difference in *luxS* transcript between the E2348/69 wild-type and *bipA*$^-$ mutant strains. The transcription start point (+1) of *luxS* refers to a position 81 nucleotides upstream of the start codon, which is in accordance with the predicted promoter region for *luxS* from *E. coli* K-12 MG1655, database accession number ECAE353. Lane 1, E2348/69; lane 2, AG2(pAJG9); lane 3, AG2.

growing *E. coli* MG1655 in DMEM to an OD_{600} of 1.2, then preparing the culture supernatant as previously detailed. Pre-conditioned medium was diluted 50:50 with fresh pre-warmed DMEM, and bacteria were grown in DMEM or pre-conditioned medium to an OD_{600} of 0.5-0.7. Whole cell extracts were prepared and resolved by PAGE before transfer to nitrocellulose and blotting with antibodies specific for either EspA or Tir (Figure 6.5). The results indicate that growth of *bipA*⁻ EPEC in media preconditioned by growth of a bacterial strain proficient in producing AI-2, does not allow a *bipA*⁻ strain to express Tir or EspA. However, the interpretation of this result is limited due to the fact that we only had antibodies specific to components of LEE4 and LEE5, which are activated in an indirect manner by quorum sensing through *ler* (Sperandio *et al.*, 1999). Due to the limitations of the experiment, this observation does not rigorously rule out the involvement of quorum sensing in BipA control.

In agreement with the findings of Sperandio *et al.* (1999), the levels of expression of EspA and Tir were not increased in E2348/69 grown in pre-conditioned medium instead of DMEM. Interestingly, it was noted that wild-type, *bipA*⁻ and transcomplemented strains grew much faster in pre-conditioned medium relative to their growth in DMEM. To confirm that this was not due to the presence of a higher starting inoculum - i.e. the presence of bacteria in pre-conditioned medium not removed by the centrifugation and filtering process, absorbance readings of the pre-conditioned medium were measured against DMEM at the start and end of the incubation period, both OD_{600} readings were zero. The reason for increased growth rate is not known but it is tempting to speculate that increased growth rate is attributable to quorum sensing through autoinducers secreted into the media by MG1655.

6.5 BipA mediates the positive control of LEE encoded factors through *ler*

Recent studies indicate that Ler is central to the process of A/E lesion formation, being essential for the expression of a number of LEE encoded genes, including those

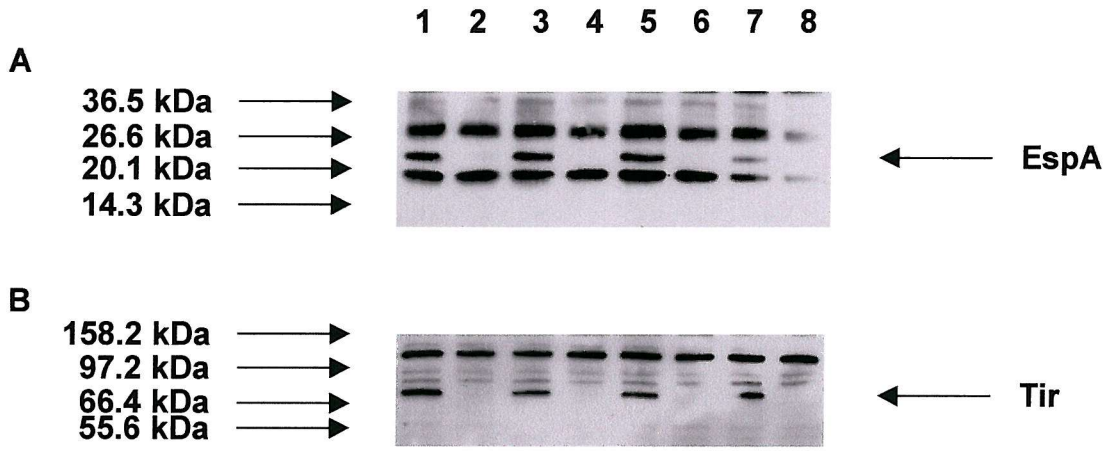


Figure 6.5. Growth of E2348/69 *bipA*⁻ strains in media pre-conditioned by growth of MG1655 does not restore EspA or Tir expression levels in this strain. Comparison of wild-type, *bipA*⁻ and transcomplemented E2348/69 grown in DMEM and pre-conditioned media. MG1655 was grown overnight in LB, diluted 1:50 into pre-warmed DMEM and grown to an OD_{600} of 1.2. Bacteria were pelletted by centrifugation and spent culture medium was passed through a 0.2 μm filter generating pre-conditioned media. Pre-conditioned media was diluted 50:50 with fresh pre-warmed DMEM; bacteria were grown in either DMEM or pre-conditioned media to an OD_{600} of 0.5-0.7. Whole cell extracts were prepared, protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to EspA (A), or Tir (B). Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lanes 1 – 4, bacteria were grown in DMEM, lanes 5 – 8, bacteria were grown in pre-conditioned media. Lanes 1 + 5, E2348/69; lanes 2 + 6, AG2; lanes 3 + 7, AG2(pAJG9); lanes 4 + 8, MG1655.

encoding the type III secretion pathway, the secreted Esp proteins, Tir and intimin (Elliott *et al.*, 2000). Ler also regulates expression of LEE located genes not required for A/E lesion formation including *rorf2*, *orf10*, *rorf10*, *orf19* and *espF* (Elliott *et al.*, 2000). In addition Ler regulates the expression of proteins encoded outside the LEE not essential for virulence, including EspC (Elliott *et al.*, 2000). As Ler is essential for the expression of LEE encoded genes it was clearly of interest to find out if BipA controlled LEE gene expression through Ler.

It was hoped that endogenous levels of the Ler protein could be measured by immunoblotting using antibodies specific to Ler. Accordingly polyclonal antibodies to a peptide corresponding to the C-terminus of EPEC Ler were produced. However, repeated attempts to clean the antibodies using an affinity purification protocol (as described in section 2.5.2 of Chapter 2) failed to produce antibodies of a sufficient calibre to detect Ler (data not shown).

To construct a plasmid expressing Ler::c-Myc fusion protein, the *ler* gene and 283 bp of upstream sequence was PCR amplified from the EPEC E2348/69 chromosome using primers AJG051 (containing translational stop codons in all three reading frames preceding the complementary upstream sequence of the *ler* gene) and AJG052 (complementary to the 3' *ler* sequence and contained nucleotides coding a c-Myc epitope). The amplified product was cloned as an *Eco*RI–*Swa*I fragment into *Eco*RI–*Xmn*I digested pACYC184 producing the plasmid pAJG10. Immunoblots of bacterial whole cell lysates prepared from the *E. coli* strain DH5α expressing pAJG10, probed with antibodies specific for c-Myc demonstrated expression of c-Myc tagged Ler in this background (data not shown).

The plasmid pAJG10 was introduced into E2348/69 and its *bipA*⁻ derivative and whole cell extracts were prepared for a comparison of expression levels of Ler::c-Myc. Immunoblots of bacterial whole cell extracts probed with antibodies specific for intimin indicated an increase in intimin expression levels in AG2(pAJG10) compared to the plasmid free *bipA*⁻ mutant (Figure 6.6). Intimin levels in

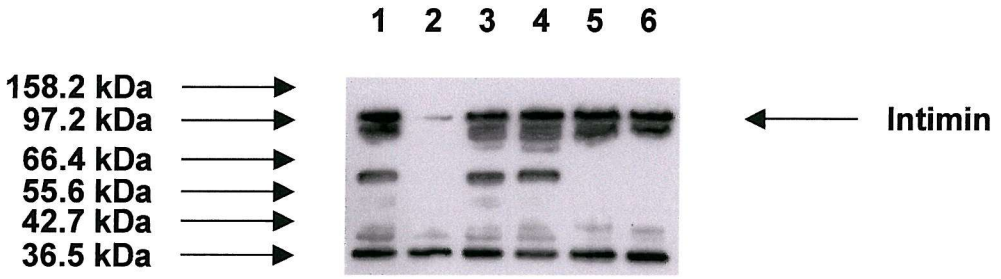


Figure 6.6. A cloned copy of *ler* increases intimin expression in *bipA*⁻ E2348/69 strains. Immunoblot of bacterial whole cell extracts, indicating an increase in intimin expression levels in AG2(pAJG10), compared to the plasmid free *bipA*⁻ mutant. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7, bacteria were pelletted by centrifugation, and whole cell extracts were prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to intimin. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, AG2(pAJG4); lane 4, AG2(pAJG9); lane 5, E2348/69(pAJG10); lane 6, AG2(pAJG10).

AG2(pAJG10) were restored to near wild-type levels, while the level of intimin in E2348/69(pAJG10) was not elevated above wild-type expression. The presence of the 57 kDa truncated intimin polypeptide is not apparent in either strain expressing the plasmid, this may not be Ler mediated, but is more likely a result of poor transfer due to an air bubble in this region. The key finding, however, is that expression of Ler::c-Myc fusion protein in a *bipA*⁻ mutant restores intimin expression.

6.5.1 Transcription of the *ler* gene is reduced in a *bipA* null mutant

If BipA was directly involved in controlling the expression of Ler, it was possible that this would be a post-transcriptional process; in the *bipA*⁻ strain we would not expect *ler* mRNA to be efficiently translated. However, the requirement for BipA may be overcome by the presence of *ler* on a multicopy plasmid. To ensure that transcription was driven only from the *ler* promoter cloned upstream of the *ler* gene in the vector pAJG10, stop codons in all three reading frames were incorporated. To further prevent any transcriptional read-through the Ω interposon bearing a streptomycin/spectinomycin resistance marker flanked by strong transcriptional translational terminators (Prentki and Krisch, 1984) was inserted upstream of the promoter region of *ler* in the plasmid pAJG10, as a 2.1 kb *Eco*RI fragment from the plasmid pHP45Ω (Prentki and Krisch, 1984), into the *Eco*RI site of pAJG10 generating the plasmid pAJG14. Expression levels of Ler::c-Myc in wild-type and *bipA*⁻ mutant strains expressing pAJG10 or pAJG14 were measured by immunoblotting whole cell extracts of bacteria grown in DMEM to an OD_{600} of 0.6-0.7, with antibodies specific to c-Myc. There was no difference in the expression of Ler::c-Myc from pAJG10 or pAJG14 in either a *bipA*⁺ or *bipA*⁻ background (Figure 6.7). Collectively these results suggest that *ler* was expressed from its own promoter, and was not enhanced by other promoters on the plasmid, as there was no decrease in expression after insertion of the Ω interposon.

For direct comparison, transcript levels of *ler* were measured from RNA samples produced from the same cultures used for immunoblotting. No differences in the

transcript levels of *ler* were detected in *bipA*$^+$ or *bipA*$^-$ strains bearing either pAJG10 or pAJG14 (Figure 6.8). Although the level of native Ler could not be measured, analysis of native *ler* transcript levels in plasmid-free wild-type and *bipA*$^-$ strains indicated that the *ler* transcript was markedly reduced in the *bipA*$^-$ background.

We also tested the levels of IHF and H-NS in wild-type and *bipA*$^-$ mutant strains bearing pAJG10 and also in the plasmid free strains (Figure 6.9 (A) and (B) respectively). Consistent with previous findings, the immunoblots indicated no difference in IHF or H-NS levels between E2348/69 *bipA*$^+$ and *bipA*$^-$ strains with or without pAJG10. These results indicate that a cloned copy of *ler* does not influence the level of IHF or H-NS. The observation of no difference in H-NS expression levels in *bipA*$^+$ and *bipA*$^-$ strains grown in DMEM further extends the observation reported in section 4.9 of Chapter 4. IHF levels have previously been shown not to be altered in *bipA*$^+$ or *bipA*$^-$ grown in DMEM (see Figure 6.3).

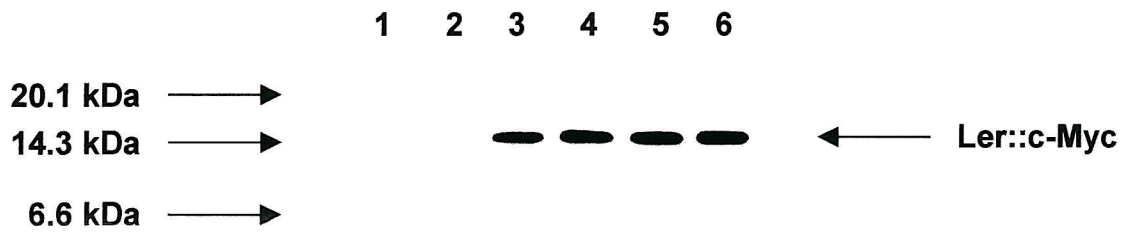


Figure 6.7. Expression of Ler::c-Myc in E2348/69 *bipA*$^{+}$ and *bipA*$^{-}$ strains. Expression levels of Ler::c-Myc were compared in wild-type and *bipA*$^{-}$ mutants expressing either pAJG10 or pAJG14. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7, bacteria were pelletted by centrifugation, and whole cell extracts were prepared. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to c-Myc. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, E2348/69(pAJG10); lane 4, AG2(pAJG10); lane 5, E2348/69(pAJG14); lane 6, AG2(pAJG14).

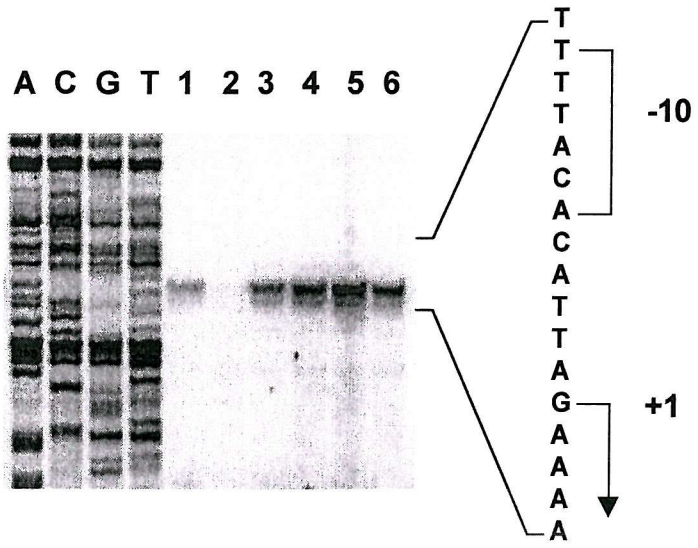


Figure 6.8. Primer extension analysis was performed with RNA extracted from E2348/69 wild-type and *bipA*⁻ mutant strains, and the same strains carrying either pAJG10 or pAJG14, and a primer complementary to the start region of *ler* (LER2). As a reference, an M13mp18 DNA sequencing ladder is shown in lanes A, C, G, T. The transcript levels indicate the absence of a *ler* transcript in the *bipA*⁻ mutant E2348/69 strain, and also demonstrate no significant difference in the *ler* transcript levels in the *bipA*⁺ and *bipA*⁻ strains expressing either pAJG10 or pAJG14. The transcription start point (+1) corresponds to position 3913 in the LEE sequence (Accession number AF022236), which is in accordance with that proposed by Elliott *et al.* (2000). The –10 box and transcription start point are indicated by a bracket and an arrow, respectively. Lane 1, E2348/69; lane 2, AG2; lane 3, E2348/69(pAJG10); lane 4, AG2(pAJG10); lane 5, E2348/69(pAJG14); lane 6, AG2(pAJG14). (Experiment performed by Dr Michele Farris).

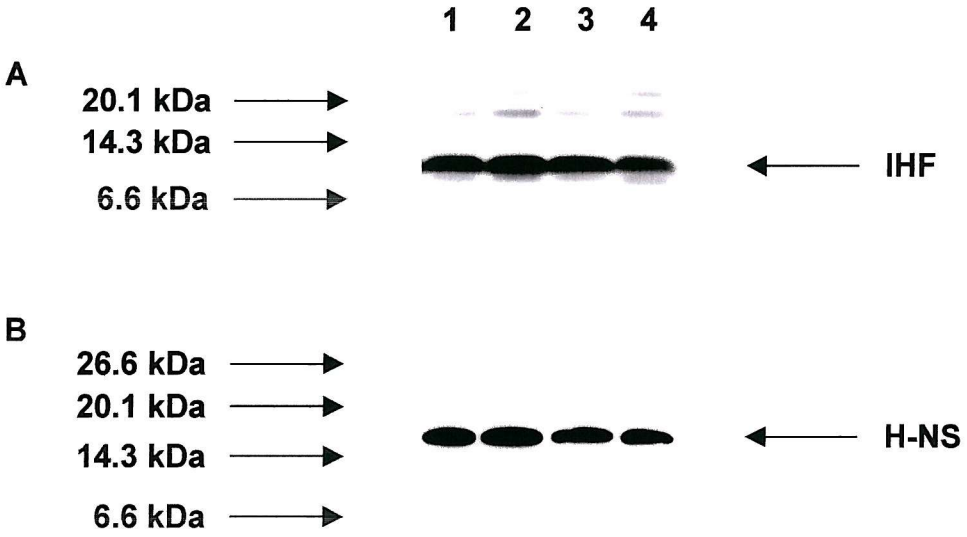


Figure 6.9. The expression levels of two DNA binding proteins involved in the regulation of the LEE, IHF and H-NS are the same in E2348/69 *bipA*$^{+}$ and *bipA*$^{-}$ backgrounds with or without a cloned copy of *ler*. Immunoblots of whole cell extracts of wild-type and *bipA*$^{-}$ mutant E2348/69 strains expressing pAJG10, indicate no difference in expression levels of IHF or H-NS between strains harbouring the plasmid and those without. Bacteria were grown in DMEM to an OD_{600} of 0.6-0.7, bacteria were pelletted by centrifugation, and whole cell extracts were prepared as previously detailed. Protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to IHF (A) or H-NS (B). Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Lane 1, E2348/69; lane 2, AG2; lane 3, E2348/69(pAJG10); lane 4, AG2(pAJG10).

6.6 Discussion

The LEE-encoded regulator Ler is pivotal to the expression of most genes on this pathogenicity island and is already known to be subject to control by IHF and Per. These studies have shown that *ler* transcripts are abolished in the absence of BipA. It is probable that BipA mediated control on the LEE is exerted indirectly at the level of transcription. In the absence of Ler, transcription of the LEE operons is repressed by the global regulator H-NS that binds to silencing regulatory sequences, SRS elements in their promoter regions, thereby blocking transcription (Bustamante *et al.*, 2001). It should be noted that the expression of Tir, intimin and the Esps were not completely abolished in *bipA*$^-$ EPEC strains. This may suggest a basal level of transcription of these genes, and therefore a possible incomplete repression by H-NS. The level of the DNA binding protein H-NS was unaltered between the *bipA*$^+$ and *bipA*$^-$ E2348/69 under a variety of growth conditions (see Figures 4.9 and 6.10). Thus this basal expression does not appear to be due to differences in expression of H-NS between the *bipA*$^+$ and *bipA*$^-$ strains.

Recent studies by other groups indicate that Ler is required for EPEC to secrete Esp proteins into the supernatant and for production of the Esp filament (Friedberg *et al.*, 1999; Elliott *et al.*, 2000). In addition to lack of Esp secretion, Esp proteins are not found at detectable levels in whole cell extracts, and Tir is not secreted by *ler*$^-$ mutants and is not translocated into HEp-2 cells (Friedberg *et al.*, 1999; Elliott *et al.*, 2000). Moreover, intimin levels are markedly reduced in the absence of *ler* (Friedberg *et al.*, 1999; Elliott *et al.*, 2000). The autotransported protein EspC is also not present in *ler*$^-$ mutants (Elliott *et al.*, 2000). These findings are in close agreement with those of this study, which has demonstrated that BipA is absolutely required for the endogenous *ler* transcription. In accordance Tir, intimin, and Esp expression levels are dramatically reduced in *bipA*$^-$ strains. Friedberg *et al.* (1999) and Elliott *et al.* (2000), also report the inability to fully complement all of these defects by the introduction of a cloned copy of *ler*, an observation also found in these studies. Possibly this reflects a critical gene dosage requirement.

Several groups have shown that *ler*⁻ mutants are still able to form microcolonies similar to those of the wild-type, but are defective in the formation of A/E lesions on cultured epithelial cells (Friedberg *et al.*, 1999; Elliott *et al.*, 2000). The strain is unable to accumulate actin beneath the bacteria although, a shadow FAS after 6 hours incubation, suggesting the ability to form A/E lesions was not completely abolished in the strain, but severely reduced (Friedberg *et al.*, 1999: Elliott *et al.*, 2000). This observation is in accordance with those from these studies and those of Farris *et al.* (1998). We previously reported that increased expression of BipA enhances actin remodelling and leads to the hyperformation of pseudopods in MAR001-derived strains (Farris *et al.*, 1998). Interestingly Elliott *et al.* (2000), report that *ler*⁻ complemented strains exhibit a FAS reaction enhanced over that of the wild-type. It is tempting to speculate that the over-expression of *ler* may account for this phenotype, and that overexpression of BipA, following cloning of its gene onto a multi-copy vector, is likely to indirectly increase the expression of Ler. However, in contrast Friedberg *et al.* (1999), report that an EPEC *ler*⁻ mutant transcomplemented strain appeared to recruit less actin than the wild-type.

Previous studies have suggested that the expression of *LEE4* is only modestly increased in *E. coli* K-12 (Sperandio *et al.*, 1999; Elliott *et al.*, 2000), while Ler is absolutely necessary for full transcription of *LEE4* in EPEC (Elliott *et al.*, 2000; indirect evidence from these studies). Elliott *et al.* (2000), propose the presence of an EPEC specific accessory factor that represses *LEE4*, and that Ler acts as a derepressor of expression from this operon.

The presence of a cloned copy of *ler* in the *bipA*⁻ derivative of E2348/69 restored intimin expression levels. This complementation was not to wild-type levels, and the truncated intimin polypeptides were not present. The presence of pAJG10 resulted in only a modest increase in intimin expression. Moreover, the prominent intimin breakdown product of around 57 kDa was absent in the E2348/69 carrying pAJG10. It should be noted that transfer of this region was impaired by an air bubble, and therefore we cannot preclude that the absence of the breakdown product is not due to

an artefact of transfer opposed to a Ler mediated effect. Further experiments are required to resolve this point.

Recent studies indicate that Ler is involved in regulating the expression of a number of non-LEE encoded genes (Elliott *et al.*, 2000). For example, Ler positively regulates the expression of EspC. Thus, it seems probable that the positive control of EspC expression by BipA is mediated through the latter control of *ler*. EPEC containing the EAF plasmid express BFP which are involved in the formation of microcolonies, Elliott *et al.* (2000), also demonstrated that there is no difference in BfpA expression in wild-type or *ler*⁻ mutant cells. Thus, assuming that BipA mediates its control on LEE virulence gene expression through Ler, we anticipate that BfpA expression will be unaltered in the *bipA*⁻ cells. Recent studies also indicate that *ler*⁻ mutants of EPEC produce additional non-BFP fimbriae with novel morphologies (long fine fimbriae, rigid bent fimbriae and short fine fimbriae; Elliott *et al.* 2000). It is intriguing that a *bipA*⁻ mutant in *Salmonella* Enteritidis also expresses novel fimbriae (A. White and C. D. O'Connor unpublished observations). While *Salmonella* strains do not contain the LEE, Ler is distantly related to proteins that are members of the H-NS family of DNA binding proteins. Thus, it is tempting to speculate that the negative regulation of fimbriae by the *bipA*⁻ mutant in *Salmonella* Enteritidis may be an indirect effect mediated by a DNA binding protein like Ler.

