Current Topics in Microbiology and Immunology

Gad Frankel · Eliora Z. Ron *Editors*

Escherichia coli, a Versatile Pathogen

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Escherichia coli, a Versatile Pathogen

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Foreword

The species *Escherichia coli* represents well-known microorganisms that are used as "working horses" in molecular biology, genomics, and biotechnology. It has long been recognized that *Escherichia coli* is also a group of organisms with high pathogenic potential both intestinally and extraintestinally. In addition, *E. coli* is a member of the microbiome in humans, animals, and even plants. In summary, *E. coli* is an organism with countless faces and the present book addresses various aspects of this organism.

Bacteria of *Shigella* species are strongly related to *E. coli*, so one could claim that *Shigella* organisms belong to the *E. coli* species. In the first chapter, Ilia Belotserkovsky and Philippe Sansonetti describe the cell biology of *E. coli* like *Shigella* pathogens causing infections of the gut. Claire Jenkins describes enteroaggregative *E. coli* bacteria, which have the capacity to colonize the gut and to induce gut-associated infectious diseases. Her findings are presented in the second chapter.

Shigella and a number of *E. coli* pathogens possess gene clusters encoding for a Type III secretion system (T3SS). In his chapter, Gad Frankel describes the Type III system machinery of EPEC, which enables the transport of proteins from microorganisms. The Type III secretion machineries allow the transfer of effector molecules to the outside and into intestinal host cells. Intestinal pathogenic *E. coli* uses this mechanism to stimulate diseases. The chapter of Abigail Clements describes the roles of the infected *E. coli* effectors, while the chapter written by Helge Karch deals with enterohemorrhagic *E. coli* (EHEC), which play an important role in public health issues. EHEC bacteria are able to induce gut-associated infections. Furthermore, the Shiga toxins—produced by EHEC—are responsible for diseases outside the gut, e.g., the kidney.

In addition to intestinal infections, *E. coli* strains may also induce extraintestinal diseases, such as infections of the urinary tract and systemic infections. Eliora Ron's chapter introduces the various types of extraintestinal pathogens containing the capacity to induce diseases in humans and animals. The analysis of these pathogens under the "One Health" aspect is of utmost importance, since *E. coli* is a

"melting pot" for gene transfer both among various strains of *E. coli* as well as of other bacterial species.

Uri Gophna, an expert in genetic analysis of *E. coli,* describes in his chapter evolutionary processes and the emerging drug resistance in *E. coli*—another important topic in the biology of this microorganism. Next to its role as intestinal and extraintestinal pathogens, *E. coli* act is also a commensal bacterium in the gut of many species. Various sequence types of *E. coli* play a role in drug resistance, gene transfer, and pathogenicity. Joseph Paitan illustrates these aspects in his chapter.

As mentioned, *E. coli* strains are serious pathogens. Therefore, it is necessary to develop vaccines in order to combat intestinal and extraintestinal infections. In her chapter, Mariagrazia Pizza describes these efforts undertaken in the development of vaccines against different types of *E. coli*.

Summarizing the articles published in this book on *E. coli,*, it is clear that these highly diverse organisms play an important role in many areas from public health to biotechnology and other fields. I strongly recommend this book for further reading and discussions.

Halle, Germany

Jörg Hacker
President of the German Academy of
Sciences Leopoldina—National
Academy of Sciences

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Shigella and Enteroinvasive *Escherichia Coli*

Ilia Belotserkovsky and Philippe J. Sansonetti

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Abstract *Shigella* and enteroinvasive *Escherichia coli* (EIEC) are gram-negative bacteria responsible for bacillary dysentery (shigellosis) in humans, which is characterized by invasion and inflammatory destruction of the human colonic epithelium. Different EIEC and *Shigella* subgroups rose independently from commensal *E. coli* through patho-adaptive evolution that included loss of functional genes interfering with the virulence and/or with the intracellular lifestyle of the bacteria, as well as acquisition of genetic elements harboring virulence genes. Among the latter is the large virulence plasmid encoding for a type three secretion system (T3SS), which enables translocation of virulence proteins (effectors) from

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the bacterium directly into the host cell cytoplasm. These effectors enable the pathogen to subvert epithelial cell functions, promoting its own uptake, replication in the host cytosol, and dissemination to adjacent cells while concomitantly inhibiting pro-inflammatory cell death. Furthermore, T3SS effectors are directly involved in *Shigella* manipulation of immune cells causing their dysfunction and promoting cell death. In the current chapter, we first describe the evolution of the enteroinvasive pathovars and then summarize the overall knowledge concerning the pathogenesis of these bacteria, with a particular focus on *Shigella flexneri*. Subversion of host cell functions in the human gut, both epithelial and immune cells, by different virulence factors is especially highlighted.

1 Introduction

Bacillary dysentery (or shigellosis) is clinically characterized by severe bloody and mucous diarrhea associated with fever and abdominal cramps. These symptoms reflect invasion of bacteria into colonic and rectal mucosa, provoking a strong inflammatory response that leads to destruction of the colonic epithelium. Life-threatening complications may also occur including hypoglycemia, bacteremia, septicemia, hemolytic uremic syndrome leading to acute renal failure and toxic megacolon (a lower intestinal occlusion accompanied by perforation and peritonitis) (van den Broek et al. 2005). Unlike other enteric infections (i.e., rotavirus, enterotoxigenic *E. coli* (ETEC), and *Vibrio cholerae*) that are marked by severe watery diarrhea, shigellosis is less likely to induce major purge; hence, dehydration and electrolyte imbalance are less frequent. Shigellosis remains one of the leading causes of morbidity and mortality mostly in low-income countries especially among children under 5 years old in endemic regions (Kotloff et al. 2013). In addition, bacillary dysentery contributes to malnutrition causing severe growth retardation in young children (van den Broek et al. 2005).

The etiological agents of shigellosis are *E. coli*-related bacteria which, historically, were divided into *Shigella* species (with four subgroups) and enteroinvasive *Escherichia coli* (EIEC) species, depending on several clinical and biochemical differences. However, with the development of molecular tools and the rise of the genomics era, it became clear that these species belong to the same genus as well as other pathogenic and commensal *E. coli* (discussed below). The unique feature of dysentery-causing strains is the ability to invade host cells, which requires specific molecular adaptations from the bacterial side and induces a particular immune response from the host side. In the current chapter, we first briefly describe the evolution of enteroinvasive *E. coli* subgroups and then focus on the virulence factors that enable these bacteria to invade and colonize the intestinal mucosa through manipulation of both epithelium and immune system. Since *Shigella flexneri* is the most studied subgroup, it is used as an example throughout this review while other subgroup specific factors are occasionally discussed.

2 Evolution of Enteroinvasive Pathovars of *E. Coli*

Kiyoshi Shiga made the first characterization of bacteria causing bacillary dysentery in 1898. He noticed the similarities of this strain to *E. coli* (or *Bacillus coli* as it was called back then) and in order to distinguish this clinically relevant strain from non-virulent *E. coli* he named it *Bacillus dysenterie* (Bensted 1956).

In the following years, more strains were isolated by several researchers and in the 1940s a *Shigella* genus was established comprising four species: *S. dysenteriae, S. flexneri, S. boydii, and S. sonnei* (Ewing 1949; Bensted 1956). Each species can be further subdivided into several serotypes (15 of *S. dysenteriae,* 14 of *S. flexneri,* 20 of *S. boydii,* and a single serotype of *S. sonnei*), based on antibody recognition of the different structures of the lipopolysaccharide (LPS) O-antigen repeat units exposed on the outer membrane of the bacteria. However, in 1944, strains of *E. coli* capable of invading the mucosa of the colon similarly to *Shigella* were identified and called enteroinvasive *E. coli* (EIEC), in contrast to other pathogenic strains of *E. coli* that did not penetrate the mucosa (such as enterohemorrhagic, enteropathogenic, and enterotoxigenic *E. coli*, *i.e.,* EHEC, EPEC, and ETEC). Generally, EIEC shows very similar characteristics to *Shigella* (and sometimes even share similar serotypes) with a milder virulence and a higher infectious dose required (DuPont et al. 1989). In 1958, *Shigella* was defined as a non-motile bacterium (with the exception of a few *S. flexneri* serotype six strains) that does not produce gas from fermentable carbohydrates and that is much less active in the utilization of different carbohydrates compared to *E. coli* (Edwards and Ewing 1986). Interestingly, *S. sonnei* stands apart from the rest of the *Shigellae*, being able to utilize more diverse sources of carbon. Biochemically, EIEC is much more similar to *Shigella* than to nonpathogenic *E. coli* (Farmer and Davis 1985) and is distinguishable from *Shigella* only by higher prevalence of the ability to ferment mucate and utilize serine, xylose or sodium acetate (Doyle 1989). With the rise of the molecular era and the accumulation of whole genome sequences, it became possible to investigate the relatedness between different species and to get insights into their evolution. It became evident that both *Shigella* and EIEC are genetically very similar and have a very high similarity to non-virulent *E. coli*, which taxonomically would put them all into one genus (Lan et al. 2004; Pettengill et al. 2015 and more). These two pathovars are estimated to have arisen independently on multiple occasions from commensal *E. coli,* while the earliest event for *Shigella* is estimated to have happened about 50,000–270,000 years ago (Pupo et al. 2000; Pettengill et al. 2015), coevolving with early humans. This comes well along with the fact that *Shigella* naturally infects only *Homo sapiens* (with the exception of a few non-human primates). Yet, while *Shigella* and EIEC are so similar genetically to other *E. coli*, what makes the striking clinical difference between the commensal *E. coli* and the enteroinvasive strains? When comparing the genomes of *Shigella*/EIEC to commensal *E. coli* K12, which are different by about 1.5% only, two main features are clearly noticeable: gain of virulence factors and loss of functional genes interfering with virulence.

First, all enteroinvasive strains contain an invasion plasmid (pINV) and pathogenicity islands (PIs) on the chromosome, acquired by horizontal gene transfer from another genus. This is evident from a different codon usage, abundance of insertion sequences and the GC content of the genes found on these genetic elements (e.g., the GC content of genes on pINV is below 40% while the rest of *Shigella* genome is around 50%). The invasion plasmid is relatively large (approximately 220 kb) and harbors around 100 genes. However, it possesses a core of 30 kb that is necessary and sufficient for the enteroinvasive phenotype of the bacteria (Buchrieser et al. 2000). About 50 genes found in this region encode for structural and auxiliary proteins of a type three secretion system (T3SS) that comprises a syringe-like structure (the type three secretion apparatus, T3SA) and proteins translocated through it, called effectors. The T3SA spans the two membranes of the bacterium and is inserted into the membrane of the target cell, thus allowing translocation of virulence factors from the pathogen directly into the host cytoplasm (see chapter "Type Three Secretion" for more details). There are around 25 T3SS effectors encoded on the virulence plasmid and 5–7 more encoded on the chromosome. In addition to the virulence plasmid, there are three main PIs and a multidrug resistance locus often found on the chromosome of *Shigella*/EIEC, encoding for about 35 virulence-associated and antibiotic resistance proteins [reviewed in (Parsot 2005; Ogawa et al. 2008; Schroeder and Hilbi 2008; Parsot 2009)]. The function of the different virulence factors is discussed in the following sections.

In contrast to virulence factor acquisition, a second feature characterizing *Shigella*/EIEC pathogenicity is the loss of function of some genes, so-called black holes. This loss was suggested to occur due to either interference of these genes with virulence or adaptation of the bacterium to the intracellular lifestyle with available nutrients (Maurelli et al. 1998). In fact, while close to 200 genes were obtained, about 900 genes are missing or were inactivated in *S. flexneri* during the divergence from commensal *E. coli* (Jin et al. 2002; Wei et al. 2003). One reason for gene loss or inactivation is that their products interfere with the function of the newly acquired virulence factors. Such an example is the outer membrane protease OmpT that interferes with the polar localization of the actin nucleator IcsA, which is necessary for intracellular movement of *Shigella* and hence for its spread and virulence (discussed below). Another example is the lysine decarboxylase (encoded by *cadA*) that catalyzes the production of the polyamine cadaverine, which was shown to inhibit the function of *Shigella* enterotoxins (Sansonetti et al. 1983; Maurelli et al. 1998). Furthermore, it was found that de novo synthesis of nicotinamide adenine dinucleotide (NAD) is inactivated in *Shigella* since its precursor (quinolinate) inhibits bacterial virulence (Prunier et al. 2007). Interestingly, despite the potential benefits of directed motility and attachment to host cells, *Shigella*/EIEC does not synthesize functional flagella and fimbria (Bravo et al. 2015). The most convincing explanation for such loss is that both surface-exposed structures are potent activators of the host innate immunity, which might interfere with the mucosal colonization process (Sakellaris et al. 2000; Ramos et al. 2004). In addition, *Shigella*/EIEC could afford loosing their autonomous motility due to an acquired ability of host-derived actin-based intracellular movement (discussed

below). An alternative explanation for gene inactivation is the abundance of nutrients inside the host, which renders many bacterial metabolic pathways (e.g., lactose fermentation) dispensable (Ito et al. 1991; Yang et al. 2005). Overall, the importance of these "black holes" for virulence is further supported by the fact that different *Shigella*/EIEC strains possess various independent mutations (also called patho-adaptive mutations) in the same "anti-virulent" gene clusters. A good example of such convergent evolution is the *cad* locus: While in some strains of *S. flexneri* and EIEC this locus is completely absent, it is present but inactive due to either insertion sequences or replacement with a prophage in *S sonnei* and some other EIEC strains (Day et al. 2001; Casalino et al. 2003; Casalino et al. 2005).

In summary, *Shigella* and EIEC along with other pathogenic *E. coli* are all taxonomically part of the *E. coli* genus based on sequence similarity. However, based on biochemical properties, invasive lifestyle, and clinical manifestations, *Shigella* and EIEC can be separated from other *E. coli* and designated as a cohort of enteroinvasive *E. coli* pathovars. Nevertheless, they do not represent an evolutionarily separate group but rather result from a convergent evolution leading to invasive patho-adaptation. For the sake of simplicity and as most of research work focused on pathovars initially called *Shigella*, this name is used throughout this review.

3 Colonization of the Intestinal Lumen and Preparation of the Virulence Arsenal

Shigella is directly transmitted from person to person by the fecal–oral route or via ingestion of contaminated food and water. Upon ingestion, the acidic environment of the stomach induces expression of bacterial periplasmic proteins that contribute to acid resistance of *Shigella* (Porter and Dorman 1994), enabling its survival at pH 2.5 for at least 2 h (Gorden and Small 1993). After reaching the intestine, *Shigella* encounters a population of microorganisms comprising over 1000 different species at a very high density of up to 10^{12} bacteria per gram of feces in the colon (Martins dos Santos et al. 2010), which is the preferential infection site for *Shigella*. Given the extremely low infectious dose [between 10 and 100 bacteria (DuPont et al. 1989)], it is evident that *Shigella* might have evolved mechanisms to compete for its niche while being vastly outnumbered by the gut microbiota. In fact, several studies stressed the inhibitory role of microbiota in *Shigella* infection [Reviewed in (Anderson et al. 2016)]. At least one of such mechanisms allowing survival in such a dense habitat is the secretion by some *Shigella* isolates (especially *S. sonnei*) of a small inhibitory protein called colicin (encoded by *shiD* in PI-1 on the chromosome), which targets phylogenetically related bacteria (Calcuttawala et al. 2015).

Another obstacle on the way of *Shigella* to the epithelial surface is the mucus layer that covers the gastrointestinal tract, reaching a thickness of 1 mm in the colon. It is made primarily of mucins, which are high-molecular weight glycoproteins linked through intermolecular disulfide bonds. Besides creating a physical barrier between the epithelium and the microbiota, this entity is enriched in

antimicrobial peptides (AMPs) and secretory immunoglobulins A (sIgA) that restrict bacterial growth, especially in the dense deeper part of the mucus layer. Since *Shigella* predominantly lacks motility, it has evolved other ways to reach the intestinal epithelial cells (IEC). First, *Shigella* preferentially binds mucus from human colon (as opposed to mucus from other parts of the gut and mucus from other mammals), suggesting an explanation for its highly specialized host and tissue tropism (Izhar et al. 1982; Sudha et al. 2001). This binding occurs through weak glycan–glycan interactions between the heavily glycosylated mucins and the highly abundant O-antigen sugar repeats that decorate the outer layer of *Shigella*'s LPS. Second, *Shigella* is predicted to encode at least one of the SPATE Serine Protease Autotransporters of Enterobacteriaceae related mucinases called Pic and EatA, which are hypothesized to pave the way for this pathogen to penetrate the mucus layer (Haider et al. 1993; Henderson et al. 1999; Patel et al. 2004).

While passing through the gastrointestinal tract, *Shigella* receives important signals that modulate the function of its virulence factors. The major transcriptional regulator of virulence genes is VirF whose expression is inhibited by the histone-like nucleoid-structuring (H-NS) repressor under conditions of low temperature and low osmolarity. Once ingested, the temperature shift leads to VirF expression that in turn induces another transcription factor, VirB, which directly controls the synthesis of important virulence genes, including those encoding for the T3SS (Maurelli and Sansonetti 1988; Porter and Dorman 1994; Durand et al. 2000). Once assembled, the T3SA is not yet ready to target host cells until it binds bile salts via its needle tip protein IpaD in the intestinal lumen, which introduces a conformational change and exposes the IpaB protein on the tip of the "secretory needle." IpaB together with IpaC forms a pore (called the translocon) inside the host membrane through which subsequent T3SS effector injection proceeds [(Dickenson et al. 2011), discussed below]. An additional level of control over the T3SS function is the sensing of oxygen through the fumarate and nitrate reductase transcriptional regulator FNR. Anaerobic conditions in the intestine mediate suppression of Spa32 and Spa33 structural components of the T3SA while detection of O_2 in the close vicinity of epithelial cells releases this suppression, promoting construction of longer T3SA needles (Marteyn et al. 2010). Needle length is critical for the ability to target the host cell as the surface of *Shigella* is heavily decorated with long LPS molecules that otherwise mask T3SA needles (West et al. 2005).

