CHAPTER 1

INTRODUCTION

The work presented in this thesis deals with the identification of a possible vaccine candidate/diagnostic marker as well as a therapeutic molecule which could potentially prevent the clinical consequences of a gram-negative bacterial pathogen, Enteropathogenic *Escherichia coli* (EPEC). EPEC is one of the major causes of childhood diarrhoea in developing countries. An introduction to the molecular mechanisms of EPEC pathogenesis, epidemiology, clinical manifestations and potential diagnostic and vaccine candidates is presented in this chapter.

1.1 DIARRHOEAL DISEASES AND MORTALITY

Diarrhoea is a consequence of gastrointestinal infections and kills 2.2 million people, especially children under the age of 5 in developing countries, every year. The predominant reservoir of infection is contaminated water. The major water-borne disease causing bacteria are *Escherichia* (Guion et al 2008), *Vibrio, Salmonella, Shigella* and *Yersinia* sp. Other infectious agents include viruses like coxsackie virus (Monlux et al 1975) and rotaviruses (Gratacap-Cavallier et al 2000) and protozoa like *Entamoeba histolytica, Cryptosporidium parvum* and *Giardia lamblia* (Marshall et al 1997).

Diarrhoeal deaths due to bacterial infections constitute for more than 35% of mortality in children under the age of five (www.who.int/child-adolescent-health/New_Publications/CHILD_HEALTH/EPI/Improving_
Diarrhoea_Estimates.pdf). *Salmonella, Shigella, Campylobacter* and *Vibrio* constitute about 13.6% of this mortality rate while the remaining 22% is caused by diarrhoeagenic *E. coli*.

### 1.2 DIARRHOEAGENIC *E. COLI*

*Escherichia coli* is the predominant facultative anaerobe of the human colonic flora. It inhabits the lumen within hours of birth and adapts itself as a normal flora in the intestine. But during cases of immunocompromised conditions, *E. coli* can cause infections in humans. The adaptability of the organism leads to breaching of the intestinal barriers and dissemination throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrhoeal disease (Nataro and Kaper, 1998). *E. coli* strains are broadly classified on the basis of serotypes. *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles. Further classification is based on expression of virulent genes, toxins and invasive properties. The different types of pathogenic *E. coli* include Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Diffuse Adherent *E. coli* (DAEC), Vero-cytotoxin (Shiga toxin) producing *E. coli* (VTEC or STEC) and Enteroaggregative *E. coli* (EAEC). These are commonly referred as ‘pathotypes’. Except for EPEC, all the other pathotypes have potential invasive properties in intestinal cells. Table 1.1 represents diarrhoeagenic *E. coli* pathotypes and clinical consequences.
Table 1.1 Diarrhoeagenic *E. coli* pathotypes and clinical consequences

<table>
<thead>
<tr>
<th><em>E. coli</em> Pathotype</th>
<th>Abbreviation</th>
<th>Definition</th>
<th>Type of Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verocytotoxin (Shiga toxin)-producing <em>E. coli</em></td>
<td>VTEC or STEC</td>
<td><em>E. coli</em> that produce verocytotoxin (Shiga toxin) VT1 and/or VT2</td>
<td>Diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome</td>
</tr>
<tr>
<td>Enterotoxigenic <em>E. coli</em></td>
<td>ETEC</td>
<td><em>E. coli</em> that produce heat stable enterotoxin (STh,STp) and/or heat labile (LT)</td>
<td>Acute watery diarrhea</td>
</tr>
<tr>
<td>Attaching and Effacing <em>E. coli</em></td>
<td>A/EEC</td>
<td><em>E. coli</em> that attach to and efface the microvilli of enterocytes, but do not produce high levels of verocytotoxins</td>
<td>Acute or persistent diarrhea</td>
</tr>
<tr>
<td>Enteropathogenic <em>E. coli</em></td>
<td>EPEC</td>
<td>Sub-type of A/EEC, usually of particular serotypes that mostly contain an EPEC adherence factor plasmid and often produce bundle-forming pilus (BFP)</td>
<td>Acute or persistent diarrhea</td>
</tr>
<tr>
<td>Enteroaggregative <em>E. coli</em></td>
<td>EAEC</td>
<td><em>E. coli</em> that exhibit a pattern of aggregative adherence to tissue culture</td>
<td>Acute, watery, often protracted diarrhea</td>
</tr>
<tr>
<td>Diffuse Adherent <em>E. coli</em></td>
<td>DAEC</td>
<td><em>E. coli</em> that exhibit a pattern of diffuse adherence to tissue culture</td>
<td>Acute or persistent diarrhea</td>
</tr>
<tr>
<td>Enteroinvasive <em>E. coli</em></td>
<td>EIEC</td>
<td><em>E. coli</em> that share virulence determinants with <em>Shigella</em> sp.</td>
<td>Acute, often inflammatory diarrhoea; dysentry</td>
</tr>
</tbody>
</table>

1.3 ENTEROPATHOGENIC E. COLI

Enteropathogenic E. coli is one of the major causes of childhood diarrhoea worldwide, especially in developing countries (Clarke et al 2003). It accounts for more than 30% of diarrhoeal cases in countries like Brazil (Gomes et al 1991), Kenya (Senerwa et al 1991), South Africa (Robins-Browne et al 1982), Mexico (Cravioto et al 1988; Cravioto et al 1990). Many cases of EPEC diarrhoea have also been recently reported in industrialized nations like Germany (Kozub-Witkowski et al 2008). Historically, EPEC strains were defined in terms of their negative characteristics, particularly their inability to produce enterotoxins or to demonstrate Shigella-like invasiveness. Virulence of EPEC depends primarily on the induction of a characteristic lesion in which the bacteria make intimate contact with the apical plasma membrane, causing localized destruction of the intestinal brush border and distortion of the apical enterocyte membrane. This results in gross cytoskeletal rearrangements, particularly the formation of an actin-rich cup-like pedestal at the site of bacterial contact and is known as ‘attaching and effacing’ (AE) lesion (Moon et al 1983). The AE class of E. coli was finally defined as those that harbour the Locus of Enterocyte Effacement or the LEE pathogenicity island and the absence of shiga toxin (stx) genes. EPEC was also distinguished as typical and atypical based on the presence and absence of the EPEC Adherence Factor (EAF) plasmid respectively.

Typical EPEC strain O119:H6 which expresses flagella H6 and intimin β is unusual and classified as unusual (Whittam and McGraw 1996). Knutton et al in 1989 observed an unusual phenotype inflicted on to host cells by AE lesion producing E. coli. They observed dense actin accumulation beneath sites of bacterial adherence through Fluorescence Actin Staining (FAS) test (Knutton et al 1989). This observation laid foundation for the search of new virulence genes that might be involved in EPEC pathogenesis. The adherent factor intimin was the first virulence factor that was identified
This was followed by the identification of LEE (McDaniel et al 1995) and then the components of the type III secretion system of T3SS (Garmendia et al 2005).