Mutations in the gene for the DNA binding protein H-NS have previously been shown to repress flagella synthesis and therefore motility (Soutourina *et al.*, 1999; Ko and Park, 2000). Mutation of *ler* has been shown not to affect motility of EPEC (Elliott *et al.*, 2000). Thus we presume that the negative regulation of flagella mediated cell motility by BipA is not mediated through Ler, although it could be mediated through a related DNA-binding protein.

The results described in this chapter further extend the information about the molecular events that control gene expression in EPEC. A complex regulatory cascade involving growth and environmental conditions, quorum sensing, regulators

such as Ler, H-NS, IHF and Per, and the ribosome binding GTPase BipA are involved in co-ordinating the synthesis of virulence proteins involved in forming the characteristic A/E lesion of EPEC.

BipA is a GTPase that is stimulated in the presence of 70S ribosomes (R. Owens and C.D.O'Connor unpublished observation). Therefore it is probable that BipA operates post-transcriptionally at the level of the ribosome, where we suspect that it may regulate the translation of a subset of mRNAs. The present studies have demonstrated that *ler* transcripts are abolished in *bipA*⁻ EPEC. While we cannot formally rule out a direct DNA binding capacity of BipA, degradation of *ler* mRNA or regulation through quorum sensing, it is more likely that BipA operates indirectly. The simplest hypothesis is that it controls the translation of a DNA binding protein – 'Factor X' – that in turn regulates *ler* transcription. An up-dated figure indicating the regulatory mechanisms of the LEE is shown in Figure 6.10. The next chapter describes initial attempts to uncover the identity of this mystery Factor X.

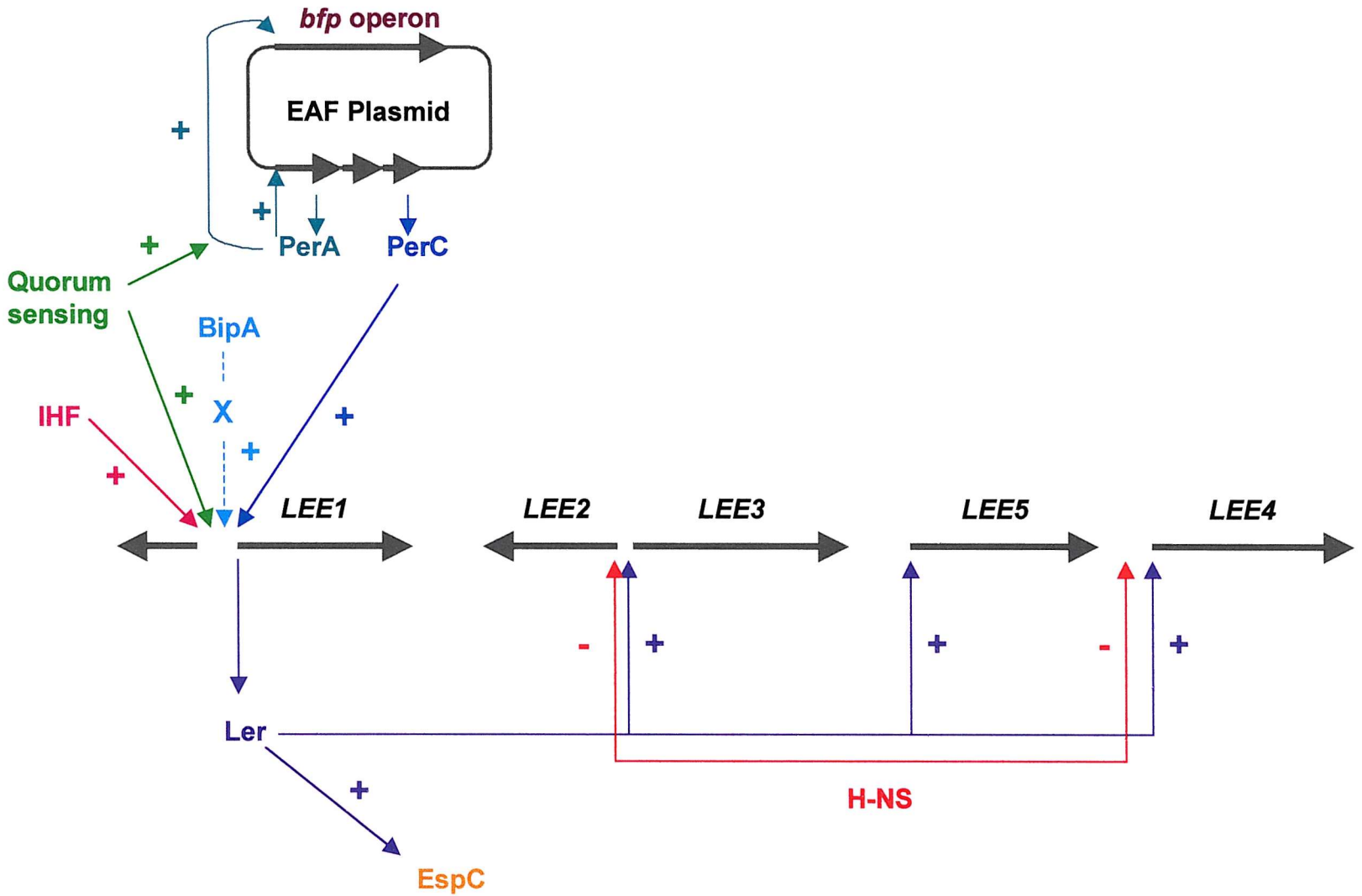


Figure 6.10. Updated model of the regulatory cascade of LEE gene expression

CHAPTER 7

CONSTRUCTION OF EPEC STRAINS WITH REPORTER GENE FUSIONS AND THEIR USE TO SEARCH FOR 'FACTOR X'

CHAPTER 7: CONSTRUCTION OF EPEC STRAINS WITH REPORTER GENE FUSIONS AND THEIR USE TO SEARCH FOR 'FACTOR X'

7.1 Introduction

Several lines of evidence suggest that BipA interacts with ribosomes: (i) The homology of BipA with ribosome binding GTPases; (ii) the presence of a motif only seen in ribosome binding GTPases; (iii) Experimental observations of interactions with the ribosome. In view of this evidence, it is unlikely that BipA acts as a transcription factor to regulate *ler*. Rather, it is more likely that it may control the expression of *ler* indirectly by controlling the translation of another component, possibly a transcription factor. The latter would subsequently activate the expression of the *ler* gene either directly, or by competing with or displacing a repressor. Having systematically looked for BipA mediated control on the LEE and ruled out regulation by known factors, we sought a method to identify the real direct target of BipA. For convenience, this target is referred to here as 'Factor X'.

To address this question, *bipA*$^{+}$ and *bipA*$^{-}$ derivatives containing a chromosomally located *espD::lacZ* fusion were prepared using strains in which the endogenous *lacZ* gene had been deleted. The resulting derivatives were used in a transposon mutagenesis screen to search for insertion events that increased the expression of *espD*, as monitored by an increase in β-galactosidase activity.

This chapter describes the construction of *lacZ* reporter gene fusions with the *espD* gene, and their use in transposon mutagenesis in an attempt to identify proteins that are primary targets for the BipA GTPase, and through which BipA controls *ler* gene expression and subsequently A/E lesion formation.

7.2 Experimental design and construction of appropriate strains

Through the introduction of a transposon into the *bipA*⁻ mutant it was hoped that we could select for a transposon inserted upstream of the gene for Factor X. If the transposon had a strong promoter reading into the Factor X gene, it was possible that Factor X would be expressed in the absence of *bipA*. To search for Factor X, a transcriptional fusion of the reporter *lacZ* gene to the *espD* gene would be constructed. Transposon insertion events upstream of Factor X would subsequently be monitored by the increase in transcription of *lacZ*, as indicated by the presence of dark blue colonies on X-gal containing medium.

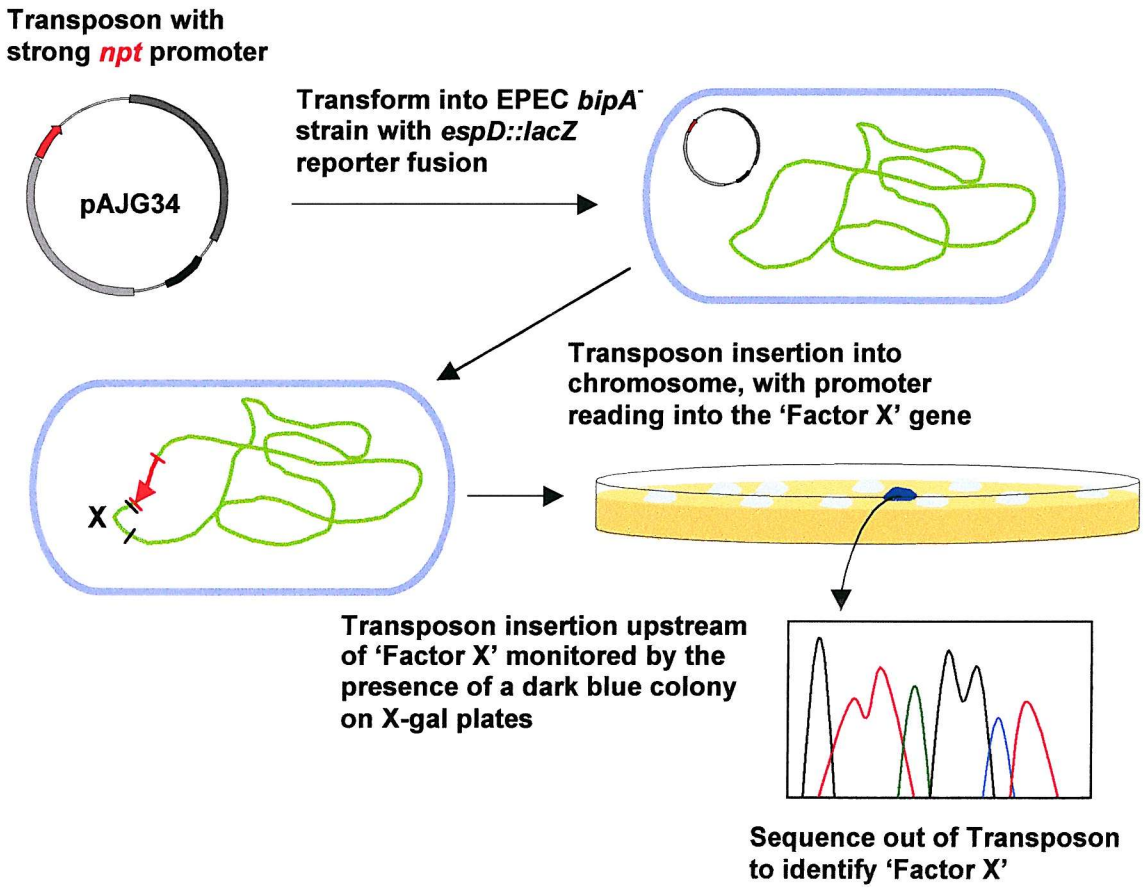


Figure 7.1. A diagram summarising the transposon mutagenesis approach outlined in the above passage and described in more detail in section 2.2.29 of Chapter 2. (Figure adapted form Epicentre, http://www.epicentre.com/transtrat.htm).

As a prerequisite, it was first necessary to remove the endogenous *lacZ* gene from the E2348/69 wild-type and *bipA*⁻ null mutant strains, so that β-galactosidase activity was attributable to the *lacZ* fusion with *espD*, and not to endogenous *lacZ* activity. Fusions in both *bipA*⁺ and *bipA*⁻ strains were produced to enable a comparison of β-galactosidase activity between the wild-type and the *bipA*⁻ mutants, to assess the likely increase in β-galactosidase expression attributable to the transposon-mediated Factor X expression.

Primers AJG055 and AJG080 (Table 2.4) were used to PCR amplify the *lacI* and *lacY* regions flanking the Δ*lacZ* gene of E2348/69Δβ-gal. The latter carries a Δ*lacZ* gene in which the open reading frame has been replaced with an *Xba*I site (R. Haigh, personal communication). The amplified product was cloned as a *Xho*I-*Sma*I fragment into *Sal*I-*Sma*I digested and gel extracted pAJG19 generating plasmid pAJG21. (A schematic showing the production of pAJG21 is shown in Figure 7.2). pAJG21 was then moved by electroporation into S17-1(λ*pir*), and a non-polar in-frame deletion mutation of *lacZ* was constructed in both EPEC E2348/69 and AG2 by allelic exchange with the chromosomally encoded *lacZ* gene. Suitable constructs were selected on the basis of white colouration on LB X-gal plates. The structures of isolates with a chromosomally located Δ*lacZ* in the E2348/69 and AG2 strains were confirmed by PCR using primers AJG055 and AJG069 (Table 2.4), (AJG069 maps 3' to *lacY*, and therefore outside of the region cloned into pAJG21). Further confirmation was obtained by Southern blotting of both *Xba*I and *Sal*I digested chromosomal DNA and probing with a 5' ^{32}P labelled 540 bp PCR product of *lacI* prepared by the PCR amplification of E2348/69 chromosomal DNA using Primers AJG087 and AJG088 (Table 2.4). A representative E2348/69Δ*lacZ* construct was named AG8, and a representative AG2Δ*lacZ* construct was named AG14.

An *espD::lacZ* transcriptional fusion was produced in the *lacZ*⁻ EPEC *bipA*⁺ and *bipA*⁻ strains using standard genetic techniques (Sambrook *et al.*, 1989) in conjunction with the crossover PCR method (Link *et al.*, 1997) and GATEWAY™ Cloning Technology (Gibco BRL Life Technologies). The 5' region of the *espD* gene from

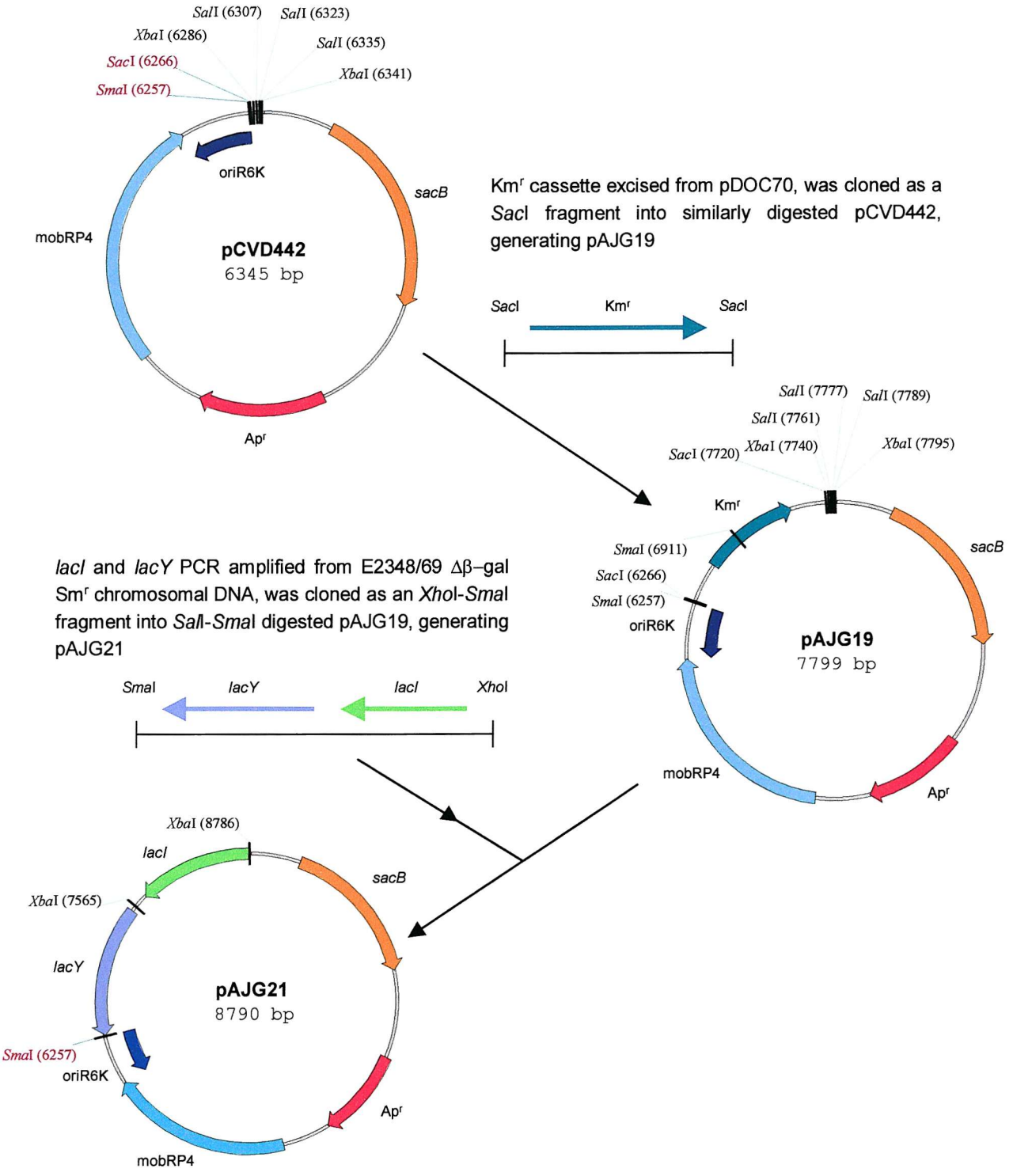


Figure 7.2. Schematic indicating the production of a suicide vector containing a *lacI-lacY* region used to produce Δ*lacZ* E2348/69 wild-type and *bipA* null mutant strains.

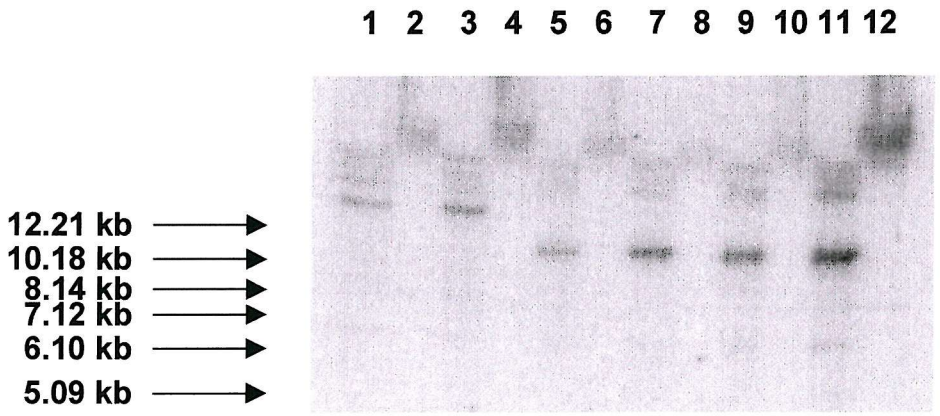


Figure 7.3. Construction of non-polar Δ*lacZ* mutants, of *bipA*$^{+}$ and *bipA*$^{-}$ E2348/69 strains. Southern hybridisation analysis, using E2348/69, AG2 and mutated derivatives of these strains. Chromosomal DNA was digested with either *Sal*I or *Xba*I and probed with a 5' ^{32}P labelled PCR product of *lacI*. *Xba*I cuts within the Δ*lacZ* region (*lacZ* has been replaced by an *Xba*I site), and *Sal*I cuts outside of the *lacI*, *lacZ*, *lacY* operon. Digestion of a Δ*lacZ* strain with *Sal*I should produce a fragment roughly 3 kb smaller than digestion of the wild-type. Lane 1, E2348/69 *Sal*I digested; lane 2, E2348/69 *Xba*I digested; lane 3, AG2 *Sal*I digested; lane 4, AG2 *Xba*I digested; lane 5, AG8 *Sal*I digested; lane 6, AG8 *Xba*I digested; lane 7, AG9 *Sal*I digested; lane 8, AG9 *Xba*I digested; lane 9, AG14 *Sal*I digested; lane 10, AG14 *Xba*I digested; lane 11, AG17 *Sal*I digested; lane 12, AG17 *Xba*I digested.

E2348/69 was amplified by PCR using primers AJG111 and AJG112 (Table 2.4), whereas the 3' region of the *espD* gene was amplified by PCR using primers AJG113 and AJG114 (Table 2.4). Primer AJG111 contained the *att*B2 sequence while primer AJG114 contained the *att*B1 sequence. As well as complementary regions to the *espD* gene, primers AJG112 and AJG113 were also complementary to each other over a stretch of 46 nucleotides. The latter contained restriction sites for cloning, a consensus ribosome-binding site and an ATG start codon. The two PCR products were mixed, the complementary regions annealed and primed at the 3' overlapping region, for a 3' extension of the complementary strand. The annealed structure was amplified by PCR using primers AJG111 and AJG114 and the resulting in-frame mutation of *espD* was transferred to the vector pPA3065 by recombination, using a slightly modified procedure supplied by Gibco BRL Life Technologies (described in Chapter 2 section 2.2.28). A suitable construct designated pAJG35 was sequenced using primers AJG111, AJG112, AJG113 and AJG114.