4 Subversion of Intestinal Epithelial Cells

4.1 Diarrhea-Inducing Toxins

One of the hallmarks of shigellosis is the production of bloody mucoid stools. However, most patients develop an initial phase of watery diarrhea, which is, at least partially, triggered by two types of toxins encoded on PI-2 and secreted by several *Shigella* strains during infection. The first type comprises *Shigella*

enterotoxin 1 and 2 (ShET1 and ShET2) encoded by *set1A* and *set1B* genes, respectively (Fasano et al. 1995; Nataro et al. 1995). While the mechanism of their action is still unknown, at least ShET2 was shown to be secreted through the T3SA (Farfan et al. 2011). Another toxin causing accumulation of fluids in the intestinal lumen is the SigA serine protease autotransporter that is able to cleave the intracellular alpha-fodrin altering the cytoskeleton of epithelial cells, although its contribution to the production of watery diarrhea is not clear (Al-Hasani et al. 2009).

Unlike the above-mentioned toxins, Shiga toxin is produced exclusively by *S. dysenteriae* type 1 and Shiga-like toxins (SLTs) are produced by certain serotypes of EHEC from prophage sequences. Shiga toxin is extremely cytotoxic against a wide variety of cell types (e.g., epithelial, endothelial, leukocytic, lymphoid, and neuronal cells) and is responsible for the development of vascular lesions in the colon, the kidney, and the central nervous system. Shiga toxin possesses an AB5 structure with an enzymatically active A–subunit non-covalently associated with five identical B-subunits. B-subunits mediate binding to the toxin receptor, a neutral glycolipid of the globo-series, globotriaosylceramide (Gb3) (Lingwood 2003). Subsequently, the toxin follows the host cell retrograde pathway to reach the ribosome-enriched endoplasmic reticulum where the A-subunits inhibit protein synthesis due to their activity as highly specific *N*-glycosidases that cleave a single adenine residue from the 28S rRNA component of eukaryotic ribosomes [reviewed in (Tesh 2010)].

4.2 Invasion to the Colonic Epithelium

4.2.1 Attachment to ECs

Most pathogens have developed numerous molecular devices to adhere and firmly attach to host cells. However, *Shigella* seems to be devoid of any common adhesins, pilli, fimbriae, etc. Weak glycan–glycan interactions that serve this pathogen to bind mucus through the LPS might also contribute to its adsorption to cellular surfaces thanks to the dense glycocalyx decorating human cells (Day et al. 2015). Additionally, two ubiquitously expressed proteins were suggested to individually serve as receptors that promote *Shigella* invasion: CD44 and α5β1 integrin (Watarai et al. 1996; Skoudy et al. 2000). These are transmembrane surface proteins that bind components of the extracellular matrix (hyaluronic acid and fibronectin, respectively) and were suggested to be bound by IpaB (for CD44) and IpaB/C/D (for α5β1 integrin) T3SA components. In any case, firm adhesion and subsequent invasion into the cells are not possible without a fully functional T3SS on the bacterium side and operative actin cytoskeleton machinery on the cell side. It is then possible that weak initial glycan–glycan interactions allow the bacterium to stay in contact with the host cell long enough to insert its T3SA needle into the plasma membrane and to inject T3SS effectors that induce actin rearrangement and membrane ruffling, ultimately securing the bacterium onto the cell surface

(discussed below). Besides, activation of the T3SS in response to bile salts triggers a stronger adhesion of bacteria to IECs, depending on the surface-exposed auto-transporter IcsA (VirG) in a yet undiscovered mechanism (Brotcke Zumsteg et al. 2014). Additionally, bile salts induce secretion of OspE1 and OspE2 proteins that remain bound to the outer membrane of *Shigella* and increase its adherence to polarized cells (Faherty et al. 2012).

4.2.2 Engulfment by IECs

In order to colonize the colonic mucosa, *Shigella* must first cross the epithelial barrier. At least two non-mutually exclusive pathways have been suggested. In both cases, however, *Shigella* triggers its uptake into non-phagocytic cells due to the action of T3SS effectors translocated into the host cells (Fig. 1). The first pathway suggests that *Shigella* initially invades microfold (M) cells, a specialized IEC subset whose role is to sample particles from the gut lumen and present them to the underlying mucosal lymphoid tissue (transcytosis). Once passed through the M cells, *Shigella* invades IECs from their baso-lateral side with a very high efficiency (Mounier et al. 1992). An alternative pathway of *Shigella* entry proposes direct interaction with the apical side of IECs through finger-like protrusions called filopodia. Usually found in the intercellular junctions, these sensory cellular organelles probe the environment to establish adhesion structures (Romero et al. 2011). Bacterial capture by the tip of the filopodia triggers its retraction toward the cell body where the invasion eventually occurs.

In both pathways, the critical step of *Shigella* invasion into the host cell is the T3SS-dependent induction of actin rearrangement and plasma membrane ruffling that leads to internalization of the bacterium in a macropinocytic-like pathway. This process is executed by the concerted action of T3SS effectors activating host tyrosine kinases and Rho GTPases. Upon contact with the host cell, IpaB interacts directly with lipids of the plasma membrane leading to the recruitment of IpaC to the surface of the bacterium. Subsequently, both IpaB and IpaC are secreted into the host plasma membrane to form a pore (translocon) required for injection of other effectors into the host cell cytosol (Epler et al. 2009). Interestingly, IpaB directly binds cholesterol and translocon formation, as well as overall invasion of *Shigella* into host cells, is suggested to be dependent on the integrity of the cholesterol-enriched membrane microdomains (*i.e.,* rafts) (Lafont et al. 2002; van der Goot et al. 2004) in which both suggested receptors—CD44 and $\alpha5\beta1$ integrin—reside. In addition to its role in translocon formation, IpaC induces actin polymerization by activation of signaling via Src tyrosine kinase (Bougneres et al. 2004) and Cdc42 (Tran Van Nhieu et al. 1999). Furthermore, IpaC directly induces actin polymerization in vitro (Kueltzo et al. 2003). The IpgB1 and IpgB2 effectors belonging to WXXE family modulate actin dynamics by mimicking RhoG and RhoA GTPases, respectively (Table 1). However, these effectors also exert guanidine exchange factor (GEF) activity toward Rac, Cdc42 (for IpgB1), and RhoA (for IpgB2) actin regulators [reviewed in (Carayol and Tran van Nhieu

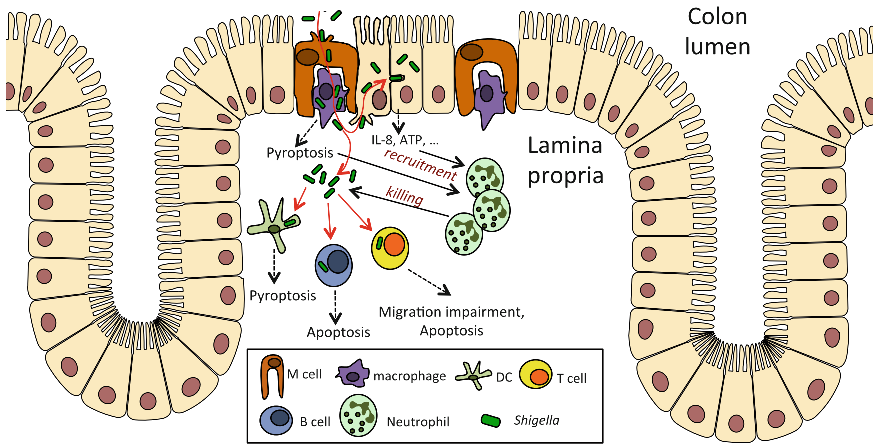

Fig. 1 Interaction of *Shigella* with epithelium and immune cells during invasion.*Shigella* translocates across the colonic epithelium via specialized M cells. Then bacteria are phagocytosed by macrophages, which are often found in close vicinity to M cells, but escape from the resulting phagosome, promote macrophage pyroptosis, and are released to the *lamina propria*. Having access to the baso-lateral side of the IECs, *Shigella* efficiently invades these cells and disseminates in the epithelium using actin-based motility. The release of pro-inflammatory cytokines (*i.e.,* IL-8) and "danger-associated" molecules (*i.e.,* ATP) by IECs and pyroptotic macrophages provokes the recruitment of neutrophils to the site of infection. The antimicrobial activity of neutrophils enables *Shigella* elimination but also causes massive tissue destruction that promotes further invasion of bacteria from the gut lumen (not shown in the scheme). Additionally, *Shigella* subverts immune cells such as DCs, B, and T lymphocytes, which it encounters in the *lamina propria*, the colonic lymphoid follicles and, with the progression of infection, the mesenteric lymph nodes. Bacteria ingested by DCs induce rapid cell death by pyroptosis. T cells lose their ability to migrate upon contact with *Shigella,* and those who are invaded eventually die while B cells undergo apoptosis even without being invaded by the bacteria (see text for details)

2013)]. The IpgD phosphatase also participates in induction of membrane ruffling through hydrolysis of a phosphatidyl-inositol 4,5-bisphosphate lipid, which is found in the inner leaflet of the plasma membrane and serves as a docking point for proteins that connect the cortical actin to the membrane (Niebuhr et al. 2000). De-connecting the cortical actin from the plasma membrane might favor its remodeling, thus allowing long membrane protrusions. Another effector—IpaA—binds vinculin and promotes its association with actin filaments, thus mediating localized actin depolymerization and reduction of the adhesion between cells and the extracellular matrix. This process contributes to *Shigella* anchoring to the membrane ruffles (Tran Van Nhieu et al. 1997) and to completion of the invasion process by assisting the closure of the bacteria-containing vacuole (Bourdet-Sicard et al. 1999). Lastly, the invasion process provokes an increase in intracellular calcium that is necessary for actin polymerization. Subsequently, dense actin filaments in the vicinity of *Shigella* confines a zone with slower diffusion rates enabling prolonged local pulses of calcium and probably assisting in effector concentration close to the entry site (Tran van Nhieu et al. 2013).

Table 1 T3SS effectors of *Shigella* involved in subversion of host cell processes

Effector	Activity/ Function	Host cell target	Effect on epithelial cells	Effect on inflammation and immune cells
IpaB	Forms pore (translocon)	Cholesterol CD44	Bacterial invasion and vacuolar rupture	Bacterial escape from phagosome; Pyroptosis of MΦs; Inhibition of EC-mediated inflammation
IpaC	Forms pore (translocon) Actin polymerization	Actin Src Cdc42	Bacterial invasion and vacuolar rupture	Bacterial escape from phagosome
IpaD	T3SS needle "plug"	TLR2		MΦ and T cell apoptosis
IpaA	Actin rearrangement	Vinculin	Bacterial invasion Reduction of cell–matrix adhesion	
IpgB1	Actin rearrangement	Rac	Bacterial invasion	
IpgB2	Actin rearrangement	RhoA	Bacterial invasion	
IpgD	$PI4,5P_2$ conversion to PI5P	$PI4,5P_2$	Bacterial invasion and vacuolar rupture Apoptosis inhibition	Inhibition of T cells migration; DAMPs secretion inhibition in EC
VirA	Microtubules degradation Inhibition of NFκB signaling	Tubulin Calpastatin Rab1	Bacterial intra-/ intercellular dissemination Apoptosis inhibition Golgi disruption	Inhibition of EC-mediated inflammation
OspF	Inhibition of MAPK signaling	ERKp38		Inhibition of EC-mediated inflammation
OspG	Inhibition of NFκB signaling	Ubiquitin	Apoptosis inhibition	Inhibition of EC-mediated inflammation
OspI	Inhibition of NFκB signaling	UBC13 E2	Apoptosis inhibition	Inhibition of EC-mediated inflammation
OspZ	Inhibition of NFκB signaling	P65	Apoptosis inhibition	Inhibition of EC-mediated inflammation

(continued)

Table 1 (continued)

Effector	Activity/ Function	Host cell target	Effect on epithelial cells	Effect on inflammation and immune cells
IpaH9.8	Inhibition of NFκB signaling	NEMO/ IKKγ U2AF35	Apoptosis inhibition	Inhibition of EC-mediated inflammation
IpaH4.5,	Inhibition of NFκB signaling	p65	Apoptosis inhibition	Inhibition of EC-mediated inflammation
IpaH0722	Inhibition of NFκB signaling	TRAF2	Apoptosis inhibition	Inhibition of EC-mediated inflammation
OspC3	Inflammasome inhibition	Caspase 4	Pyroptosis inhibition	Inhibition of EC-mediated inflammation
IpaJ	Membrane trafficking inhibition	ARF1	Golgi disruption	Inhibition of EC-mediated inflammation
IpaH7.8	Inflammasome activation	GLMN		Pyroptosis of MΦs

Although the complete picture of the whole invasion process is still not completely understood, it is evident that *Shigella* has developed a variety of effectors that dynamically modulate host actin to promote bacterium engulfment inside otherwise non-phagocytic cells.

4.3 *Intracellular Movement, Autophagy Escape, and Dissemination*

Following internalization, *Shigella* rapidly (<15 min) lyses the surrounding vacuole in a T3SS-dependent manner; however, the exact mechanism of this process is still elusive. It was demonstrated that the translocon components IpaB and IpaC have been suggested to directly create holes in the membrane of the vacuole (High et al. 1992; Barzu et al. 1997; Blocker et al. 1999; Hume et al. 2003; Harrington et al. 2006). A recent study found that the phosphatase activity of IpgD is necessary for recruitment of Rab-11 positive recycling endosomes to the *Shigella* containing vacuole, thus promoting its rupture through a yet-to-be identified mechanism (Mellouk et al. 2014).

Once inside the cytosol, *Shigella* hijacks the host actin machinery for its intracellular movement. The central bacterial mediator of actin polymerization is IcsA (VirG) that recruits and activates neuronal Wiskott–Aldrich syndrome protein (N-WASP), which in turn recruits the Arp2/3 complex. Together they act as a nucleating factor of actin polymerization that propels the bacterium due to the

localization of IcsA at one pole of *Shigella* (Egile et al. 1999). In addition to the actin-nucleating complex, the T3SS-secreted cysteine protease VirA assists the intracellular movement of *Shigella* by degrading the dense microtubule network (Yoshida et al. 2002).

Any intracellular bacterium faces the challenge of being trapped and directed to degradation by a process called autophagy. This is a complex system used by the cell for sequestering cytoplasmic content in de novo generated double-membrane vesicles that subsequently fuse with lysosomes. This process not only allows recycling of cytosolic components but also enables targeting and destruction of intracellular pathogens thanks to the specific recognition of common pathogenic components. When associated with the host membrane upon cell entry, *Shigella* is targeted by the autophagy machinery in an IcsA-dependent manner. However, due to the not-well understood action of another surface protein IcsB, the bacterium succeeds in escaping using the power of polymerizing actin tails (Ogawa et al. 2005; Mostowy et al. 2010; Campbell-Valois et al. 2015).

In addition to intracellular movement and autophagy escape, actin-based motility enables *Shigella* to spread to adjacent epithelial cells through the formation of protrusions in the cell membrane that preferentially occurs at tri-cellular junctions (Fukumatsu et al. 2012). Interestingly, when a bacterium is found in the cytosol, its T3SS is inactive but once pushed against the plasma membrane it is reactivated, thus allowing a new cycle of cellular invasion to proceed in the neighboring cell (Kuehl et al. 2014; Campbell-Valois et al. 2014).

Proliferation inside host cells requires dramatic adaptation of the pathogen to a new environment, in which access to several nutrients such as iron is limited and is under the control of the host. Due to its intrinsic toxicity, iron is normally sequestered inside the cytosol by dedicated proteins. *Shigella* expresses several siderophores (PI-encoded IucA-D, IutA) and several iron transport systems (such FecA-E, FecI, FecR) that are able to scavenge the intracellular iron for the bacterial benefit [reviewed in (Payne et al. 2006)].

4.4 Epithelial Integrity Preservation

Successful replication of *Shigella* inside IECs induces different stresses and, together with detection of intracellular bacteria (discussed in the next section), leads to cell death and destruction of the colonic epithelium. Occurring too early, this does not only abolish the replicative niche of the bacteria, but also prevents cell-to-cell spread. Therefore, *Shigella* evolved to interfere with these processes at different levels.

The reaction of the cell to the infection and the accumulating damage is to induce two parallel processes: inflammation (discussed in the next section) and programed cell death (apoptosis). The master regulator of both processes is the transcriptional factor nuclear factor kB (NF-kB). Several different signaling pathways in the cell lead to its activation and *Shigella* evolved to interfere with many of

them via the action of specific plasmid-encoded T3SS effectors, including OspF, OspG, OspI, OspZ, IpaH9.8, IpaH4.5, IpaH0722 [reviewed in (Ashida et al. 2015; Killackey et al. 2016)]. The above-mentioned PI4,5P phosphatase effector IpgD generates PI5P that contributes to epidermal growth factor receptor (EGFR) activation, which sustains the PI3 K/Akt pro-survival signaling by inducing Mdm2-mediated proteasomal targeting of the pro-apoptotic factor p53 (Pendaries et al. 2006). Another effector, VirA, also inhibits the p53 pathway by binding to the calpain inhibitor calpastatin, thus activating the calpain protease that degrades p53 (Bergounioux et al. 2012). An additional way for epithelial cells to fight intracellular infection is the induction of inflammasome complexes that lead to pro-inflammatory cell death. *Shigella* interferes with the non-canonical caspase-4 mediated pathway of inflammasome induction through secretion of the T3SS effector OspC3 that sequesters caspase-4, thus preventing its activation (Kobayashi et al. 2013). Besides preventing cell death, *Shigella* also utilizes strategies to prevent IEC shedding, which is one of the defense mechanisms against intracellular pathogens. To this end, OspE1/2 increases cell anchoring to the extracellular matrix by binding to the integrin-like kinase (Kim et al. 2009).

5 Subversion of the Immune System

Every pathogen that breaches the natural barrier of the host has to face the different facets of the immune system. Some pathogens (including *Shigella*) evolved an intracellular lifestyle that aids in the evasion from humoral immunity (*i.e.,* antibodies, complement, reactive oxygen species). However, proliferation of bacteria inside host cells is detected through various pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively), thus provoking inflammation and immune cell recruitment to fight the infection. In this section, we discuss how *Shigella*, with the help of its virulence factors (Table 1), many of which are already described in the previous section, subvert both innate and adaptive immunity.