The virulence and AE lesion producing capability of EPEC is mainly attributed to the LEE. It is a 35.6 kb pathogenicity island that comprises 5 polycistronic operons and 41 open reading frames. It codes for most of the virulence factors like EspA, EspB, EspD, EspF, Map, intimin and its receptor Tir (translocated intimin receptor). The LEE also codes for the regulators Ler, GleA and GleR and the chaperones CesAB, CesD, CesD2, CesF and CesT (Garmendia et al 2005). The gene expression of all these virulence factors is regulated by the LEE-encoded-regulator or Ler, which in turn, is regulated by per regulon, which is present in the EAF plasmid that also encodes the bundle forming pilus or BFP. It has been demonstrated that Ler is required for expression of the LEE operons LEE2, LEE3, LEE4, and LEE5 and the LEE genes espF, espG, and map (Clarke et al 2003). The ler gene encodes a distant homologue of H-NS (for histone-like nucleoid-structuring protein), a nucleoid-associated protein that is frequently involved in the response of enterobacteria to environmental stimuli (Atlung and Ingmer, 1997). H-NS binding to promoter sequences represses the expression of LEE2, LEE3, LEE4, LEE5, and espG at both 27 and 37°C; however, it represses the expression of LEE1 (including ler) only at 27°C. Thus, when a culture of wild-type bacteria is shifted from 27 to 37°C, H-NS repression of ler expression is alleviated, and it was proposed that Ler antagonization of H-NS then leads to expression of LEE2, LEE3, LEE4, LEE5 and espG. Use of transcriptional reporters in EPEC ler and hns single mutants and ler hns double mutants has demonstrated that this hypothesis is mainly correct; however, in an hns mutant, which should be derepressed for LEE5 expression at all temperatures, there was still a requirement for Ler, indicating that it must also activate this operon by another, unknown mechanism (Umanski et al 2002).
1.3.1 Type III Secretion System and EPEC Secreted Proteins

Type III secretion system (T3SS) is exclusively associated with virulence and aid in protein secretion in a variety of plant and animal pathogens. Many protein components of T3SS have shown sequence similarities to those of flagella basal bodies (Hueck 1998). The supermolecular structure of EPEC T3SS revealed similarity to that of *Shigella* (Sekiya et al 2001). EPEC proteins EspA, EspB, EspD are proteins secreted in a T3SS-dependent manner (Hayward et al 2006) (Figure 1.1) and are required for AE lesion formation in host epithelium (Donnenberg et al 1993; Foubister et al 1994; Kenny et al 1996) and Tir (Kenny et al 1997).

![EPEC T3SS mediated disruption of host cellular functions](image)

Adapted from Hayward et al 2006.

**Figure 1.1** EPEC T3SS mediated disruption of host cellular functions
Tir (known as Hp90 previously) was initially identified as a host protein that gets tyrosine phosphorylated (residue Y474) during EPEC infection (Figure 1.2). It was later that Kenny et al (1997) showed that Hp90 was actually a bacterial protein that was translocated into the host cell during infection. Tir forms a hairpin loop topology upon insertion into the host cell membrane, thereby acting as a receptor for intimin. Clustering of Tir is mediated by intimin and is necessary for Y474 phosphorylation at the C-terminal domain and actin polymerisation (Campellone et al 2004). The N-terminal domain of Tir binds several focal adhesion proteins like α-actinin,
talin, vinculin and cortactin (Garmendia et al 2005). TccP of EHEC is the only protein other than Tir that can induce actin rich pedestals in host cells (Garmendia et al 2004).

EspA and the T3SS form the ‘molecular syringe’ to facilitate transport of virulence factors like EspB, EspD and Tir from the bacterium to the host. The EspA forms the hollow conduit through which other effectors are transferred into the host (Crepin et al 2005). The N-terminal of EspA plays a major role in protein stability, interaction with CesAB chaperone and filament biogenesis (Singh et al 2008). EspA plays a role in the initial attachment of atypical EPEC strains (Cleary et al 2004). Very recently, a second chaperone after CesAB, L0017 has been identified and implicated in EspA assembly (Su et al 2008).

EspB and EspD provide the pore-forming function on the host cell membrane (Fivaz and van der Goot, 1999). Distinct functional domains of EspB play roles in protein translocation (Luo and Donnenberg 2006). In addition, EspB is assumed to play additional roles like translocation to the cytoplasm and causing actin redistribution (Taylor et al 1999). EspB has also been shown to interact directly with α-catenin which, in turn, is recruited to the site of actin accumulation (Kodama et al 2002). Besides, EspB is known to interact with α1-antitrypsin (Knappstein et al 2004). Tir, a 78kDa bacterial protein is translocated to the host and in the process, gets phosphorylated at serine and tyrosine residues and finally inserts into the host cell membrane as a 90kDa receptor for intimin. It also interacts with α-catenin and Nck for pedestal formation (Bhavsar et al 2007; Goosney et al 2000; Gruenheid et al 2001). Recently, it has been shown that Tir-intimin interaction is based on three hot spot residues and beta-hairpin structure present in Tir (Ross and Miller 2007). EspB is also assumed to play a role in anti-inflammatory properties of EPEC since an EspB mutant produced exaggerated immune
responses from the host (Sharma et al 2006). EspB was also found to be co-expressed with maltoporin (Kumar et al 2001). EspD protein influences the formation of surface appendages in host cells and localises to the host cell membrane (Kresse et al 1999).

*Escherichia coli* (EPEC) type III effector protein EspF is required for inducing cell death in host cells (Crane et al 2001). Recently, it was found that EspF nucleates a multiprotein signaling complex composed of eukaryotic sorting nexin 9 (SNX9) and neuronal Wiskott-Aldrich syndrome protein (N-WASP). The high affinity association between EspF and SNX9 induces membrane remodeling in host cells. These membrane-remodeling events are directly coupled to N-WASP/Arp2/3-mediated actin nucleation. EspF dynamically localises to membrane-trafficking organelles in a spatiotemporal pattern that correlates with SNX9 and N-WASP activity in living cells. EspF-dependent assembly of SNX9 and N-WASP promotes EPEC pathogenesis and gastrointestinal disease (Alto et al 2007).

The Map or Mitochondria associated protein is a multi-functional protein that is involved both in host cell death (Boya et al 2001) and in filopodia formation (Kenny et al 2002). Recently, it was shown that Map binds to, induces proteolysis of, and co-localises with EBP50/NHERF1 (Na+/H+ exchanger regulatory factor regulator 1) during infection of cultured epithelial cells (Simpson et al 2006) and also alters mitochondrial morphology (Papatheodorou et al 2006). EspH is another LEE-encoded effector that promotes filopodia formation and elongated pedestals in COS cells (Tu et al 2003). EspG shows significant homology to the host cell invasion protein, VirA of *S. flexneri* and enteroinvasive *E. coli* (Elliott et al 2001). EspG and another VirA homologue, now termed EspG2 present in the second pathogenicity island, inhibits luminal membrane chloride transport via
modulation of surface DRA, the major luminal anion exchanger (Gill et al 2007).