Primers AJG109 and AJG120 were used to PCR amplify the *lacZ* gene from MG1655 chromosomal DNA, which was subsequently cloned as a *Not*I-*Asc*I fragment into similarly digested pAJG35, thereby generating the plasmid pAJG36. (Primer AJG109 contained the *lacZ* ribosome binding site as well as complementary regions to the *lacZ* gene sequence). The kanamycin resistance cassette was amplified by PCR from pDOC70 using the primers AJG121 and AJG110 and cloned as an *Asc*I-*Pme*I fragment into similarly digested pAJG36. This generated the plasmid pAJG39. (A schematic showing the production of pAJG39 is shown in Figure 7.4). pAJG39 carrying the *espD::lacZ::km*^{r} fragment was electrotransformed into S17-1(λ*pir*), and subsequently transferred to AG8 and AG14, with selection for allelic exchange with the chromosomally encoded *espD* gene (Donnenberg and Kaper, 1991). A number of suitable constructs were selected on the basis of their kanamycin resistant blue phenotype on LB + kanamycin + X-gal plates. The structure of isolates with a chromosomally located *espD::lacZ::km*^{r} in AG8 and AG14 was confirmed by PCR using primers AJG135, AJG098, AJG136 and AJG099 (Table 2.4). Further conformation was obtained by Southern blotting of both *Bgl*II and *Sca*I digested

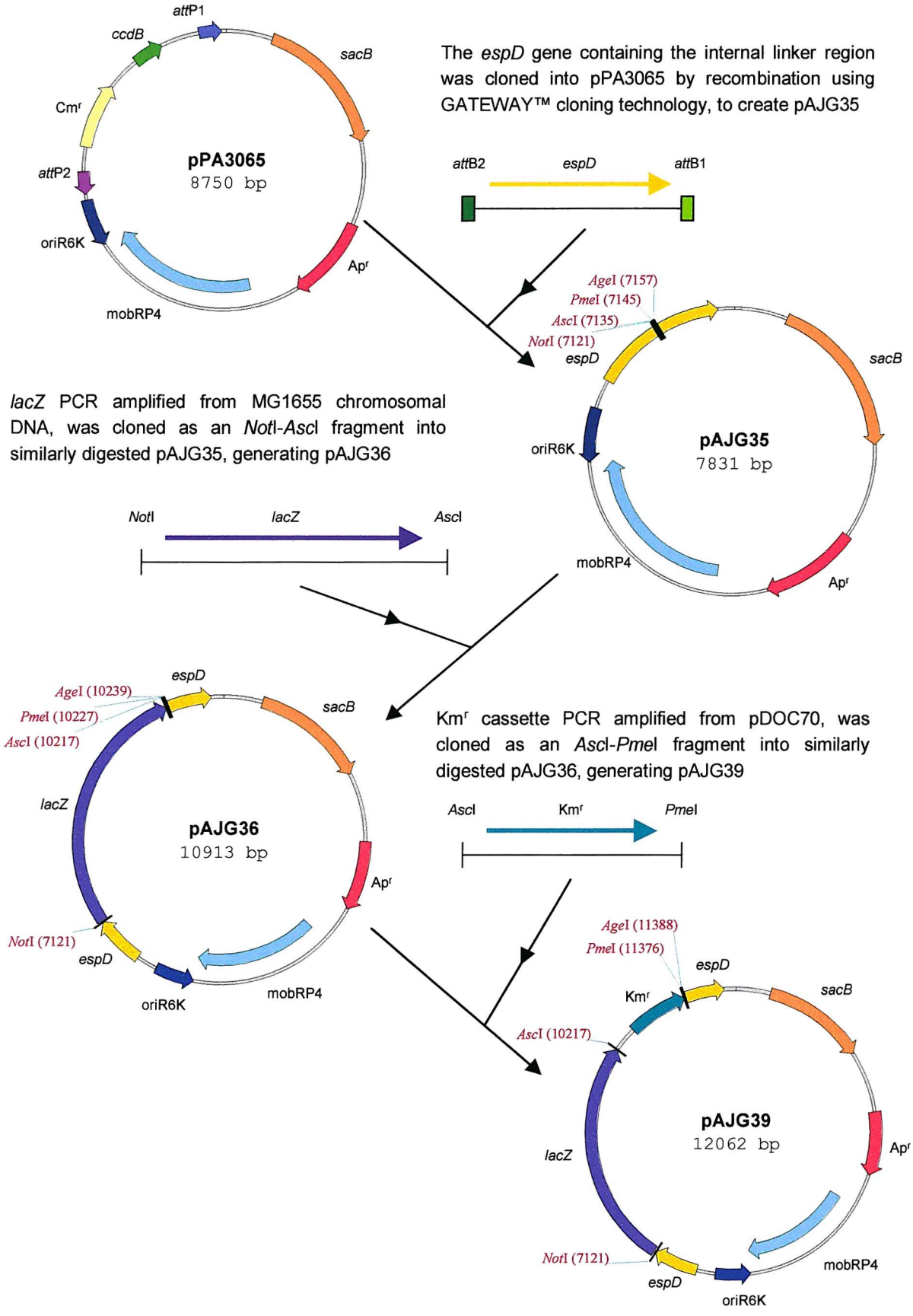


Figure 7.4. Schematic indicating the production of a suicide vector containing a transcriptional fusion of *lacZ* with *espD*.

chromosomal DNA and probing with a 5' ^{32}P labelled 369 bp PCR product of the 3' region of *espD*, prepared by PCR amplification of the EPEC E2348/69 chromosome using AJG139 and AJG141 (Table 2.4). A representative AG8*espD::lacZ::km*^{r} strain was named AG22, and a representative AG14*espD::lacZ::km*^{r} strain was named AG25.

7.3 Comparison of β-galactosidase expression of AG22 and AG25

The gene for EspD is encoded on the operon *LEE4* along with genes for other Esps, which are transcribed as a single polycistronic message (Elliott *et al.*, 1998; Mellies *et al.*, 1999). The present studies have shown that EspD expression is reduced in *bipA*$^-$ strains, as is *ler* transcription. Transcription of *espA* is also abolished in the *bipA*$^-$ strain (M. Farris unpublished observations) and, since *espA* and *espD* are in the same operon, we anticipate that the transcription of *espD* would also be reduced. β-galactosidase assays conducted with strains AG22 and AG25 grown in conditions to mimic the gastrointestinal tract, (DMEM at 37°C, 5% CO_2), established over a five fold reduction in transcription of *espD* (as measured by β-galactosidase activity of the *espD::lacZ* transcriptional fusion) in the *bipA*$^-$ strain (Figure 7.6). This confirms the production of chromosomally encoded *espD::lacZ* reporter fusions and substantiates the reduced transcription of *espD* in E2348/69 *bipA*$^-$ strains.

To allow visualisation of *lac*$^+$ transposon insertion mutants of AG25, we tested β-galactosidase expression from strains AG22 and AG25 on a range of media promoting maximal and sub-optimal expression conditions. It was reasoned that maximal expression in AG22 would occur under conditions of growth in DMEM, while under identical conditions expression in a *bipA*$^-$ strain, AG25, would be low due to the absence of Factor X. MacConkey plates, sucrose-TB plates, LB plates, and M9 Minimal Media plates - the latter three containing X-gal – were tested along with various DMEM X-gal containing plates. The latter included: DMEM with 0.45% glucose, DMEM with 0.45% glucose and no phenol red (to improve visualisation of blue colonies), DMEM with no glucose instead supplemented with sodium succinate

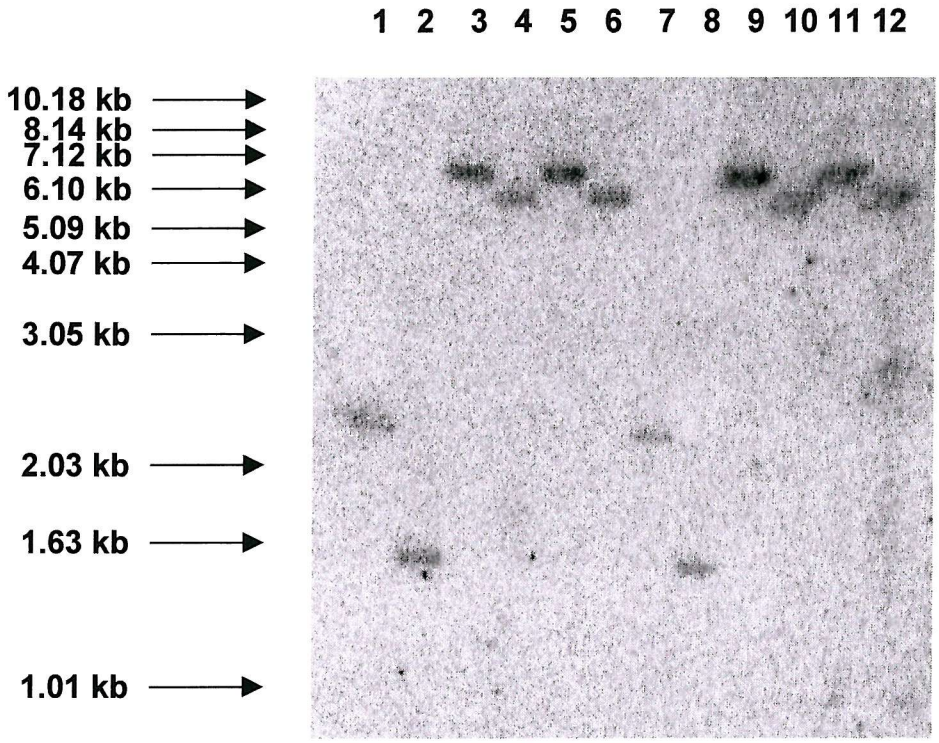


Figure 7.5. Construction of *espD::lacZ* mutants, of Δ*lacZ bipA*$^{+}$ and *bipA*$^{-}$ E2348/69 strains. Southern hybridisation analysis, using AG8, AG14, and strains in which the *lacZ* gene has been placed under the control of the promoter for the *espD* gene. Chromosomal DNA was digested with either *Bgl*II or *Sca*I and probed with a 5' ^{32}P labelled PCR product of *espD*. Digestion with *Bgl*II should produce a fragment of 2343 bp if wild-type and 6567 bp if an *espD::lacZ::km*^{r} fusion, while digestion with *Sca*I should produce a fragment of 1549 bp if wild-type and 5773 bp if an *espD::lacZ::km*^{r} fusion. Lane 1, AG8 *Bgl*II digested; lane 2, AG8 *Sca*I digested; lane 3, AG22 *Bgl*II digested; lane 4, AG22 *Sca*I digested; lane 5, AG23 *Bgl*II digested; lane 6, AG23 Sc*a*I digested; lane 7, AG14 *Bgl*II digested; lane 8, AG14 *Sca*I digested; lane 9, AG24 *Bgl*II digested; lane 10, AG14 *Sca*I digested; lane 11, AG25 *Bgl*II digested; lane 12, AG25 *Sca*I digested.

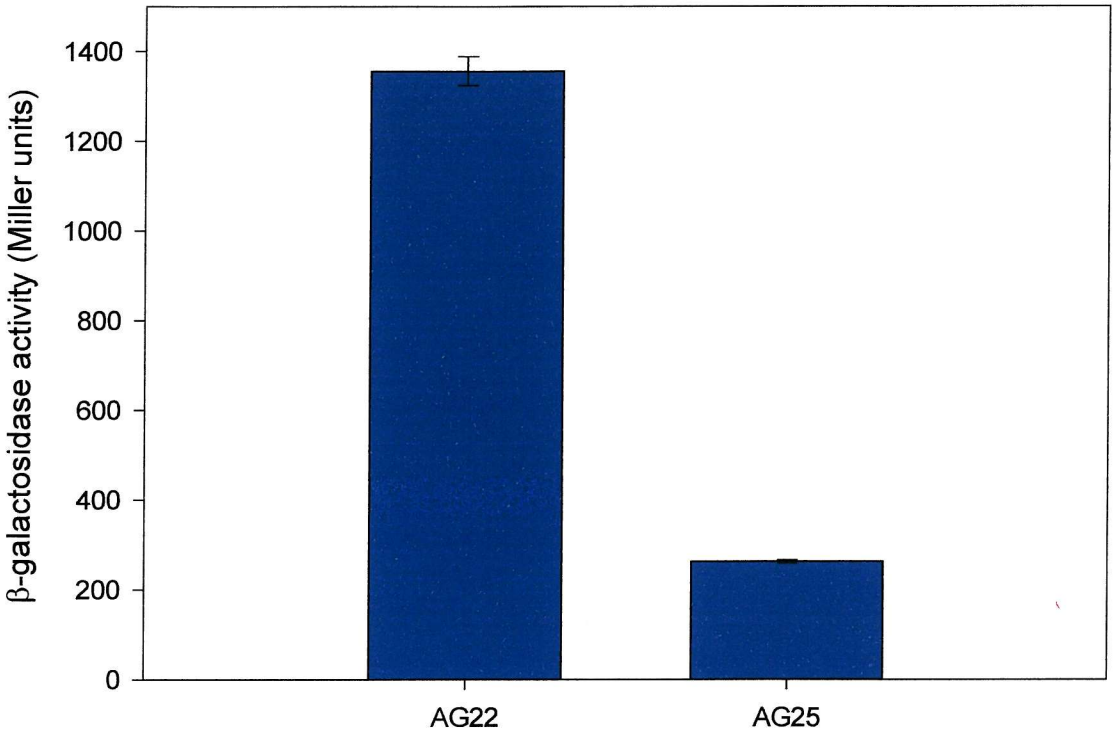


Figure 7.6. Comparison of β-galactosidase activity from *bipA*$^{+}$ and *bipA*$^{-}$ *espD::lacZ* fusions. Bacteria were grown shaking in LB at 37°C overnight, diluted 1:50 into pre-warmed DMEM and grown to an OD_{600} of 0.6-0.7 standing at 37°C, 5% CO_2. β-galactosidase assays were performed as detailed in section 2.7.6 of Chapter 2. The graph shows the average β-galactosidase activity (Miller units) from three experiments and the standard deviation plotted as error bars.

to a concentration of 0.4% as a carbon source, DMEM plates were incubated at 37°C and 5% CO_2, all other plates were incubated at 37°C. The differences in β-galactosidase expression between AG22 and AG25 (Figure 7.7) were not as pronounced as anticipated.

We observed over a five-fold difference in β-galactosidase levels in mid-log phase cultures grown in DMEM. However, the same strains plated onto X-gal containing media did not reflect such a discernable difference. Interestingly, some of the plates offering greatest difference in β-galactosidase activity, as detected by a visual screen of colonies on a plate, were media with sub-optimal conditions for expression of LEE encoded virulence factors. The reason for this is unclear. LB, M9 Minimal Media and DMEM sodium succinate X-gal medium plates were chosen for transposon mutagenesis studies.

7.4 Production of a transposon delivery system

To search for Factor X, we required a transposon that, if inserted upstream in the correct orientation, would result in constitutive expression of the downstream gene(s). Therefore, Tn5B50, which carries the *npt* promoter in the transposon delivery vector pRTP1, was chosen (Simon *et al.*, 1989; Lee *et al.*, 1992). The plasmid pRTP1::Tn5B50 is a delivery system for the transposon into cells, the rationale behind the use of pRTP1 as a vehicle for transposon delivery is based on the fact that the vector carries a gene encoding the Sm-sensitive allele of the gene for ribosomal protein S12, rendering an otherwise Sm-resistant strain Sm sensitive (Stibitz *et al.*, 1986). The use of this transposon system for mutagenesis requires the selection for a spontaneous Sm resistant mutant strain in which the streptomycin resistant allele of *rps*L is phenotypically recessive to the streptomycin-sensitive *rps*L allele on the pRTP1 derivatives. Transposition of Tn5B50 onto the genome of an Sm resistant strain and subsequent loss of plasmid can be selected for by growth in the presence of streptomycin and tetracycline (Lee *et al.*, 1992).

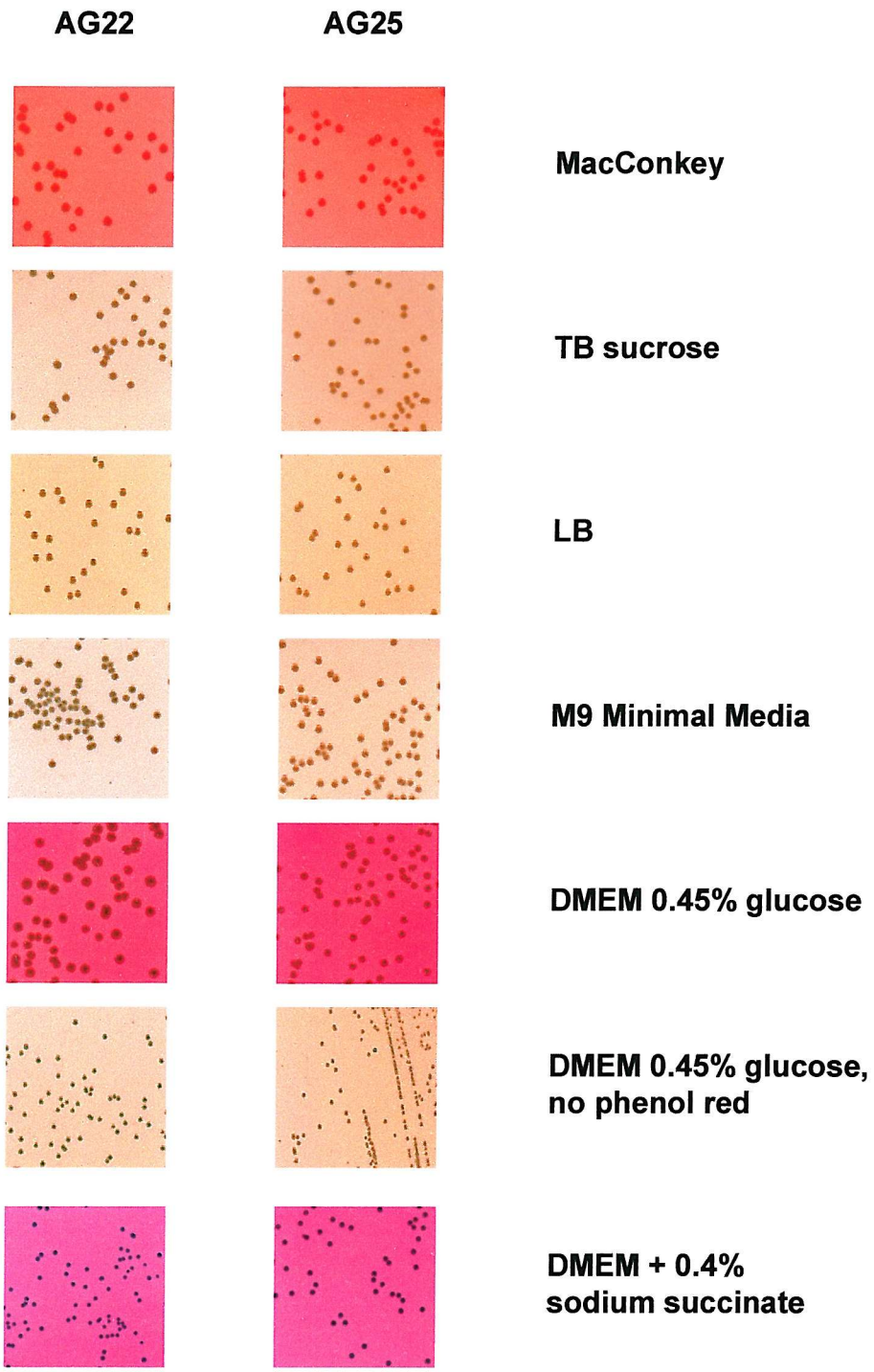


Figure 7.7. AG22 and AG25 were plated onto growth media as shown, containing X-gal (with the exception of MacConkey plates) to select media for transposon mutagenesis studies. All plates were incubated at 37°C, plates produced from DMEM were incubated in the presence of 5% CO_2. LB, M9 minimal medium and DMEM sodium succinate, were identified as producing the greatest colour difference between AG22 and AG25 and were chosen for transposon mutagenesis studies.

The plating of E2348/69 wild-type and *bipA*⁻ mutant strains on Sm plates indicated that these strains were already Sm resistant. The EAF plasmid cured strain JPN15 and the BipA null mutant derivative of this strain were not Sm resistant. Thus either our E2348/69 wild-type strain was made Sm resistant prior to us receiving it, or the EAF plasmid contains an Sm resistance allele. Due to the fact that the selection procedure of pRTP1::Tn5B50 depends on a particular type of resistance allele, it was decided to modify pRTP1::Tn5B50 and to place the *sacB* gene encoding levansucrase which is lethal in the presence of sucrose (Gay *et al.*, 1985) on the vector for counter selection. Transposition of Tn5B50 onto the genome of the recipient strain and subsequent loss of the plasmid was selected by growth on Tc plates followed by replica plating onto TB sucrose Tc plates. Primers AJG127 and AJG128 were used for the PCR amplification of the *sacB* gene together with around 300 bp of upstream sequence from the plasmid pCVD442. The PCR product was digested with *Bgl*II and cloned into *Bam*HI digested pRTP1::Tn5B50 (a gift from C. Lee). Following ligation, the mix was re-digested with *Bam*HI to linearise any pRTP1::Tn5B50 that had self-ligated and electrotransformed into DH5α. Suitable constructs were selected using ampicillin + tetracycline plates, followed by replica plating onto ampicillin + tetracycline and sucrose-TB ampicillin + tetracycline plates. Colonies that grew on the former but not the latter were further screened for the presence of the *sacB* gene by PCR using primers AJG127 and AJG128 (Table 2.4). A representative clone was named pAJG34

7.5 Transposon mutagenesis of *espD::lacZ* fusion constructs

AG25, E2348/69 *bipA*⁻ strain with the *espD::lacZ* was transformed with pAJG34, and bacteria containing the plasmid were selected for by growth on Ap, Tc plates. The transposon mutagenesis approach was performed as previously detailed (Lee *et al.* 1992, see section 2.2.29 of chapter 2). In short, a 1:50 dilution of an overnight culture of AG25(pAJG34), grown in LB Tc medium, was grown to an OD_{600} of 1.5. Serial dilutions were prepared and plated onto LB X-gal Tc plates, following growth at 37°C overnight colonies were replica plated onto sucrose-TB X-gal Tc plates and

grown at 37°C overnight. Colonies from these plates were further replica plated onto LB, M9 Minimal Media and DMEM sodium succinate plates containing X-gal and Tc. The LB and M9 plates were left at 37°C, while the DMEM plates were left at 37°C, 5% CO_2 overnight. Blue colonies were identified using a visual screen (Figure 7.8). Colonies from the sucrose-TB plates were also replica plated onto LB Ap Tc plates to measure the frequency of colonies that still contained the plasmid. It turned out that 3% of the 1000 colonies counted were still ampicillin resistant. The transposon mutagenesis approach yielded blue colonies on all three media that are candidates for further investigation. As anticipated from preliminary studies, the level of discrimination on DMEM plates was quite low. There were a greater number of intense blue colonies on the sub-optimal M9 minimal media plates. This may reflect transposon insertion events upstream of LEE activators, and therefore the constitutive expression of *espD::lacZ*. However, the frequency for this appears too high. We would expect that the constitutive expression of Per, Ler, and IHF would also produce blue colonies. Equally, disruption of the *hns* reading frame would be expected to allow constitutive expression of the LEE. Candidate colonies dark blue on all three media potentially harbours an insertion event upstream of a gene involved in controlling LEE gene transcription. The site of transposition could be determined by sequencing the flanking regions of the transposon.

7.6 The transcriptional regulator SlyA complements intimin expression in the E2348/69 *bipA*⁻ strain

The SlyA family of transcriptional regulators activates virulence factor expression in pathogens such as *Salmonella* and *Yersinia*. It has recently been shown that over-expression of SlyA in EPEC E2348/69 induces the untargeted secretion of the EPEC proteins EspA, EspB, EspC, EspD, EspF and Tir into LB at 37°C (S. Swift, in preparation). Factor X could be a transcription regulator, so the ability of SlyA to complement the BipA mutation in E2348/69 in terms of expression of LEE encoded genes was investigated. E2348/69 and its *bipA*⁻ mutant were transformed with pII30,

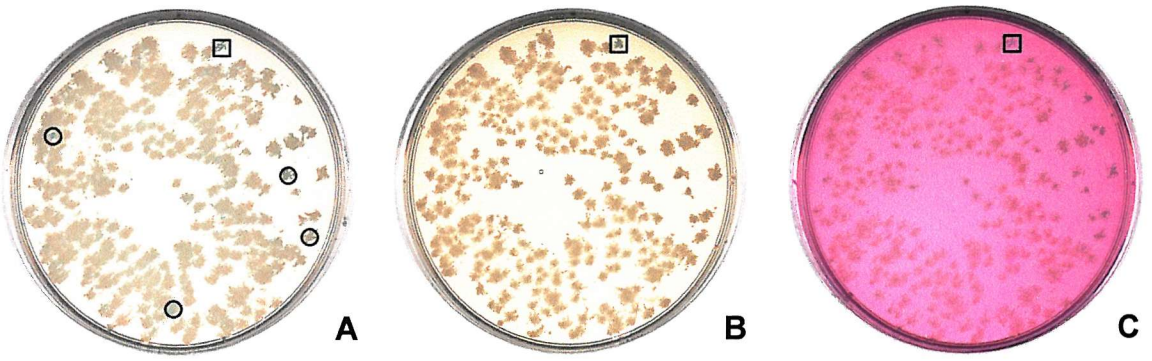


Figure 7.5. Identification of positive β-galactosidase colonies on X-gal containing media. AG25 plated on M9 Minimal Media (A), LB (B), and DMEM sodium succinate (C) plates containing X-gal following transposon mutagenesis. The figure highlights blue colonies from the background 'white' colonies. Darker blue colonies on each plate are identified by a square (□) while those just blue on the M9 Minimal Media plate are identified by a circle (○).

a plasmid in which *slyA* control is uncoupled from its native promoter and is under the control of the IPTG inducible *trc* promoter. SlyA induction protocols are detailed elsewhere. In brief; a 1:20 dilution of overnight culture grown in LB was incubated at 37°C for 3 hours to an OD_{600} of around 1.5, in the presence and absence of IPTG. Controls of *bipA*$^+$ and *bipA*$^-$ strains, grown in the presence and absence of IPTG and containing the cloning vector pTrc99A, were also grown. Whole cell extracts were prepared; protein samples were separated by PAGE, transferred onto nitrocellulose and probed with antisera specific to intimin. Increased levels of intimin expression were observed in the E2348/69 wild-type and *bipA*$^-$ mutant strains upon induction of *slyA* expression from pII30 with concentrations of IPTG 0.1mM and greater (Figures 7.9 and 7.10 respectively). The controls demonstrate that this is due to the induction of *slyA* and not the presence of the plasmid or IPTG. This observation suggests that SlyA can complement the *bipA*$^-$ defect. Whether this is as a result of a cloned copy of a known transcriptional activator like SlyA (these studies have indicated that a cloned copy of *per* and *ler* can complement the *bipA*$^-$ defect), or whether BipA controls SlyA expression remains to be seen. Figures 7.9 and 7.10 indicate the presence of intimin breakdown products upon the induction of *slyA* with 0.1 mM IPTG and proteolysis was greater in the *bipA*$^+$ and *bipA*$^-$ strains expressing *slyA*. The pattern of intimin breakdown products in the *bipA*$^-$ strain is different to those in the *bipA*$^+$ strain. Specifically, there was the absence of the prominent band around 57 kDa that is observed with *bipA*$^+$ strains. Interestingly, the intimin expression levels in wild-type *slyA* expressing strains were not greatly increased from wild-type expression levels. Moreover, increased breakdown products were observed in the plasmid expressing strain, further suggesting there may be a threshold level for intimin. The band around 36 kDa, which is decreased in wild-type E2348/69 expressing *slyA*, is not an intimin breakdown product, as this band is also present in MG1655 whole cell extracts probed with anti-intimin antibodies (Figure 6.2 (C)).