5.1 Manipulation of the Host Innate Immunity

5.1.1 Non-specific Barrier Subversion

The first non-specific line of defense in the gut is the mucus (discussed above), which, besides being a physical barrier between the microbiota and the ECs, also contains antimicrobial peptides (AMPs) that play a crucial role in intestinal homeostasis and protection against pathogens (Hancock and Diamond 2000; Sansonetti 2004). Usually, secretion of AMPs is augmented during interaction with pathogens. However, upon *Shigella* infection, the production of several peptides

(*i.e.,* LL-31, human β-defensins 1 and 3) is down-regulated (Islam et al. 2001; Sperandio et al. 2008). More specifically, mucin-producing polarized human intestinal ECs infected with *Shigella* are affected in mucin gene transcription (particularly MUC5AC), protein glycosylation, and secretion. The modified mucus is unable to form a thick protective gel staving off the bacteria from IEC surface and instead creates a thin layer that sticks to the apical side of the cell, thus favoring even more the infection by the bacteria (Sperandio et al. 2013).

Another non-specific barrier is the complement system, which is found in tissues and body fluids. Components of this system are able to bind to the bacterial surface, thus leading to opsonization and lysis (Janeway et al. 2009). However, the presence of the long polymers of O-antigen at the bacterial surface is important for *S. flexneri* resistance to direct complement-mediated serum killing, probably by binding the complement away from the bacterial membrane (Hong and Payne 1997). Interestingly, *S. sonnei*, which has a relatively short LPS (20–25 units, versus up to 100 units of *S. flexneri*), possesses instead a high-molecular weight capsule. This capsule is characterized by structural similarity to the LPS O-antigen and exerts similar function in preventing complement-mediated killing (Caboni et al. 2015).

5.1.2 Intestinal Epithelial Cell Subversion

In addition to their function in nutrient absorption, IECs also play an important role in recognizing invasive pathogens and recruiting immune cells to the site of infection. Therefore, the epithelium is considered as a part of the host innate immunity. Extracellular *Shigella* can be recognized via the binding of LPS to Toll-like receptor 4 (TLR4), which is mostly found on the plasma membrane of myeloid cells, provoking a pro-inflammatory response (Poltorak et al. 1998; Hoshino et al. 1999). However, ECs detect mainly intracellular *Shigella* through the binding of bacterial cell wall peptidoglycan subunits to cytoplasmatic Nod-like receptors NOD1 and NOD2, thus leading to both mitogen-activated protein kinase (MAPK) and NF-kB pro-inflammatory signaling. Activation of these pathways leads to secretion of different pro-inflammatory cytokines and chemokines, including interleukin-8 (IL-8) that recruits neutrophils, which are required for eventual pathogen elimination but also contribute to massive tissue destruction. As mentioned above, several T3SS effectors are used by *Shigella* to interfere with these signaling cascades at different levels, thus reducing the inflammation and helping pathogen proliferation.

Furthermore, secretion of pro-inflammatory molecules (as well as antimicrobial peptides and mucus) by infected cells relies on effective secretory mechanisms. The Golgi apparatus is a central organelle involved in secretion of proteins by eukaryotic cells. Upon infection by *Shigella*, the Golgi apparatus is completely disrupted by the action of three T3SS effectors: VirA, IpaJ, and IpaB. VirA and IpaJ target Rab and ARF GTPases, respectively, two major regulators of membrane trafficking whose inactivation leads to Golgi fragmentation (Dong et al. 2012; Burnaevskiy et al. 2013; Burnaevskiy et al. 2015). IpaB also contributes to Golgi

disruption through its ability to bind and redirect cholesterol from inner membranes toward the entry site of *Shigella* in the plasma membrane (Mounier et al. 2012).

Apart from PAMPs, damaged and stressed cells release DAMPs (such as ATP and uric acid) through plasma membrane hemi-channels, which eventually triggers inflammation. The T3SS effector IpgD produces PI5P that prevents hemi-channels opening and therefore reduces the inflammatory response (Puhar et al. 2013).

5.1.3 Phagocytic Cell Subversion

After crossing of the epithelial lining, bacteria encounter phagocytic cells that are present in intestinal lymphoid follicles, especially macrophages (MΦs) that are located beneath the M cells. During the early stages of infection, *Shigella* is indeed phagocytosed by resident macrophages and dendritic cells (DCs). However, these cells rapidly die when infected with *Shigella* in vitro (Zychlinsky et al. 1992; Edgeworth et al. 2002). In fact, *Shigella* escapes from the phagocytic vacuole into the cytosol using its T3SS, similarly to the way it happens in IECs. Subsequently, bacteria multiply within invaded macrophages and dendritic cells and induce their pro-inflammatory cell death called pyroptosis. This process is initiated by the recognition of the T3SA needle protein components MxiH and MxiI by neuronal apoptosis inhibitory proteins (NAIP). NAIP proteins induce NOD-like receptor (NLRC4)-dependent inflammasome activation that leads to pyroptotic death of MΦs, resulting in secretion of the pro-inflammatory cytokines IL-1β and IL-18 (reviewed in (Bergsbaken et al. 2009). Additionally, besides contributing to translocon formation, IpaB is able to form an ion channel allowing an influx of potassium ions (among others) that is recognized by the NLRC4-inflammasome, further enhancing the pyroptosis process (Senerovic et al. 2012). Furthermore, the IpaH7.8 T3SS effector induces NLRC3 and NLRC4 inflammasomes (Suzuki et al. 2014). Recently, another pathway resulting in MΦ death following *Shigella* infection describes apoptosis activation via the IpaD T3SA-needle tip protein [discussed below (Arizmendi et al. 2016)].

Apparently, the rapid killing of phagocytic cells seems to be beneficial to *Shigella*, especially due to the fact that once released from dying MΦs in the *lamina propria*, bacteria can invade epithelial cells from the baso-lateral side (which is a highly efficient process compared to infection of the apical side of IECs). On the other hand, pyroptosis-associated neutrophil recruitment eventually promotes the clearance of *Shigella* bacteria. Indeed, several observations suggest that *Shigella* invests resources in dampening neutrophil recruitment and activation. First, *Shigella* expresses a PI-encoded virulence factor, called ShiA, that hinders neutrophil recruitment in a yet-to-be discovered mechanism (Ingersoll et al. 2003). Second, during proliferation in epithelial cells *Shigella* alters the acetylation levels of its LPS, as compared to “free-living” extracellular bacteria. The decrease of LPS acetylation reduces its immuno-stimulatory capacity toward phagocytic cells, thus significantly reducing TLR4-mediated inflammasome activation, IL-1β release from infected MΦs and the capacity to induce oxidative burst in neutrophils (Paciello et al. 2013).

Controversially, recruitment of neutrophils during the initial steps of infection might contribute to bacterial colonization by destabilization of the epithelial barrier, thus allowing *Shigella* to reach the baso-lateral side of the IECs while bypassing the M cells (Sansonetti et al. 1999). Another ambiguity is that although neutrophils indeed efficiently trap and kill *Shigella* (Weinrauch et al. 2002; Brinkmann et al. 2004), they also undergo T3SS-dependent necrosis (François et al. 2000). Another important virulence factor enabling *Shigella* to avoid killing by neutrophils and MΦs is the iron-containing superoxide dismutase FeSOD encoded by the *sodB* gene found on the chromosome (Franzon et al. 1990). This enzyme converts superoxide radicals, produced by phagocytic cells as a part of their bactericidal activity, into H_2O_2 and O_2 (McCord and Fridovich 1978). Overall, the interplay between neutrophils and *Shigella* in disease progression is still not clear.

5.2 Manipulation of the Host Adaptive Immunity

Shigella-specific immunity elicited upon natural infection is characterized by the induction of a humoral response directed mainly against LPS and to a lesser extent against some protein effectors (Islam et al. 1995b; Phalipon et al. 1995; Levine et al. 2007). Protective immunity is serotype specific, pointing out the O-antigen of the polysaccharide part of LPS as the target for protective antibodies. However, antibody-mediated protection arises only after several episodes of infection, is of short duration, and is poorly efficient in limiting reinfection, particularly in young children (Raqib et al. 2000; Raqib et al. 2002). Considering the instructive role of innate immunity in the acquired immune response (Luster 2002; Iwasaki and Medzhitov 2010), modulation of the host innate immune responses by *Shigella* certainly affects the development of a protective adaptive immunity. Indeed, several lines of evidence suggest that *Shigella*-induced acute inflammation contributes to the impairment of the adaptive immune response by the production of immunosuppressive cytokines (such as IL-10 and TGF-β) (Raqib et al. 1995; Phalipon and Sansonetti 2007; Sperandio et al. 2008; Sellge et al. 2010). In addition, since NF-κB is also a major regulator of the adaptive immune response, inhibition of the NF-κB signaling by *Shigella* in IEC (as described above) harms not only the innate, but also the adaptive immunity. Moreover, high levels of MΦ, DC, B cell, and T cell death occurs in the *lamina propria* as seen in colonic biopsies of *Shigella*-infected individuals (Islam et al. 1995a; Zychlinsky et al. 1996; Raqib et al. 2002). In fact, *Shigella* invades activated T cells, multiplies in their cytosol and eventually causes cell death in vitro. Furthermore, not only invaded but also T cells just being in contact with *Shigella* lose their ability to migrate toward chemo-attractants in vitro and to migrate inside lymph nodes in vivo. This phenomenon is achieved thanks to the T3SS effector IpgD that is delivered into the lymphocyte cytoplasm (along or aside of invasion), resulting in hydrolysis of PI4,5P at the plasma membrane. This leads to dephosphorylation of the ERM proteins and their inability to re-localize at one T cell pole upon chemokine stimulus (Konradt et al. 2011;

Salgado-Pabón et al. 2013). Since constant scanning of antigen presenting cells by T lymphocytes in the lymph node is a key feature enabling T cell activation, proliferation, and differentiation, by interfering with this process *Shigella* dampens the specific immune response and memory development. Moreover, *Shigella* is also able to affect the second facet of the adaptive immunity, *i.e.,* the B lymphocytes, by invading and killing these cells or just by inducing TLR2-mediated apoptosis through delivery of T3SA needle tip protein IpaD (Nothelfer et al. 2014).

6 Concluding Remarks

Since their discovery over a hundred years ago, an extensive investigation of *Shigella* and EIEC species has yielded an impressive amount of knowledge regarding different aspects of their epidemiology, physiology, and pathogenicity mechanisms. However, they still remain among the top four causes of life-threatening diarrheal diseases among children under 5 years old in sub-Saharan Africa and South Asia (Kotloff et al. 2013), causing between 28,000 and 34,400 deaths per year in 2011 and 2013, respectively (Lanata et al. 2013; GBD GBD 2013a DALYS and HALE Collaborators et al. 2015). Furthermore, mortality among *Shigella*-infected patients above 5 years old adds about 40,000 deaths per year, overall accounting for about 88.4 million cases of Shigellosis (Lamberti et al. 2014; GBD GBD 2013b Mortality and Causes of Death Collaborators 2015). Besides mortality, these infections significantly contribute to Shigellosis-associated disability-adjusted life years: 7 million in 2010 (Murray et al. 2012).

Without any animal reservoir, *Shigella* is transmitted from person to person directly through hands and indirectly through contaminated water and food. Shigellosis is indeed associated with poor sanitation and hygiene and limited access to clean drinking water, except for *S. sonnei* whose rates increase with economic development [reviewed in (Anderson et al. 2016)]. The variety of species and serotypes associated with shigellosis makes it possible for reinfections to occur locally or during travel to areas where other serotypes predominate. Moreover, since *Shigella* succeeds to impair the development of long-lived protective immunity, several infections of the same individual with the same serotype are possible. Healthy individuals with mild infections usually recover without specific treatment, but because *Shigella* invades the colonic mucosa, it often causes dysentery, which is not amenable to oral rehydration. Antibiotic treatment is recommended for dysentery, severe shigellosis and individuals with compromised immune systems. However, many studies reveal high rates of resistance to at least one common antibiotic such as ampicillin, tetracycline, and chloramphenicol (Sadeghabadi et al. 2014; Khaghani et al. 2014; Cui et al. 2015; Bhattacharya et al. 2015). While these strains can be successfully treated with quinolones and fluoroquinolones, the emergence of multidrug-resistant strains of *Shigella*, that are resistant also to the latter antibiotics, further complicates the treatment, making

prevention of infection critical (Bhattacharya et al. 2014; Aggarwal et al. 2016; Yang et al. 2016; Poramathikul et al. 2016).

While a comprehensive approach to diarrhea prevention and control is the ideal solution, water and sanitation infrastructure development might still be out of reach for many low-income countries. Therefore, a broadly protective vaccine would become an ideal solution for *Shigella*-associated burden especially in these countries. Although several studies in animals and humans have demonstrated the feasibility of such a vaccine, there is no licensed vaccine against *Shigella* yet [reviewed in (Mani et al. 2016)]. We believe that in order to develop an efficient vaccine, as well as new approaches in shigellosis prevention and treatment, we must first complete our knowledge regarding the different steps of *Shigella* pathogenesis and immune evasion. In fact, the first step of the infection, namely the colonization, is the least explored. It is still unclear how such a small inoculum of 10–100 bacteria is able to cause a disease, despite *Shigella* being a non-motile bacterium, which is also devoid of any classical adhesins. Does it proliferate in the human gut and where? What is the role of non-specific protective factors of the intestine such as microbiota, mucus, and antimicrobial peptides in controlling the colonization process? An additional poorly investigated but intriguing aspect is the ability of *Shigella* to infect humans asymptomatically, a situation that can reach up to 60% in endemic regions and during outbreaks (Cohen et al. 1989; Guerrero et al. 1994; Qadri et al. 1995; Becker et al. 2015). It is unclear which factors define the balance between clinically expressed and subclinical infection. Deep sequencing techniques could help to compare the microbiota composition in stools of both groups. Another aspect is the role of innate immunity: Does it promotes bacterial spread (and thus acute dysentery) or limits it? What is the dynamics of adaptive immunity development and how *Shigella* impairs this process? Although recent advances in understanding the interaction of *Shigella* with each cell type separately have shed some light on these processes, we still miss the global picture of pathogenesis and immune subversion. One of the major obstacles in answering these questions is the lack of an appropriate and convenient animal model for shigellosis. One possible solution is the use of guinea pigs since *Shigella* is able to infect the colon of these animals, similarly to humans, provoking an acute rectocolitis (Shim et al. 2007; Barman et al. 2011). Detailed microscopic examination of the infected colons during *Shigella* infection (using *ex*- and in vivo techniques) combined with single-cell analysis of the infected tissues could ameliorate our understanding of the infection process and the role of the innate immunity. However, there are no adequate genetic tools and sufficient knowledge of the immune system in guinea pigs. Human xenograft in SKID mice is another useful model although it is very demanding (Zhang et al. 2001). Alternatively, several new approaches arise to study infection processes at the organ level using intestine-derived organoids [reviewed in (Zhang and Sun 2016)] and microfluidics with the so-called Organs on chip, which recapitulate the epithelial barrier and some of the immune system components (Kim et al. 2016). Ideally, extensive analysis of colonic biopsies and blood samples from naturally infected humans combined with clinical and epidemiological data in endemic regions could be particularly useful to understand the pathogenic processes.

In summary, further deciphering of the molecular mechanisms underlying colonization, epithelial barrier breaching and immune system subversion as well as integrating these data into a global picture of *Shigella* pathogenesis might eventually fill the gap between basic sciences and the ability to translate these findings into disease-control measures.

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Enteroaggregative *Escherichia coli*

Claire Jenkins

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Abstract Enteroaggregative *Escherichia coli* (EAEC, formerly known as "EAggEC") cause acute or persistent watery diarrhoea (with or without mucus) in children, predominantly in low-income countries, and are associated with travellers' diarrhoea in children and adults in middle and high income countries. The diverse nature of EAEC is such that not all strains cause disease. Conversely, certain strains of EAEC possess additional virulence determinants associated with the ability to cause severe diarrhoea and other symptoms, which might be life-threatening in vulnerable patients. The EAEC virulence factors described to date are either encoded on the large virulence plasmid of EAEC (plasmid of aggregative adherence) or on pathogenicity islands on the chromosome. Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix followed by isolation of the organism and confirmation of the presence of EAEC-associated genes using PCR. The variability of the plasmid structure and virulence gene sequences and the possibility that this mobile genetic element may be lost has necessitated the inclusion of chromosomal markers in the molecular screening assays. There is evidence in the literature of foodborne transmission of EAEC, but

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currently no evidence of a zoonotic reservoir. Fimbriae-mediated adhesion and biofilm formation are likely to be involved in both clinical manifestations of infection and attachment to foodstuffs. Multidrug resistance appears to be common in EAEC and geographically widespread. Whole-genome sequencing has revealed the mosaic genomic structure of EAEC and provided evidence that horizontal gene transfer and recombination are the driving force for acquisition of novel genome features and potentially novel pathogenic mechanisms. This has significant public health implications in terms of the diversity and pathogenesis of EAEC and its ability to colonise and cause disease in the human host.

1 Introduction

Enteroaggregative *Escherichia coli* (EAEC, formerly known as “EAggEC”) cause acute or persistent watery diarrhoea (with or without mucus) in children, predominantly in low-income countries (Okeke and Nataro 2001), and are associated with travellers’ diarrhoea in children and adults in middle- and high-income countries (Wilson et al. 2001). Other symptoms include nausea and vomiting, anorexia, borborygmi and tenesmus (Huang et al. 2006). In low-income countries, the propensity of EAEC to cause persistent diarrhoea for more than two weeks is associated with significant morbidity.