### 1.3.1.1 Role of non-LEE effectors in EPEC pathogenesis

Among the non-LEE encoded effectors, EspC, glyceraldehyde 3-phosphate dehydrogenase, NleA and Cif, EspC is coded by the *espC* gene present in the second pathogenicity island along with EspG2 and has been found to be an enterotoxin (Mellies et al 2001) and the serine protease motif of the effector was found to be the cause of cell damage (Navarro-Garcia et al 2004). EspC also displays protease activity on human haemoglobins (Drago-Serrano et al 2006). The lambdoid phage encoded Cif promotes cell cycle arrest in host cells by blocking G2/M transition (Marches et al 2003). Recently, it was shown that NleA of EPEC compromises Sec23/24 complex, a component of the mammalian COPII protein coat that shapes intracellular protein transport vesicles (Kim et al 2007). The glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has recently been implicated in the pathogenesis of gram- negative bacteria. The enzyme was associated with Caco-2 cells after EPEC cell adhesion and purified GAPDH bound to human plasminogen and fibrinogen (Egea et al 2007).
### Table 1.2 EPEC virulence genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alternative or previous name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bfpA</em></td>
<td></td>
<td>BFP biogenesis, major pilus sub-unit-bundlin</td>
</tr>
<tr>
<td><em>bfpB</em></td>
<td></td>
<td>BFP biogenesis-outer membrane</td>
</tr>
<tr>
<td><em>bfpC</em></td>
<td></td>
<td>BFP biogenesis-inner membrane</td>
</tr>
<tr>
<td><em>bfpD</em></td>
<td></td>
<td>BFP biogenesis</td>
</tr>
<tr>
<td><em>bfpE</em></td>
<td></td>
<td>BFP biogenesis-transporter</td>
</tr>
<tr>
<td><em>bfpF</em></td>
<td></td>
<td>BFP bundle dissociation</td>
</tr>
<tr>
<td><em>bfpG</em></td>
<td></td>
<td>BFP biogenesis-outer membrane</td>
</tr>
<tr>
<td><em>bfpH</em></td>
<td></td>
<td>Function unknown</td>
</tr>
<tr>
<td><em>bfpI</em></td>
<td></td>
<td>BFP biogenesis-inner membrane</td>
</tr>
<tr>
<td><em>bfpJ</em></td>
<td></td>
<td>BFP biogenesis-inner membrane</td>
</tr>
<tr>
<td><em>bfpK</em></td>
<td></td>
<td>BFP biogenesis-inner membrane</td>
</tr>
<tr>
<td><em>bfpL</em></td>
<td></td>
<td>BFP biogenesis-inner membrane</td>
</tr>
<tr>
<td><em>bfpU</em></td>
<td></td>
<td>BFP biogenesis-periplasmic</td>
</tr>
<tr>
<td><em>bfpP</em></td>
<td></td>
<td>BFP biogenesis-prepilin peptidase</td>
</tr>
<tr>
<td><em>bfpT</em></td>
<td><em>perA</em></td>
<td>Regulator of <em>bfp</em> and <em>ler</em></td>
</tr>
<tr>
<td><em>bfiV</em></td>
<td><em>perB</em></td>
<td>Accessory factor for PerA</td>
</tr>
<tr>
<td><em>bfpW</em></td>
<td><em>perC</em></td>
<td>Accessory factor for PerA</td>
</tr>
<tr>
<td><em>escC</em></td>
<td><em>sepC</em></td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td><em>escD</em></td>
<td><em>sepE</em></td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td><em>escF</em></td>
<td><em>orf28</em></td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td><em>escJ</em></td>
<td><em>sepD</em></td>
<td>T3SS biogenesis</td>
</tr>
</tbody>
</table>
Table 1.2 (Continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alternative or previous name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>escN</td>
<td>sepB</td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td>escR</td>
<td>sepI</td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td>escS</td>
<td>sepH</td>
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</tr>
<tr>
<td>escT</td>
<td>sepG</td>
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</tr>
<tr>
<td>escU</td>
<td>sepF</td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td>escV</td>
<td>sepA</td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td>sepQ</td>
<td></td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td>sepZ</td>
<td></td>
<td>T3SS biogenesis</td>
</tr>
<tr>
<td>eae</td>
<td>eaeA</td>
<td>Adhesin binds Tir-intimin</td>
</tr>
<tr>
<td>tir</td>
<td></td>
<td>Translocated intimin receptor-initiates actin pedestal formation</td>
</tr>
<tr>
<td>espA</td>
<td></td>
<td>Forms filaments between bacteria and host delivery of effector molecules</td>
</tr>
<tr>
<td>espB</td>
<td>eaeB</td>
<td>Translocation pore; host cytosolic effector</td>
</tr>
<tr>
<td>espD</td>
<td></td>
<td>Translocation pore</td>
</tr>
<tr>
<td>espF</td>
<td>orf30</td>
<td>Tight junction disruption; cell death</td>
</tr>
<tr>
<td>espG</td>
<td>rorf2</td>
<td>Microtubule disruption</td>
</tr>
<tr>
<td>map</td>
<td>orf19</td>
<td>Mitochondrial membrane disruption; filopodia formation</td>
</tr>
<tr>
<td>ler</td>
<td>orf1</td>
<td>LEE and EspC regulator</td>
</tr>
<tr>
<td>cesD</td>
<td>sepE</td>
<td>Chaperone for EspB and EspD</td>
</tr>
<tr>
<td>cesT</td>
<td>orf21</td>
<td>Chaperone for Tir</td>
</tr>
<tr>
<td>cesF</td>
<td>rorf10</td>
<td>Chaperone for EspF</td>
</tr>
</tbody>
</table>

Adapted from Clarke et al 2003
1.3.2 The Four Stage Model of EPEC Pathogenesis

A four stage model for the pathogenic mechanism of EPEC has been proposed (Clarke et al 2003) (Figure 1.3).

In the first stage, EPEC cells express bundle-forming pili (BFP), intimin, and (EspA filaments) the expression of these determinants is dependent on both plasmid and chromosomal genes.

Figure 1.3 Four stage model of EPEC pathogenesis

In the second stage, EPEC cells adhere to the epithelial cell via BFP and EspA filaments, and a type III secretion system injects the translocated intimin receptor (Tir) and other effector molecules directly into the host cell. Effector molecules activate cell-signaling pathways, causing alterations in the host cell cytoskeleton and resulting in the depolymerization of actin and the loss of microvilli. Tir is modified and inserts into the host membrane.
In the third stage, the EspA filaments are lost from the bacterial cell surface; the bacterial adhesin intimin binds to the modified Tir, resulting in intimate attachment; and accumulation of actin and other cytoskeletal elements occurs beneath the site of bacterial adherence.

In the fourth stage, massive accumulation of cytoskeletal elements at the site of bacterial attachment results in the formation of the characteristic EPEC pedestal structure. The translocated effector molecules disrupt host cell processes, resulting in loss of tight-junction integrity and mitochondrial function, leading to both electrolyte loss and eventual cell death.