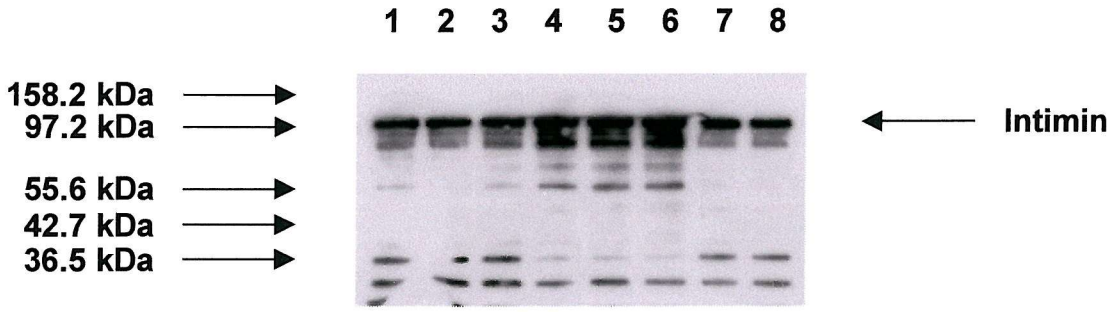


Figure 7.9. A cloned copy of *slyA* enhances intimin expression in wild-type E2348/69. Immunoblot of whole cell extracts from E2348/69, E2348/69(pII30) and E2348/69(pTrc99A) probed with anti-intimin antibodies. Bacteria were grown in LB medium supplemented with IPTG as indicated for 3 hours to an OD_{600} of 1.5. Whole cell extracts were prepared and protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to intimin. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Samples for lanes 1, 3 and 7 were grown in LB medium; samples for lanes 2, 6 and 8 were grown in LB medium containing 1.0 mM IPTG; the samples in lanes 4 and 5 were grown in LB medium supplemented with 0.1 or 0.5 mM IPTG respectively. Lanes 1 + 2, E2348/69; lanes 3 – 6, E2348/69(pII30); lanes 7 + 8, E2348/69(pTrc99A).

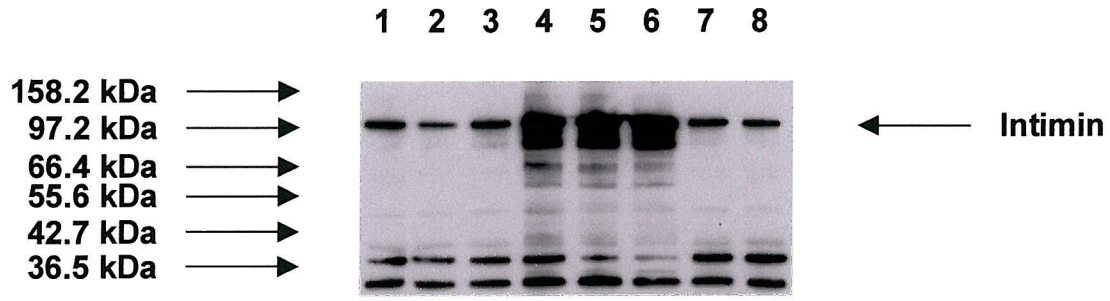


Figure 7.10. Immunoblot of whole cell extracts from AG2, AG2(pII30) and AG2(pTrc99A) probed with anti-intimin antibodies. Bacteria were grown in LB medium supplemented with IPTG as indicated, for 3 hours to an OD_{600} of 1.5. Whole cell extracts were prepared and protein samples were separated by PAGE on a 4-12% BisTris NuPAGE gel, transferred onto nitrocellulose and probed with antisera specific to intimin. Immunoreactive bands were visualised using SuperSignal chemiluminescence substrate. Samples for lanes 1, 3 and 7 were grown in LB medium; samples for lanes 2, 6 and 8 were grown in LB medium containing 1.0 mM IPTG; the samples in lanes 4 and 5 were grown in LB medium supplemented with 0.1 or 0.5 mM IPTG respectively. Lanes 1 + 2, AG2; lanes 3 – 6, AG2(pII30); lanes 7 + 8, AG2(pTrc99A).

7.7 Discussion

These studies have shown that BipA is necessary for the EPEC-directed reorganization of the cytoskeleton in host cells, a consequence of BipA positively controlling the transcription of *ler*. In the absence of *ler*, H-NS mediated negative repression of the LEE operons is maintained and virulence gene expression is suppressed at low levels. BipA has sequence homology with a number of ribosome binding GTPases. Indeed, BipA has recently been shown to associate with ribosomes and to have a GTPase activity that is stimulated by the presence of ribosomes (R. Owens and C. D. O'Connor, unpublished observations). The interpretation of these findings is that BipA operates post-transcriptionally, possibly by binding to the ribosome and directing the translation of a subset of mRNAs. Ler transcript levels were reduced in the *bipA*⁻ strain, and hence it is unlikely that BipA directly controls *ler* expression, (although this is a formal possibility and cannot be dismissed). Therefore we anticipate that BipA controls the translation of another protein, Factor X, which functions to activate/enhance the transcription of *ler*.

A *lacZ* fusion with the *LEE4* encoded *espD* gene was produced for transposon mutagenesis studies. β-galactosidase measurement with this strain further supported a decrease in the transcription of *LEE4* components in the *bipA*⁻ strain. Initial screens using a transposon mutagenesis approach, to identify Factor X suggest that the basal level of β-galactosidase production, as indicated by blue colouration on X-gal plates, may be too high to discriminate true up-regulations from a large number of colonies. It is possible that the approach could be modified to carry out an ELISA based screen of a number of colonies, monitoring β-galactosidase activity by using *o*-nitrophenyl-β-D-galactopyransoide as a substrate. Gene dosage of transcriptional activators of the LEE is an important consideration, and the constitutive expression of a factor involved in regulating *ler* may not be ideal. Thus the transposon approach using a constitutive promoter remains to be proved.

The transcriptional regulator SlyA was shown to complement the *bipA*⁻ defect in terms of intimin expression. However, whether SlyA is Factor X, or if the presence of SlyA at high levels can overcome the requirement for BipA, is unclear. Several lines of evidence argue against BipA controlling *ler* through SlyA: (i) SlyA overexpression results in uncontrolled expression of the virulence factors, while E2348/69 *slyA*⁻ mutants show little difference in activation of protein secretion from the wild-type strain (S. Swift, in preparation). Since SlyA corrects the BipA defect, we would presume SlyA to be downstream in the cascade, i.e. BipA would act on SlyA. We have clearly demonstrated that *bipA*⁻ mutants have severely abrogated *ler* transcript levels, which results in low expression of EspA, EspB, EspD, Tir and intimin. As a consequence we would expect a mutation in Factor X to show the same phenotype as a mutation in BipA. (ii) SlyA mutants exhibit the same phenotype as the parental strain, it is only on overproduction of SlyA that an effect is seen. (iii) SlyA is implicated in controlling flagella mediated motility, over-expression or deletion of SlyA suppresses flagellar-mediated swimming motility, while EPEC *bipA*⁻ mutants are more motile than the wild-type. (iv) *slyA*⁻ mutants do not affect the FAS test, while *bipA*⁻ mutants fail to trigger cytoskeletal rearrangements. From the available evidence, it would suggest that, as with the overexpression of a cloned copy of Per or Ler in the *bipA*⁻ EPEC, the overexpression of the transcriptional activator SlyA overcomes the requirement for BipA.

E2348/69 *bipA*⁻ strains expressing SlyA on a multicopy plasmid produce a pattern of intimin breakdown products. However, these are different from the pattern observed with *bipA*⁺ breakdown products. In E2348/69 wild-type there is a strong band corresponding to a putative breakdown product of around 57 kDa, which is not so prominent in the *bipA*⁻ strains containing a cloned copy of SlyA. This adds weight to BipA controlling the proteolytic degradation of intimin. It is interesting to note that overexpression of SlyA results in the induction of the autotransporter protein EspC (S. Swift, in preparation).

CHAPTER 8

A TRANSPOSABLE P_{BAD} PROMOTER AND ITS USE TO CONSTRUCT A *bipA* DERIVATIVE WITH ARABINOSE-INDUCIBLE EXPRESSION

CHAPTER 8: A TRANSPOSABLE P_{BAD} PROMOTER AND ITS USE TO CONSTRUCT A *bipA* DERIVATIVE WITH ARABINOSE-INDUCIBLE EXPRESSION

8.1 Introduction

A plasmid in which the level of BipA expression could be controlled from an inducible promoter was desired for complementation studies. Possibly the most direct approach to produce such a construct would be to PCR amplify the *bipA* gene and insert it into an already available expression plasmid. However, an alternative and more generally useful approach was to construct a transposable delivery system in which a transposon carrying a highly regulable promoter could be placed upstream of the gene of interest following its *in vitro* transposition. This chapter describes the production of a transposon carrying the highly regulable arabinose-inducible P_{BAD} promoter. To illustrate the use of the transposable promoter strategy, we have placed the *bipA* gene under the control of the P_{BAD} promoter.

The inducible high-level synthesis of proteins from their cloned genes introduced into *E. coli* is now widely used for purification and functional analysis studies (Sambrook *et al.*, 1989). Protein expression is commonly achieved by inserting the gene of interest downstream of a strong but regulable promoter in an expression vector. However, this requires prior knowledge of the location and size of the open reading frame for the gene of interest and its success is often dictated by the availability of appropriate restriction enzyme sites flanking the gene that can be used for its insertion into the expression vector. Additionally there is usually no direct selection for derivatives of the expression vector that contains the inserted gene. Although we have sequence information for *bipA*, this chapter describes an alternative approach that exploits an efficient *in vitro* transposition system based on transposon Tn5 (Goryshin and Reznikoff, 1998; Goryshin *et al.*, 2000), to place the *araC* gene and the highly controllable P_{BAD} promoter adjacent to a gene of interest. The AraC protein is both a positive and negative regulator, transcription of P_{BAD} is induced on

addition of L-arabinose, in the absence of this sugar transcription occurs at very low levels and can be repressed further by growth in the presence of glucose, which reduces the levels of 3', 5'- cyclic AMP, lowering the expression of the catabolite-repressed P_{BAD} promoter (Englesberg and Wilcox, 1974; Lee *et al.*, 1981; Lee *et al.*, 1987; Guzman *et al.*, 1995; Schleif, 2000). Insertion of the promoter immediately upstream of an open reading frame effectively renders gene expression dependent on the presence of L-arabinose. The method also facilitates expression optimisation studies by producing an array of constructs with variable distances between the P_{BAD} promoter and the gene.

Results in this chapter illustrate that placing the transcription of the gene for *bipA* under the control of the P_{BAD} promoter following *in vitro* transposition can be used to render the low temperature growth of AG2 dependent on arabinose.

8.2 Construction of a transposable promoter

For the requirements of this study and for future applications, we desired a transposon with (i) a self-contained and highly regulable promoter; (ii) suitable flanking inverted repeats that would be efficiently recognized by Tn5 transposase *in vitro*; (iii) a genetic element that would block transcription from endogenous promoter(s) located 5' to the open reading frame of interest and (iv) a selectable marker. Criteria (i) and (ii) were fulfilled by inserting a fragment of the *E. coli ara* operon, carrying the *araC* regulatory gene and the P_{BAD} promoter, between the 19 bp mosaic inverted repeats of Tn5 present in the plasmid pMOD<MCS>. The repressor/activator protein, AraC, specified by the *araC* gene blocks transcription from P_{BAD} when L-arabinose is replaced by D-glucose (Englesberg and Wilcox, 1974; Lee *et al.*, 1981; Lee *et al.*, 1987). To meet criteria (iii) and (iv) an Ω interposon, containing a streptomycin/spectinomycin resistance gene and strong transcription and translation terminators (Prentki and Krisch, 1984) was placed immediately 5' to the *araC* gene. The Omega interposon bearing a streptomycin/spectinomycin resistance marker flanked by strong transcriptional and translational terminators (Prentki and Krisch, 1984), was inserted as a 2.1 kb *Eco*RI fragment from plasmid pHP45Ω (Prentki and Krisch, 1984) into the *Eco*RI site of pMOD<MCS> generating the plasmid pAJG27. A 1.3 kb *Sac*I-*Xba*I fragment carrying *araC* and P_{BAD} from plasmid pMPM123 (Mayer, 1995) was then inserted into the *Sac*I and *Xba*I sites of plasmid pAJG27 to construct the plasmid pAJG28. The *in vitro* transposon (TnΩP_{BAD}) thus made could be conveniently excised from pAJG28 as a 3.5 kb fragment using *Pvu*II. (Figure 8.1 summarises the production of TnΩP_{BAD}).

8.3 *In vitro* transposition of pAJG9 with TnΩP_{BAD}

The *in vitro* transposition reaction was accomplished as described in section 2.2.21 of Chapter 2. Briefly, a 3.5 kb *Pvu*II fragment carrying TnΩP_{BAD} was excised from pAJG28, gel purified and mixed with a hyperactive mutant form of Tn5 transposase. The resulting protein:DNA complex was then added to pAJG9 DNA, carrying the

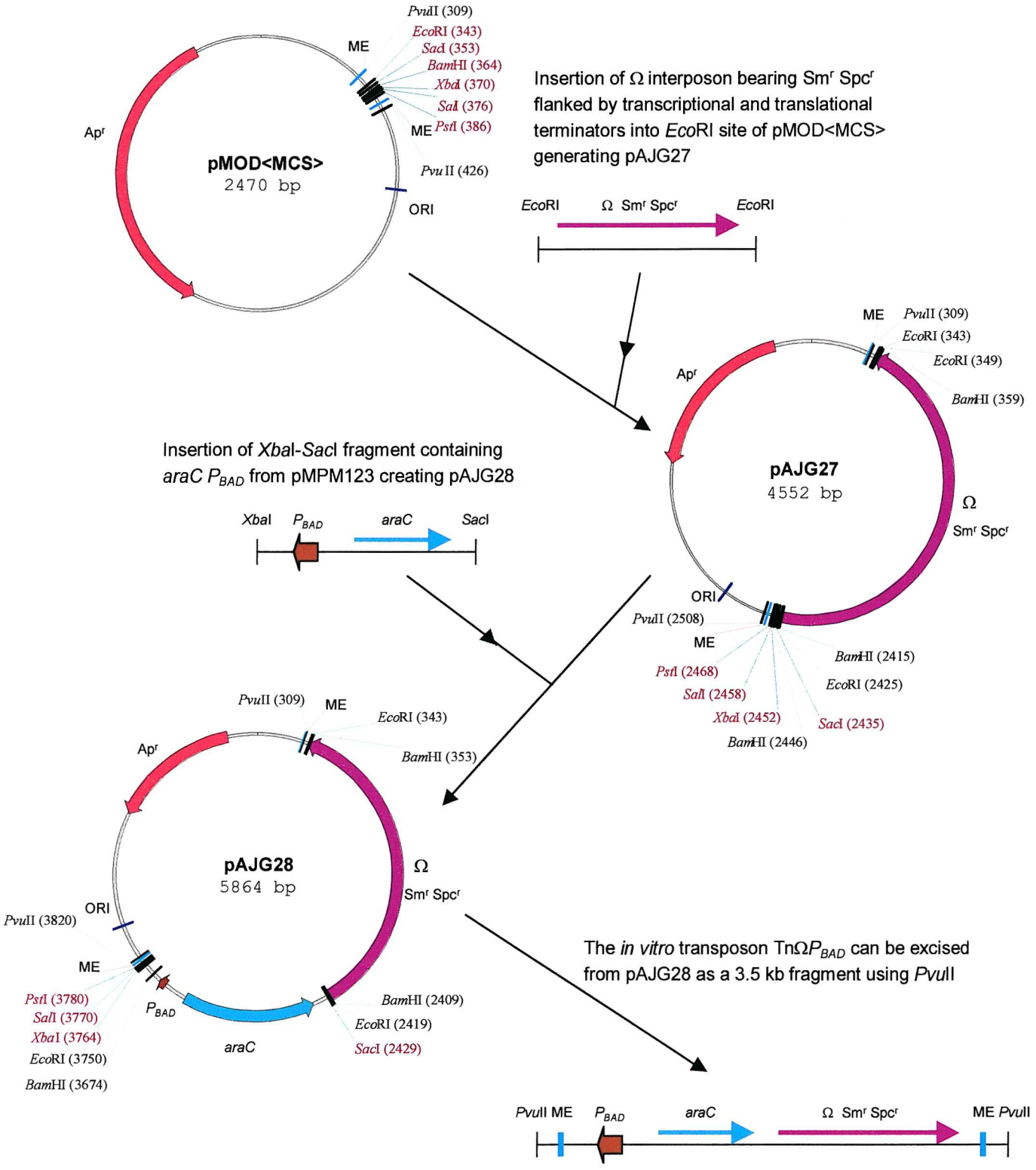


Figure 8.1. Schematic representation of the construction of the *in vitro* transposable promoter TnΩP_{BAD}. ME indicates the 19 bp mosaic ends that are recognised by Tn5 transposase. Restriction sites important for cloning are shown, unique restriction sites are depicted in brown type.

E2348/69 *bipA* gene and 387 bp of upstream sequence, and transposition was completed by the addition of Mg^{++} ions (Goryshin *et al.*, 2000; Goryshin and Reznikoff, 1998). Following the introduction of the treated plasmid DNA into *E. coli* DH5α, transformants bearing the transposable promoter were selected on plates containing spectinomycin and streptomycin.

DNA sequence analysis of three of the resulting spectinomycin and streptomycin resistant transformants showed that in one isolate the transposable promoter had inserted 443 bp upstream of the *bipA* open reading frame in the correct orientation. (Sites of insertion are illustrated in Figure 8.2)

This plasmid, pAJG31, was transformed into the E2348/69 *bipA*⁻ strain AG2, and the level of BipA expression from this plasmid was measured in the presence and absence of inducer L-arabinose. Previous arabinose induction kinetics studies using the plasmid pMPM123 (Mayer, 1995) were performed in SB medium. This media, along with LB and TB media (which had been used for previous BipA phenotypic studies, cold-shock and motility respectively), were used to monitor BipA expression. Mayer, 1995, demonstrated that expression of cloned genes in pMPM123 reached maximal induction upon addition of 0.001% L-arabinose in bacterial strains which are deleted for *ara* genes, while 1% L-arabinose is required for full induction in strains that can ferment L-arabinose. An induction range of 0.001 to 1.0% L-arabinose was used for these studies. Bacteria were grown to an OD_{600} of 0.6-0.7, whole cell extracts were prepared, protein samples separated by PAGE, transferred to nitrocellulose and probed with BipA-specific antibodies. BipA was expressed in cells grown in the presence of L-arabinose, but also when cells were grown in glucose containing medium (Figure 8.3), which is an efficient repressor of the P_{BAD} promoter (Guzman *et al.*, 1995). Previous searches have suggested that the native promoter(s) for BipA were located between the P_{BAD} promoter and the start of the open reading frame for BipA. Thus *bipA* can be transcribed from the *bipA* promoter(s) in the presence or absence of L-arabinose or D-glucose, as well as a possible contributory effect of the P_{BAD} promoter in the presence of L-arabinose. Interestingly the BipA

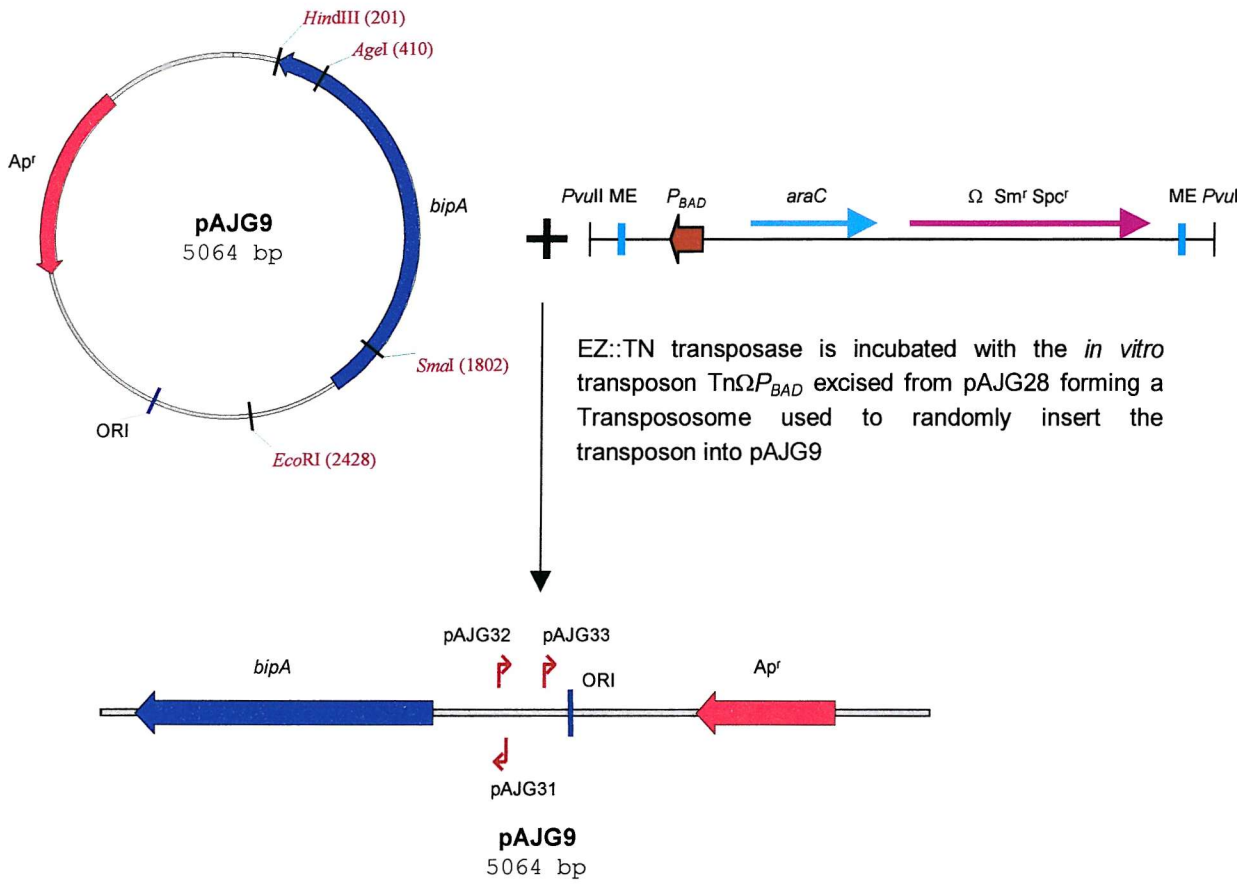


Figure 8.2. Schematic indicating the *bipA*$^{+}$ plasmid pAJG9 showing some sites of TnΩP_{BAD} insertion following its *in vitro* transposition. The designations of the plasmid derivatives carrying the *in vitro* transposon are indicated above and below the arrows indicating the sites of insertion.