The diverse nature of EAEC is such that not all strains cause disease. Conversely, certain strains of EAEC possess additional virulence determinants associated with the ability to cause severe diarrhoea and other symptoms, which might be life-threatening in vulnerable patients. EAEC were first described by Nataro et al. in 1987 and were identified by their ability to aggregately adhere to tissue culture cells in a distinct stacked-brick pattern (Fig. 1). The ability to aggregate in this way is mediated by aggregative adherence fimbriae (AAF), of which there are at least five variants (I, II, III, IV and V). Expression of AAF is mediated by the plasmid-encoded transcriptional activator AggR (Dudley et al. 2006). More recent studies use the term “typical” EAEC to refer to strains of EAEC harbouring aggR, and strains without EAEC are referred to as “atypical”.

A study of infectious intestinal disease (IID) in the UK in 1993–96 showed that EAEC were the most commonly isolated diarrhoeagenic *E. coli* in patients with symptoms of gastroenteritis presenting to a doctor (5.1%) (Wilson et al. 2001). There is evidence in the literature of foodborne transmission of EAEC, mostly through documented outbreaks and case-control studies. However, relatively little is known about the burden of EAEC in IID or about the reservoir(s) and transmission pathways.

This chapter presents an overview of EAEC with respect to clinical presentation, the pathogenicity mechanisms associated with this group and interrelationships with other *E. coli* pathotypes and provides an update of the methods for the detection, identification and characterisation of EAEC. The public health risk of EAEC infections arising from the presence of EAEC in the food chain and antimicrobial

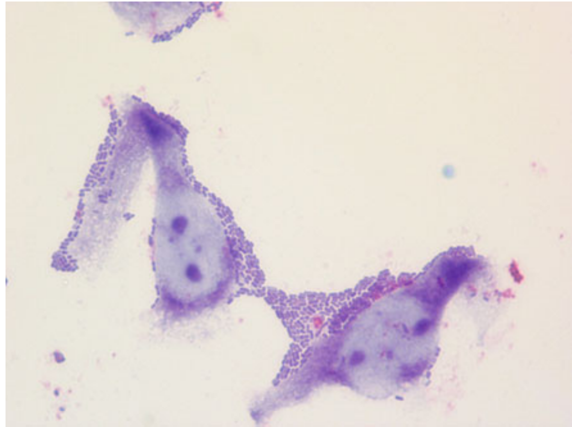

Fig. 1 EAEC were first identified by their ability to aggregately adhere to tissue culture cells in a distinct stacked-brick pattern (Courtesy of Marie Chattaway, Gastrointestinal Bacterial Reference Unit, Public Health England, London, UK)

resistance is assessed, and recent insights into this emerging gastrointestinal pathogen from the analysis of whole-genome sequencing data are summarised.

2 Pathogenicity Mechanisms

Pathogenesis of EAEC is complex as strains are heterogeneous. Case-control studies have documented the prevalence of putative virulence genes but, for the most part, have been unable to correlate the presence of specific genes to disease. The current model of EAEC pathogenesis comprises three steps (Fig. 2):

- Adherence to the intestinal mucosa via aggregative adherence fimbriae,
- Increased mucus production leading to extensive biofilm formation on the surface of the enterocytes, and
- Secretion of toxins and induction of the inflammatory response.

The EAEC virulence factors described to date are either encoded on the large virulence plasmid of EAEC, designated plasmid of aggregative adherence (pAA) or on pathogenicity islands on the chromosome (Table 1). The key virulence regulator of EAEC is AggR, a member of the AraC/XylS family of bacterial transcriptional regulators, and the defining factor for typical EAEC strains. *aggR* is located on the pAA plasmid and controls a number of genes encoding putative virulence factors located on the pAA and additional factors located on the chromosome. Expression of the aggregative adherence fimbriae (AAF), dispersin, the dispersin translocator Aat, and the Aai type VI secretion system, is all regulated by AggR (Morin et al. 2013).

Initial attachment of EAEC to the intestinal mucosa is mediated by AAFs. AAFs are regarded as the principle adhesin of EAEC and are found exclusively in this pathotype (Jønsson et al. 2015). AAFs were first described with respect to their role in the formation of the characteristic stacked-brick aggregative pattern on HEp-2

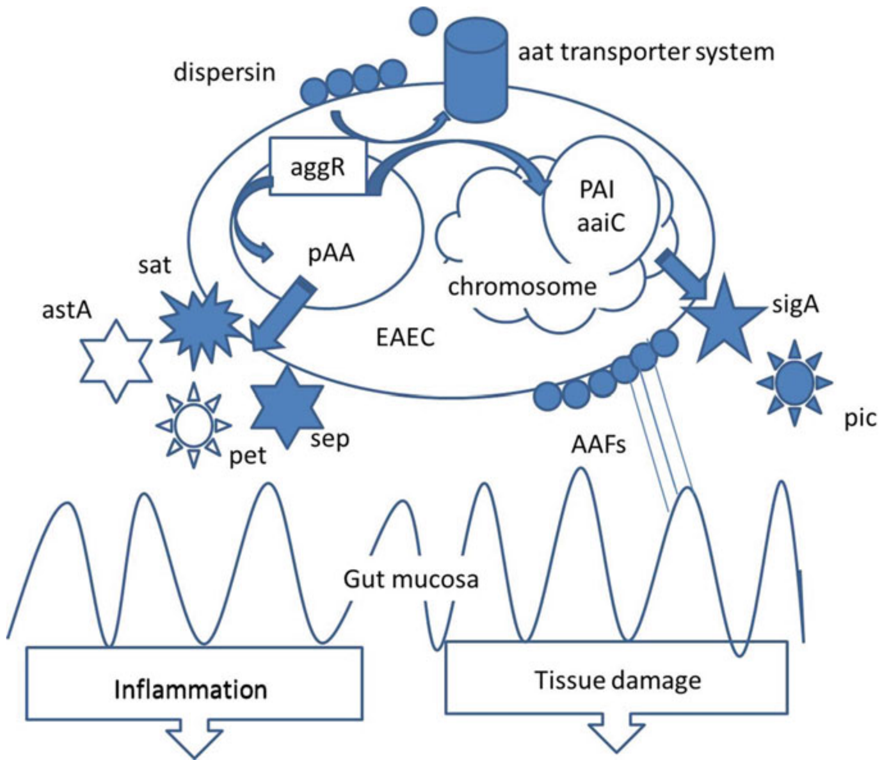

Fig. 2 Current model of EAEC pathogenesis (Adapted from a figure by Erik Juncker Boll, Department of Microbiological infection and Control, Statens Serum Institute, Copenhagen, Denmark)

cells (Nataro et al. 1987). Following adhesion to the epithelial surface, the AAFs have also been associated with epithelial inflammation in vitro, such as interleukin secretion, disruption of epithelial junctions and triggering migration of polymorphonuclear leucocytes (Harrington et al. 2005; Boll et al. 2012). Currently, five different AAF variants have been identified (AAF I–V), all showing a high level of conservation of their accessory genes, despite low level of amino acid identity among the pilin subunits (Jønsson et al. 2015).

The AAFs are members of the chaperone–usher fimbrial group, common to many Gram-negative bacteria. The operon consists of four proteins: the usher, the chaperone, the micro-pilin subunit and major pilin subunit. AAFs have a high isoelectric point (pI 8.9–9.4) relative to other adhesins of the chaperone–usher family. In the gut, where the pH ranges from 6 to 7.4, the AAFs carry a high positive charge, which may play a role in binding (Jønsson Ph.D. Thesis, 2017).

The gene encoding dispersin (aap) is located on the pAA lying immediately upstream of the AggR transcriptional activator and is under AggR control (Sheikh et al. 2002). Dispersin is a positively charged small protein that binds

Table 1 Genes and toxins often found in the EAEC pathotype

Common EAEC factor	Description	Location
aggR	Master regulator for EAEC plasmid virulence genes, including aggregative adherence factors, fimbriae AAF/I-AAF/V, and a large cluster of chromosomal genes inserted on a pathogenicity island at the PheU locus	pAA
aatA-P	Encodes proteins responsible for transporting the dispersin protein out of the outer membrane of EAEC	pAA
aap	Encodes a 10 kDa secreted protein named dispersin and is responsible for "dispersing" EAEC across the intestinal mucosa	pAA
aggA	Encodes AAF/I mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
aafA	Encodes AAF/II, mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
agg3A	Encodes AAF/III haemagglutination of erythrocytes	pAA
agg4A	Encodes AAF/IV mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
agg5A	Encodes AAF/V mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
aaiA-Y	PAI encoding a type VI secretion system (T6SS)	chromosome
pet	A 108 kDa autotransporter protein that functions as a heat-labile enterotoxin and cytotoxin	pAA
sigA	IgA protease-like homologue, enterotoxin and cytotoxin	Chromosome
pic	Mucinase, immunomodulation, colonisation, lectin-like haemagglutinin	Chromosome
sepA	*Shigella* extracellular enterotoxin	pAA
sat	Secreted autotransporter toxin. Enterotoxin and cytotoxin, impairment of tight junctions, autophagy	pAA
astA	*astA* encodes the enteroaggregative heat-stable toxin (EAST-1), which has physical and mechanistic similarities to *E. coli* STa enterotoxin	pAA

non-covalently to the lipopolysaccharide of the outer membrane of EAEC. It participates in formation of a surface coat that acts to disperse the bacteria, partially counteracting aggregation mediated by aggregative adherence fimbriae permitting the AAFs to extend from the surface of the bacterium (Jønsson Ph.D. Thesis, 2017).

In addition to the virulence genes on the pAA, a number of pathogenicity islands (PAIs) have been identified on the chromosome of EAEC. One of these islands consists of 25 contiguous genes (aaiA-Y), activated by AggR and located on a 117 kb PAI inserted at pheU in EAEC (Dudley et al. 2006). Many of these genes have homologues in other Gram-negative bacteria and were recently proposed to constitute a type VI secretion system (T6SS). Distribution studies indicated that aaiA and aaiC are commonly found in EAEC isolates worldwide, particularly in strains defined as typical EAEC. These data support the hypothesis that AggR is a

global regulator of EAEC virulence determinants on both the chromosome and the plasmid, and builds on the hypothesis that T6SS is an important mediator of pathogenesis (Dudley et al. 2006).

Another PAI is designated SHE (also found in *Shigella flexneri*) and encodes the Serine Protease Autotransporter Pic and ShET1 enterotoxins (Jønsson Ph.D. Thesis, 2017). Serine Protease Autotransporters of Enterobacteriaceae (SPATEs) are a family of extracellular proteases thought to play a role in EAEC pathogenesis. The SPATEs are named for their serine protease motif that confers proteolytic capability and are secreted via a type V secretion system. SPATEs are implicated in immune evasion, mucosal damage and colonisation. The most commonly found SPATEs in EAEC include: plasmid-encoded toxin (Pet), protein involved in intestinal colonisation (Pic), secreted autotransporter toxin (Sat), *Shigella* IgA-like protease homology (SigA) and *E. coli*-secreted protein (EspP) (Boisen et al. 2009). All SPATEs found in EAEC are located on the chromosome, except for Pet which is located on the pAA.

EAEC strains often produce an enteroaggregative heat-stable toxin (EAST1) encoded by the plasmid-encoded *astA* genes and haemolysin E (HlyE), but like ShET1, these toxins are not specific to EAEC (Harrington et al. 2006).

3 Interrelationships with Other *E. Coli* Pathotypes

EAEC are one of the six diarrhoeagenic *E. coli* (DEC) pathotypes defined by their pathogenicity gene profiles (Tozzoli and Scheutz 2014). These are enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), Shiga toxin-producing *E. coli* (STEC), and EAEC. *E. coli* can also cause extra-intestinal (ExPEC) infections in humans, primarily urinary tract (caused by uropathogenic *E. coli*) and sepsis/meningitis (caused by neonatal meningitis *E. coli*).

In recent years, the more widespread use of molecular techniques has revealed that many strains of *E. coli* harbour virulence genes associated with more than one pathogenic group. Most of the *E. coli* virulence factors are encoded by genes carried on mobile genetic elements (e.g. plasmids, phages and pathogenicity islands), and the horizontal gene transfer of such elements is the driver for the continuous emergence of new pathotypes (Tozzoli et al. 2014).

The Stx-producing EAEC O104:H4 strain that caused the large outbreak of HUS in Germany in 2011 outbreak carried the EAEC genes *aggR*, *aggA*, *set1*, *pic* and *aap* as well as a prophage encoding the *stx2* gene (Bielaszewska et al. 2011). This outbreak highlighted the threat to public health associated with strains of *E. coli* comprising more than one single pathotype; however, strains of *E. coli* comprising multiple pathotypes had been described previously. Such strains were first reported as the causative agent of a small HUS outbreak that occurred in France at the beginning of the 1990s (Morabito et al. 1998), where patients were infected with an *E. coli* O111:H2 strain showing the ability to adhere to cultured cells with the stacked-brick

adhesion mechanism (Nataro and Kaper 1998) and able to produce Stx2 (Morabito et al. 1998). Furthermore, sporadic cases of infection with Stx-producing EAEC strains of serotype O104:H4 were retrospectively described in the time period 2000–2010 soon after the German outbreak (King et al. 2012). Subsequently, a sporadic HUS case caused by a Stx-producing EAEC O111:H21 and a small outbreak of infection with a Stx-producing EAEC O127:H4 occurred in Northern Ireland in 2012 (Dallman et al. 2012) and in Italy in 2013 (Tozzoli et al. 2014), respectively.

The observation that the genomic backbone of Stx-producing EAEC is similar to that of non-Stx-producing EAEC, indicates that these strains may emerge following the acquisition of an Stx-carrying phage from a ruminant reservoir by strains of EAEC from human sewage (Tozzoli et al. 2014). Countries where EAEC infections are endemic and treatment of human sewage is limited may represent a source for the emergence of the Stx-producing EAEC pathotype. It has been proposed that the occurrence of the EAEC/STEC pathotype *E. coli* may be an ongoing, low-frequency event. The occurrence of outbreaks probably relates primarily to epidemiological opportunities for propagation and dissemination of the organisms in food or infected carriers.

Other combinations of EAEC pathotypes have been detected, such as those present in isolates possessing EAEC-associated genes together with ExPEC-associated traits as described in the *E. coli* serotypeO78:H10 responsible for causing an outbreak of UTI in Denmark (Olesen et al. 2012). The outbreak strain carried a range of virulence genes including *fimH* (type I fimbriae; ubiquitous in *E. coli*); *fyuA*, *traT* and *iutA* (associated with extra-intestinal pathogenic *E. coli*); and *sat*, *pic*, *aatA*, *aggR*, *aggA*, ORF61, *aaiC*, *aap* and ORF3 (associated with EAEC). In a study of ESBL-producing *E. coli*, eight multidrug-resistant ESBL-producing EAEC were isolated from urine specimens and one from a blood culture (Chattaway et al. 2014a, b). The multidrug-resistant EAEC isolates belonged to sequence type (ST) 38, predominantly associated with urinary tract infections. It is clear that the spectrum of pathogenic *E. coli* types is continuous rather than a rigid list of separated groups.

4 Methods for the Detection, Identification and Characterisation

Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from these matrices, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes using PCR. Following the outbreak of Stx-producing EAEC O104:H4 in 2011, the STEC European Union Reference Laboratory (EU-RL) developed a molecular methodology to screen food samples and faecal specimens for the presence of EAEC by the detection of *aggR* and *aaiC* (http://www.iss.it/vtec/index.php?lang=2&anno=2017&tipo=3).

In the 1980s, EAEC were described as exhibiting a characteristic “stacked-brick” pattern on adhesion to HEp-2 cells monolayers (Nataro et al. 1987). Since then the HEp-2 adhesion assay has been considered the gold standard for the identification of the EAEC. Although regarded as a sensitive and specific assay for the identification of this *E. coli* pathogroup, this approach is cumbersome and requires experienced personnel, specialised facilities making it an unsuitable assay for a routine testing. Molecular methods have largely replaced the phenotypic adhesion assay for the identification and characterisation of EAEC. A number of different PCR protocols are available, targeting a wide variety of genes. Given the recognised heterogeneity of EAEC, the different PCR assays produce variable results when compared to the phenotypic adhesions assay.

Early studies established evidence that the aggregative adhesion properties of EAEC were associated with the pAA plasmid, and the design of molecular screening tools was directed towards the use of sequences from this plasmid (Vial et al. 1988). Baudry et al. developed a DNA probe, CVD432, which showed a high degree of correlation with the phenotypic assay (Baudry et al. 1990), although a number of subsequent studies conducted using the CVD432 probe for screening EAEC strains isolated from cases of diarrhoea in different geographic locations showed more variable results (Okeke and Nataro 2001).

In 1995, the first PCR tool was developed based on the sequence of the *Eco*RI/*Pst*I fragment of pCVD432 plasmid, later found to correspond to a gene encoding the aggregative autotransporter, *aat* (Schmidt et al. 1995). A number of subsequent studies showed limited correlation between the molecular hybridisation and PCR assays suggesting that, in spite of the initial strong association of the presence of the plasmid with the ability to induce the stacked-brick pattern of adhesion, there was a certain degree of variability in the plasmid structure (Dutta et al. 1999; Tsai et al. 2003). More recent studies have been aimed at a more complete characterisation of the plasmid itself, and assays based on the detection of more than one marker have been deployed (Czeczulin et al. 1999; Cerna et al. 2003; Jenkins et al. 2006; Scheutz et al. 2014).

The variability of the plasmid structure and sequence, and the possibility that this mobile genetic element may be lost, has led to the conclusion that chromosomal markers should be included in the molecular screening assays (Jenkins et al. 2006; Scheutz et al. 2014). Following extensive genotyping of EAEC in different studies (Jenkins et al. 2006; Boisen et al. 2012), it was recognised that, similarly to the plasmid-associated genes, no chromosomal markers are present in 100% of EAEC. Some markers have been identified as being significantly associated with EAEC isolated from symptomatic cases, such as the SPATE toxin SepA (Boisen et al. 2012). As described above, the STEC EU-RL PCR assay for screening food samples and faecal specimens targets the pAA-encoded *aggR* and *aaiC* which is located on the chromosome. This assay is recommended for clinical diagnostic use.

An increasing number of diagnostic microbiology laboratories are implementing a multiplex gastrointestinal (GI) PCR approach for the detection of GI pathogens in clinical cases and foods, including target for EAEC. These assays provide a rapid, standardised, cost-effective pan-pathogen approach for the detection of bacteria

associated with GI infection and, moving forward, will improve the surveillance of EAEC disease.