1.4 ROLE OF ADHERENCE AND INFLAMMATION IN EPEC PATHOGENESIS

1.4.1 Adherence - a Major Step in Pathogenesis of Non-Invasive Bacteria

The four stage model of EPEC pathogenesis reveals the importance of adherence in the pathogenesis of a non-invasive bacterium like EPEC. Among the various effector molecules described earlier, some play a role in adherence. One of the most important steps in initiating an infection is the ability of bacteria to adhere to host cells (Finlay and Falkow 1997). Adhesion involves strong and highly specific interactions with receptors on the cell surface (Ofek and Doyle 1994). The criteria that a bacterial product must fulfil to be classified as an adhesin are broadly based on its specificity and saturability (Nougayrede et al 2003).

Intimin, BFP, EspA, flagella and Efa1/LifA have been proposed as putative adherence factors for EPEC. Intimin is an established adhesin that interacts with Tir and helps EPEC to form an ‘intimate’ attachment with the
host intestinal epithelium (Cleary et al 2004). Other putative receptors for intimin have also been suggested, including nucleolins (Sinclair and O'Brien, 2002) for the intimin of the related pathogen enterohaemorrhagic E. coli (EHEC) and β1 integrins (Frankel et al 1996), although other investigations suggest that intimin does not bind β1 integrins (Liu et al 1999). An atypical EPEC strain with intimin subtype omicron was recently shown to possess invasive properties in HeLa cells (Hernandes et al 2007).

BFP was shown to aid in inter-bacterial interactions and formation of three-dimensional microcolonies (Giron et al 1991). The role of BFP in cellular adherence and dispersal in vitro (Knutton et al 1999) and in vivo in human volunteers (Bieber et al 1998) has been demonstrated using a bfpF mutant. Putative receptors have been proposed for BFP with respect to the inhibition of localised adherence. They include N-acetylgalactosamine (Scaletsky et al 1988), fucosylated tetra- and penta-saccharides (Cravioto et al 1991), N-acetyl lactosamine conjugated with bovine serum albumin (BSA) and Lewis X-BSA (Hyland et al 2007; Vanmaele et al 1999). Phosphatidyl ethanolamine was found to directly interact with BFP and the interaction was completely abolished by anti-BFP antiserum (Barnett Foster et al 1999). However, in an in vitro organ culture model, intimin was found to be essential for two-dimensional microcolony formation and there was little difference in adherence patterns between wild type and bfp mutant strains (Hicks et al 1998).

EspA is a part of the type III translocon apparatus coded by the LEE. It forms a filamentous structure that helps in the transportation of effector molecules from the bacterium to the host (Knutton et al 1998). EspA interacts with another LEE protein, EspB, which is believed to form a pore in the host cell membrane, thereby acting as a receptor for EspA. EspB is one of the secretory virulence factors, along with EspD, that is expressed during the
initial reversible adherence of EPEC to intestinal cells. EspB/D translocate through EspA and form pores in the epithelial membrane, thereby facilitating the entry of other virulence factors such as Tir, Map and EspF into the host cell. Mutants deficient in EspB fail to adhere and to cause gross cytoskeletal rearrangements in host epithelia (Tacket et al 2000; Warawa et al 1999), but EspB is not necessarily required for EspA interaction with host cells (Hartland et al 2000); other possible receptors are yet to be validated. EspA and BFP have been shown to play a role in biofilm formation (Moreira et al 2006).

Flagella-mediated adherence has been suggested by the recent observation that a fliC (flagellin) gene mutant was deficient in adherence (Giron et al 2002). Electron microscopy studies have revealed that flagella aid in the contact of aggregated microcolonies to host cells. EPEC H6 and H7 flagella have recently been shown to bind mucin (Erdem et al 2007) Similarly, EPEC lymphostatin (LifA) (Klapproth et al 2000) and the products of the efa1 (Nicholls et al 2000) and toxB (Tatsuno et al 2001) genes in EHEC have been shown to play a role in adherence, but the putative receptors for these proteins still remain to be identified. A putative non-fimbrial adhesin present in atypical EPEC has recently been reported (Scaletsky et al 2005).

The transition metal zinc, at concentrations that produced little or no inhibition of EPEC growth, caused decreased expression of EPEC protein virulence factors, such as bundle-forming pilus (BFP), EPEC secreted protein A, and other EPEC secreted proteins, and reduced EPEC adherence to cells in tissue culture by reducing the abundance of the RNAs encoded by the bfp gene, by the plasmid-encoded regulator per, by the locus for the enterocyte effacement (LEE)-encoded regulator ler, and by several of the esp genes (Crane et al 2007).
1.4.2 Molecular Architecture of Host Immune System

Inflammatory response is divided into the first line defence called innate immunity and a later developing immunity, i.e. adaptive immunity. The innate immune system mounts the initial response to tissue invasion. The primary cellular components of the innate immune system are macrophages, dendritic cells, natural killer (NK) cells and neutrophils (Granucci et al 2003). Macrophages are bone-marrow-derived phagocytic cells that are important for tissue homeostasis and are found in virtually all tissues of the body. Macrophages are crucial to immunosurveillance against invading pathogens and malignancies. Macrophages have a primary function to discriminate between self and non-self, and they participate in host defence against microorganisms and cancer. Macrophages also play important roles in the innate, acquired and inflammatory responses. Macrophages can function as accessory cells, presenting antigen and providing co-stimulatory ligands (e.g., CD80, CD86, and CD48) and co-stimulatory cytokines (e.g., IL-1β and IL-12) to the infiltrating T cells. Macrophages can be activated to produce pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6, chemoattractant cytokines such as IL-8, macrophage inflammatory proteins and pro-inflammatory products of arachidonic acid metabolism and toxic reactive oxygen and nitrogen intermediates.

1.4.3 Pro-Inflammatory Cytokines

Pro-inflammatory cytokines are multifunctional groups of proteins that frequently have overlapping functions (Dinarello 2001). Blockade of a single cytokine will often have limited effects on the overall inflammatory response (Dinarello 1997).
1.4.3.1 Tumor Necrosis Factor (TNF)

TNFα is released from neutrophils and macrophages upon first encounter with bacterial pathogens. Recognition of pathogens via toll-like receptors leads to TNFα expression along with other proinflammatory cytokines (Medzhitov 2001). TNFα expression also leads to activation of macrophages, which improves phagocytosis and antigen presentation. Thus, early TNFα expression is important for innate responses to infection and to improve interactions between macrophages and T cells. TNFα is produced by many cells including T-cells, macrophages and NK cells (Tracey and Cerami, 1994). TNFα stimulates the production of many inflammatory cytokines such as IL-1β, IFN-γ, NO, iNOS etc. (Cheshire and Baldwin 1997). Over expression of this cytokine is generally assumed to play an important role in chronic inflammatory diseases.