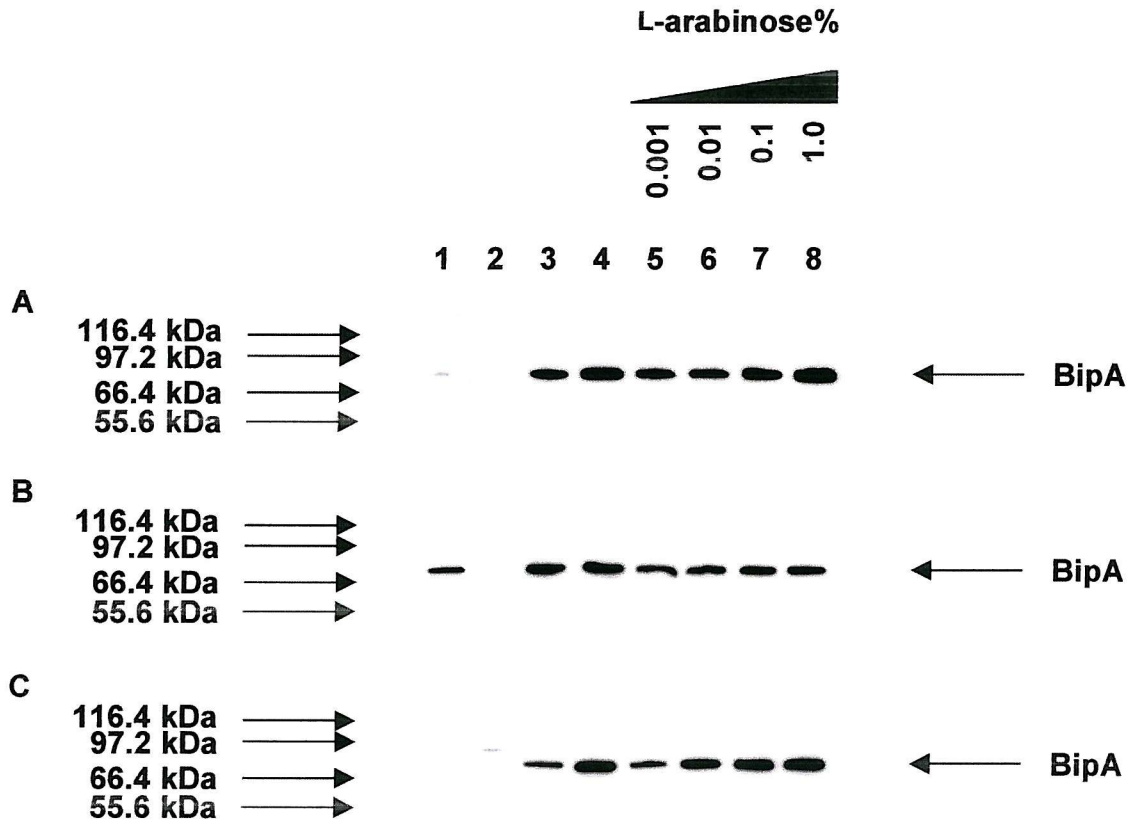


Figure 8.3. BipA protein expression from AG2(pAJG31) in the presence or absence of inducer L-arabinose. Immunoblots of whole cell extracts from E2348/69, AG2 and AG2(pAJG31) grown in (A) LB medium, (B) SB medium, (C) TB medium. Bacteria were grown to an OD_{600} of 0.6-0.7; whole cell extracts were prepared as previously detailed. Protein samples were separated by PAGE on 4-12% BisTris NuPAGE gels, transferred onto nitrocellulose and probed with anti-BipA antibodies. Lanes 1 + 2 contain extracts from E2348/69 and AG2 respectively while lanes 3 – 8 contain extracts of AG2(pAJG31). Samples for lanes 1 – 3 were grown with no additions to the medium, whereas the sample in lane 4 had the addition of 0.4% D-glucose. The samples in lanes 5 – 8 were grown in media supplemented with 0.001, 0.01, 0.1 or 1.0% L-arabinose respectively.

expression profile does not change as the concentration of L-arabinose is increased; also BipA expression in the wild-type E2348/69 appears to differ in different media. Growth of E2348/69 in SB media seems to indicate higher wild-type BipA expression than growth of the bacteria in LB, which showed reduced BipA expression, and TB media in which wild-type BipA expression is not detected.

8.4 Placing the cloned E2348/69 *bipA* gene under the control of the P_{BAD} promoter

To remove the *bipA* promoter region in pAJG31, a 2.0 kb *Sal*I-*Pst*I fragment was excised from pAJG31 and replaced with a 1.5 kb *Sal*I-*Pst*I fragment, PCR amplified from the EPEC E2348/69 chromosome using the primers AJG095 and AJG096 (Table 2.4). Primer AJG095 contained stop codons in all three reading frames; restriction sites for spacing and a consensus Shine-Dalgarno sequence preceding the GTG start codon of *bipA*. The resulting plasmid was designated pAJG38, and the *bipA* gene was sequenced on both strands to confirm its structure. Figure 8.4 shows a schematic of the production of a *bipA* derivative with arabinose-inducible expression.

BipA expression before and after induction of the P_{BAD} promoter was tested. In contrast to the results obtained with pAJG31, immunoblotting showed that BipA expression due to pAJG38 was very tightly repressed in D-glucose-grown cells but was readily induced by addition of L-arabinose (Figure 8.5). BipA was not induced in the absence of any sugar, indicating the tight repression by the P_{BAD} promoter in the absence of L-arabinose. Expression of the *bipA* gene reached maximal induction upon addition of 0.1% L-arabinose in SB and TB media, possibly 1.0% L-arabinose in LB media. Interestingly the maximal induction was not greater than wild-type expression levels in SB media. As previously demonstrated, the levels of wild-type BipA induction were low in LB and TB medium compared to SB medium. There appeared to be an unspecific band cross-reacting around 84 kDa, that was repressed by 0.4% glucose, and concentrations of L-arabinose greater than 0.001% in TB media, (this band is just visible in lane 2 of Figure 8.3C)

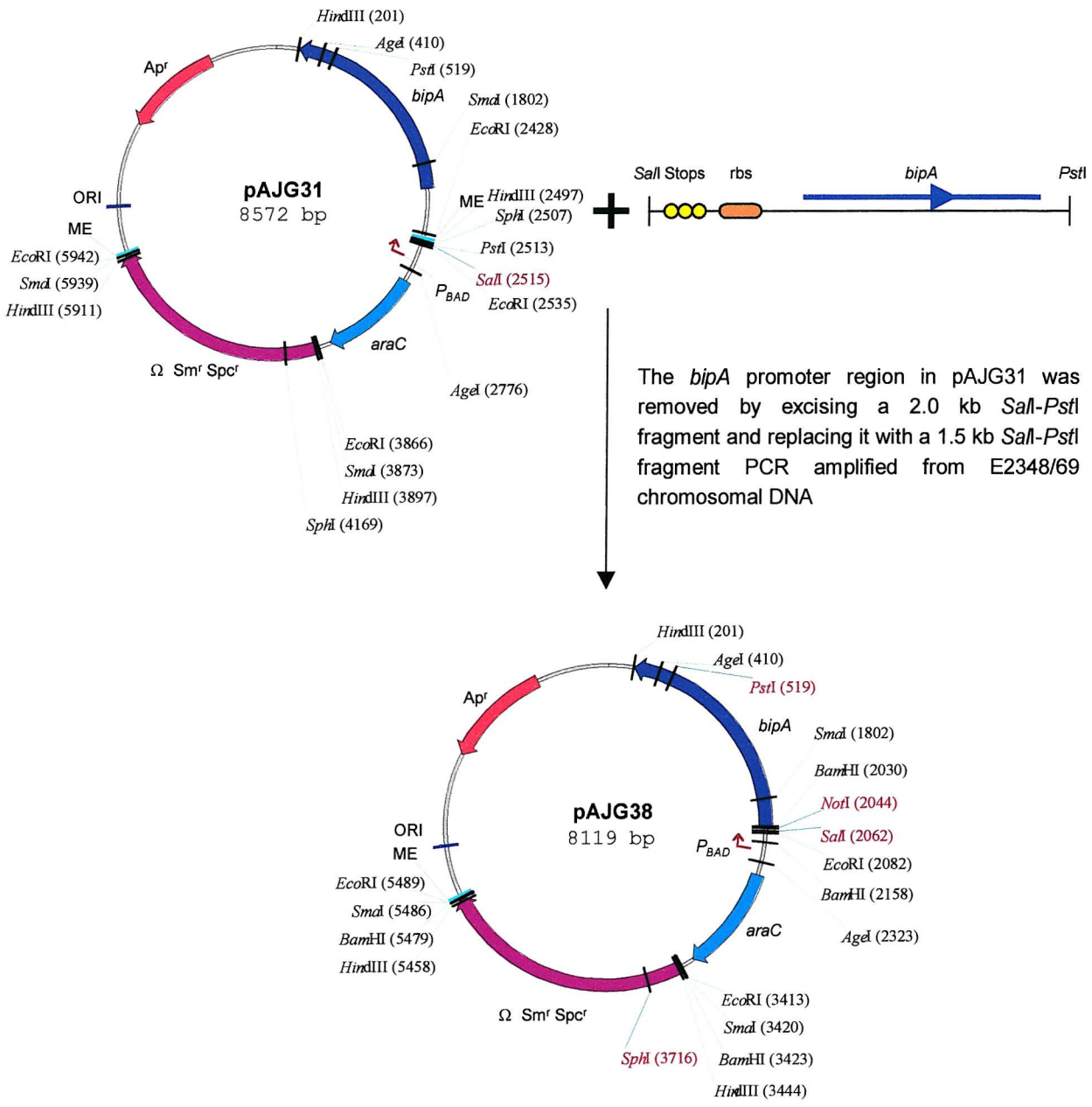


Figure 8.4. Schematic indicating the production of a *bipA* derivative with arabinose-inducible expression. Restriction sites important for cloning are shown, unique restriction sites are depicted in brown type.

Figure 8.5. BipA protein expression from AG2(pAJG38) in the presence or absence of inducer L-arabinose. Immunoblots of whole cell extracts from E2348/69, AG2 and AG2(pAJG38) grown in (A) LB medium, (B) SB medium, (C) TB medium. Bacteria were grown to an OD_{600} of 0.6-0.7; whole cell extracts were prepared as previously detailed. Protein samples were separated by PAGE on 4-12% BisTris NuPAGE gels, transferred onto nitrocellulose and probed with anti-BipA antibodies. Lanes 1 + 2 contain extracts from E2348/69 and AG2 respectively while lanes 3 – 8 contain extracts of AG2(pAJG38). Samples for lanes 1 – 3 were grown with no additions to the medium, whereas the sample in lane 4 had the addition of 0.4% D-glucose. The samples in lanes 5 – 8 were grown in media supplemented with 0.001, 0.01, 0.1 or 1.0% L-arabinose respectively.

8.4.1 Construction of an *E. coli* strain with L-arabinose-dependent growth at low temperatures

These studies (also R. Haigh and P. Williams unpublished observations) indicate that the BipA protein is required for the growth of both EPEC and *E. coli* K-12 at temperatures below 30°C. Having demonstrated the induction of BipA from pAJG38 in the presence of L-arabinose, and repression in the absence of this sugar, or in the presence of D-glucose, we reasoned that growth of AG2(pAJG38) should be dependent on L-arabinose at low temperature. To confirm this, AG2(pAJG38) was tested for growth at 27°C and 37°C on LB, SB and TB plates. In keeping with the immunoblotting results (Figure 8.5), AG2(pAJG38) failed to form colonies at 27°C after 12 hours incubation on plates containing D-glucose or low concentrations of L-arabinose. In contrast it grew as well as the equivalent *bipA*$^+$ strain when L-arabinose was present at concentrations of ≥0.1% (Figure 8.6 shows a representative example, plates for the other media showed the same trend). These results demonstrate the efficacy of the P_{BAD} transposable promoter, and demonstrate that it is capable of very tight control of BipA expression, even when present on a multi-copy plasmid. In keeping with the immunoblot results for AG2(pAJG31), this strain grew as well as the equivalent *bipA*$^+$ strain at 27°C in the presence or absence of either sugar (data not shown).

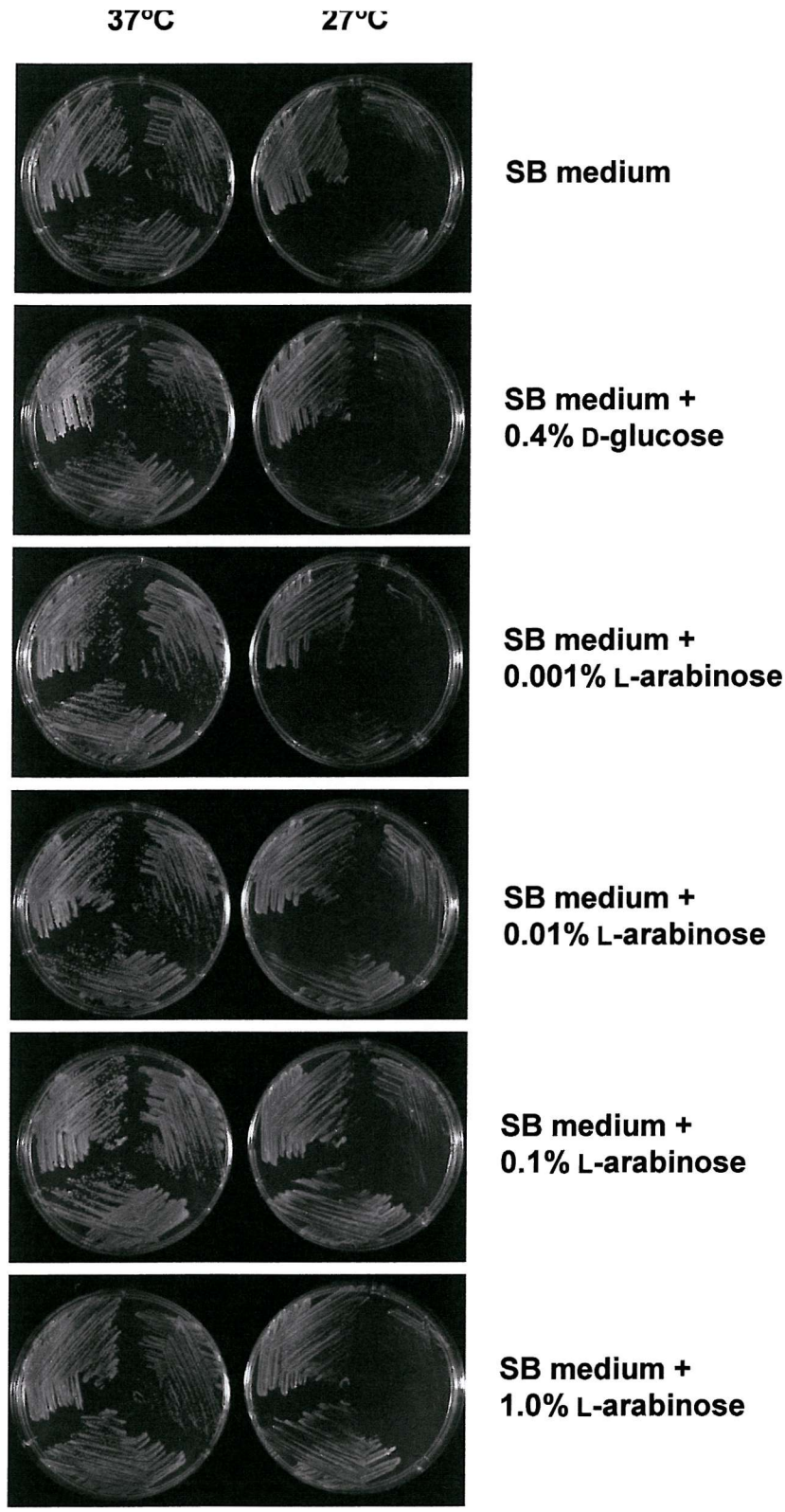


Figure 8.6. Arabinose dependent growth of AG2(pAJG38) at low temperatures. SB plates containing E2348/69, AG2 and AG2(pAJG38) were supplemented with D-glucose or L-arabinose at the concentrations shown, and incubated for 12 hours at 37°C or 27°C. The top left segment of each plate corresponds to E2348/69, whereas the top right and bottom segments correspond to AG2 and AG2(pAJG38), respectively.

8.5 Discussion

It is often desirable to have a system in which the expression of a gene of interest can be tightly regulated, allowing induction and repression under certain conditions. We sought to place a cloned copy of the E2348/69 *bipA* gene under the control of a tightly regulated and inducible promoter. Some commonly used negatively regulated promoters such as the isopropyl-D-thiogalactoside (IPTG) *tac* promoter, are less tightly regulated than positively regulated promoters (Judson and Mekalanos, 2000). Thus the positively regulated P_{BAD} promoter, which is activated by the product of the *araC* gene in the presence of L-arabinose, was chosen (Guzman *et al.*, 1995). A Tn5-derived *in vitro* transposition system was used to place the EPEC E2348/69 *bipA* gene under the control of P_{BAD} on a multi-copy plasmid. As well as demonstrating the tight regulation of BipA expression, the use of TnΩP_{BAD} was demonstrated by generating an E2348/69 strain in which growth at low temperatures is dependent on a threshold concentration of L-arabinose.

Although immunoblot analysis showed an induction profile characteristic of an induction range that led to a maximal level of BipA expression, and the cold-sensitive growth phenotype indicated a threshold level of induction, followed by an L-arabinose concentration dependent increase. Induction of P_{BAD} by L-arabinose is not linear but an all-or-nothing induction due to the fact that the transporter for the inducer is under the control for the inducer itself. (Siegele and Hu, 1997). Intermediate concentrations of the inducer give rise to subpopulations of cells that are fully induced and fully uninduced (Siegele and Hu, 1997). This can be overcome by placing the expression of *araE*, which codes for a low-affinity, high capacity transporter of L-arabinose, under independent control (Khlebnikov *et al.*, 2000). This may be desirable to produce a homogenous population of induced bacteria.

As well as producing an inducible *bipA* construct we wanted to produce a transposable promoter system for future general applications. Other advantages of the system produced in these studies include its stability – unlike normal transposons

TnΩP_{BAD} lacks a transposase gene and hence cannot transpose once it is established. It is also useful to be able to vary the distance between a gene and the promoter. This can change the expression by over 1000-fold in different constructs (Roberts *et al.*, 1979). Thus, it is often prudent to analyse a range of derivatives, in which a promoter is located at varying distances from the genes of interest, to optimise gene transcription. The Tn5-based transposable promoter approach described in this chapter is potentially useful in this respect as it displays little target specificity and hence simultaneously produces multiple derivatives with variable distances between the gene of interest and the P_{BAD} promoter. A further advantage is that the procedure does not require prior knowledge of the location of the open reading frame for the gene of interest, provided the gene product can be readily assayed. Finally, fragment insertion is polar, due to the presence of the Ω interposon on the transposon, thus this transposon approach may prove useful for defining operons, as it allows the expression of co-transcribed genes to be uncoupled.

CHAPTER 9

GENERAL DISCUSSION

CHAPTER 9: GENERAL DISCUSSION

9.1 General Discussion

Previous proteomic studies have uncovered a novel GTPase termed BipA that regulates a number of stress- and virulence-associated processes in *E. coli* and *Salmonella*. Since BipA has orthologues in non-pathogenic as well as pathogenic organisms it is likely that it has a general role, and controls pathways other than those involved in virulence. Previous studies have indicated that in the poorly characterised EPEC strain MAR001, BipA regulates several important pathogen-host cell interactions and is tyrosine phosphorylated (Farris *et al.*, 1998). The available evidence suggests that the BipA GTPase is a global regulatory protein that may operate via a totally novel mechanism.

Owing to the involvement of BipA in regulating the A/E lesion of EPEC, the key aims of these studies were to explore the phenotypes of BipA null mutants in more detail and to pinpoint the role of BipA in A/E lesion formation. To achieve these aims it was necessary to clone a biologically active form of *bipA* and to construct *bipA*$^-$ mutants in E2348/69 and JPN15. Several lines of evidence suggest that the active wild-type form of the *bipA* gene has been cloned. Similarly, three lines of evidence indicate that the *bipA* null mutants have been successfully constructed; evidence for the involvement of BipA in virulence is also presented, along with areas for future investigation.

9.2 Cloning and sequence analysis of the *bipA* gene from EPEC strain E2348/69

The successful cloning of an active EPEC homologue of *bipA* was confirmed during the functional characterisation of the *bipA* gene using such biological assays as cold shock, motility, and expression of LEE encoded virulence factors. Interestingly these

studies have also highlighted that EPEC BipA, C-terminally truncated by 16 amino acids retains functional activity.

9.3 Construction of *bipA* null mutants

bipA$^-$ mutants were successfully produced in the EPEC strain E2348/69 and the plasmid-cured derivative, JPN15. The production of these mutants was confirmed by (*i*) PCR amplification, using primers to chromosomal regions of DNA upstream and downstream of *bipA*; (*ii*) Southern hybridisation using a range of restriction endonucleases; (*iii*) Western blotting. No BipA specific band was seen in the immunoblots of whole cell extracts using polyclonal antisera to BipA, whereas the protein was readily detected in the positive control samples.

EPEC *bipA*$^-$ mutants exhibited different phenotypes to the wild-type strain in assays such as cell motility, cold sensitivity of growth and differences in the synthesis of key effector proteins. However, when the *bipA* gene was supplied *in trans* to these mutants on a high copy number plasmid, the wild-type phenotype was restored. This confirmed that an active form of the *bipA* gene had been successfully cloned and that a lesion in *bipA* was responsible for the mutant phenotype.

9.4 The role of EPEC BipA in growth

Although no differences in the growth rate of wild-type and *bipA*$^-$ EPEC were observed during growth into stationary phase at 37°C, in either nutrient rich or depleted media, these studies have highlighted that EPEC and *E. coli* K-12 BipA, are essential for growth at low temperature. However, this is not the case for *bipA*$^-$ mutants of *Salmonella* Typhimurium or *Salmonella* Enteritidis, which do not show a reduced growth phenotype at 27°C. Thus, BipA may not be an essential gene in *Salmonella* or, alternatively, the factor(s) that are affected by BipA depletion in *E. coli* are functionally redundant.