5 Clinical Symptoms and Burden of Disease

EAEC are commonly associated with acute and chronic diarrhoeal illness among children in both developing and developed and/or industrialised regions and travellers with diarrhoea. The incubation period of diarrhoeagenic EAEC is typically between 8 and 18 h (Harrington et al. 2006). Infection with EAEC usually presents clinically as watery diarrhoea, often with mucus, nausea and vomiting, with or without fever (Huang et al. 2003). Other less common symptoms include anorexia, borborygmi and tenesmus. Additionally, there is evidence to suggest that the odds of developing post-infectious irritable bowel syndrome (IBS) are dramatically increased after acute infectious gastroenteritis with EAEC has been discussed (Sobieszczańska et al. 2007). A predominant feature of EAEC infection in low-income countries is the propensity to cause persistent diarrhoea for more than 2 weeks, making these bacteria a significant cause of mortality (Huang et al. 2006). The most significant public health concern stemming from EAEC infections in children in low-income countries is malnourishment, as persistent EAEC infections lead to chronic inflammation, which damages the intestinal epithelium and reduces its ability to absorb nutrients.

Studies suggest EAEC are a major cause of diarrhoeal disease, and it has been estimated that between 2 and 68% of patients with diarrhoea are infected with EAEC (Nataro et al. 1998; Wilson et al. 2001; Kahali et al. 2004). In the UK IID study in 1993–96, EAEC were the most commonly isolated enterovirulent *E. coli* in patients with symptoms of gastroenteritis presenting to a doctor (5.1%) (Wilson et al. 2001). In the second IID study in 2008–09, EAEC were isolated from more than 1.9% of cases in the population and 1.4% of cases presenting to a doctor (Tam et al. 2012). Data from the IID studies confirmed previous conclusions that concluded that the current definition of EAEC by plasmid gene detection includes true pathogens and non-pathogenic variants (Chattaway et al. 2013).

6 The Zoonotic Potential of EAEC and Contamination of the Environment

Reports of animals being a reservoir of EAEC are often based on the presence of genes that are not specific for EAEC, such as *astA*, in specimens from both healthy and sick animals. Most reports originate from parts of the world where pollution by human faecal waste is common (Table 2). Studies using EAEC-specific targets have found no evidence of EAEC in animals (Cassar et al. 2004).

Following the outbreak of Stx-producing EAEC O104:H4 in Germany in 2011, 2000 colonies from faecal samples of 100 cattle from 34 different farms, all located in the HUS outbreak region of Northern Germany, were screened for genes associated with the O104:H4 HUS outbreak strain (*terD*, *rfb*(O104), *fliC*(H4)), STEC (*stx1*, *stx2*, *escV*), EAEC (*pAA*, *aggR*, *astA*) and ESBL production (*bla*(CTX-M), *bla*(TEM), *bla*(SHV)) (Wieler et al. 2011). No EAEC were detected. In a similar study undertaken in France after the 2011 outbreak, 1468 cattle were analysed for faecal carriage of the Stx-producing *E. coli* O104:H4 outbreak strain by PCR assays targeting *stx2*, *wzxO104*, *fliCH4* and *aggR* genetic markers. None of the faecal samples contained the four markers simultaneously, indicating that cattle in France were not likely to be a reservoir of O104:H4, but results of the test for *aggR* were not reported (Auvray et al. 2012). In a recent study in Japan, no EAEC isolates, as assessed by the presence of *aggR*, were detected (Akiyama et al. 2015). To date, there is no evidence that EAEC have a zoonotic reservoir.

Contamination of the environment by EAEC, particularly watercourses, can occur in parts of the world where human sanitary systems are insufficient, and there is a high incidence of EAEC in people (Table 2). Prolonged survival of EAEC for at least several weeks in wet and dry substrates appears to be possible, and environmental contamination may also be a pathway for EAEC on salads and other vegetable produce (Table 2).

7 Foodborne Transmission

There is evidence in the literature of foodborne transmission of EAEC, mostly through documented outbreaks and case-control studies (Table 3). In Japan, a major outbreak caused by EAEC O untypeable:H10 in 1993 involving up to 2500 cases mainly in schoolchildren was associated with school lunches (Itoh et al. 1997). In the UK in the 1990s, four EAEC outbreaks associated with restaurants, a charity Christmas dinner and a conference were reported but no specific food vehicle was identified in any of these outbreaks (Smith et al. 1997). The 2011 German outbreak of EAEC O104:H4 was epidemiologically linked to contaminated fenugreek seeds (Frank et al. 2011). In June 2013, a foodborne outbreak was caused by EAEC isolated from kippered trotters mixed with vegetables, 22 cases and four asymptomatic food handlers, who probably contaminated the food (Shin et al. 2015) (Table 3).

In two further foodborne outbreaks of gastroenteritis that occurred 10 days apart among individuals who had meals at the restaurant of a farm holiday resort in Italy in 2007, an EAEC strain of serotype O92:H33 was isolated from six participants and one member of staff. A retrospective cohort study indicated a pecorino cheese made with unpasteurised sheep milk as a possible source of infection (Scavia et al. 2008), but since the outbreak EAEC strain was only isolated from food handlers, cross-contamination of the food product cannot be excluded, nor can contamination of food by asymptomatic excretors.

Table 2 Reports of evidence of EAEC detected in animals, food and the environment

Animal	Country	Findings	Reference
• Cattle (n = 304) • Chickens (n = 350) • Pigs (n = 263)	Burkina Faso	*astA* in 7% of cattle, 6% of chicken and 3.2% of pig samples	Kagambega et al. (2012)
• Pigs (n = 50)	South African	20.5% of *E. coli* from pigs had *astA*	Mohlatlole et al. (2013)
• Antelopes • Cattle	Zambia	*astA* was detected more frequently in antelopes (83.3%) than in cattle (33.3%)	Kuroda et al. (2013)
• Diarrhoeic dog	Germany		Breitwieser (1999)
• Dogs and cats • Poultry manure	Brazil		Puno-Sarmiento et al. (2013, 2014)
Food	Country	Findings	Reference
100 poultry carcases	Burkina Faso	*aggR* detected in 13 isolates of *E. coli*	Kagambega et al. (2012)
120 samples of beef and edible intestines	Korea	5 (4%) isolates	Kagambega et al. (2012)
Okra		ESBL-producing strain of EAEC	Zurfluh et al. (2015)
Environment	Country	Findings	Reference
Village-wide outbreak in 1996	India	Epidemiologically associated with the consumption of water from open well	Pai et al. (1997)
Survey of natural water	Bangladesh	ETEC, EPEC and STEC pathotypes were detected consistently, but genes from the EIEC and EAEC pathotypes were only found occasionally, and never in the rainy season or in winter	Akter et al. (2013)
Longitudinal study in a high population density, urban setting sampling domestic rainwater harvest tanks and in river water samples	South Africa	EAEC was found in 16% of 80 samples	Dobrowsky et al. (2014)

(continued)

Table 2 (continued)

Environment	Country	Findings	Reference
Pre-treated water in a drinking water treatment plant	Taiwan	EAEC-associated genes were found in 3.6% of 55 water samples, alongside high levels of other potentially pathogenic *E. coli*	Huang et al. (2011)
Urban floodwater	Australia	*astA* (69%) and *aggR* (29%) genes, carried by EAEC, were frequently detected in *E. coli* isolates	Sidhu et al. (2013)

Table 3 Outbreaks of EAEC and the AMR profile of the outbreak strain

Outbreak	Resistance profile	Comments	Reference
Urinary tract infection of multiresistant *E. coli* O78:H10, Denmark, 1991	Ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracyclines and trimethoprim		Olesen et al. (2012)
Shiga toxin (Stx)-producing EAEC O104:H4 outbreak, EU, USA and Canada, 2011	Ampicillin, amoxicillin/ clavulanic acid, piperacillin/ sulbactam, piperacillin/ tazobactam, cefuroxime, cefotaxime, cefpirome and ceftazidime, and also was resistant to streptomycin, nalidixic acid, tetracyclines and trimethoprim and the sulphonamides but was susceptible to the carbapenems	The strain contained an 88.5-kb IncI1-ST31 plasmid —pESBL-EA11— that encoded bla-CTX-M-15 and bla-TEM. Although not considered important in treatment of affected persons in this outbreak, the presence of resistance genes may have contributed to the development and spread of the causative organism	Bielaszewska et al. (2011), Rasko et al. (2011), Scheutz et al. (2014), EFSA (2011)
Multipathogen foodborne outbreak, UK, 2013	Of 20 EAEC isolates characterised, a range of resistance profiles were identified, ranging from nalidixic acid alone through to ampicillin, sulphonamides, streptomycin, nalidixic acid, ceftazidime, cefataxime, ceftiofur and cefpirome	Ten EAEC serotypes were identified in faecal samples recovered from patients in the large and complex multipathogen foodborne outbreak in the UK in February/March 2013	Dallman et al. (2014)
Outbreak of *E. coli* O untypeable: H10 in Japan in 1993 associated with school lunches, in which over 2600 children were affected	All isolates were susceptible to nalidixic acid, chloramphenicol, streptomycin, kanamycin and cephalothin but were resistant to ampicillin		Itoh et al. (1997)

In an outbreak of gastrointestinal foodborne illness associated with a Street Spice festival in the UK in 2011 and involving over 400 persons, 29 cases of *Salmonella* infection were confirmed. As most cases had reported symptoms characteristic of EAEC infection, such as abdominal cramps and persistent diarrhoea, further investigations were carried out retrospectively using a GI PCR assay. A high proportion of specimens were positive for the aggR target, and EAEC were cultured from 20 cases (Dallman et al. 2014). Risk factors associated with illness included eating foods from one particular vendor and eating a food item containing uncooked curry leaves. Although the *E. coli* count in colony forming unit (cfu) per ml from the curry leaves associated with the outbreak was high (>1000 cfu/ml), the testing algorithm at that time did not include tests specific for EAEC and EAEC were not cultured from the food samples. Strains of EAEC were detected in the food handlers, and contamination of the food by the food handlers was thought to be the most likely source (Table 3).

The infection status of food handlers, including asymptomatic carriage of EAEC, and hygienic conditions applied during the handling and processing of foodstuffs in some countries appears to be an important factor in contamination of foods at retail, catering or household level (Oundo et al. 2008). Multiple EAEC adherence factors are involved in the interaction of EAEC with leaves, and similar colonisation factors are used to bind such to the gut mucosa and leaf surfaces (Berger et al. 2009). It is thought that prolonged survival of organisms on dry fenugreek seeds may have been involved in the Stx-producing EAEC O104:H4 outbreak (EFSA BIOHAZ Panel 2011).

8 Biofilm Formation

Bacterial biofilms are structured communities of bacterial cells enclosed in a self-produced polymer matrix (consisting of proteins, exopolysaccharide and nucleic acid) attached to biological and non-biological surfaces. Biofilms allow bacteria to survive and thrive in hostile environments as well as being associated with chronic or persistent infections. Bacteria within biofilms can withstand host immune responses and are less susceptible to antimicrobials and disinfectants.

EAEC form thick biofilms on the intestinal mucosa, and most EAEC strains form a biofilm on glass or plastic surfaces when grown in cell culture medium with high sugar and osmolarity. AAFs bind extracellular matrix proteins and show species specificity in terms of erythrocyte agglutination, suggesting that this binding specificity could impact on the efficiency and selectivity of biofilm formation. Transposon mutagenesis confirmed the involvement of genes known to be required for AAF/II expression, as well as the *E. coli* chromosomal *fis* gene, a DNA-binding protein that is involved in growth phase-dependent regulation, in biofilm formation (Sheikh et al. 2001). The incompatibility group (Inc) I1 plasmid of EAEC C1096 encodes a type IV pilus that contributes to plasmid conjugation, epithelial cell adherence and adherence to abiotic surfaces, including via biofilm formation (Dudley et al. 2006).

When subjected to low iron conditions, an EAEC strain (042) showed a decrease in biofilm formation. Conversely, an increase in biofilm formation was observed for clinical EAEC strains cultured in restricted iron conditions, but the reduction of iron concentration inhibited the aggregative adherence to HEp-2 cells of all EAEC strains tested. Low iron availability may therefore modulate biofilm formation and adhesive properties of EAEC as a result of redox stress (Alves et al. 2010).

AAF-mediated adhesion and biofilm formation are likely to be involved in both clinical manifestations of infection and attachment to foodstuffs, such as lettuce after irrigation or washing using water that has become contaminated with human faecal waste (Berger et al. 2009; Castro-Rosas et al. 2012). Uropathogenic strains in particular may make use of biofilm formation to persist on epithelial surfaces and canulae (Boll et al. 2013). A high proportion of EAEC strains associated with travellers' diarrhoea produce biofilms, as well as being highly antimicrobial-resistant (Mohamed et al. 2007; Mendez Arancibia et al. 2009).

9 Antimicrobial Resistance

Although gastrointestinal symptoms associated with EAEC may persist for weeks, infection is usually self-limiting and the standard recommended treatment is oral rehydration therapy. However, the symptoms can be debilitating and have a high socio-economic impact, especially in low-income settings, and treatment may be sought if the diarrhoea and abdominal pain are severe and/or prolonged. Multidrug resistance appears to be common in EAEC and geographically widespread.

Isolates of EAEC exhibiting high incidence of resistance to co-trimoxazole, ampicillin and tetracyclines were detected in studies carried out in Africa and Asia (Oundo et al. 2008; Chen et al. 2014). During a study in India between 2006 and 2007, an increase in isolates with resistance to quinolones was observed (Raju and Ballal 2009). Resistance to ampicillin, cefotaxime (encoded by a CTX-M-15 β-lactamase), gentamicin, co-trimoxazole, nalidixic acid and ciprofloxacin has been reported in EAEC isolates from travellers from India returning to Spain (Vila et al. 2001; Guiral et al. 2011). In studies in Central and South America from 2006 to 2007, the most common *E. coli* pathogens in cases of diarrhoea were EAEC (14%), of which greater than 90% of isolates were resistant to antimicrobials (Ochoa et al. 2009).

In Europe, of 160 strains of *E. coli* identified as EAEC isolated from patients in the UK with infectious intestinal disease or gastroenteritis between 1993 and 1996, over 50% were resistant to one or more of eight antimicrobials, and 30 (19%) were resistant to four or more drugs with one strain being resistant to eight antimicrobials (Wilson et al. 2001). Multidrug-resistant isolates of EAEC have been described elsewhere in Europe, notably in Poland and Spain (Sobieszczańska et al. 2003; Mendez Arancibia et al. 2009)

The most frequently used first-line antimicrobials which have traditionally been used for the treatment of travellers' diarrhoea are ampicillin, co-trimoxazole,

tetracyclines (doxycycline) and quinolones, due to their ready availability and inexpensive cost (Kong et al. 2015). As EAEC have become increasingly resistant to various antibiotics, selection of an appropriate antibiotic should take into account the region of the world where the infection was acquired, as there are different antimicrobial susceptibility patterns for each geographical region. EAEC infections have been successfully treated with ciprofloxacin and other fluoroquinolones, although this group of antimicrobials is not in general regarded as suitable for use in children. The emergence of multiple antimicrobial-resistant strains often coupled with resistance to quinolones and third-generation cephalosporins has compromised treatment in some regions (Kong et al. 2015). The use of antimicrobials to eliminate carriage of Stx-producing strains from patients or food handlers is still considered a controversial treatment because of the risk of promoting the development of HUS by stimulating Stx production (Siefert and Tarr 2012).

Of note for EAEC is the high occurrence of resistance to antimicrobials in comparison with other *E. coli* pathotypes associated with food production animals, specifically STEC. Although AMR has been identified in STEC from both human infections (Day et al. 2017) and from cattle and beef products (Ennis et al. 2012), resistance does appear to be less common than in EAEC isolates from cases of human infection. Possible explanations for this anomaly may be related to either differences in the innate propensity of STEC and EAEC strains to acquire and maintain plasmids encoding for AMR, or to antimicrobial selective pressure, with patients with EAEC infections more likely to have been exposed to antimicrobials than cattle, the major reservoir of STEC.

10 Whole-Genome Sequencing

Whole-genome sequencing analysis has provided further evidence that EAEC are a heterogeneous group of pathogens with respect to their genotypic characteristics. This high level of genetic diversity is apparent at every level from the population structure, to the genomic architecture of the pAA plasmid, and the presence and absence of putative virulence genes and their variants on the plasmid and the chromosome (Jenkins et al. 2005; Rasko et al. 2008; Dallman et al. 2014).

MLST and WGS data provide evidence that prevailing "successful" EAEC lineages have evolved independently many times and are dispersed throughout the entire *E. coli* population (Fig. 3). Pupo et al. (2000) suggested that strains of *E. coli* act as genetic repositories with the ability to acquire DNA from multiple sources and the ability to act as donors. The successful lineages, as defined by MLST complex, appear to be globally distributed. There is some evidence that certain lineages may be more pathogenic than others (Chattaway et al. 2014a, b). ClonalFrame analysis showed that EAEC mutation and recombination rates vary across the lineages and that both events play an important part in the evolution of EAEC. Although the dataset was limited, Chattaway et al. (2014a, b) showed that recombination rate was higher in the STs associated with disease. Analysis of WGS

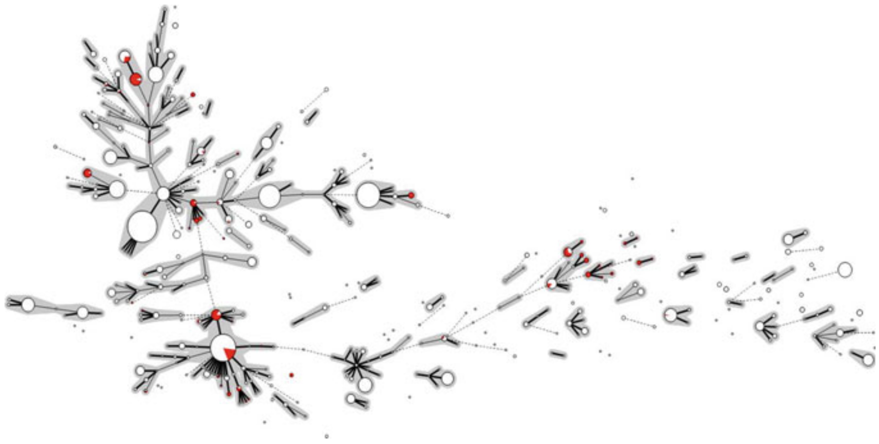

Fig. 3 Minimum spanning tree illustrating that EAEC lineages (highlighted in red) has evolved independently many times and is dispersed throughout the *E. coli* population (Courtesy of Marie Chattaway, Gastrointestinal Bacterial Reference Unit, Public Health England, London, UK)

data indicates that prophage and phage elements play a significant role in the evolution of certain *E. coli* pathovars (Rasko et al. 2008).