1.4.3.2 Interleukin-1β

IL-1β is one of the prototypic multifunctional cytokines, which can affect almost all types of cells and induce its effect frequently with other cytokines and mediator molecules. IL-1β was first discovered as a major mediator of inflammation. It is evident that this cytokine has numerous functions related to host defence mechanisms. IL-1β is produced by various types of cells, including macrophages, dendritic cells, B cells, and T cells. In the immune system, IL-1β is known to activate lymphocytes, monocytes, macrophages, and NK cells (Dinarello 1997). IL-1β has diverse potentiating effects on the proliferation, differentiation, and functioning of diverse non-adaptive cells (NK cells, macrophages, granulocytes, etc.) as well as specific immunocompetent cells (T and B cells).
1.4.3.3 Interleukin-8

One of the most remarkable properties of IL-8 is the variation of its expression levels. Normally, IL-8 protein is barely secreted from noninduced cells, but its production is rapidly induced by a very wide range of stimuli encompassing proinflammatory cytokines such as tumor necrosis factor (TNF) or IL-1, bacterial, viral products and cellular stress. Stimuli such as TNF or IL-1 can increase IL-8 secretion by about 100 fold while bacteria can stimulate a ten fold increase in IL-8 expression (Hoffmann et al 2002). Maximal IL-8 expression is achieved when its gene promoter is derepressed, NF-κB and JNK pathways are activated and the resulting mRNA is rapidly stabilised by the p38 MAPK pathway.

1.4.3.4 Toll-like Receptor 4

Toll-like receptor 4 or TLR4 is essential for lipopolysaccharide (LPS) recognition (Figure 1.4). LPS stimulation of macrophages results in the decreased expression of the LPS receptor complex composed of TLR4 and MD-2, a co-factor that facilitates LPS binding (Akashi et al 2000).
1.4.3.5 Toll-like Receptor 2

TLR2 recognizes a variety of microbial components. These include lipoproteins, lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria. TLR2 reportedly recognizes LPS preparations from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pylori* (Smith et al 2003). These LPS structurally differ from the typical LPS of Gram-negative bacteria.
recognized by TLR4 in the number of acyl chains in the lipid A, thereby conferring differential recognition (Netea et al 2002).

1.4.4 EPEC and Inflammation

EPEC being one of the major causes of childhood diarrhoea, the immune response to EPEC and its virulence determinants has been demonstrated using various cellular inflammatory markers. IgA offers the primary immune defence against EPEC. Colostrum IgA is transmitted from mother to child and effectively prevents EPEC infections (Carbonare et al 1997). EPEC has been shown to induce NF-κB, the nuclear transcription factor, which in turn, stimulates the transcription of the chemokine IL-8 and this leads to the recruitment of polymorphonuclear leucocytes (PMNs) to the site of infection (Savkovic et al 1996; Savkovic et al 1997). EPEC effector molecules like EspA, Intimin, EspB and BfpA have been shown to be very potent initiators of immune response (Martinez et al 1999). Flagellin has also been shown to induce IL-8 in host epithelia (Zhou et al 2003). IL-8, the CXC chemokine plays a crucial role in the recruitment of immune cells to the site of EPEC infection. The PMNs and intestinal macrophages form the first line of defence. Pathogens have to disrupt tight junction barriers and benefit from it by means of inflammation.

An important step in EPEC pathogenesis was revealed recently when it was shown that EPEC inactivates immune responses before compromising epithelial barrier function. Also, effective IL-8 stimulation is suppressed even when Caco-2 cells were stimulated with EPEC antigen, flagellin (Ruchaud-Sparagano et al 2007). EPEC has also been shown to inhibit phagocytosis by macrophages. This is an important step in bacterial persistence in the intestine, since antiphagocytosis would also reduce EPEC antigen presentation by phagocytic cells, thereby delaying immune response
Lipopolysaccharide (LPS or ‘O’ antigen) is a major effector of inflammatory mediators and is released during bacterial multiplication or cell lysis (Hewett and Roth 1993). LPS is also released in high amounts during antibiotic treatment (Frieling et al 1997).

1.4.4.1 Lipopolysaccharide (LPS)

LPS is a major constituent of the outer membrane of gram-negative bacteria and is the only lipid constituent of the outer leaflet; a single E. coli cell contains approximately $3.5 \times 10^6$ LPS molecules. Other components of the bacterial outer membrane are glycerolphospholipids in the inner leaflet and inner membrane and proteins (e.g., pore proteins such as OmpA in E. coli), some of which are firmly associated with the LPS molecules. LPS is an essential compound of the cell wall and is mandatory for bacterial viability. LPS is not toxic when it is incorporated into the bacterial outer membrane, but after release from the bacterial wall, its toxic moiety, lipid A, is exposed to immune cells, thus evoking an inflammatory response. LPS and other cell wall constituents are released from the bacterial cells when they multiply but also when bacteria die or lyse. Various endogenous factors like complement and bactericidal proteins can cause disintegration of bacteria, resulting in the release of LPS. In addition, some antibiotics are known to cause the release of LPS from bacteria (Van Amersfoort et al 2003).

EPEC LPS has been shown to elicit a mass immune response in lamina propria fibroblasts and markedly enhanced neutrophil migration in vitro. An array of pro-inflammatory cytokines like TNFα, IL-1β, IL-8 and cell adhesion molecules (CAMs) were shown to be induced by EPEC LPS (Chakravortty and Kumar, 1999). Healthy adults produce antibody response against selected EPEC ‘O’ antigen serotypes (Zapata-Quintanilla et al 2006). Since EPEC inactivates intestinal immune response and inhibits phagocytosis,
intestinal persistence and bacterial multiplication would release large amounts of LPS.

1.5  ANTIBIOTIC TREATMENT

1.5.1  Classes of Antibiotics for Bacterial Infections

1.5.1.1  Penicillins

The penicillins are the oldest class of antibiotics and are classed as the beta-lactam antibiotics. They are generally bacteriocidal. The penicillins can be further subdivided. The natural pencillins are based on the original penicillin G structure; penicillinase-resistant penicillins include methicillin and oxacillin. Aminopenicillins such as ampicillin and amoxicillin have an extended spectrum of action compared with the natural penicillins; extended spectrum penicillins are effective against a wider range of bacteria. These generally include coverage for *Pseudomonas aeruginosa* and may provide the penicillin in combination with a penicillinase inhibitor. Penicillins act by inhibiting the peptidoglycan synthesis of the bacterial cell wall.

1.5.1.2  Cephalosporins

Cephalosporins and the closely related cephapenems and carbapenems, like the pencillins, contain a beta-lactam chemical structure. They have the same mode of action as other beta-lactam antibiotics. The "cepha" drugs are subgrouped into 1st, 2nd, 3rd and 4th generations. Each generation has a broader spectrum of activity than the one before. In addition, cefoxitin, a cephapenem, is highly active against anaerobic bacteria, which offers utility in treatment of abdominal infections. The 3rd generation drugs like cefotaxime, ceftriaxone, cross the blood-brain barrier and
may be used to treat meningitis and encephalitis. Cephalosporins are the usually preferred agents for surgical prophylaxis.

1.5.1.3 Fluoroquinolones

The fluoroquinolones are synthetic antibacterial agents, and not derived from bacteria. The parent of the group is nalidixic acid. The quinolones were earlier used only to treat urinary tract infections. The fluoroquinolones are broad-spectrum bacteriocidal drugs which are well distributed into bone tissue, and so well absorbed that in general they are as effective by the oral route as by intravenous infusion. They inhibit the bacterial DNA gyrase or the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription. For many gram-negative bacteria DNA gyrase is the target, whereas topoisomerase IV is the target for many gram-positive bacteria.