9.5 The role of EPEC BipA in virulence-related processes

The cloned *bipA* gene and null mutants of *bipA* have been used to explore the involvement of the factor in two key virulence-related processes, namely, flagellar-mediated cell motility and the expression of proteins that are required for an attaching and effacing lesion.

Motility is an essential virulence attribute for many gastrointestinal pathogens, not least because it allows colonization of many inaccessible niches (Ottemann and Miller, 1997). As with a previous *bipA*⁻ mutant in an EPEC strain of a different serotype, it was found that removal of *bipA* (in E2348/69 or JPN15) resulted in hypermotility, and increased secretion of flagellin into the culture media. Interestingly, similar *bipA*⁻ mutants of *E. coli* K-12, *Salmonella* Typhimurium and *Salmonella* Enteritidis display the opposite phenotype, i.e. they have slightly reduced motility relative to the parent strain (A. Grant, N. Kinsella, A. White and C.D. O'Connor, unpublished observations). Thus BipA appears to regulate a highly conserved set of genes in a very different manner in EPEC as opposed to *E. coli* K-12 and *Salmonella*. Farris *et al.* (1998) showed that in EPEC, BipA negatively regulates not just flagellin, but also the number of flagellar filaments, and cell motility in general. They therefore propose that BipA operates early on in the flagellar assembly pathway. The most probable point of regulation is at the level of the master operon of the flagellar gene hierarchy (i.e. the class I genes, *flhCD*). Although the precise mode of control remains to be elucidated, the evidence obtained in the present study would appear to rule out BipA-mediated control of H-NS protein a key regulator of *flhCD*, as no significant difference in its level was detected between the wild-type and *bipA*⁻ mutant.

The second virulence-related process that was investigated was the formation of an attaching and effacing lesion. This process, which is a hallmark of EPEC infection, requires the co-ordinated expression and secretion of several key effector proteins

into host cells, including Tir, EspA, EspB and EspD. Additionally, the A/E lesion can only form if EPEC cells display intimin on their cell surfaces.

The results presented in Chapter 6 indicate that BipA is required for the LEE-directed rearrangement of actin in host cells infected with EPEC strain E2348/69. Further studies showed that BipA positively regulates the secreted effector proteins EspA, EspB, EspD and Tir, as well as EspC encoded outside of the LEE. BipA also regulates intimin and possibly its degradation, although further studies are required to delineate whether BipA regulates a protease that is responsible for the down regulation of intimin late on in infection, and whether this protease is EspC. Thus, BipA co-ordinately regulates several processes implicated in EPEC pathogenesis making it a key regulator of EPEC virulence.

The down-regulation of EspA, EspB, EspD, Tir and intimin that occurred in *bipA*$^-$ derivatives of E2348/69 was also observable in another *bipA*$^-$ derivative that lacked the EAF plasmid. This strongly suggests that BipA does not operate via the *per* regulatory system (specified by the EAF plasmid). BipA is not required for the expression of another positive regulator of the LEE, IHF. However, these studies do not preclude the possibility that BipA negatively regulates the expression of a factor that prevents IHF from binding to its recognition site upstream of *ler*, thus preventing IHF mediated activation of *ler*. Analysis of *ler* transcripts showed that BipA mediates the positive control of LEE encoded factors through *ler*. *ler* transcripts are abolished in EPEC *bipA*$^-$ strains and, in the absence of Ler, H-NS mediated negative repression of the LEE is maintained and virulence gene expression is suppressed at low levels. Based on the available evidence of the mechanism of BipA, it is most likely that BipA operates indirectly. The simplest hypothesis is that BipA controls the translation of a DNA binding factor – 'Factor X' that then regulates *ler* transcription. This is also the simplest explanation for the studies of Rowe *et al.* (2000), who showed that BipA regulates the transcription of genes involved in the synthesis of the *E. coli* K5 capsule.

Further studies are required to characterise 'Factor X'. It would be informative to carry out mobility-shift assays using a DNA fragment carrying the *ler* regulatory region to determine if a DNA binding protein 'Factor X' is missing in the *bipA*⁻ EPEC mutant. If this could be established then a major focus of future research would be to purify 'Factor X' and to determine if BipA regulated it at the transcriptional or post-transcriptional level.

9.6 The BipA regulatory mechanism

Nogueira and Springer, (2000) suggest that a true regulator should be able to activate or repress gene expression in either a direct, or indirect response to the environment or the physiological state of the cell. Collectively, these studies, those by Farris *et al.* (1998) and Rowe *et al.* (2000) suggest that BipA is indeed a global regulator. BipA is unrelated to other known virulence or global regulatory proteins, but shares sequence similarity to GTPases that interact with ribosomes, suggesting that it may regulate target proteins by a novel mechanism operating at the level of the ribosome. Recently several global regulators have been shown to operate post transcriptionally, controlling gene expression at the level of translation and mRNA stability (Nogueira and Springer, 2000). However, none of the known regulators that operate post-transcriptionally use mechanisms that are consistent with the properties of BipA.

BipA can bind to ribosomes in a guanine nucleotide independent manner, although, its GTPase activity is stimulated in the presence of 70S ribosomes (R. Owens and C. D. O'Connor, unpublished observations). Indeed, BipA appears to bind to either the same site or an overlapping site on the ribosome as EF-G, albeit the affinity of EF-G for the ribosome is greater than that of BipA (R. Owens and C. D. O'Connor, unpublished observations). The present studies have highlighted that the GTPase activity of BipA is critical for controlling motility and cold shock, as observed by the inability of the EPEC BipA mutant, G77V, to transcomplement the *bipA*⁻ strains. However, in terms of the mechanism of action of BipA, the significance of the sequence similarity to ribosome binding GTPases, and the stimulation of GTPase

activity in the presence of ribosomes is unclear. It is possible that the ribosome activates BipA. The 'inactive' BipA GTPase may bind to the 70S ribosome, the ribosome could then catalyse a conformational change of BipA with the hydrolysis of GTP to GDP, and the then 'active' BipA-GDP could act elsewhere. However, this would be a unique mechanism of action, as other GTP binding proteins, are active in the GTP bound form and inactive in the GDP bound form (Kjeldgaard *et al.*, 1996). An alternative hypothesis is that BipA affects some aspect of the translation process; For example, BipA could act as an elongation factor for the translation of a subset of mRNAs.

The initiation codon in mRNA is generally AUG (methionine). Occasionally, however, AUG is replaced by GUG (valine) or UUG (leucine). BipA has an unusual start codon GUG. Thus, one hypothesis could be that BipA acts as a translation factor for proteins with unusual start codons including itself. It is intriguing that a number of genes involved in control of motility such as *flbB*, *hdfR* and *flhD* have GUG start codons, although this could be purely coincidental. *lacI* which codes for the Lac repressor starts with GUG. Thus if BipA was required as an elongation factor for unusual start codons, differences in β-galactosidase expression might be expected on X-gal containing medium between wild-type and *bipA*$^-$ strains. The lack of a discernible difference in the intensity of blue colouration of EPEC wild-type and *bipA*$^-$ strains grown on X-gal containing medium suggests this hypothesis is unlikely (A. Grant, unpublished observations). However, it is possible that additional mechanisms compensate for decreased translational initiation of *lacI* mRNA, and further investigation is merited.

Previous studies on TypA/BipA suggest that the protein repeatedly co-purified with preparations of RNA polymerase (Freestone *et al.*, 1995). Repeated attempts to confirm that BipA copurifies with RNA polymerase, or alternatively is a subunit of RNA polymerase have been unsuccessful (A. Grant, M. Farris and C. D. O' Connor, unpublished observations). Nonetheless, given the reported copurification with RNA

polymerase, and the effects on transcription of *ler* and genes involved in K5 capsule formation, it cannot be discounted that BipA affects transcription.

9.7 Final comment

Evidence is mounting to suggest that BipA may use an unprecedented mechanism to regulate the expression of target proteins - some of which seem to be important for virulence. The production and characterisation of the BipA mutants in this study places us in an ideal position to elucidate the role of the protein in disease caused by a major class of gastrointestinal pathogens. Future studies will undoubtedly uncover additional processes that are regulated by BipA, both in pathogenic and non-pathogenic bacteria. However, of even greater interest will be the elucidation of the mechanism of action of this novel global regulator.

CHAPTER 10

BIBLIOGRAPHY

CHAPTER 10: BIBLIOGRAPHY

A

Abe A., de Grado M., Pfuetzner R.A., Sánchez-SanMartin C., DeVinney R., Puente J.L., Strynadka C.J. and Finlay B.B. (1999) Enteropathogenic *Escherichia coli* translocated intimin receptor, Tir, requires a specific chaperone for stable secretion. *Molec. Microbiol.* **33**, 1162-1175

Adelstein R.S. and Eisenberg E. (1980) Regulation and kinetics of the actin-myosin-ATP interaction. *Ann. Rev. Biochem.* **49**, 921-956

Adu-Bobie J., Frankel G., Bain C., Gonçalves A.G., Trabulsi L.R., Douce G., Knutton S. and Dougan G. (1998) Detection of intimins α, β, γ,and δ, four intimin derivatives expressed by attaching and effacing microbial pathogens. *J. Clin. Microbiol.* **36**, 662-668

Ævarsson A. (1995) Structure-based sequence alignment of elongation factors Tu and G with related GTPases involved in translation. *J. Mol. Evol.* **41**, 1096-1104

Afflerbach H., Schröder O. and Wagner R. (1998) Effects of the *Escherichia coli* DNA-binding protein H-NS on rRNA synthesis *in vivo*. *Molec. Microbiol.* **28**, 641-653

Aiba H., Adhya S. and de Crombrugghe B. (1981) Evidence for two functional *gal* promoters in intact *Escherichia coli*. *J. Biol. Chem.* **256**, 11905-11910

Akira S. and Kishimoto T. (1997) NF-IL6 and NF-κB in cytokine gene regulation. *Adv. Immunol.* **65**, 1-46

Alton, K.N. and Vapnek D. (1979) Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn*9*. *Nature*. **282**, 20-27

Arigoni F., Talabot F., Peitsch M., Edgerton M.D., Meldrum E., Allet E., Fish R., Jamotte T., Curchod M-L. and Loferer H. (1998) A genome-based approach for the identification of essential bacterial genes. *Nature. Biotechnol.* **16**, 851-856

Atlung T. and Ingmer H. (1997) H-NS: a modulator of environmentally regulated gene expression. *Molec. Microbiol.* **24**, 7-17

Azam T.A., Iwata A., Nishimura A., Ueda S. and Ishiama A. (1999) Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J. Bacteriol.* **181**, 6361-6370

Azam T.A. and Ishihama A. (1999) Twelve species of the nucleoid-associated protein from *Escherichia coli*. *J. Biol. Chem.* **274**, 33105-33113

B

Bain C., Keller R., Collington G.K., Trabulsi L.R. and Knutton S. (1998) Increased levels of intracellular calcium are not required for the formation of attaching and effacing lesions by enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **66**, 3900-3908

Bakal C.J. and Davies J.E. (2000) No longer an exclusive club: eukaryotic signalling domains in bacteria. *Trends. Cell. Biol.* **10**, 32-38

Baldwin T.J., Brooks F., Knutton S., Manjarrez-Hernandez H.A., Aitken A. and Williams P.H. (1990) Protein phosphorylation by protein kinase C in Hep-2 cells infected with enteropathogenic *Escherichia coli*. *Infect. Immun.* **58**, 761-765

Baldwin T.J., Ward W., Aitken A., Knutton S. and Williams P.H. (1991) Elevation of intracellular free calcium levels in Hep-2 cells infected with enteropathogenic *Escherichia coli*. *Infect. Immun.* **59**, 1599-1604

Baldwin T.J., Lee-Delaunay M.B., Knutton S. and Williams P.H. (1993) Calcium-calmodulin dependence of actin accretion and lethality in cultured Hep-2 cells infected with enteropathogenic *Escherichia coli*. *Infect. Immun.* **61**, 760-763

Baldwin T.J. (1998) Pathogenicity of enteropathogenic *Escherichia coli*. *J. Med. Microbiol.* **47**, 283-293

Barker H.C. (1997) Antimicrobial peptides derived from the human bactericidal/permeability-increasing protein (BPI): Structural determinants and mechanism of action.

Barker H.C., Kinsella N., Jaspe A., Friedrich T. and O' Connor C.D. (2000) Formate protects stationary-phase *Escherichia coli* and *Salmonella* cells from killing by a cationic antimicrobial peptide. *Molec. Microbiol.* **35**, 1518-1529

Batchelor M., Prasannan S., Daniell S., Reece S., Connerton I., Bloomberg G., Dougan G., Frankel G. and Matthews S. (2000) Structural basis for recognition of the translocated intimin receptor (Tir) by intimin from enteropathogenic *Escherichia coli*. *EMBO J.* **19**, 2452-2464

Beamer L.J., Carroll S.F. and Eisenberg D. (1997) Crystal structure of human BPI and two bound phospholipids at 2.4 Angstrom resolution. *Science.* **276**, 1861-1864

Beamer L.J., Carroll S.F. and Eisenberg D. (1999) The three-dimensional structure of human Bactericidal/Permeability-Increasing protein. *Biochem. Pharmacol.* **57**, 225-229

Ben-Ami G., Ozeri V., Hanski E., Hofmann F., Aktories K., Hahn K.M., Bokoch G.M. and Rosenshine I. (1998) Agents that inhibit Rho, Rac, and Cdc42 do not block formation of actin pedestals in HeLa cells infected with enteropathogenic *Escherichia coli*. *Infect. Immun.* **66**, 1755-1758

Beran R.K. and Simons R.W. (2001) Cold-temperature induction of *Escherichia coli* polynucleotide phosphorylase occurs by reversal of its autoregulation. *Molec. Microbiol.* **39**, 112-125

Berlutti F., Casalino M., Zagaglia C., Assunta Fradiani P., Visca P. and Nicoletti M. (1998) Expresion of the virulence plasmid-carried apyrase gene (*apy*) of enteroinvasive *Escherichia coli* and *Shigella flexneri* is under the control of H-NS and the VirF and VirB regulatory cascade. *Infect. Immun.* **66**, 4957-4964

Berridge M.J. (1993) Inositol triphosphate and calcium signalling. *Nature.* **361**, 315-325

Bertin P., Terao E., Hee Lee E., Lejeune P., Colson C., Danchin A. and Collatz E. (1994) The H-NS protein is involved in the biogenesis of flagella in *Escherichia coli*. *J. Bacteriol.* **176**, 5537-5540

Bertin P., Benhabiles N., Krin E., Laurent-Winter C., Tendeng C., Turlin E., Thomas A., Danchin A. and Brasseur R. (1999) The structural and functional organization of H-NS-like proteins is evolutionarily conserved in Gram-negative bacteria. *Molec. Microbiol.* **31**, 319-329

Beutin L., Gleier K., Kontny I., Echeverria P. and Scheutz F. (1997) Origin and characteristics of enteroinvasive strains of *Escherichia coli* isolated in Germany. *Epidemiol. Infect.* **118**, 199-205

Bieber D., Ramer S.W., Wu C-Y., Murray W.J., Tobe T., Fernandez R. and Schoolnik G.K. (1998) Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. *Science.* **280**, 2114-2118

Blattner F.R., Plunkett G.I., Bloch C.A., Perna N.T., Burland V., Riley M., Collado-Vides J., Glasner J.D., Rode C.K., Mayhew G.F., Gregor J., Wayne-Davis N., Kirkpatrick H.A., Goeden M.A., Rose D.J., Mau B. and Shao Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science.* **277**, 1453-1474

Bolivar F., Rodriguez R.L., Greene P.J., Betlach M.C., Heyneker H.L. and Boyer H.W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene.* **2**, 95-113

Bourne H.R., Sanders D.A. and McCormick F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature.* **349**, 117-127

Bretscher A. and Weber K. (1980) Villin is a major protein of the microvillus cytoskeleton which binds both G and F actin in a calcium-dependent manner. *Cell.* **20**, 839-847

Britton R.A., Powell B.S., Dasgupta S., Sun Q., Margoli W., Lupski J.R. and Court D.L. (1998) Cell cycle aarrest in Era GTPase mutants: a potential growth rate-regulated checkpoint in *Escherichia coli. Molec. Microbiol.* **27**, 739-750

Bustamante V. H., Santana F.J., Calva E. and Puente J.L. (2001) Transcriptional regulation of type III secretion genes in enteropathogenic *Escherichia coli*: Ler antagonizes H-NS-dependent repression. *Molec. Microbiol.* **39**, 664-678

C

Canil C., Rosenshine I., Ruschkowski S., Donnenberg M.S., Kaper J.B. and Finlay B.B. (1993) Enteropathogenic *Escherichia coli* decreases the transepithelial electrical resistance of polarized epithelial monolayers. *Infect. Immun.* **61**, 2755-2762

Cantarelli V.V., Takahashi A., Akeda Y., Nagayama K. and Honda T. (2000) Interaction of enteropathogenic or enterohemorrhagic *Escherichia coli* with HeLa cells results in translocation of cortactin to the bacterial adherence site. *Infect. Immun.* **68**, 382-386

Celli J., Deng W. and Finlay B.B. (2000) Enteropathogenic *Escherichia coli* (EPEC) attachment to epithelial cells: exploiting the host cell cytoskeleton from the outside. *Cellular. Microbiol.* **2**, 1-9

Chang A.C.Y. and Cohen S.N. (1978) Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**, 1141-1156

Chen S-M., Takiff H.E., Barber A.M., Dubois G.C., Bardwell J.C.A. and Court D.L. (1990) Expression and characterization of RNase III and Era proteins. *J. Biol. Chem.* **265**, 2888-2895

Chikami G.K., Fierer J. and Guiney D.G. (1985) Plasmid-mediated virulence in *Salmonella dublin* demonstrated by use of a Tn*5-oriT* construct. *Infect. Immun.* **50**, 420-424

Chilcott G.S. and Hughes K.T. (1998) The type III secretion determinants of the flagella anti-transcription factor, FlgM, extend from the amino-terminus into the anti-σ^{28} domain. *Molec. Microbiol.* **30**, 1029-1040

Chinali G. and Parmeggiani A. (1982) Differential Modulation of the Elongation-Factor-G GTPase activity by tRNA bound to the ribosomal A-site or P-site. *Eur. J. Biochem.* ***125***, 415-421

Clark M.A., Hirst B.H. and Jepson M.A. (1998) M-cell surface β1 integrin expression and invasin-mediated targeting of *Yersinia pseudotuberculosis* to mouse Peyer's patch M cells. *Infect. Immun.* **66**, 1237-1243

Collazo C.M. and Galan J.E. (1997) The invasion-associated type-III protein secretion system in *Salmonella*. *Gene*. **192**, 51-59

Collington G.K., Booth I.W. and Knutton S. (1998a) Rapid modulation of electrolyte transport in Caco-2 cell monolayers by enteropathogenic *Escherichia coli* (EPEC) infection. *Gut*. **42**, 200-207

Collington G.K., Booth I.W., Donnenberg M.S., Kaper J.B. and Knutton S. (1998b) Enteropathogenic *Escherichia coli* virulence genes encoding secreted signalling proteins are essential for modulation of Caco-2 cell electrolyte transport. *Infect. Immun.* **66**, 6049-6053

Cornelis G.R. and Wolf-Watz H. (1997) The *Yersinia* YOP virulon: a bacterial system for subverting eukaryotic cells. *Molec. Microbiol.* **23**, 861-867

Covarrubias A.A. and Bastarrachea F. (1983) Nucleotide sequence of the *glnA* control region of *Escherichia coli*. *Mol. Gen. Genet.* **190**, 171-175

Crane J.K. and Oh J.S. (1997) Activation of host cell protein kinase C by enteropathogenic *Escherichia coli*. *Infect. Immun.* **65**, 3277-3285

Czerucka D., Dahan S., Mograbi B., Rossi B. and Rampal P. (2000) *Saccharomyces boulardii* preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic *Escherichia coli*-infected T84 cells. *Infect. Immun.* **68**, 5998-6004

Czerucka D., Dahan S., Mograbi B., Rossi B. and Rampal P. (2001) Implication of mitogen-activated protein kinases in T84 cell responses to enteropathogenic *Escherichia coli* infection. *Infect. Immun.* **69**, 1298-1305

D

Deibel C., Krämer S., Chakraborty T. and Ebel F. (1998) EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein. *Molec. Microbiol.* **28**, 463-474

Delahay R.M., Knutton S., Shaw R.K., Hartland E.L., Pallen M.J. and Frankel G. (1999) The coiled-coil domain of EspA is essential for the assembly of the type III secretion translocon on the surface of enteropathogenic *Escherichia coli*. *J. Biol. Chem.* **274**, 35969-35974

Dersch P., Kneip S. and Bremer E. (1994) The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. *Mol. Gen. Genet.* **245**, 255-259

DeVinney R., Knoechel D.G. and Finlay B.B. (1999a) Enteropathogenic *Escherichia coli*: cellular harassment. *Curr. Opin. Microbiol.* **2**, 83-88

DeVinney R., Gauthier A., Abe A. and Finlay B.B. (1999b) Enteropathogenic *Escherichia coli*: a pathogen that inserts its own receptor into host cells. *Cell. Mol. Life Sci.* **55**, 961-976

DeVinney R., Stein M., Reinscheid D., Abe A., Ruschkowski S. and Finlay B.B. (1999c) Enterohemorrhagic Escherichia coli O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. *Infect. Immun.* **67**, 2389-2398

Djafari S., Ebel F., Deibel C., Krämer S., Hudel M. and Chakraborty T. (1997) Characterization of an exported protease from Shiga toxin-producing *Escherichia coli*. *Molec. Microbiol.* **25**, 771-784

Donnenberg M.S., Donohue-Rolfe A. and Keusch G.T. (1989) Epithelial cell invasion: An overlooked property of enteropathogenic *Escherichia coli* (EPEC) associated with the EPEC adherence factor. *J. Infect. Dis.* **160**, 452-459

Donnenberg M.S. and Kaper J.B. (1991) Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect. Immun.* **59**, 4310-4317

Donnenberg M.S., Girón J.A., Natarto J.P. and Kaper J.B. (1992a) A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* is associated with localized adherence. *Molec. Microbiol.* **6**, 3427-3437

Donnenberg M.S. and Kaper J.B. (1992b) Enteropathogenic *Escherichia coli*. *Infect. Immun.* **60**, 3953-3961

Donnenberg M.S., Yu J. and Kaper J.B. (1993a) A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. *J. Bacteriol.* **175**, 4670-4680

Donnenberg M.S., Tacket C.O., James S.P., Losonsky G., Nataro J.P., Wasserman S.S., Kaper J.B. and Levine M.M. (1993b) Role of *eaeA* gene in experimental enteropathogenic *Escherichia coli* infection. *J. Clin. Invest.* **92**, 1412-1417

Donnenberg M.S., Kaper J.B. and Finlay B.B. (1997) Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *TIMS.* **5**, 109-114

Donnenberg M.S. (2000) Pathogenic strategies of enteric bacteria. *Nature.* **406**, 768-774

Dower W.J., Miller J.F. and Ragsdale C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucl. Acids. Res.* **16**, 6127-6145

Dytoc M., Fedorko L. and Sherman P.M. (1994) Signal transduction in human epithelial cells infected with attaching and effacing *Escherichia coli in vitro*. *Gastro.* **106**, 1150-1161

E

Echeverria P., Sethabutr O., Serichantalergs O., Lexomboon U. and Tamura K. (1992) *Shigella* and enteroinvasive *Escherichia coli* infections in households of children with dysentery in Bangkok. *J. Infect. Dis.* **165**, 144-147

Elliot S.J., Wainwright L.A., McDaniel T.K., Jarvis K.G., Deng Y.K., Lai L.-C., McNamara B.P., Donnenberg M.S. and Kaper J.B. (1998) The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Molec. Microbiol.* **28**, 1-4