The pAA is regarded as a defining feature of EAEC, but recent WGS analysis has shown the pAA is associated with a wide range of plasmid replicon types and that it has a diverse genomic architecture (Dallman et al. 2014). WGS data can also be used to determine the presence or absence of all the major putative EAEC virulence genes, including *aggR, aat, aap, sepA, sigA, pic,* aggregative adherence fimbrial (AAF) types I–V and, more recently, a putative isopentenyl isomerise (IDI) enzyme (Rasko et al. 2011). WGS data have also been used to determine the integrity of the chromosomally encoded AAI operon and to provide information on antibiotic resistance (Dallman et al. 2014).

As yet, WGS is not used routinely for the detection of EAEC either from human faecal samples or from foods; however, the technology is progressing rapidly and there is potential of WGS to be used for such purposes (Loman et al. 2013). Multilocus sequence typing (MLST) and whole-genome sequencing (WGS) data have made a significant contribution to our understanding of the evolution and pathogenic potential of enteroaggregative *E. coli* (EAEC). The mosaic genomic structure of EAEC facilitates horizontal gene transfer, and recombination is the driving force for acquisition of novel genome features and potentially novel pathogenic mechanisms. The EAEC pan-genome is considered open and is still evolving by gene acquisition and diversification. This has significant public health implications in terms of the diversity and pathogenesis of EAEC and its ability to colonise and cause disease in the human host.

11 Summary

1. EAEC are a heterogeneous group of pathogens with respect to both phenotypic and genotypic characteristics. The current model of EAEC pathogenesis involves the initial adherence to the intestinal mucosa via aggregative adherence fimbriae under the control of the transcriptional regulator, AggR, biofilm formation on the surface of the enterocytes, secretion of toxins and induction of the inflammatory response. Key virulence factors are encoded on the pAA or PAI located on the chromosome.
2. Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from these matrices, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes using PCR. The STEC EU-RL PCR assay for screening food samples and faecal specimens targets the pAA-encoded *aggR* and *aaiC* which is located on the chromosome, and is recommended for clinical diagnostic use.
3. EAEC are commonly associated with acute and chronic diarrhoeal illness among children in both developing and developed and/or industrialised regions and travellers with diarrhoea. Studies suggest EAEC are a major cause of diarrhoeal disease. Increasing number of diagnostic microbiology laboratories are implementing a PCR approach for the detection of EAEC in clinical cases and foods, and this will improve the surveillance of EAEC disease.
4. There is no evidence that EAEC have a zoonotic reservoir but contamination of the environment can occur in parts of the world where human sanitary systems are insufficient and there is a high incidence of EAEC.
5. There is evidence in the literature of foodborne transmission of EAEC, and the infection status of food handlers, including asymptomatic carriage of EAEC, and hygienic conditions applied during the handling and processing of foodstuffs in some countries may be an important factor in contamination of foods at retail, catering or household level.
6. The ability to form biofilms is linked to the severity of human disease and is likely to be involved in environmental survival.
7. Multidrug resistance appears to be common in EAEC and geographically widespread. The emergence of multiple antimicrobial-resistant strains often coupled with resistance to quinolones and third-generation cephalosporins has compromised treatment in some regions.
8. Whole-genome sequencing analysis has provided evidence that EAEC exhibit a high level of genetic diversity and that prevailing "successful" EAEC lineages have evolved independently many times and are dispersed throughout the entire *E. coli* population.
9. The mosaic genomic structure of EAEC facilitates horizontal gene transfer, and recombination is the driving force for acquisition of novel genome features and potentially novel pathogenic mechanisms. The emergence of mixed EAEC/STEC pathotype *E. coli* is likely to be an ongoing low-frequency event and has significant public health implications.

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The Type III Secretion System of Pathogenic *Escherichia coli*

Sabrina L. Slater, Agnes M. Sågfors, Dominic J. Pollard, David Ruano-Gallego and Gad Frankel

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Abstract Infection with enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC), enteroinvasive *E. coli* (EIEC) and *Shigella* relies on the elaboration of a type III secretion system (T3SS). Few strains also encode a second T3SS, named ETT2. Through the integration of coordinated intracellular and extracellular cues, the modular T3SS is assembled within the bacterial cell wall, as well as the plasma membrane of the host cell. As such, the T3SS serves as a conduit, allowing the chaperone-regulated translocation of effector proteins directly into the host cytosol to subvert eukaryotic cell processes. Recent technological advances revealed high structural resolution of the T3SS apparatus and how it could be exploited to treat enteric disease. This chapter summarises the current knowledge of the structure and function of the *E. coli* T3SSs.

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1 Introduction

A healthy gastrointestinal (GI) tract encounters 9 L of fluid per day but only 100–200 ml of fluid is retained in the stools. Certain pathogenic microorganisms can alter the movement of ions and water in the gut towards net secretion, resulting in diarrhoea. With an estimated 2–4 billion incidents per year, acute diarrhoea is a significant contributor to morbidity and mortality worldwide, posing an immense burden on global health (Bern et al. 1992; Hodges and Gill 2010; Kosek et al. 2003; Snyder and Merson 1982). In low- to middle-income countries, diarrhoea is estimated to account for up to 760,000 infant deaths per annum (World Health Organisation), placing infectious diarrhoea within the five most common causes of death in children under the age of five (Liu et al. 2015).

Among bacterial agents of diarrhoeal disease, several pathotypes of *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp. depend on a specialised macromolecular syringe, the Type III Secretion System (T3SS), to cause disease. Through the T3SS, Gram-negative bacteria deliver virulence factors—also named 'effector proteins'—into host cells or bacterial competitors. The T3SS—also known as the injectisome—spans the inner and outer bacterial membranes and punctures the host plasma membrane like a syringe to translocate the effector proteins directly from the bacteria into the cytosol of host enterocytes. This enables the pathogen to control host cell signalling pathways, creating an environmental niche in which to thrive. In some pathogenic strains of *E. coli*, the T3SS is responsible for the translocation of over 25 effector proteins. The roles that these effector proteins play in pathogenesis are discussed in chapter "Modulation of Host Cell Processes by T3SS Effectors".

The term 'Type Three Secretion System' was coined in 1991 following the observation that *Yersinia* 'Yop' proteins were translocated into host cells in a general secretory pathway (Sec)-independent manner. Fewer than 10 years later, the *Salmonella* T3SS was first visualised using negative staining and electron microscopy (Kubori et al. 1998) soon followed by the *E. coli* T3SS (Sekiya et al. 2001). This chapter presents the contribution of this remarkable nanomachine to the pathogenesis of certain pathotypes of *E. coli* and reviews current knowledge on the assembly, regulation, function and importance of the T3SS. Advances in studying individual components and the impact of effector secretion on host cells have revealed fascinating complexity and sophistication to this system, indicating that our understanding of the injectisome during human infection is far from complete.

2 Enteric *E. coli* and the LEE-Encoded T3SS

Three currently defined pathotypes of *E. coli* rely on the T3SS and effector proteins to infect the human gut: enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), of which enterohaemorrhagic *E. coli* (EHEC) is a subtype, and enteroinvasive *E. coli* (EIEC) (Clements et al. 2011; Gaytán et al. 2016).

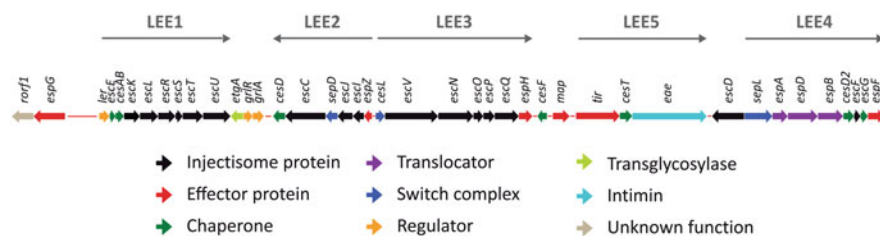

Fig. 1 **The EPEC Locus of Enterocyte Effacement (LEE)**. This genomic island is organised into five main operons. It harbours all the necessary genes to assemble a functional T3SS, as well as regulators and core effector proteins. Minor differences are found in the EHEC and *C. rodentium* LEE

EPEC-induced diarrheoa primarily affects children under the age of 2 in low-income countries (Chen and Frankel 2005), while EHEC is also capable of causing haemorrhagic colitis and haemolytic uraemic syndrome in infected patients. EIEC shares both its virulence mechanism and clinical symptoms with *Shigella* spp., and it is believed that current EIEC strains represent an intermediate between *E. coli* and *Shigella* spp. The majority of T3SS components and effectors of EIEC and *Shigella* are encoded on the virulence plasmid pINV, while the injectisomes of EPEC and EHEC along with chaperones, the adhesin intimin, core effector proteins, a lytic transglycosilase and regulatory proteins are encoded within a genomic pathogenicity island termed the Locus of Enterocyte Effacement (LEE) (Pallen et al. 2005). Additionally, typical EPEC strains carry the large virulence EPEC adherence factor (EAF) plasmid, which encodes the bundle forming pilus (BFP) operon as well as the plasmid-encoded regulator (Per) operon, encoding PerA, PerB and PerC, which regulate LEE expression (Gomez-Duarte and Kaper 1995).

The LEE is essential for disease in these pathotypes and comprises 41 conserved genes that allow the T3SS-dependent colonisation of mammalian hosts. Its low G +C content (38% compared to 50% for the whole genome) suggests it was acquired via horizontal gene transfer. LEE islands are also found in the closely related species rabbit EPEC (REPEC), *Escherichia albertii* (Hyma et al. 2005) and *Citrobacter rodentium*, with high degrees of similarity both in terms of gene repertoire and organisation (Petty et al. 2011) (Fig. 1). The structural components of the T3SS are encoded on operons LEE1, LEE2, LEE3 and LEE4, except EscD, encoded on its own ORF. They have been demonstrated to be sufficient to assemble a functional injectisome (Ruano-Gallego et al. 2015).

3 Regulation of T3SS Expression

Upon ingestion of contaminated food or water, acid resistance of the pathogens facilitates the survival of the bacteria through the low pH of the stomach (Nguyen and Sperandio 2012), and environmental signals throughout the intestine are sensed

to gradually turn on the virulence factors of the pathogens (Connolly et al. 2015; Furniss and Clements 2017). These signals include temperature (Umanski et al. 2002), host signals (De Nisco et al. 2018) and microbiota signals (Carlson-Banning and Sperandio 2018).

At the site of infection, the host hormones adrenaline and noradrenaline and the quorum-sensing molecules auto-inducers 2 and 3 are produced by the gastrointestinal cells and sensed by A/E pathogens to induce the expression of the T3SS (Hughes and Sperandio 2008; Russell et al. 2007; Sperandio et al. 1999). The interplay between the host and microbiome is of significant importance to protect the intestine against infections, as A/E pathogens can sense and take advantage of an unbalanced gut environment. For example, a diet that is poor in fibre causes rapid microbiota-derived degradation of intestinal mucin and thus promotes both a more oxygenic environment and the availability of by-products like the short fatty acids succinate, butyrate and fucose (Desai et al. 2016; Pacheco et al. 2012), all of which activate expression of the T3SS.

Environmental signals are largely integrated by A/E pathogens through sensor kinases *(*Moreira et al. 2016) including quorum sensing that affects internal specific and global transcriptional regulators: PerC (EPEC)/PchABC (EHEC) or the histone-like nucleoid-structuring protein (H-NS) (Bustamante et al. 2001), among others (Martínez-Santos et al. 2012). The coordinate effects of these signals activate LEE transcription as well as distinct fimbrial and non-fimbrial adhesins that participate in the initial attachment to enterocytes. Most of these signals converge to regulate the transcription of the first gene encoded in operon LEE1: the LEE-encoded regulator (*ler*) (Bingle et al. 2014; Mellies et al. 1999). Constitutively expressed at low levels, Ler activates the transcription of operons LEE2-LEE5 counteracting the inhibitory effect of H-NS (Bustamante et al. 2001; Elliott et al. 2000; Winardhi et al. 2014), but also functions as a repressor of LEE1 itself (Berdichevsky et al. 2005; Bhat et al. 2014), creating a negative feedback loop. Extra-LEE genes that are known to be regulated by Ler in EPEC include non-LEE-encoded effectors (Nle) and espC (Mellies et al. 2001), which encodes an autotransporter extracellular serine protease that is thought to play various roles in pathogenicity (Navarro-Garcia et al. 2014; Salinger et al. 2009). In contrast, the EHEC homologue of espC, espP, is not Ler-regulated.

In addition to Ler, other LEE-encoded regulators of the T3SS expression include GrlR (the negative regulator) and GrlA (the positive regulator) (Jimenez et al. 2010; Russell et al. 2007). GrlA interacts with the promoter of LEE1 to activate its transcription, however GrlR is able to inhibit this activation by binding directly to GrlA (Padavannil et al. 2013). The action of ClpXP protease on GrlR releases GrlA under T3SS-inducing conditions (Iyoda and Watanabe 2005), but additional post-translational modifications may be necessary for full activation (Alsharif et al. 2015).

4 Assembly of the T3SS

The assembly of the T3SS requires input from transcriptional regulators, chaperones, environmental cues and molecular switches. Though not yet comprehensively understood, T3SS construction can be categorised into four stages: (i) assembly of the basal body and export apparatus, (ii) assembly of the inner rod and needle, (iii) assembly of the filament and translocon and (iv) secretion of effectors. The

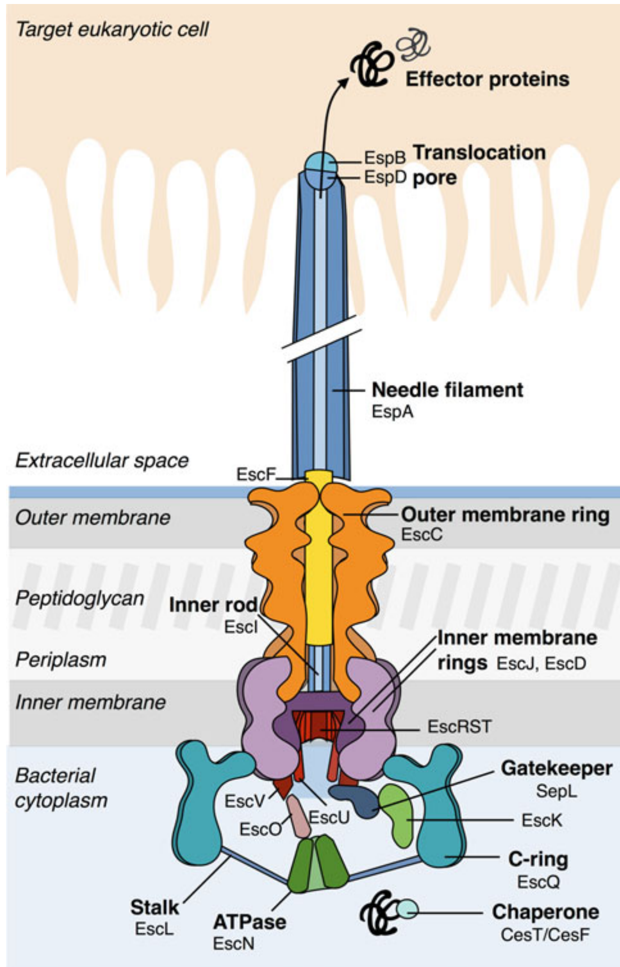

Fig. 2 Schematic representation of the *E. coli* T3SS. The T3SS consists of a number of coaxial ring-like structures. It comprises an export apparatus (red) a base complex and a needle filament (blue) through which unfolded effectors are channelled. At the distal end of the injectisome is a pore that inserts into the host membrane. The cytoplasmic ATPase complex (green) powers injectisome assembly as well as the translocation of effector proteins

Table 1 Injectisome components of *E. coli*, *C. rodentium*, *Salmonella*, *Shigella* and *Yersinia*

Functional role	*E. coli* and *C. rodentium*	*Salmonella* SPI-1	*Shigella*	*Yersinia*
Cytoplasmic components				
Cytoplasmic regulator	SepD	SpiC	–	–
Sorting platform component	EscK	OrgA	MxiK	YscK
Stator	EscL	OrgB	MxiN	YscL
Gatekeeper	SepL	InvE	MxiC	YopN
Cytosolic C-ring	EscQ	SpaO	Spa33 (SpaO)	YscQ
Needle length regulator	EscP	InvJ	Spa32 (SpaN)	YscP
Stalk	EscO	InvI	Spa13	YscO
Cytosolic ATPase	EscN	InvC	Spa47 (SpaL)	YscN
Chaperones				
For early substrates	EscE; EscG	–	–	YscE; YscG
For middle substrates	CesAB; CesD; CesD2	SicA	IpgC	LcrG; SycD; SycB
For effectors/late substrates	CesT; CesF	SicP; SigE; InvB	IpgE; IpgA; Spa1	YscB; YsaK; SycE; SycT; SycH; SycN
Basal body				
Outer membrane secretion ring	EscC	SpiA/InvG	MxiD	YscC
Inner membrane ring	EscD	SpiB/PrgH	MxiG	YscD
Inner rod	EscI	PrgJ	MxiI	YscI
Inner membrane ring component	EscJ	PrgK	MxiJ	YscJ
Inner rod	EscI	PrgJ	MxiI	YscI
Inner membrane-associated core	EscR	SpaP	Spa24 (SpaP)	YscR
Inner membrane core	EscS	SpaQ	Spa9 (SpaQ)	YscS
Inner membrane core	EscT	SpaR	Spa29 (SpaR)	YscT

(continued)

Table 1 (continued)

Functional role	*E. coli* and *C. rodentium*	*Salmonella* SPI-1	*Shigella*	*Yersinia*
Inner membrane core	EscU	SpaS	Spa40 (SpaS)	YscU
Major export apparatus	EscV	InvA	MxiA	YscV
Extracellular components				
Needle	EscF	PrgI	MxiH	YscF
Filament/tip	EspA (filament)	SipD (tip)	IpaD (tip)	LcrV (tip)
Translocation pore	EspB	SipB	IpaB	YopB
Translocation pore	EspD	SipC	IpaC	YopD

complete *E. coli* injectisome is 23 nm long, 8–9 nm wide and is comprised of around 3.5 MDa of protein (Fig. 2). Throughout this chapter, *E. coli* nomenclature will be used, though the *Salmonella, Shigella* and *Yersinia* nomenclature can be found in Table 1.