1.5.1.4 Aminoglycosides

Aminoglycosides are a group of antibiotics that are effective against certain types of bacteria. They include amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, tobramycin, and apramycin. Aminoglycosides that are derived from bacteria of the Streptomyces genus are named with the suffix -mycin, while those which are derived from Micromonospora are named with the suffix -micin. Aminoglycosides work by binding to the bacterial 30S ribosomal subunit (some work by binding to the 50S subunit), inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site and also causing misreading of mRNA, leaving the bacterium unable to synthesize proteins vital to its growth. Aminoglycosides are particularly useful for their effectiveness in treating Pseudomonas aeruginosa infections.
1.6 ANTIMICROBIAL RESISTANCE

Most microorganisms can reproduce rapidly and bacteria can freely exchange genes by conjugation, transformation and transduction between widely divergent species. This horizontal gene transfer, coupled with high mutation rate and may other means of genetic variation, allows microorganisms to swiftly evolve (via natural selection) to survive new environments and respond to environmental stresses. This rapid evolution is important in medicine, as it has led to the recent development of pathogenic bacteria that are resistant to most antibiotics (Enright et al 2002). Gram negative pathogens show increased resistance (Liassine 2000) since they have the ability to rapidly acquire resistance. Antimicrobial resistance is an increasing problem across hospitals worldwide, especially in intensive care settings, where nosocomial infections are 5-10 times more likely to occur than on the general wards (Arnold et al 2007).

The introduction of potent antimicrobial agents has increased survival among patients with gram negative sepsis, but mortality still varies between 20 and 50% especially in patients with shock (Rangel-Frausto 2005). Gram-negative bacterial infections constitute an emerging threat because of the development of multidrug-resistant organisms. There is a relative shortage of new drugs in the antimicrobial development pipeline that have been tested in vitro and evaluated in clinical studies (Vergidis and Falagas 2008).

1.7 EPEC AND ANTIBIOTIC TREATMENT

Antibiotics like amikacin, garamycin, colistin and cefotaxime are generally prescribed for EPEC infections (Ruczkowska et al 1990). But several cases of antimicrobial resistance in EPEC have been observed in different parts of the world including Sudan (Ahmed et al 2000), Germany
(Guerra et al 2006; Lim et al 1992), United States (Moyenuddin et al 1989), Kenya (Senerwa et al 1991), Uruguay (Vignoli et al 2005) and Tanzania (Vila et al 1999) with resistance patterns spread across penicillins, cephalosporins and aminoglycosides. Antibiotic resistance in EPEC is also a major challenge since bacterial persistence inactivates sodium D-glucose co-transporter (SGLT-1) leading to decreased water absorption in the microvilli, thereby leading to persistent, watery diarrhoea (Dean et al 2006).

Generally, EPEC strains are susceptible to quinolone antibiotics like ciprofloxacin and nalidixic acid, but reports have shown that EPEC strains, especially, the O111 serotypes exhibit increased resistance to quinolones (Guerra et al 2006; Levine and Rennels, 1978; Schroeder et al 2002).

![Figure 1.5 Gram negative bacterial outer membrane](image)

Adapted from Alexander and Rietschel, 2001.

**Figure 1.5 Gram negative bacterial outer membrane**

This antimicrobial resistance may be attributed to the nature of the outer membrane of gram negative bacteria (Figure 1.5). It provides a hydrophilic surface and acts as a permeability barrier to external agents (Alexander and Rietschel 2001). This is mainly due to the presence of LPS. The outer membrane also contains hydrophilic channels known as porins,
which allow nutrients with relatively small molecular weight (< 600 Daltons) to enter the inner parts of the cell. These water-filled pores generally exclude the entry of hydrophobic substances (Nikaido 2003). The influx of many lipophilic compounds is prevented by multidrug efflux pumps which are energized by a proton motive force (Poole 2002). The different families of pumps involved in resistance are FQ, fluoroquinolone; CM, chloramphenicol; TC, tetracycline; ML, macrolides, MD, multidrug. While NorA is a multidrug transporter, it exports only FQs (and biocides) as clinically relevant agents (Poole 2005) (Figure 1.6).

Adapted from Poole, 2005.

**Figure 1.6 Multidrug efflux pumps on the gram-negative bacterial outer membrane**

Quinolone drugs are a large and widely used class of synthetic antibacterial compounds. First-generation quinolones include nalidixic acid, a drug which was suspended from use in the United States in 2006. Subsequent generations have been modified to increase spectrum and potency. The most significant modification has been the addition of a fluorine atom at position C-6 in drugs such as ciprofloxacin (CFX), which results in a considerable increase in activity (Heddle and Maxwell, 2002). The target enzyme for quinolones in *E. coli* is the enzyme DNA gyrase (Sugino et al 1977).
DNA gyrase is a type II topoisomerase that is able to alter the topological state of DNA by cleaving both strands, passing a double strand of DNA through the gap and resealing the ends. In the presence of ATP, this results in negative supercoiling, a reaction unique to gyrase (Champoux 2001). The crystal structures of the 43-kDa N-terminal domain of GyrB (responsible for ATP hydrolysis and capturing a DNA strand) and a 59-kDa fragment of the 64-kDa N-terminal domain of GyrA containing the residues for DNA binding and cleavage and the quinolone resistance-determining region [QRDR; residues 67 to 106] have been solved (Morais Cabral et al 1997; Wigley et al 1991). Resistance to quinolones commonly arises in DNA gyrase via mutations in the QRDR. Such mutations include GyrA (Ser83 to Trp), which gives 20-fold resistance to a wide range of quinolones. Mutation of Asp87 to Asn or Val is also a frequent spontaneous mutation that occurs in the QRDR region that leads to quinolone resistance (Barnard and Maxwell, 2001). This region of GyrA is close to the active site, where the DNA is bound, and close to the tyrosine at position 122, where the phosphotyrosine link between enzyme and DNA is formed. Quinolone drugs bind to gyrase-DNA complexes, but only weakly to either gyrase or DNA. It is thought that quinolones bind to a pocket consisting of the QRDR of GyrA and the region of distorted DNA bound to it. Quinolone is thought to interact with the DNA-enzyme complex in this pocket (Heddle and Maxwell, 2002). The Ser83 mutation in the GyrA subunit has been observed in EPEC O111 strains that have been associated with quinolone resistance (Guerra et al 2006; Schroeder et al 2002).

The coumarins (e.g. novobiocin, chlorobiocin) are naturally occurring compounds, isolated from certain strains of Streptomyces, which inhibit the ATPase activity of the B protein of DNA gyrase (Boehm et al 2000). Coumarins are also produced as secondary metabolites by plants (Lewis and Ausubel 2006). Coumarin resistance in E. coli has been attributed
to mutations Asp426 to Asn and Lys447 to Glu (Yamagishi et al. 1981; Yamagishi et al. 1986). The GyrB residues in the 47kDa C-terminal domain form part of a quinolone binding pocket that includes DNA and the quinolone resistance-determining region in GyrA and large conformational changes during the catalytic cycle of the enzyme allow these regions to come into close proximity (Figure 1.7). Therefore, this might be the possible reason for association of quinolone resistance with GyrB mutations (Heddle and Maxwell 2002).