Elliot S.J., Hutcheson S.W., Dubois M.S., Mellies J.L., Wainwright L.A., Batchelor M., Frankel G., Knutton S. and Kaper J.B. (1999) Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. *Molec. Microbiol.* **33**, 1176-1189

Elliott S.J., Sperandio V., Giron J.A., Shin S., Mellies J.L., Wainwright L., Hutcheson S.W., McDaniel T.K. and Kaper J.B. (2000) The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **68**, 6115-6126

Elsbach P. and Weiss J. (1988) Phagocytic cells: oxygen-independent antimicrobial systems. Inflammation: Basic Principles and Clinical Correlates (Gallin J.I., Goldstein I.M. and Snyderman R. eds.) Raven Press, New York. **24**, 445-465

Elsbach P. and Weiss J. (1993) Bactericidal/permeability increasing protein and host defense against gram-negative bacteria and endotoxin. *Curr. Opin. Immunol.* **5**, 103-107

Engelhorn M. and Geiselmann J. (1998) Maximal transcriptional activation by the IHF protein of *Escherichia coli* depends on optimal DNA bending by the activator. *Molec. Microbiol.* **30**, 431-441

Englesberg E. and Wilcox G. (1974) Regulation: positive control. *Ann. Rev. Genet.* **8**, 219-242

Eslava C., Navarro-García F., Czeczulin J.R., Henderson I.R., Cravioto A. and Nataro J.P. (1998) Pet, an autotransporter enterotoxin from enteroaggregative *Escherichia coli*. *Infect. Immun.* **66**, 3155-3163

F

Farris M., Grant A., Richardson T.B. and O'Connor C.D. (1998) BipA: a tyrosine-phosphorylated GTPase that mediate interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. *Molec. Microbiol.* **28**, 265-279

Fields P.I., Groisman E.A. and Heffron F. (1989) A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science*. **243**, 1059-1062

Finlay B.B., Rosenshine I., Donnenberg M.S. and Kaper J.B. (1992) cytoskeletal composition of attaching and effacing lesions associated with enteropathogenic *Escherichia coli* adherence to HeLa cells. *Infect. Immun.* **60**, 2541-2543

Finlay B.B. and Falkow S. (1997) Common themes in microbial pathogenicity revisited. *Microbiol. Molec. Biol. Rev.* **61**, 136-169

Fondacaro J.D. and Henderson L.S. (1985) Evidence for protein kinase C as a regulator of intestinal electrolyte transport. *Am. J. Physiol.* **249**, G422-G426

Foubister V., Rosenshine I., Donnenberg M.S. and Finlay B.B. (1994a) The *eaeB* gene of enteropathogenic *Escherichia coli* is necessary for signal transduction in epithelial cells. *Infect. Immun.* **62**, 3038-3040

Foubister V., Rosenshine I. and Finlay B.B. (1994b) A diarrheal pathogen, Enteropathogenic *Escherichia coli* (EPEC), triggers a flux of inositol phosphates in infected epithelial cells. *J. Exp. Med.* **179**, 993-998

Frankel G., Candy D.C.A., Everest P. and Dougan G. (1994) Characterization of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohemorrhagic *Escherichia coli*, *Citrobacter freundii* and *Hafnia alvei*. *Infect. Immun.* **62**, 1835-1842

Frankel G., Candy D.C.A., Fabiani E., Adu-Bobie J., Gil S., Novakova M., Phillips A.D. and Dougan G. (1995) Molecular characterization of a carboxy-terminal eukaryotic-cell-binding domain of intimin from enteropathogenic *Escherichia coli*. *Infect. Immun.* **63**, 4323-4328

Frankel G., Lider O., Hershkoviz R., Mould A.P., Kachalsky S.G., Candy D.C.A., Cahalon L., Humphries M.J. and Dougan G. (1996) The cell-binding domain of intimin from enteropathogenic *Escherichia coli* binds to β_1 integrins. *J. Biol. Chem.* **271**, 20359-20364

Frankel G., Phillips A. D., Rosenshine I., Dougan G., Kaper J. B. and Knutton S. (1998) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subverisve elements. *Molec. Microbiol.* **30**, 911-921

Freestone P., Grant S., Toth I. and Norris V. (1995) Identification of phosphoproteins *in Escherichia coli*. *Molec. Microbiol.* **15**, 573-580

Freestone P., Trinei M., Clarke S.C., Nyström T. and Norris V. (1998) Tyrosine phosphorylation in *Escherichia coli*. *J. Mol. Biol.* **279**, 1045-1051

Friedberg D., Umanski T., Fang Y. and Rosenshine I. (1999) Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli*. *Molec. Microbiol.* **34**, 941-952

Friedman D.I. (1988) Integration Host Factor: A Protein for All Reasons. *Cell.* **55**, 545-554

G

Gallegos M-T., Schleif R., Bairoch A., Hofmann K. and Ramos J.L. (1997) AraC/XylS family of transcriptional regulators. *Microbiol. Molec. Biol. Rev.* **61**, 393-410

Gauthier A., de Grado M. and Finlay B.B. (2000) Mechanical fractionation reveals structural requirements for enteropathogenic *Escherichia coli* Tir insertion into host membranes. *Infect. Immun.* **68**, 4344-4348

Gay P., Coq D.L., Steinmetz M., Berkelman T. and Kado C.I. (1985) Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J. Bacteriol.* **164**, 918-921

Giladi H., Igarashi K., Ishihama A. and Oppenheim A.B. (1992) Stimulation of the phage λ pL promoter by integration host factor requires the carboxy terminus of the α-subunit of RNA polymerase. *J. Mol. Biol.* **227**, 985-990

Giladi H., Koby S., Prag G., Engelhorn M., Geiselmann J. and Oppenheim A.B. (1998) Participation of IHF and a distant UP element in the stimulation of the phage λ P_L promoter. *Molec. Microbiol.* **30**, 443-451

Girón J.A., Ho A.S.Y. and Schoolnik G.K. (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science*. **254**, 710-713

Gomez-Duarte O.G. and Kaper J.B (1995) A plasmid encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. *Infect. Immun.* **63**, 1767-1776

Goosney D.L., de Grado M. and Finlay B.B. (1999) Putting *E. coli* on a pedestal: a unique system to study signal transduction and the actin cytoskeleton. *Trends. Cell Biol.* **9**, 11-14

Goosney D.L., DeVinney R., Pfuetzner R.A., Frey E.A., Strynadka N.C. and Finlay B.B. (2000) Enteropathogenic *E. coli* translocated intimin receptor, Tir, interacts directly with α-actinin. *Curr. Biol.* **10**, 735-738

Goryshin I.Y. and Reznikoff W.S. (1998) Tn5 *in vitro* transposition. *J. Biol. Chem.* **273**, 7367-7374

Goryshin I.Y., Jendrisak J., Hoffman L.M., Meis R. and Reznikoff W.S. (2000) Insertional transposon mutagenesis by electroporation of released transposition complexes. *Nat. Biotechnol.* **18**, 97-100

de Grado M., Abe A., Gauthier A., Steele-Mortimer O., DeVinney R. and Finlay B.B (1999) Identification of the intimin-binding domain of Tir of enteropathogenic *Escherichia coli*. *Cellul. Microbiol.* **1**, 7-17

Gray B.H. and Haseman J.R. (1994) Bactericidal activity of synthetic peptides based on the structure of the 55-kilodalton bactericidal protein from human neutrophils. *Infect. Immun.* **62**, 2732-2739

Guzman L-M., Belin D., Carson M.J. and Beckwith J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**, 4121-4130

H

Hacker J. and Kaper J.B. (2000) Pathogenicity islands and the evolution of microbes. *Ann. Rev. Microbiol.* **54**, 641-679

Haigh R., Baldwin T., Knutton S. and Williams P.H. (1995) Carbon dioxide regulated secretion of the EaeB protein of enteropathogenic *Escherichia coli*. *FEMS. Microbiol. Lett.* **129**, 63-68

Hartland E.L., Batchelor M., Delahay R.M., Hale C., Matthews S., Dougan G., Knutton S., Connerton I. and Frankel G. (1999) Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. *Molec. Microbiol.* **32**, 151-158

Hartland E. L., Daniell S.J., Delahay R.M., Neves B.C., Wallis T., Shaw R.K., Hale C., Knutton S. and Frankel G. (2000) The type III protein translocation system of enteropathogenic *Escherichia coli* involves EspA-EspB protein interactions. *Molec. Microbiol.* **35**, 1483-1492

Hecht G. and Koutsouris A. (1999) Enteropathogenic *E. coli* attenuates secretagogue-induced net intestinal ion transport but not Cl^- secretion. *Am. J. Physiol.* **276**, G781-G788

Helander A., Hansson G.C. and Svennerholm A.M. (1997) Binding of enterotoxigenic *Escherichia coli* to isolated enterocytes and intestinal mucus. *Micro. Pathogen.* **23**, 335-346

Hentschel U., Steinert M. and Hacker J. (2000) Common molecular mechanisms of symbiosis and pathogenesis. *Trends. Microbiol.* **8**, 226-231

Herschlag D. (1995) RNA chaperones and the RNA folding problem. *J. Biol. Chem.* **270**, 20871-20874

Hicks S., Frankel G., Kaper J.B., Dougan G. and Phillips A.D. (1998) Role of intimin and bundle-forming pili in enteropathogenic *Escherichia coli* adhesion to pediatric intestinal tissue *in vitro*. *Infect. Immun.* **66**, 1570-1578

Higgins L.M., Frankel G., Connerton I., Gonçalves N.S., Dougan G. and MacDonald T.T. (1999) Role of bacterial intimin in colonic hyperplasia and inflammation. *Science*. **285**, 588-591

Hill S.M., Phillips A.D. and Walker-Smith J.A. (1991) Enteropathogenic *Escherichia coli* and life threatening chronic diarrhoea. *Gut.* **32**, 154-158

Hofman P., Piche M., Far D.F., Le Negrate G., Selva E., Landraud L., Alliana-Schmid A., Boquet P. and Rossi B. (2000) Increased *Escherichia coli* phagocytosis in neutrophils that have transmigrated across a cultured intestinal epithelium. *Infect. Immun.* **68**, 449-455

Hommais F., Krin E., Laurent-Winter C., Soutourina O., Malpertuy A., Le Caer J-P., Danchin A. and Bertin P. (2001) Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. *Molec. Microbiol.* **40**, 20-36

Hueck C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Mol. Microbiol. Rev.* **62**, 379-433

Hughes K.T., Gillen K.L., Semon M.J. and Karlinsey J.E. (1993) Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science.* **262**, 1277-1280

Hurme R. and Rhen M. (1998) Temperature sensing in bacterial gene regulation – what it all boils down to. *Molec. Microbiol.* **30**, 1-6

I

Iino T. (1977) Genetics of structure and function of bacterial flagella. *Ann. Rev. Genet.* **11**, 161-182

Ilan O., Bloch Y., Frankel G., Ullrich H., Geider K. and Rosenshine I. (1999) Protein tyrosine kinases in bacterial pathogens are associated with virulence and production of exopolysaccharide. *EMBO J.* **18**, 3241-3248

Inada T., Kawakami K., Chen S-M., Takiff H.E., Court D.L. and Nakamura Y. (1989) Temperature-Sensitive Lethal Mutant of Era, a G Protein in *Escherichia coli. J. Bacteriol.* **171**, 5017-5024

Inuzuka M. and Helsinki D.R. (1978) Requirement of a plasmid encoded protein for replication *in vitro* of plasmid R6K. *Proc. Natl. Acad. Sci. USA* **75**, 5381-5385

Ismaili A., Philpott D.J., Dytoc M.T. and Sherman P.M. (1995) Signal transduction responses following adhesion of verocytotoxin-producing *Escherichia coli. Infect. Immun.* **63**, 316-3326

J

Jerse A.E., Yu J., Tall B.D. and Kaper J.B. (1990) A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc. Natl. Acad. Sci. USA* **87**, 7839-7842

Jerse A.E. and Kaper J.B. (1991) The *eae* gene of Enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. *Infect. Immun.* **59**, 4302-4309

Jiang W., Hou Y. and Inouye M. (1997) CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. *J. Biol. Chem.* **272**, 196-202

Johnstone B.H., Handler A.A., Chao D.K., Nguyen V., Smith M., Ryu S.Y., Simons E.L., Andreson P,E. and Simons R.W. (1999) The widely conserved Era G-protein contains an RNA-binding domain required for Era function *in vivo*. *Molec. Microbiol.* **33**, 1118-1131

Jones P.G., Mitta M., Kim Y., Jiang W. and Inouye M. (1996) Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **93**, 76-80

Judson N. and Mekalanos J.J. (2000) TnAraOut, A transposon-based approach to identify and characterize essential bacterial genes. *Nature. Biotechnol.* **18**, 740-745

K

Kalman D., Weiner O.D., Goosney D.L., Sedat J.W., Finlay B.B., Abo A. and Bishop J.M. (1999) Enteropathogenic *Escherichia coli* acts through WASP and Arp2/3 complex to form actin pedestals. *Nature. Cell. Biol.* **1**, 389-391

Karch H., Schubert S., Zhang D., Zhang W., Schmidt H., Ölschläger T. and Hacker J. (1999) A genomic island, termed high-pathogenicity island, is present in certain non-O157 shiga toxin-producing *Escherichia coli* clonal lineages. *Infect. Immun.* **67**, 5994-6001

Karlinsey J.E., Tanaka S., Bettenworth V., Yamaguchi S., Boos W., Aizawa S-I. and Hughes K.T. (2000a) Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. *Molec. Microbiol.* **37**, 1220-1231

Karlinsey J.E., Lonner J., Brown K.L. Hughes K.T. (2000b) Translation/secretion coupling by type III secretion systems. *Cell.* **102**, 487-497

Kauffmann F. (1947) The serology of the *coli* group. *J. Immunol.* **57**, 71-100

Kaziro Y., Itoh H., Kozasa T., Nakafuku M. and Satoh T. (1991) Structure and function of signal transducing GTP-binding proteins. *Ann. Rev. Biochem.* **60**, 349-400

Kelly G., Prasannan S., Daniell S., Fleming K., Frankel G., Dougan G., Connerton I. and Matthews S. (1999) Structure of the cell-adhesion fragment of intimin from enteropathogenic *Escherichia coli*. *Nat. Struct. Biol.* **6**, 313-318

Kenny B. and Finlay B.B. (1995) Protein secretion by enteropathogenic *Escherichia coli* is essential for transducing signals to epithelial cells. *Proc. Natl. Acad. Sci. USA.* **92**, 7991-7995

Kenny B., Lai L.C., Finlay B.B. and Donnenberg M.S. (1996) EspA, a protein secreted by enteropathogenic *Escherichia coli*, is required to induce signals in epithelial cells. *Molec. Microbiol.* **20**, 313-323

Kenny B. and Finlay B.B. (1997) Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signalling events, including tyrosine phosphorylation of phospholipase C-γ1. *Infect. Immun.* **65**, 2528-2536

Kenny B., DeVinney R., Stein M., Reinschield D.J., Frey E.A. and Finlay B.B. (1997a) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell.* **91**, 511-520

Kenny B., Abe A., Stein M. and Finlay B.B. (1997b) Enteropathogenic *Escherichia coli* protein secretion is induced in response to conditions similar to those in the gastrointestinal tract. *Infect. Immun.* **65**, 2606-2612

Kenny B. (1999) Phosphorylation of tyrosine 474 of the enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. *Molec. Microbiol.* **31**, 1229-1241

Kenny B. and Jepson M. (2000) Targeting of an enteropathogenic *Escherichia coli* (EPEC) effector protein to host mitochondria. *Cellular. Microbiol.* **2**, 579-590

Kenny B. and Warawa J. (2001) Enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule does not undergo full modification when introduced into host cells by EPEC-independent mechanisms. *Infect. Immun.* **69**, 1444-1453

Khlebnikov A., Risa Ø., Skaug T., Carrier T.A. and Keasling J.D. (2000) Regulatable arabinose-inducible gene expression system with consistent control in all cells of a culture. *J. Bacteriol.* **182**, 7029-7034

Kjeldgaard M., Nyborg J. and Clark B.F.C. (1996) The GTP binding motif: variations on a theme. *FASEB.* **10**, 1347-1367

Klapproth J-M.A., Donnenberg M.S., Abraham J.M., Mobley H.L.T. and James S.P. (1995) Products of enteropathogenic *Escherichia coli* inhibit lymphocyte activation and lymphokine production. *Infect. Immun.* **63**, 2248-2254

Klapproth J-M.A., Donnenberg M.S., Abraham J.M. and James S.P. (1996) Products of enteropathogenic *E. coli* inhibit lymphocyte production by gastrointestinal lymphocytes. *Am. J. Physiol.* **271**, G841-G848

Klapproth J-M.A., Scaletsky I.C.A., McNamara B.P., Lai L-C., Malstrom C., James S.P. and Donnenberg M.S. (2000) A large toxin from pathogenic *Escherichia coli* strains that inhibits lymphocyte activation. *Infect. Immun.* **68**, 2148-2155

Klauser T., Pohlner J. and Meyer T.F. (1993) The secretion pathway of IgA protease-type proteins in gram-negative bacteria. *BioEssays.* **15**, 799-805

Knutton S., Lloyd D.R. and McNeish A.S. (1987a) Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. *Infect. Immun.* **55**, 69-77

Knutton S., Lloyd D.R. and McNeish A.S. (1987b) Identification of a new fimbrial structure in enterotoxigenic *Escherichia coli* (ETEC) serotype 0148:H28 which adheres to human intestinal mucosa: a potentially new human ETEC colonization factor. *Infect. Immun.* **55**, 86-92

Knutton S., Baldini M.M., Kaper J.B. and McNeish A.S. (1987c) Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to Hep-2 cells. *Infect. Immun.* **55**, 78-85

Knutton S., Baldwin T., Williams P.H. and McNeish A.S. (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells: Basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **57**, 1290-1298

Knutton S., Adu-Bobie J., Bain C., Phillips A.D., Dougan G. and Frankel G. (1997) Down regulation of intimin expression during attaching and effacing Enteropathogenic *Escherichia coli* adhesion. *Infect. Immun.* **65**, 1644-1652

Knutton S., Rosenshine I., Pallen M.J., Nisan I., Neves B.C., Bain C., Wolff C., Dougan G. and Frankel G. (1998) A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J.* **17**, 2166-2176

Knutton S., Shaw R.K., Anantha R. P., Donnenberg M.S. and Zorgani A.A. (1999) The type IV bundle-forming pilus of enteropathogenic *Escherichia coli* undergoes dramatic alterations in structure associated with bacterial adherence, aggregation and dispersal. *Molec. Microbiol.* **33**, 499-509

Ko M. and Park C. (2000) H-NS-Dependent Regulation of Flagellar Synthesis Is Mediated by a LysR Family Protein. *J. Bacteriol.* **182**, 4670-4672

Kutsukake K., Ohya Y. and Iino T. (1990) Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. *J. Bacteriol.* **172**, 741-747

L

La Teana A., Brandi A., Falconi M., Spurio R., Pon C.L. and Gualerzi C.O. (1991) Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proc. Natl. Acad. Sci. USA.* **88**, 10907-10911

Laemmli U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-685

Lai L-C., Wainwright L.A., Stone K.D. and Donnenberg M.S. (1997) A third secreted protein that is encoded by the enteropathogenic *Escherichia coli* pathogenicity island is required for transduction of signals and for attaching and effacing activities in host cells. *Infect. Immun.* **65**, 2211-2217

Laurent-Winter C., Ngo S., Danchin A. and Bertin P. (1997) Role of *Escherichia coli* histone-like nucleoid structuring protein in bacterial metabolism and stress response identification of targets by two-dimensional electrophoresis. *Eur. J. Biochem.* **244**, 767-773

Lee N.L., Gielow W.O. and Wallace R.G. (1981) Mechanism of *araC* autoregulation and the domains of two overlapping promoters, P_c and P_{BAD}, in the L-arabinose regulatory region of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **78**, 752-756

Lee N., Francklyn C. and Hamilton E.P. (1987) Arabinose-induced binding of AraC protein to $araI_2$ activates the *araBAD* operon promoter. *Proc. Natl. Acad. Sci. USA.* **84**, 8814-8818

Lee C.A., Jones B.D. and Falkow S. (1992) Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. *Proc. Natl. Acad. Sci. USA*. **89**, 1847-1851

Levine M.M. and Edelman R. (1984) Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: Epidemiology and pathogenesis. *Epidemiol. Rev.* **6**, 31-51

Levine M.M., Nataro J.P., Karch H., Baldini M.M., Kaper J.B. and Black R.E. (1985) The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on plasmid encoding an enteroadhesiveness factor. *J. Infect. Dis.* **152**, 550-559

Levine M.M. (1987) *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J. Infect. Dis.* **155**, 377-389

Lin Y-P., Sharer J.D. and March P.E. (1994) GTPase-Dependent Signalling in Bacteria: Characterization of a Membrane-Binding Site for Era in *Escherichia coli*. *J. Bacteriol.* **176**, 44-49

Link A.J., Phillips D. and Church G.M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: Application to open reading frame characterization. *J. Bacteriol.* **179**, 6228-6237

Little R.G., Kelner D.N., Lim E., Burke D.J. and Conlon P. (1994) Functional domains of recombinant bactericidal permeability-increasing protein ($rBPI_{23}$). *J. Biol. Chem.* **269**, 1865-1872

Liu H., Magoun L., Luperchio S., Schauer D.B. and Leong J.M. (1999) The Tir-binding region of enterohaemorrhagic *Escherichia coli* intimin is sufficient to trigger actin condensation after bacterial-induced host cell signalling. *Molec. Microbiol.* **34**, 67-81

Lodish H., Baltimore D., Berk A., Zipursky S.L., Matsudaira P. and Darnell J. (1995) Molecular Cell Biology. Scientific American Books.

Lopez P.J., Marchand I., Joyce S.A. and Dreyfus M. (1999) The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. *Molec. Microbiol.* **33**, 188-199

Lu Q. and Inouye M. (1998) The Gene for 16S rRNA methyltransferase (*ksgA*) functions as a multicopy suppressor for a cold-sensitive mutant of Era, an essential Ras-like GTP binding protein in *Escherichia coli*. *J. Bacteriol.* **180**, 5243-5246

Luo Y., Frey E.A., Pfuetzner R.A., Creagh A.L., Knoechel D.G., Haynes C.A., Finlay B.B. and Strynadka N.C.J. (2000) Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. *Nature*. **405**, 1073-1077

Lupas A. (1996) Coiled coils: new structures and new functions. *TIBS*. **21**, 375-382

M

Machesky L.M. and Insall R.H. (1999) Signalling to Actin Dynamics. *J. Cell. Biol.* **146**, 267-272

Macnab R.M. (1992) Genetics and biogenesis of bacterial flagella. *Annu. Rev. Genet.* **26**, 131-158

Macnab R.M. (1996) Flagella and motility. In *Escherichia coli* and *Salmonella* and molecular biology (Edited by Neidhardt F.), p.121. ASM Press, Washington.