5 Assembly of the Basal Body and Export Apparatus

There are four major components of the injectisome: the needle, the basal body, the export apparatus and the cytoplasmic protein complexes. The order of construction is disputed within the field and between species, which may represent divergence in the assembly pathway. An 'outside-in' model has been proposed for *Yersinia* beginning with the outer membrane ring and working inwards towards the export apparatus (Diepold et al. 2010, 2011) and the reverse 'inside-out' model has been described for *Salmonella* and *E. coli* (Wagner et al. 2010). This 'inside-out' model begins with nucleation of the inner membrane export apparatus components EscR, EscS and EscT forming a stable complex. EscRST recruits the export gate EscV oligomeric ring and the autoprotease EscU, completing the 5-member export apparatus EscRSTUV (Diepold et al. 2011). Autoproteolytic cleavage of EscU is an important signal for the union of the basal body and the export apparatus. The basal body ring components are exported via the Sec pathway, inserted within the bacterial membranes and come together with the export apparatus. A homo-oligomeric EscD ring at the inner membrane encircles the EscJ ring, which does not possess a transmembrane region but is instead tethered to the inner membrane. In the periplasm, EscD interacts with the outer membrane ring of EscC (Creasey et al. 2003a; Ogino et al. 2006) and is presumed to bind the EscJ ring. EscC belongs to the secretin family of proteins found in several bacterial secretion systems. Unlike other T3SS where the insertion and oligomerisation of the secretin are directed by pilotin, a lipoprotein not identified in A/E pathogens, EscC is believed to be regulated by other T3SS components (Gauthier and Finlay 2003). Assembly and coupling of the membrane rings to the export complex are followed by the formation of the EscQ cytosolic ring sorting complex, which recruits EscN and EscL, two parts of the three-component ATPase complex (Biemans-Oldehinkel et al. 2011). The ATPase complex is only activated upon the conformational change in EscL induced by the binding of EscO to EscN (Romo-Castillo et al. 2014). The function of the ATPase complex will be discussed in a later section.

Formation of the basal body is the only stage of assembly where components are directed by the Sec pathway. Once assembled, the estimated dimensions of the EPEC T3SS basal body are 16.7 $\pm$ 1.9 nm wide at the outer membrane ring, 18.1 $\pm$ 2.5 nm wide at the inner membrane ring and 31.4 $\pm$ 4.3 nm tall (Sekiya et al. 2001). This immature structure can secrete the so-called early- and mid-substrates required for construction of the inner rod, needle, tip and translocon.

6 The C-Ring/Sorting Complex

Multiple EscQ subunits make up the C-ring/sorting complex that sits at the foot of the basal body (Biemans-Oldehinkel et al. 2011; Pallen et al. 2005). In the flagellar T3SS, the C-ring is a three-component structure that has been attributed to generating flagellar torque and rotational switching, though whether this translates to the injectisome is debated. However, based on data from the *Salmonella* T3SS, the C-ring has been proposed to form a so-called 'sorting platform' (Lara-Tejero et al. 2011). Co-immunoprecipitation experiments show that the *Salmonella* EscQ homologue is predominantly associated with translocon proteins, but after deletion of translocator substrates is instead occupied by late effector proteins. These experiments also showed its interaction with the ATPase complex subunit homologues of EscN and EscL (Biemans-Oldehinkel et al. 2011). Most EscQ homologues have an internal translation site, giving rise to both the full length protein and a truncated C-terminal product, homologous to two of the three flagellar C-ring components (Bzymek et al. 2012; Lorenz et al. 2012; Notti et al. 2015). However, it is not clear whether EscQ expressed by A/E pathogens is produced in this way, and it is currently thought that the sorting platform is composed of EscQ, EscL and EscK, a crucial protein for substrate secretion (Soto et al. 2017).

7 The ATPase Complex

EscN, EscL and EscO together constitute the T3SS ATPase complex. It is located at the base of the export apparatus and has structural similarity to both the flagellar and the F1 ATPase. At the core of this complex is the multifunctional, multi-domain protein EscN. The EscN N-terminus facilitates its hexameric self-oligomerisation, while the central domain harbours a conserved ATPase domain and the C-terminus is the proposed recognition site for T3SS substrates. Aside from energising the secretion process, EscN is thought to serve as a docking site for the chaperone-substrate complex (Gauthier and Finlay 2003; Thomas et al. 2004), enabling the ATP hydrolysis-dependent uncoupling of these complexes, and unfolding of the protein which is to be secreted. As the needle pore is 2–3 nm wide this unfolding is essential for T3SS assembly. Binding of EscO to EscN promotes a change of conformation in EscL that activates EscN (Biemans-Oldehinkel et al. 2011; Romo-Castillo et al. 2014). Due to the structural similarity to the F1 ATPase, it is assumed that effector translocation is powered by a combination of ATP hydrolysis and proton motive force (Ibuki et al. 2011; Imada et al. 2007; Romo-Castillo et al. 2014; Zarivach et al. 2007). There is currently not enough data to confirm that EscN is the sole energiser of the T3SS, and due to a lack of high quality structural data, the mechanics of energising is still poorly understood.

8 Inner Rod and Needle Assembly

Once the basal body and the export apparatus join, EscI monomers assemble to create a hollow inner rod. EscI is also thought to have a role in substrate regulation, a theory supported by the fact that EscI interacts with EscU and EscP, components that contribute to substrate recognition and regulation. As the T3SS must penetrate the bacterial peptidoglycan layer, the specialised lytic transglycosilase EtgA, a peptidoglycan degrading enzyme, is required for this step of the assembly (García-Gómez et al. 2011). EtgA interacts directly with EscI, a relationship shown to enhance EtgA enzymatic activity (Burkinshaw et al. 2015). The EscF polymer, which forms the needle of the T3SS, is believed to be generated simultaneously with EscI (Gaytán et al. 2016). To prevent premature self-polymerisation of EscF, co-chaperones EscE and EscG bind EscF in the bacterial cytoplasm (Sal-Man et al. 2013).

At 23 nm long and 8–9 nm wide (Ogino et al. 2006; Sekiya et al. 2001), the EPEC injectisome is the shortest T3SS, compared to *Salmonella enterica* (25–80 nm), *Shigella flexneri* (45–50 nm), *Yersinia pestis* (41 nm) and *Yersinia enterocolitica* (58 nm). Despite this variation between bacterial genera, needle length tends to be conserved within species, perhaps reflecting its role in determining host tropism. Needle completion also controls the switch from the secretion of early substrates to middle and late substrates for the assembly of the filament and translocon structures (Buttner 2012; Minamino et al. 2004). In EPEC, the specificity switch occurs when EscP interacts with EscU, causing a conformational change in EscU that ultimately signals a substrate switch from injectisome components to translocated proteins (Feria et al. 2012). There are several suggested mechanisms for this switch. One hypothesis, named the infrequent ruler model, proposes that EscP is occasionally secreted in an elongated form and its passage through the needle is hindered by EscF subunits. Thus, as the needle grows, the chance of EscP interacting with EscU in the cytosol is increased (Feria et al. 2012). This is an adaptation of the ruler model first published in 2003, wherein EscP is anchored to both the tip of the growing filament and the basal body: once EscP is stretched to its maximum capacity, EscF is no longer incorporated into the needle and the substrate switches (Journet et al. 2003). In both models, the length of EscP determines substrate switching, hence the name 'ruler protein'. In an alternative model, the inner rod regulates the needle length and timing of the substrate-switching event. Overexpression of the *Yersinia* inner rod protein results in shorter needles, while mutations presumed to slow inner rod assembly cause an elongated needle (Wood et al. 2008). Accordingly, EPEC EscI has also been shown to interact with EscP and EscU (Creasey et al. 2003a; Sal-Man et al. 2012). It is likely that needle length and substrate switching is in fact controlled by a combination of these models.

9 Filament and Translocon Assembly

The T3SS EspA filament is an extension of the EscF needle structure that is polymorphous among EPEC and EHEC isolates (Daniell et al. 2001a; Neves et al. 2003a). After translocation through the T3SS, EspA subunits self-polymerise via their C-terminal coiled-coil domains, with the completed filament averaging 90 nm in length (Daniell et al. 2001b; Delahay et al. 1999; Knutton et al. 1998). These coiled-coil domains are not only important for filament assembly, but also for prohibiting the immature polymerisation of EspA in the bacterial cytosol, where they are bound by the chaperone CesAB (Yip et al. 2005). Unlike the needle, the filament displays a more variable length seemingly dependent on the availability of EspA subunits, and can be up to 700 nm long (Crepin et al. 2005; Sekiya et al. 2001). The filament has proposed roles in bacterial adhesion and sensing of mammalian cells (Cleary et al. 2004). Interestingly, once intimate attachment has been achieved the EspA filament is disassembled by an unknown mechanism, and is absent from the mature A/E lesions (Dahan et al. 2004; Knutton et al. 1998).

The translocon is assembled by hetero-oligomerisation of EspB and EspD with 6–8 subunits, and has a pore size of 3–5 nm (Ide et al. 2001). It is responsible for puncturing the mammalian cell membrane, with both components predicted to possess transmembrane domains. Additionally, EspD comprises a C-terminal coiled-coil domain, which is necessary for A/E lesion formation (Daniell et al. 2001b). It was recently shown that host cells may be able to sense this puncturing, as contact with the EPEC injectisome was sufficient to induce activation of NF-κB, which in turn is subverted by anti-inflammatory effectors (Litvak et al. 2017).

10 The Roles of Chaperones in T3SS Assembly and Secretion

Chaperones are required in the bacterial cytoplasm throughout the assembly of the T3SS, and for the translocation of late effectors during the infection of host cells. They tend to be small acidic proteins and hold roles in delivering subunits and effectors to the export complex, preventing homo- and hetero-oligomerisation or degradation of substrates in the cytoplasm. Chaperones are also thought to play a role in defining the substrate hierarchy. Eight chaperones have been characterised in the LEE, and are classified according to their substrate: those that are specific for one effector protein (Class IA: CesF and CesL), several effectors (Class IB: CesT), translocators (Class II: CesAB, CesD and CesD2), and needle subunits (Class III: EscE and EscG) (reviewed in Gaytán et al. 2016; Izoré et al. 2011).

Together, class II chaperones control the secretion of translocators EspA, EspB, EspD. With the exception of CesD2, which only has one substrate (EspD) (Neves et al. 2003b), these chaperones work in coordination. Secretion of EspB and EspD requires CesD (Wainwright and Kaper 1998). EspB also interacts with CesAB, as

does EspA, prior to secretion (Creasey et al. 2003b; Yip et al. 2005). In addition to CesAB, EspA is also chaperoned by CesA2 (Su et al. 2008). Both class III chaperones, EscG and EscE, are important for EscF secretion, as discussed above (Sal-Man et al. 2013). EscF is sometimes referred to as an 'early substrate', while the translocators EspA, EspB and EspD are 'mid-substrates' (Table 1).

Upon the completion of T3SS assembly, a disputed external signal leads to a second substrate switch from translocators to effectors, which are injected into host cells via the completed T3SS. This event involves the regulatory SepD-SepL-CesL complex. SepD and SepL, dubbed the gatekeeper proteins, simultaneously promote translocator secretion while preventing the premature translocation of late effectors (Deng et al. 2004, 2015; O'Connell et al. 2004). While the mechanism of promoting translocator secretion is unclear, it is thought that the interaction between SepL and the effector protein Tir is responsible for preventing effector secretion. Under the established effector hierarchy, Tir is the first effector to be translocated, and thus its interaction with SepL prevents translocation of any other effectors (Thomas et al. 2007; Wang et al. 2008). The Tir binding site of SepL is shared with the inner membrane ring protein EscD, perhaps playing a role in relief from the suppression of late effector secretion (Wang et al. 2008). Furthermore, in A/E pathogens removal of calcium from growth medium switches the secretion specificity from translocators to effectors (Deng et al. 2005; Gaytán et al. 2017; Ide et al. 2003; Kenny et al. 1997); under normal calcium conditions, the ruler protein EscP binds extracellular calcium flowing into the incomplete injectisome, stabilising its interaction with SepL and inhibiting effector secretion (Shaulov et al. 2017). However, contact between EspA/B/D and the host membrane does not play a role in specificity switching, as previously thought (Gaytán et al. 2017).

11 The ETT2: A Second T3SS?

In 2001, analysis of the EHEC O157:H7 genome revealed a second type III secretion system cluster, designated the *E. coli* Type Three Secretion System 2 (ETT2). The ETT2 locus appears to have been inserted into the *E. coli* genome immediately upstream tRNA glyU locus, spanning 27.5 kb and 35 open reading frames (ORFs) (Ren et al. 2004). It was initially noted that the ETT2 is remarkably homologous to the *S. enterica* serovar Typhimurium T3SS, SPI-1, with some genes sharing up to 64% identity with their *Salmonella* counterparts. This homology guided further analysis of each ORF, leading to the discovery that several genes in the ETT2 contain frameshift mutations, rendering it non-functional. However, fragments of the ETT2 have been identified in a variety of enteric *E. coli* isolates from several mammalian and avian sources (Osawa et al. 2006; Zhou et al. 2014). The isoform of each ETT2 identified is now categorised from 'type A' to 'type K' per its completeness, where type A encodes all 35 ORFs (Cheng et al. 2012). Interestingly, the ETT2 is considered intact in the emerging enteropathogen *E. albertii,* which gives a more specific idea of when the locus was acquired (Ooka

et al. 2015). As such, ETT2 classification may provide an insight into the phylogenetic origin of *E. coli* isolates.

A secretion-competent ETT2 has never been identified, therefore the question of its function remains unsolved. In earlier works, it was suggested that proteins encoded by the ETT2 could complement the function of the LEE-encoded T3SS This now seems unlikely, given the mutational attrition within ORFs in the ETT2 and the lack of ETT2-specific effectors. Interestingly, deletion of ETT2 genes impacts several aspects of bacterial virulence (Ideses et al. 2005), although the mechanistic details behind these observations remain unclear. Two ETT2 encoded regulators, EtrA and EivF, repress LEE expression and therefore reduce bacterial adherence (Zhang et al. 2004). This could explain why an *ΔetrA* mutant was also deficient in intracellular survival (Wang et al. 2017). A third regulator, EtrB, was shown to activate LEE expression by direct interaction with Ler, the master regulator of the LEE pathogenicity island (Luzader et al. 2016). More recently, Wang et al. showed that deletion of the ETT2 putative ATPase EivC inhibited the flagellar motility of the avian pathogenic *E. coli* strain APCE94. The lack of motility was attributed to downregulation of the flagellum and upregulation of fimbrial genes. Additionally, the *ΔeivC* strain had significantly decreased intracellular survival compared to WT APCE94 (Wang et al. 2016). Together, these observations suggest that the ETT2 is not just an artefact, as it was once assumed.

12 In Vitro and in Vivo Tools to Study the *E. coli* T3SS

Significant strides have been made delineating the molecular mechanisms that underpin T3SS-dependent virulence in enteric *E. coli*, aided by technical advances in fluorescence microscopy, electron cryotomography and single-cell super-resolution techniques. Our structural understanding of T3SS architecture is becoming increasingly clear as crystal structures of components are being solved, and defined functions during infection are being assigned to T3SS components and effectors. Numerous studies in vivo have demonstrated the importance of the T3SS for A/E virulence, including a study with human volunteers that confirmed the requirement for structural T3SS components (EspB and EspA) for the development of diarrhoea (Donnenberg et al. 1993; Tacket et al. 2000). However, furthering our understanding of the T3SS requires robust in vitro and in vivo models that do not rely on the availability of human samples. Intestinal biopsies have historically been used for in vitro organ culture (IVOC) models to demonstrate how A/E pathogens attach to the apical portion of intestinal crypts (Shaw et al. 2005). However, for biochemical assays, in vitro studies typically use cultured cells (such as HeLa cells or polarised Caco-2 cells) (Wong et al. 2011).

In vivo models of A/E pathogenesis allow studies of the complex interplay between the pathogen and its host as well as the intestinal microbiota, which is known to play a vital role in disease development. A variety of surrogate species have been infected with EPEC and EHEC, including *Caenorhabditis elegans*, pigs,

baboons, macaques, infant rabbits, ferrets and cows (Ritchie 2014; Law et al. 2013), but these techniques inevitably suffer from the bacterium not representing a natural pathogen. Therefore, closely related animal-specific A/E pathogens serve as a better proxy for human infection: the use of REPEC is limited as it causes high mortality (Milon et al. 1999), but *C. rodentium* is an indispensable tool for studying A/E pathogenesis. *C. rodentium* causes transmissible murine colonic hyperplasia with A/E lesions indistinguishable from those caused by EPEC and EHEC (Collins et al. 2014; Schauer et al. 1995). In general, murine models benefit from the availability of inbred or genetically manipulated mice that can be maintained under germ-free or controlled pathogenic conditions. The *C. rodentium* model continues to give invaluable insights into the physiological outcomes of the translocation of effector proteins in vivo and can be combined with biochemical techniques such as mass spectrometry to uncover strategies used by A/E pathogens to circumvent immune responses and cause disease.