![Interaction of ciprofloxacin (fluoroquinolone) with E. coli DNA Gyrase](image)

Adapted from Heddle and Maxwell, 2002.

**Figure 1.7 Interaction of ciprofloxacin (fluoroquinolone) with E. coli DNA Gyrase**

### 1.8 ANTIBIOTICS AND THEIR SIDE EFFECTS

Even though antibiotics are the cornerstone for the prevention and treatment of numerous diseases, they unfortunately have the potential to cause adverse events.
Hypersensitivity in penicillins is common, and cross allergenicity with cephalosporins has been reported. Several cephalosporins and related compounds have been associated with seizures. Cefmetazole, cefoperazone, cefotetan and ceftriaxone may be associated with a fall in prothrombin activity and coagulation abnormalities (Hantson et al 1999). Pseudomembranous colitis has been reported with cephalosporins and other broad spectrum antibiotics (Neu 1982). Certain drugs in this class may cause renal toxicity (Tune 1990). Some of the fluoroquinolones such as lomefloxacin has been associated with increased photosensitivity. All drugs in this class have been associated with convulsions (Domagala 1994).

Aminoglycosides cause nephrotoxicity and ototoxicity. These problems can occur even with normal doses. Dosing should be based on renal function, with periodic testing of both kidney function and hearing (Mingeot-Leclercq et al 1999; Mingeot-Leclercq and Tulkens 1999).

The unguided usage of the available antibiotics and self-medication by the public without awareness on the dosages has substantially increased the risk of bacteria becoming resistant to the existing antibiotics. There has not been a single class of antibiotic that has come into the commercial grounds for around half a century. Even though reports show hopes of new class of antibiotics (Rose and Rybak 2006), it is inevitable to foresee that the clever, already antibiotic-resistant microbes will become resistant to any new class of antibiotics as well. Adverse antibiotic-induced reactions are a concern not only because they cause host injury but also because they interrupt and complicate therapy and often necessitate alternative, more expensive agents that have the ability to foster the emergence and spread of drug-resistant organisms (Gleckman and Czachor 2000). This necessitates the probing of all humanly possible resources for new compounds that could have antibacterial effects that are safe in vivo.
1.9 EPEC DIAGNOSIS AND TREATMENT

Novel insights into the molecular mechanisms of EPEC pathogenesis have led to the identification of an array of markers as possible targets for diagnosis. Two of them include intimin and BFP (Franzolin et al 2005). Their role in pathogenesis is significant due to their involvement in EPEC adherence to host tissue. Gram-negative bacterial outer membrane serves as a potential target for identifying putative diagnostic targets. Previous work from our laboratory has shown that outer membrane preparations of EPEC are cytopathic (Kumar et al 2001) and play a major role in cell signalling and inflammation (Malladi et al 2004a and 2004b; Puthenedam et al 2007). Porins form a considerable amount of outer membrane constituents. Porins as diagnostic targets have shown potential with *Shigella* (Biswa et al 2007) and *Salmonella* (Vordermeier et al 1987). Outer membrane protein A or OmpA of EHEC has been shown to induce dendritic cells (Torres et al 2006). Apart from their role in inducing inflammation, porins have also be implicated in bacterial adherence (Hara-Kaonga and Pistole, 2004; Sperandio et al 1995). The maltose inducible porin, LamB, has been shown to aid in adherence (Rocha-De-Souza et al 2001). Porins have also been implicated in the development of multi-drug resistance in bacterial pathogens (Torres et al 2007). Hence, there is an imperative requirement of alternative forms of therapy to combat multi-drug resistance and side effects of antibiotic therapy.

1.10 PLANT–DERIVED ANTIBACTERIALS

Medicinal plants represent a rich source from which antimicrobial agents may be obtained. They are the major source of drugs for the treatment of various health disorders especially in rural areas of Pakistan, India, China, Afghanistan, Iran and other countries of this region. The use of plant based
medicines (local medicine) dates back to 4000-5000 B.C. India has a rich heritage of possessing ancient systems of medicine for treating illnesses in the form of Ayurveda, Siddha and Unani. These have identified a large number of plants with therapeutic efficiencies (Tripathi et al 2008). Nowadays huge number of allopathic medicines also contains plant based ingredients that are used for their preparation by different companies. There are about 400,000 species of higher plants in the world, as compared to animal’s species that are about 5-10 million. The plant materials contain thousands of chemicals such as resins, rubbers, gums, waxes, dyes, flavors, fragrances, proteins, amino acids, bioactive peptides, phytohormones, sugar, tannins, alkaloids, terpenoids, flavonoids and bio pesticides which act against diseases and infections of humans and animals when properly used. A great variety of organic compounds produce by plants are not directly involved in primary metabolic processes of growth and development but secondary metabolites. These substances serve as plant defense mechanisms against predation by microorganisms, insects, herbivores (Cowan 1999). The active principle of many drugs found in plants are secondary metabolites of which at least 12,000 have been isolated which is less than 10% of its total.

Medicine is becoming increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics are becoming ineffective. Natural-products chemists and microbiologists think that the multitude of potentially useful phytochemical structures which could be synthesized chemically is at risk of being lost irretrievably (Borris, 1996). In the early days of antibiotics, antimicrobial substances were found only from bacterial and fungal resources and plants were hardly looked up to for their antimicrobial potential. After a remarkable decrease in pace of the arrival of new classes of antimicrobial compounds, the probing of plants for antibacterial agents had set in with rigour and greater pace. The supportive literature from traditional medicine and usage of herbal formulations by
public also must have instigated the clinical microbiologists to probe into plant compounds. It was reported that in 1996, sales of botanical medicines increased 37% over 1995 (Klink 1997).

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman 1963). Most of them are secondary metabolites (Schultes 1978). In many cases, these substances serve as plant defence mechanisms against predation. Some give plants their odours (terpenoids); others (quinones and tannins) are responsible for plant pigment; some of the compounds are responsible for plant flavour (like the terpenoid capsaicin from chilli peppers), and some spices that are used by humans to season food yield useful medicinal compounds. Cowan (1999) in his extensive research classifies compounds under a few major categories which show significant antimicrobial activity.

1.10.1 Phenolics and Polyphenols

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. The common herbs tarragon and thyme both contain caffeic acid, which is effective against bacteria (Brantner et al 1996). Catechol and pyrogallol both are hydroxylated phenols shown to be toxic to microorganisms. Catechol has two OH groups and pyrogallol has three. The sites and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Geissman 1963). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds,
possibly through reaction with sulphhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman 1987). Phenolic compounds possessing a C3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial. Eugenol is a well-characterized representative found in clove oil and is considered bacteriostatic against bacteria (Gaysinsky et al 2005).

1.10.2 Quinones

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds are coloured and are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin. Their presence in henna gives that material its dyeing properties (Fessenden, and Fessenden 1982). In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern et al 1996) often leading to inactivation of the protein and loss of function because of which the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. Hypericin, an anthraquinone from St. John's wort (Hypericum perforatum) was initially used as an antidepressant, and Duke reported that it had general antimicrobial properties (Duke 1985).