Macnab R.M. (1999) The bacterial flagellum: Reversible rotary propellor and type III export apparatus. *J. Bacteriol.* **181**, 7149-7153

Malstrom C. and James S. (1998) Inhibition of murine splenic and mucosal lymphocyte function by enteric bacterial products. *Infect. Immun.* **66**, 3120-3127

Manago G. M. (1999) Messenger RNA stability and its role in control of gene expression in bacteria and phages. *Ann. Rev. Genet.* **33**, 193-227

Manjarrez-Hernandez H.A., Amess B., Sellers L., Baldwin T.J., Knutton S., Williams P.H. and Aitken A. (1991) Purification of a 20 kDa phosphoprotein from epithelial cells and identification as a myosin light chain. *FEBS*. **292**, 121-127

Manjarrez-Hernandez H.A., Baldwin T.J., Aitken A., Knutton S. and Williams P.H. (1992) Intestinal epithelial cell protein phosphorylation in enteropathogenic *Escherichia coli* diarrhoea. *Lancet*. **339**, 521-523

Mannion B.A., Kalatzis E.S., Weiss J. and Elsbach P. (1989) Preferential binding of the neutrophil cytoplasmic granule-derived bactericidal/permeability increasing protein to target bacteria. *J. Immunol*. **142**, 2807-2812

March P.E. and Inouye M. (1985) GTP-binding membrane protein of *Escherichia coli* with sequence homolgy to initiation factor 2 and elongation factors Tu and G. *Proc. Natl. Acad. Sci. USA*. **82**, 7500-7504

McDaniel T.K. and Kaper J.B. (1997) A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Molec. Microbiol.* **23**, 399-407

McDaniel T.K., Jarvis K.G., Donnenberg M.S. and Kaper J.B. (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc. Natl. Acad. Sci. USA.* **92**, 1664-1668

McNamara B.P. and Donnenberg M.S. (1998) A novel proline-rich protein, EspF, is secreted from enteropathogenic *Escherichia coli* via the type III export pathway. *FEMS Microbiol. Lett.* **166**, 71-78

McNamara B.P. and Donnenberg M.S. (2000) Evidence for specificity in type 4 pilus biogenesis by enteropathogenic *Escherichia coli*. *Microbiol.* **146**, 719-729

Meier T., Peery R.B., Jaskunas R. and Zhao G. (1999) 16S rRNA is bound to Era of *Streptococcus pneumoniae*. *J. Bacteriol.* **181**, 5242-5249

Meier T., Peery R.B., McAllister K.A. and Zhao G. (2000) Era GTPase of *Escherichia coli*: binding to 16S rRNA and modulation of GTPase activity by RNA and carbohydrates. *Microbiol.* **146**, 1071-1083

Mellies J.L., Elliot S.J., Sperandio V., Donnenberg M.S. and Kaper J.B. (1999) The Per regulon of from enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Molec. Microbiol.* **33**, 296-306

Mellies J.L., Navarro-Garcia F., Okeke I., Frederickson J., Nataro J.P. and Kaper J.B. (2001) *espC* pathogenicity island of enteropathogenic *Escherichia coli* encodes an enterotoxin. *Infect. Immun.* **69**, 315-324

Mercurio F. and Manning A.M. (1999) Multiple signals converging on NF-κB. *Curr. Opin. Cell. Biol.* **11**, 226-232

Miller J.H. (1977) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, New York.

Minamino T., González-Pedrajo B., Yamaguchi K., Aizawa S-I. and Macnab R.M. (1999) FliK, the protein responsible for flagellar hook length control in *Salmonella* is exported during hook assembly. *Molec. Microbiol.* **34**, 295-304

Minamino T. and Macnab R.M. (2000) Interactions among components of the *Salmonella* flagellar export apparatus and its substrates. *Molec. Microbiol.* **35**, 1052-1064

Moon H., Whipp S., Argenzio R., Levine M. and Giannella R. (1983) Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect. Immun.* **41**, 1340-1351

N

Nataro J.O., Scaletsky I.C.A., Kaper J.B., Levine M.M. and Trabulsi L.R. (1985) Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. *Infect. Immun.* **48**, 378-383

Nataro J.P., Yikang D., Yingkang D. and Walker K. (1994) AggR, a transcriptional activator of aggregative adherence fimbriae I expression in enteroaggregative *Escherichia coli*. *J. Bacteriol.* **176**, 4691-4699

Nataro J.P., Seriwatana J., Fasano A., Maneval D.R., Guers L.D., Noriega F., Dubovsky F., Levine M.M. and Glen Morris JR J. (1995) Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. *Infect. Immun.* **63**, 4721-4728

Nataro J.P. and Kaper J.B. (1998) Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **11**, 142-201

Navarro García F., Eslava C., Villaseca J.M., López-Revilla R., Czeczulin J.R., Srinivas S., Nataro J.P. and Cravioto A. (1998) In vitro effects of a high-molecular-weight heat-labile enterotoxin from enteroaggregative *Escherichia coli*. *Infect. Immun.* **66**, 3149-3154

Neves B.C., Knutton S., Trabulsi L.R., Sperandio V., Kaper J.B., Dougan G. and Frankel G. (1998) Molecular and ultrastructural characterisation of EspA from different enteropathogenic *Escherichia coli* serotypes. *FEMS Microbiol. Lett.* **169**, 73-80

Nissen P., Kjeldgaard M., Thirup M., Polekhina G., Reshetnikova L., Clarke B.F.C. and Nyborg J. (1995) Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu , and a GTP analog. *Science*. **270**, 1464-1472

Nogueria T. and Springer M. (2000) Post-transcriptional control by global regulators of gene expression in bacteria. *Curr. Opin. Microbiol.* **3**, 154-158

O

Odell E.W. and Segal A.W. (1988) The bactericidal effects of the respiratory burst and the myeloperoxidase system isolated in the neutrophil cytoplasts. *BBA*. **971**, 266-274

Oswald E., Schmidt H., Morabito S., Karch H., Marches O. and Caprioli A. (2000) Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: Characterization of a new intimin variant. *Infect. Immun.* **68**, 64-71

Ottemann K.M. and Miller J.F. (1997) Roles for motility in bacterial - host interactions. *Molec. Microbiol.* **24**, 1109-1117

Owen-Hughes T.A., Pavitt G.D., Santos D.S., Sidebotham J.M., Hulton C.S.J., Hinton J.C.D. and Higgins C.F. (1992) The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. *Cell*. **71**, 255-265

P

Pahl H.L. and Baeuerle P.A. (1997) The ER-overload response: activation of NF-κB. *TIBS*. **22**, 63-67

Pallen M.J., Dougan G. and Frankel G. (1997) Coiled-coil domains in proteins secreted by type III secretion systems. *Molec. Microbiol.* **25**, 423-425

Phillips A.D. and Frankel G. (2000) Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with human intestinal organ cultures. *J. Infect. Dis.* **181**, 1496-1500

Philpott D.J., McKay D.M., Mak W., Perdue M.H. and Sherman P.M. (1998) Signal transduction pathways involved in enteropathogenic *Escherichia coli*-induced alterations in T84 epithelial permeability. *Infect. Immun.* **66**, 1680-1687

Plunkett G., Burland V., Daniels D. and Blattner F. (1993) Analysis of the *Escherichia coli* genome. III. DNA sequence of the region from 87.2 to 89.2 minutes. *Nucl. Acids. Res.* **21**, 3391-3398

Prentki P. and Krisch H.M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene*. **29**, 303-313

Prüß B.M. and Wolfe A.J. (1994) Regulation of acetyl phosphate synthesis and degradation, and the control of flagellar expression in *Escherichia coli*. *Molec. Microbiol.* **12**, 973-984

Puente J.L., Bieber D., Ramer S.W., Murray W. and Schoolnik G.K. (1996) The bundle-forming pili of enteropathogenic *Escherichia coli*: transcriptional regulation by environmental signals. *Molec. Microbiol.* **20**, 87-100

Q

Qi S.-Y., Li Y. and O'Connor C.D. (1994) The region around residue 115 of human bactericidal/permeability-increasing protein is not involved in lipopolysaccharide binding or bactericidal activity. *Biochem. J.* **298**, 711-718

Qi S.-Y., Li Y., Szyroki A., Giles I.G., Moir A. O'Connor C.D. (1995) *Salmonella typhimurium* responses to a bactericidal protein from human neutrophils. *Molec. Microbiol.* **17**, 523-531

R

Ramer S.W., Bieber D. and Schoolnik G.K. (1996) BfpB, an outer membrane lipoprotein required for the biogenesis of bundle-forming pili in enteropathogenic *Escherichia coli*. *J. Bacteriol.* **178**, 6555-6563

Reitzer L.J. and Magasanik B. (1985) Expression of *glnA* in *Escherichia coli* is regulated at tandem promoters. *Proc. Natl. Acad. Sci. USA*. **82**, 1979-1983

Rice P.A., Yang S-W., Mizuuchi K. and Nash H.A. (1996) Crystal structure of an IHF-DNA complex: A protein-induced DNA U-turn. *Cell*. **87**, 1295-1306

Rich C., Favre-Bonte S., Sapena F., Joly B. and Forestier C. (1999) Characterization of enteroaggregative *Escherichia coli* isolates. *FEMS Microbiol. Lett.* **173**, 55-61

Roberts T.M., Kacich R. and Ptashne M. (1979) A general method for maximizing the expression of a cloned gene. *Proc. Natl. Acad. Sci. USA*. **76**, 760-764

Rodnina M., Savelsgergh A., Katunin V. and Wintermeyer W. (1997) Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. *Nature*. **385**, 37-41

Rosenshine I., Donnenberg M.S., Kaper J.B. and Finlay B.B. (1992) Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. *EMBO J.* **11**, 3551-3560

Rosenshine I., Ruschkowski S. and Finlay B.B. (1996a) Expression of attaching/effacing activity by enteropathogenic *Escherichia coli* depends on growth phase, temperature, and protein synthesis upon contact with epithelial cells. *Infect. Immun.* **64**, 966-973

Rosenshine I., Ruschkowski S., Stein M., Reinscheid D., Mills D. S. and Finlay, B. B. (1996b) A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. *EMBO J.* **15**, 2613-2624

Rothbaum R., McAdams A.J., Giannella R. and Partin J.C. (1982) A clinicopathologic study of enterocyte-adherent *Escherichia coli*: A cause of protracted diarrhea in infants. *Gastroenterol.* **83**, 441-454

Rowe S., Hodson N., Griffiths G. and Roberts I.S. (2000) Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA and Integration Host Factor in regulation of Group 2 capsule gene clusters in pathogenic *E. coli*. *J. Bacteriol.* **182**, 2741-2745

S

Salyers A.A., Speer B.S. and Shoemaker N.B. (1990) New perspectives in tetracycline resistance. *Molec. Microbiol.* **4**, 151-156

Salyers A.A. and Whitt D.D. (1994) *Bacterial Pathogenesis: A molecular approach.* ASM Press, Washington.

Sambrook J., Fritsch E.F. and Maniatis T. (1989) *Molecular Cloning: A laboratory manual.* Cold Spring Harbor Laboratory, New York.

Savarino S.J., Fasano A., Robertson D.C. and Levine M.M. (1991) Enteroaggregative *Escherichia coli* elaborate a heat stable enterotoxin demonstrable in an in vitro rabbit intestinal model. *J. Clin. Invest.* **87**, 1450-1455

Savarino S.J., Fasano A., Watson J., Martin B.M., Levine M.M., Guandalini S. and Guerry P. (1993) Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. *Proc. Natl. Acad. Sci. USA.* **90**, 3093-3097

Savkovic S.D., Koutsouris A. and Hecht G. (1996) Attachment of a noninvasive enteric pathogen, enteropathogenic *Escherichia coli*, to cultured human intestinal epithelial monolayers induces transmigration of neutrophils. *Infect. Immun.* **64**, 4480-4487

Savkovic S.D., Koutsouris A. and Hecht G. (1997) Activation of NF-κB in intestinal epithelial cells by enteropathogenic *Escherichia coli*. *Am. J. Physiol.* **272**, C1160-C1167

Sayed A., Matsuyama S-I. and Inouye M. (1999) Era, an essential *Escherichia coli* small G-protein, binds to the 30S ribosomal subunit. *Biochem. Biophys. Res. Commun.* **264**, 51-54

Scaletsky I.C.A., Silva M.L. and Trabulsi L.R. (1984) Distinctive patterns of enteropathogenic *Escherichia coli* to HeLa cells. *Infect. Immun.* **45**, 534-536

Schleif R. (2000) Regulation of the L-arabinose operon of *Escherichia coli*. *Trends. Genet.* **16**, 559-565

Schröder O. and Wagner R. (2000) The bacterial DNA-binding protein H-NS represses ribosomal RNA transcription by trapping RNA polymerase in the initiation complex. *J. Mol. Biol.* **298**, 737-748

Shi X. and Bennett G.N. (1994) Plasmids bearing *hfq* and the *hns*-like gene *stpA* complement *hns* mutants in modulating arginine decarboxylase gene expression in *Escherichia coli*. *J. Bacteriol.* **176**, 6769-6775

Shimamoto T. and Inouye M. (1996) Mutational analysis of Era, an essential GTP-binding protein of *Escherichia coli*. *FEMS Microbiol. Lett.* **136**, 57-62

Siegele D.A. and Hu J.C. (1997) Gene expression from plasmids containing the *araBAD* promoter at subsaturating inducer conditions represents mixed populations. *Proc. Natl. Acad. Sci. USA.* **94**, 8168-8172

Silhavy T.J., Berman M.L. and Enquist L.W. (1984) Experiments with gene fusions. Cold Spring Harbor Laboratory, New York.

Silverman M. and Simon M. (1973) Genetic analysis and flagella mutants in *Escherichia coli*. *J. Bacteriol.* **113**, 105-113

Simon R., Quandt J. and Klipp W. (1989) New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. *Gene*. **80**, 161-169

Soutourina O., Kolb A., Krin E., Laurent-Winter C., Rimsky S., Danchin A. and Bertin P. (1999) Multiple control of flagellum biosynthesis in *Escherichia coli*: role of H-NS protein and the cyclic

AMP-catabolite activator protein complex in transcription of the *flhDC* master operon. *J. Bacteriol.* **181**, 7500-7508

Spassky A., Rimsky S., Garreau H. and Buc H. (1984) H1a, an *E. coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro*. *Nucl. Acids. Res.* **12**, 5321-5340

Sperandio V., Mellies J.L., Nguyen W., Shin S. and Kaper J.B. (1999) Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *PNAS.* **96**, 15196-15201

Sperandio V., Mellies J.L., Delahay R.M., Frankel G., Crawford J.A., Nguyen W. and Kaper J.B. (2000) Activation of enteropathogenic *Escherichia coli* (EPEC) *LEE2* and *LEE3* operons by Ler. *Molec. Microbiol.* **38**, 781-793

Spitz J., Yuhan R., Koutsouris A., Blatt C., Alverdy J. and Hecht G. (1995) Enteropathogenic *Escherichia coli* adherence to intestinal epithelial monolayers diminishes barrier function. *Am. J. Physiol.* **268**, G374-G379

Stark H., Rodnina M.V., H-J Wieden., van Heel M. and Wintermeyer W. Large-scale movement of Elongation Factor G and extensive conformational change of the ribosome during translocation. *Cell.* **100**, 301-309

Stein M., Kenny B., Stein M.A. and Finlay B.B. (1996a) Characterization of EspC, a 110-Kilodalton protein secreted by enteropathogenic *Escherichia coli* which is homologous to members of the Immunoglobulin A protease-like family of secreted proteins. *PNAS.* **178**, 6546-6554

Stein M.A., Mathers D.A., Yan H., Baimbridge K.G. and Finlay B.B. (1996b) Enteropathogenic *Escherichia coli* markedly decreases the resting membrane potential of Caco-2 and HeLa human epithelial cells. *Infect. Immun.* **64**, 4820-4825

Stibitiz S., Black W. and Falkow S. (1986) The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. *Gene.* **50**, 133-140

Stone K.D., Zhang H.Z., Carlson L.K. and Donnenberg M.S. (1996) A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for the BFP ultrastructure. *Molec. Microbiol.* **20**, 325-338

T

Tacket C. O., Sztein M.B., Losomsky G., Abe A., Finlay B.B., McNamara P., Fantry G.T., James S.P., Nataro J.P., Levine M.M. and Donnenberg M.S. (2000) Role of EspB in experimental human enteropathogenic *Escherichia coli* infection. *Infect. Immun.* **68**, 3689-3695

Taylor K.A., O'Connell C.B., Luther P.W. and Donnenberg M.S. (1998) The EspB protein of enteropathogenic *Escherichia coli* is targeted to the cytoplasm of infected HeLa cells. *Infect. Immun.* **66**, 5501-5507

Taylor K.A., Luther P.W. and Donnenberg M.S. (1999) Expression of the EspB protein of enteropathogenic *Escherichia coli* within HeLa cells affects stress fibers and cellular morphology. *Infect. Immun.* **67**, 120-125

Taylor K.A., O'Connell C.B., Thompson R. and Donnenberg M.S. (2001) The role of pyridoxal phosphate in the function of EspB, a protein secreted by enteropathogenic *Escherichia coli*. *FEBS.* **488**, 55-58

La Teana A., Brandi A., Falconi M., Spurio R., Pom C.I. and Gualerzi C.O. (1991) Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proc. Natl. Acad. Sci. USA*. **88**, 10907-10911

Thorpe C.M., Hurley B.P., Lincicome L.L., Jacewicz M.S., Keusch G.T. and Acheson D.W.K. (1999) Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. *Infect. Immun.* **67**, 5985-5993

Tobe T., Schoolnik G.K., Sohel I., Bustamante V.H. and Puente J.L. (1996) Cloning and characterization of *BfpTVW*, genes required for the transcriptional activation of *BfpA* in enteropathogenic *Escherichia coli*. *Molec. Microbiol.* **21**, 963-975

Tobe T., Tatsuno I., Katayama C.-Y.W., Schoolnik G.K. Sasakawa C. (1999) A novel chromosomal locus of enteropathogenic *Escherichia coli* (EPEC), which encodes a *bfpT*-regulated chaperone-like protein, TrcA, involved in microcolony formation by EPEC. *Molec. Microbiol.* **33**, 741-752

Tsen H.-Y. and Jian L.-Z. (1998) Development and use of a multiplex PCR system for the rapid screening of heat labile toxin I, heat stable toxin II and shiga-like toxin I and II genes of *Escherichia coli* in water. *J. Appli. Microbiol.* **84**, 585-592

Tupper A.E., Owen-Hughes T.A., Ussery D.W., Santos D.S., Ferguson D.J.P., Sidebotham J.M. Hinton J.C.D. and Higgins C.F. (1994) The chromatin-associated protein H-NS alters DNA topology *in vitro*. *EMBO J.* **13**, 258-268

U

Ueguchi C., Suzuki T., Yoshida T., Tanaka K. Mizuno T. (1996) Systematic mutational analysis revealing the functional domain organization of *Escherichia coli* nucleoid protein H-NS. *J. Mol. Biol.* **263**, 149-162

Ueno-Nishio S., Mango S., Reitzer L.J. and Magasanik B. (1984) Identification and regulation of the *glnL* operator-promoter of the complex *glnALG* operon of *Escherichia coli*. *J. Bacteriol.* **160**, 379-384

Ulshen M.H. and Rollo J.L. (1980) Pathogenesis of *Escherichia coli* gastroenteritis in man - another mechanism. *N. Engl. J. Med.* **302**, 99-101

V

Valance B.A. and Finlay B.B. (2000) Exploitation of host cells by enteropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **97**, 8799-8806

VanBogelen R.A. and Neidhardt F.C. (1990) Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **87**, 5589-5593

W

Wachter C., Beinke C., Mattes M. and Schmidt M.A. (1999) Insertion of EspD into epithelial target cell membranes by infecting enteropathogenic *Escherichia coli*. *Molec. Microbiol.* **31**, 1695-1707

Wai S.N., Takade A. and Amako K. (1996) The hydrophobic surface protein layer of enteroaggregative *Escherichia coli* strains. *FEMS Microbiol. Lett.* **135**, 17-22

Wainwright L.A. and Kaper J.B. (1998) EspB and EspD require a specific chaperone for proper secretion from enteropathogenic *Escherichia coli*. *Molec. Microbiol.* **27**, 1247-1260

Warawa J., Finlay B.B. and Kenny B. (1999) Type III secretion-dependent hemolytic activity of enteropathogenic *Escherichia coli*. *Infect. Immun.* **67**, 5538-5540

Wei B.L., Brun-Zinkernagel A-M., Simecka J.W., Prüß B.M., Babitzke P. and Romeo T. (2001) Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. *Molec. Microbiol.* **40**, 245-256

Weis W.I. and Drickamer K. (1996) Structural basis of lectin-carbohydrate recognition. *Ann. Rev. Biochem.* **65**, 441-473

Weiss J., Victor M. and Elsbach P. (1983) Role of charge and hydrophobic interactions in the action of the bactericidal permeability-increasing protein on neutrophils and gram-negative bacteria. *J. Clin. Invest.* **71**, 540-549

Wieler L. H., McDaniel T. K., Whittam T.S. and Kaper J.B. (1997) Insertion site of the locus of enterocyte effacement in enteropathogenic and enterohemorrhagic *Escherichia coli* differs in relation to the clonal phylogeny of the strains. *FEMS Microbiol. Lett.* **156**, 49-53

Williams R.M. and Rimsky S. (1997) Molecular aspects of the *E. coli* nucleoid protein, H-NS: a central controller of gene regulatory networks. *FEMS.* **156**, 175-185

Wilson K.S. and Noller H.F. (1998) Molecular movement inside the translational engine. *Cell.* **92**, 337-349

Wolff C., Nisan I., Hanski E., Frankel G. and Rosenshine I. (1998) Protein translocation into host epithelial cells by infecting enteropathogenic *Escherichia coli*. *Molec. Microbiol.* **28**, 143-155

Y

Yamada H., Muramatsu S. and Mizuno T. (1990) An *Escherichia coli* protein that preferentially binds to sharply curved DNA. *J. Biochem.* **108**, 420-425

Yamada H., Yoshida T., Tanaka K., Sasakawa C. and Mizuno T. (1991) Molecular analysis of the *Escherichia coli hns* gene encoding a DNA-binding protein, which preferentially recognizes curved DNA sequences. *Mol. Gen. Genet.* **230**, 332-336

Yanomoto T., Koyama Y., Matsumoto M., Sonoda E., Nakayama S., Uchimura M., Paveenkittiporn W., Tamura K., Yokota T. and Echeverria P. (1992) Localized, aggregative, and diffuse adherence to HeLa cells, plastic, and human small intestines by *Escherichia coli* isolated from patients with diarrhea. *J. Infect. Dis.* **166**, 1295-1310

Young G.M. Schmiel D.H. and Miller V.L. (1999) A new pathway for the secretion of virulence factors by bacteria: The flagellar export apparatus functions as a protein-secretion system. *Proc. Natl. Acad. Sci. USA.* **96**, 6456-6461

Yuhan R., Koutsouris A., Savkovic S.D. and Hecht G. (1997) Enteropathogenic *Escherichia coli*-induced myosin light chain phosphorylation alters intestinal epithelial permeability. *Gastroenterol.* **113**, 1873-1882

Z

Zangrossi S., Briani F., Ghisotti D., Regonesi M.E., Tortora P. and Dehò G. (2000) Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*. *Molec. Microbiol.* **36**, 1470-1480

Zhang A., Rimsky S., Reaban M.E., Buc H. and Belforrt M. (1996) *Escherichia coli* protein analogs StpA and H-NS: regulatory loops, similar and disparate effects on nucleic acid dynamics. *EMBO J.* **15**, 1340-1349

Zhang H.-Z., Lory S. and Donnenberg M.S. (1994) A plasmid-encoded prepillin peptidase gene from enteropathogenic *Escherichia coli*. *J. Bacteriol.* **176**, 6885-6891

Zhang H.-Z. and Donnenberg M.S. (1996) DsbA is required for stability of the type IV pili of enteropathogenic *Escherichia coli*. *Molec. Microbiol.* **21**, 787-797