1.10.3 Flavones, Flavonoids, and Flavonols

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). Since they are known to be synthesized by plants in response to microbial infection (Dixon et al 1983),
they have also been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya et al. 1996). Catechins, the most reduced form of the C3 unit in flavonoid compounds have been extensively researched due to their occurrence in oolong green teas. It was noticed some time ago that teas exerted antimicrobial activity and that they contain a mixture of catechin compounds. These compounds inhibited in vitro Vibrio cholerae O1 (Borris 1996) and Streptococcus mutans (Batista et al. 1994). The catechins inactivated cholera toxin in Vibrio (Borris 1996). The authors propose that small structural differences in the compounds are critical to their activity and pointed out another advantage of many plant derivatives: their low toxic potential. Pharmacologically active concentrations are not likely to be harmful to human hosts. Lipophilic compounds would be more disruptive of this structure. However, several authors have also found the opposite effect; i.e., the more hydroxylation, the greater the antimicrobial activity (Tsuchiya et al. 1996).

1.10.4 Tannins

Tannins are a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 to 3,000 (Haslam 1996). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam 1996). Their mode of antimicrobial action, as described in the section on quinones, may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc. The antimicrobial significance of this particular activity has not been
explored. Scalbert (Scalbert 1991) reviewed the antimicrobial properties of tannins.

1.10.5 Coumarins

Coumarins are phenolic substances made of fused benzene and pyrone rings (Thornes 1997). Hydroxycinnamic acids, related to coumarins, seem to be inhibitory to gram-positive bacteria (Fernandez et al 1996). Coumarins have also shown potential as bacterial efflux pump inhibitors (Stavri et al 2007).

1.10.6 Terpenoids and Essential Oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is C10H16, and they occur as diterpenes, triterpenes, and tetraterpenes (C20, C30, and C40), as well as hemiterpenes (C5) and sesquiterpenes (C15). When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Artemisin and its derivative -arteether, also known by the name qinghaosu, find current use as antimalarials (Vishwakarma 1990). A terpenoid constituent, capsaicin, has a wide range of biological activities in humans, affecting the nervous, cardiovascular, and digestive systems (Virus and Gebhart 1979). The evidence for its antimicrobial activity is mixed. Recently, Cichewicz and Thorpe found that capsaicin might enhance the growth of
Candida albicans but that it clearly inhibited various bacteria to differing extents (Cichewicz and Thorpe 1996). Although possibly detrimental to the human gastric mucosa, capsaicin is also bactericidal to Helicobacter pylori (Jones et al 1997). The ethanol-soluble fraction of purple prairie clover yields a terpenoid called petalostemumol, which showed excellent activity against Bacillus subtilis and Staphylococcus aureus and lesser activity against gram-negative bacteria as well as Candida albicans (Hufford et al 1993). Two diterpenes (Batista et al 1994) were found to work well against Staphylococcus aureus, V. cholerae, P. aeruginosa, and Candida spp.

1.10.7 Alkaloids

Heterocyclic nitrogen compounds are called alkaloids. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy Papaver somniferum (Fessenden and Fessenden 1982); Berberine is an important representative of the alkaloid group. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (O’Neill et al 1987). Berberine along with an associated multi-drug resistant (MDR) 5’-methoxyhydnocarpin isolated from the same plant acts as a very potent anti-bacterial (Amin et al 1969).

1.10.8 Lectins and Polypeptides

Peptides are often positively charged and contain disulfide bonds. Their mechanism of action may be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Zhang and Lewis 1997). They are toxic to yeasts and gram-negative and gram-positive bacteria (Fernandez de Caleyta et al 1972). Fabatin, a newly identified 47-residue peptide from fava
beans, appears to be structurally related to -thionins from grains and inhibits *E. coli, P. aeruginosa*, and *Enterococcus hirae* but not *Candida* or *Saccharomyces* (Zhang and Lewis 1997).

The success of plant defence mechanisms in combating pathogen infection rivals mammalian systems. Plant and mammalian systems have a lot in common when confronted with pathogenic bacterial infections. Both the groups sacrifice infected host tissues by apoptosis; produce anti- microbial peptides and attack the microbes by creating an acidic environment or by producing hydrogen peroxide and iron chelators (Lewis and Ausubel 2006). Plants have developed an enormous variety of small- molecule antimicrobials. Although their anti-microbial activity is relatively weak when compared to that of common antibiotics produced by bacteria and fungi, some of the molecules show promise as anti-microbials. Plant-derived antimicrobials include pyrithione from *Polyalthia nemoralis* (Dixon 2001) which is highly potent against bacteria and fungi. Other examples of potent antimicrobials include rhein, plumbagin, resveratrol, gossypol and coumestrol (Lewis and Ausubel 2006). Plant compounds are found to be really potent when MDR are disabled by mutation or by incorporating synthetic MDR inhibitors. For example, rhein showed no inhibitory activity against *E. coli* at 500 µg/mL, whereas disabling MDRs reduced the MIC to as low as 0.25 µg/mL (Tegos et al 2002). Apart from anti-microbial activity, some of these molecules exhibit multiple properties like anti-inflammatory and anti-cancer activity. Berberin (Kuo et al 2004), rhein (Guo et al 2002) and a plant polyphenol, quercetin (Tormakangas et al 2005) have been shown to possess dual anti-microbial and anti-inflammatory activity.
1.10.9 Diarylheptanoids

Diarylheptanoids are a class of phenolic compounds that have been shown to possess very potent anti-inflammatory properties (Lee et al. 2006). Diarylheptanoids are widely produced by members of the Zingiberaceae family, especially from the medicinally important plant, Alpinia officinarum (Yadav et al. 2003). Alpinia officinarum, also known as lesser galangal is a perennial herb found throughout the tropical and sub-tropical regions of Asia. The rhizome is widely used in India and China for the treatment of respiratory infections, gastro-intestinal disorders, cancer and arthritis (An et al. 2006). Diarylheptanoids efficiently reduce bacterial lipopolysaccharide-induced inflammation (Lee et al. 2006). They have also been therapeutically used for combating Leishmania infections (Alves et al. 2004). Phenolic compounds can also act as permeabilisers of the outer membrane by disruption of the LPS. Such phenolic compounds have been shown to possess antimicrobial properties primarily due to their disruption of outer membrane (Alakomi et al. 2007).

1.11 OBJECTIVES

Taking into consideration the three important pathogenic strategies of EPEC, viz. adherence, bacterial persistence and inflammation, the following objectives have been addressed in this thesis:

- Understanding the mechanisms of EPEC pathogenesis with reference to the role of EPEC LamB (maltoporin) in adherence to host epithelial cells and as a possible candidate for diagnosis.
• Evaluating the role of lipopolysaccharide (LPS) from EPEC in the inflammatory response during pathogenesis.

• Testing the immunomodulatory and bactericidal potential of *Alpinia officinarum* plant extract and pure compound (diarylheptanoid) on EPEC.

• Understanding the bactericidal activity of the diarylheptanoid from *Alpinia officinarum* by *in silico* modelling targeting DNA gyrase.