13 Exploiting the T3SS to Treat Disease

The genetic variety of pathogenic *E. coli* strains underpins their success but makes treatment of disease difficult. Rehydration therapy is currently the most effective way to manage symptoms of *E. coli* infection, which can become dangerous if it is not self-limiting. The traditional treatment for unidentified bacterial infections associated with diarrhoea is often antibiotics. Broad-spectrum antibiotics may kill the offending bacterium but will also affect commensals. Additionally, some antibiotics can exacerbate expression of Shiga toxins if they are present (Freedman et al. 2016), which can lead to HUS and ultimately renal failure. These problems could be bypassed by targeting the T3SS: the injectisome is not required for growth, so its inhibition should not enforce selective pressure on targeted bacteria. Further, the T3SS is only conserved in pathogenic bacteria, so commensals will not be affected by treatment, and the development of anti-T3SS drugs for *E. coli* could be adapted to target other T3SS-expressing pathogens.

There are several elements of type III secretion that can be targeted. Aside from inhibiting the basal body, vaccines can be developed against the translocon and needle, LEE regulatory elements can be manipulated, effectors can be counteracted and secretion can be inhibited (Charro and Mota 2015). One of the most studied classes of anti-T3SS compounds is salicylidene acylhydrazides (SAHs), which subvert secretion in several ways. First, SAHs interact with the inner membrane proteins of the basal body to restrict the passage of effectors. Owing to its homology with the T3SS basal body, SAHs have also been shown to block the flagellar apparatus, effectively reducing bacterial motility. Lastly, SAHs have been shown to interact with proteins involved in *E. coli* metabolism, resulting in the downregulation of T3SS gene expression (Mcshan and Guzman 2015).

Although other classes of compounds have been shown to inhibit the T3SS, their development as drugs is hindered by their toxicity to eukaryotic cells, their

similarity to existing drugs against which pathogens have gained resistance, difficulty introducing the compound into the gut, or lack of a clear bacterial target for their action. Owing to the intricacy of LEE regulation, our incomplete knowledge of T3SS effectors and the problems with anti-T3SS compounds described above, vaccines against the bacterial surface structures represent the most promising treatment candidates (O'Ryan et al. 2015). For example, self-polymerisation of EspA can be inhibited by treatment with synthetic EspA-like coiled-coil domains, which effectively outcompete endogenous EspA interactions to inhibit the secretion of effectors and A/E lesion formation (Larzábal et al. 2010). Additionally, when produced either *in planta* or in vitro and administered orally to ruminants, EspA itself is able to act as a vaccine to reduce bacterial shedding (Miletic et al. 2017; Potter et al. 2004). Indeed, many other injectisome proteins and pathogenic *E. coli* virulence factors are highly immunogenic in ruminants, including flagellin, intimin, Tir, EspB and EspD. Antibodies against these proteins have been identified in bovine and human colostrum and offer protection for both calves and human infants against EHEC colonisation (Loureiro et al. 1998; Vilte et al. 2008), often by reducing ruminants' shedding (McNeilly et al. 2015).

Although the complexity of the T3SS presents an obstacle for inhibiting its action, it is a targeted membrane-specific nanomachine capable of secreting cargo, and this itself is an exploitable property (González-Prieto and Lesser 2018). Recently, all of the structural components of the EPEC injectisome were inserted in five independent transcriptional units, or engineered LEEs (eLEE), into the genome of the commensal strain *E. coli* K-12. Rational design of each eLEEs meant their expression can be controlled, avoiding the intricate regulation found in the A/E pathogens. The resulting strain, named synthetic injector *E. coli* (SIEC), was demonstrated to assemble functional injectisomes and efficiently translocate T3-substrate proteins (Ruano-Gallego et al. 2015). Indeed, several studies have also demonstrated its use as a drug delivery system by engineering a signal sequence from an effector onto the protein of interest. This includes delivery of a nuclear-targeted recombinase for editing the genome of induced pluripotent stem cells (Bichsel et al. 2011), delivery of antigens for immunotherapy (Le Gouëllec et al. 2013) and delivery of angiogenic inhibitors for the shrinkage of tumours (Shi et al. 2016).

A current challenge in the field is shifting from studying T3SS effector proteins and injectisome components in isolation to applying a holistic approach that considers the role of effectors in context of the full effector repertoire, while also considering spatio-temporal regulatory mechanisms. Many mechanistic details on the assembly and regulation of the T3SS must still be elucidated, including fully defining the signal that triggers the translocation of effector proteins upon contact of host cells. Answering these questions will help design strategies to interfere with the system to help relieve the global burden of enteric *E. coli* infections on human health.

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Modulation of Host Cell Processes by T3SS Effectors

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Abstract Two of the enteric *Escherichia coli* pathotypes—enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC)—have a conserved type 3 secretion system which is essential for virulence. The T3SS is used to translocate between 25 and 50 bacterial proteins directly into the host cytosol where they manipulate a variety of host cell processes to establish a successful infection. In this chapter, we discuss effectors from EPEC/EHEC in the context of the host proteins and processes that they target—the actin cytoskeleton, small guanosine triphosphatases and innate immune signalling pathways that regulate inflammation and cell death. Many of these translocated proteins have been extensively characterised, which has helped obtain insights into the mechanisms of pathogenesis of these bacteria and also understand the host pathways they target in more detail. With increasing knowledge of the positive and negative regulation of host signalling pathways by different effectors, a future challenge is to investigate how the specific effector repertoire of each strain cooperates over the course of an infection.

1 Introduction

Most *Escherichia coli* strains share a common genetic backbone of approximately 4.1 Mbp. However, significant divergence has occurred over the past 4.5 million years as a consequence of Horizontal Gene Transfer (HGT). Lineage-specific acquisition of large groups of virulence genes (termed Pathogenicity Islands, PAIs), plasmids and prophages have given rise to a range of *E. coli* pathotypes with genomes up to 1 Mb larger than those of commensal *E. coli* strains. These strains, known as the pathogenic *E. coli*, have the ability to cause a broad range of diseases in different hosts (Croxen and Finlay 2010; Blattner et al. 1997).

Six distinct enteric (also known as diarrheagenic) *E. coli* pathotypes are currently recognised. Of these, enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), alongside the murine pathogen *Citrobacter rodentium* (Petty et al. 2010), the rabbit diarrheagenic *E. coli* (RDEC) (Agin et al. 1996) and the emerging pathogen *Escherichia albertii* (Huys et al. 2003; Nimri 2013) are characterised by the formation of an ultrastructural lesion on the apical surface of the intestinal epithelium known as an 'Attaching and Effacing (A/E)' lesion (Knutton et al. 1987; Moon et al. 1983). Therefore, these pathogens are collectively referred to as the A/E pathogens. A/E lesions result from the effacement of the brush border microvilli and significant rearrangement of the actin cytoskeleton underneath adherent bacteria. This ability is conferred on the A/E pathogens by a 35 kb PAI known as the Locus of Enterocyte Effacement (LEE) (McDaniel et al. 1995) which encodes a type 3 secretion system (T3SS) (Elliott et al. 1998; Jarvis et al. 1995) and a suite of effector proteins (Wong et al. 2011).

The T3SS is critically important for the virulence of the A/E pathogens, and the effectors delivered by the T3SS target many fundamental processes within the infected host cell. Different pathotypes and indeed different isolates within those pathotypes encode a unique T3SS effector repertoire, and whilst we now have an excellent understanding of how many of these effectors function individually, we know less about the cooperation or antagonism of the entire effector repertoire for each strain.

This chapter describes many of the host cell processes targeted by T3SS effectors found in the A/E pathogens. Often this work has been performed with a single EPEC or EHEC strain (or in some cases with *C. rodentium*). Throughout this chapter when we refer to the pathotype, it is with the assumption that the effectors have the same function across the A/E pathogens, unless specifically stated otherwise.

2 Pathotype Definitions: EPEC and EHEC

EPEC and EHEC are the most extensively studied of the *E. coli* pathotypes, and the original pathogenic *E. coli* strain described by Bray and colleagues in 1945 was an EPEC strain (Bray 1945). The EPEC pathotype can be subdivided into typical and atypical strains. Typical EPEC carries the EPEC adherence factor (EAF) plasmid that encodes the type IV bundle-forming pilus (BFP) responsible for the classic localised adherence phenotype on epithelial cells (Donnenberg et al. 1992). In contrast, atypical EPEC is a diverse group of isolates that are often genetically more similar to other *E. coli* pathotypes than they are to typical EPEC (Ingle et al. 2016). However, due to the presence of the LEE and the absence of shiga toxin, these strains are classified as EPEC. The current genetic definition for EPEC and EHEC is therefore based on molecular detection of the EAF plasmid (*bfp*+), the LEE PAI (*eae*+) and shiga toxin genes (*stx*+) with typical EPEC defined as bfp+, eae+, stx−, atypical EPEC as bfp−, eae+, stx− and EHEC as bfp−, eae+, stx+. Phylogenetic analysis further categorises EHEC strains into EHEC1 and EHEC2 and typical EPEC into EPEC1-4 lineages (Hazen et al. 2013; Lacher et al. 2007).

The diversity within the pathotypes includes diversity within the T3SS effectors that are encoded by each strain. The lineage of the LEE at least partly dictates the effector repertoire for a particular strain, despite many effectors being non-LEE-encoded (NLE) (Ingle et al. 2016), and whilst the prototypical EPEC strain E2348/69 encodes at least 25 T3SS effectors (Deng et al. 2012), some EPEC and EHEC strains can encode up to 50 effectors (Tobe et al. 2006). Table 1 contains a summary of the activity of all currently recognised T3SS effectors from EPEC and EHEC, and Fig. 1 shows the genetic organisation of phage-encoded effectors from the EHEC strain Sakai. Beyond the effectors already described, it is likely that additional T3SS effectors remain to be discovered from recently sequenced aEPEC strains (Ingle et al. 2016).

Table 1 Summary of T3SS effector activities

Effector[a]	Biochemical activity	Host target or partner	Reported function	References
Cif	Glutamine deamidase	Nedd8	Inhibits cell cycle progression	Cui et al. (2010)
Efa1/LifA	–	–	A/E lesion formation	Cepeda-Molero et al. (2017)
EspH	–	DH-PH domain of RhoGEFs	Actin manipulation, inhibition of phagocytosis	Dong et al. (2010), Tu et al. (2003)
EspF	–	SNX9, N-WASP, ABCF2 and various actin-binding proteins	Membrane remodelling, actin nucleation and induction of apoptosis	Marches et al. (2006), Alto et al. (2007), Nougayrede et al. (2007)
EspG	TBC-like Rab GAP	Various Rab GTPases, Arf 1/5/6 and PAKs	Inhibits protein secretion and recycling	Dong et al. (2012), Selyunin et al. (2011), Furniss et al. (2016)
EspJ	Amidation and ADP-ribosylation	Non-receptor tyrosine kinases	Inhibits phagocytosis	Young et al. (2014), Marches et al. (2008), Pollard et al. (2018)
EspK	–	–	Predicted NK-κB inhibition (based on homology to GogB^{Stm})	Vlisidou et al. (2006), Pilar et al. (2012)
EspL family	Cysteine protease	RIPK1, RIPK3, TRIF, ZBP1/DAI	Inhibits necroptosis and inflammation	Pearson et al. (2017)
EspM family	WxxxE GEF	RhoA	Stress fibre formation and cell–cell junction disruption	Alto et al. (2006), Arbeloa et al. (2008), Simovitch et al. (2010)
EspN	–	–	–	Tobe et al. (2006)
EspO family	–	ILK	Predicted cell adhesion (based on homology to OspE^{Sf})	Kim et al. (2009)
EspR family	–	–	–	Tobe et al. (2006)

(continued)

Table 1 (continued)

Effector[a]	Biochemical activity	Host target or partner	Reported function	References
EspS	–	–	–	Tobe et al. (2006)
EspT	WxxxE GEF	Rac1, Cdc42	Lamellipodia formation, host cell invasion	Alto et al. (2006), Bulgin et al. (2009)
EspV	–	–	Actin remodelling	Arbeloa et al. (2011)
EspW	–	Kif15	Actin remodelling	Sandu et al. (2017)
EspX family	–	–	–	Tobe et al. (2006)
EspY family	–	–	–	Tobe et al. (2006)
EspZ	–	CD98	Inhibits excessive cytotoxicity	Berger et al. (2012), Shames et al. (2010)
Map	WxxxE GEF	Cdc42, EBP50	Filopodia dynamics and cell barrier function	Alto et al. (2006), Berger et al. (2009)
NleA	–	Sec24, NLRP3	Inhibits protein secretion Inhibits inflammasome activation	Kim et al. (2007), Yen et al. (2015)
NleB family	Glycosyltransferase	FADD, TRADD, RIPK1	Inhibits extrinsic (death receptor-induced) apoptosis	Li et al. (2013), Pearson et al. (2013)
NleC	Zinc metalloprotease	P65, p50, p300	NF-kB inhibition	(Baruch et al. (2011), Pearson et al. (2011), Yen et al. (2010)
NleD	Zinc metalloprotease	P38, JNK	MAPK inhibition	Baruch et al. (2011)
NleE	S-adenosyl-l-methionine (SAM)-dependent cysteine methyltransferase	TAB2/3, ZRANB3	NF-κB inhibition and DNA repair	Zhang et al. (2012),Yao et al. (2014)
NleF	–	Caspase 4, 8 and 9	Inhibits intrinsic and extrinsic apoptosis	Blasche et al. (2013)

(continued)

Table 1 (continued)

Effector[a]	Biochemical activity	Host target or partner	Reported function	References
NleG family	E3 ubiquitin ligase	–	–	Wu et al. (2010)
NleH family	Ser/Thr kinase	Binds BI-1 and RPS3	Inhibition of NF-KB and intrinsic cell death signalling	Gao et al. (2009), Hemrajani et al. (2010)
NleL (EspX7)	E3 ubiquitin ligase	JNK	A/E lesion formation	Sheng et al. (2017), Lin et al. (2011), Piscatelli et al. (2011)
NleJ	–	–	–	Deng et al. (2012)
TccP	–	IRSp53, IRTKS and various actin-binding proteins	Actin rearrangement and intimate attachment	Smith et al. (2010), Weiss et al. (2009), Vingadassalom et al. (2009), Campellone et al. (2004)
Tir	–	SHIP2, Nck, IRTKS, IRSp53, PI3 K, CK18, Talin, Vinculin, α-actinin, cortactin, 14-3-3tau	Actin rearrangement and intimate attachment	Smith et al. (2010), Weiss et al. (2009), Vingadassalom et al. (2009), Batchelor et al. (2004), Freeman et al. (2000), Gruenheid et al. (2001), Mousnier et al. (2008), Patel et al. (2006), Sason et al. (2009)

[a]Family is used to indicate multiple homologues of the effector exist. In some cases, binding partners and functions have only been demonstrated for one family member

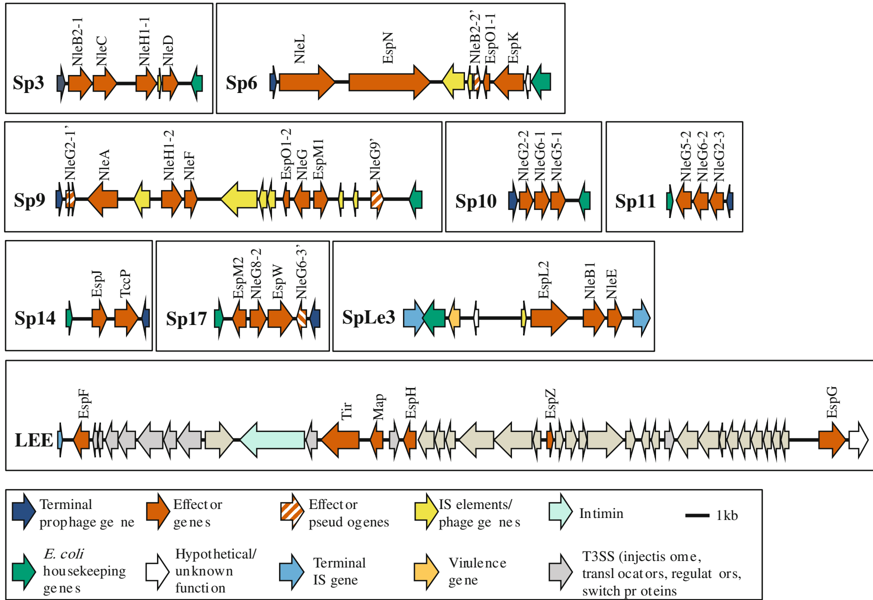

Fig. 1 Exchangeable Effector Loci (EEL) of EHEC O157. Seven EELs within prophages (Sp3, 6, 9, 10, 11, 14 and 17) and the two Pathogenicity Islands (SpLe3 and LEE) of EHEC O157 strain Sakai are depicted. Prophage EELs that are predicted to encode only effector pseudogenes have not been included (Sp4 and 12). All prophage EELs are flanked at one end by prophage genes, with only the terminal prophage gene indicated here. Similarly, the SpLE3 and LEE PI are flanked by IS elements/remnants; only the terminal ones are shown. Effectors located on non-phage EELs are not shown. Gene and EEL nomenclature follows that of Tobe et al. (2006) (for further details on the T3SS genes encoded within the LEE PI, refer to Fig. 1)

In this chapter, we discuss effectors grouped based on their ability to manipulate the actin cytoskeleton, the small guanosine triphospatases (GTPases) and innate immune signalling pathways.

3 Manipulation of Actin by Tir

Found in a wide range of eukaryotic cells, where it is often the most abundant protein in a cell, actin is involved in an array of different cellular processes. Actin is extremely dynamic and is found in both monomeric (G-actin) and various filamentous (F-actin) forms. Actin filaments together with microtubules and intermediate filaments are key components of the cell cytoskeleton, controlling cell shape and the organisation of cellular components. Considering its central role in the cell, it is unsurprising that numerous pathogens target actin and/or actin-binding proteins.

Tir, the translocated intimin receptor, is involved in the subversion of host cell actin and allows the A/E pathogens to intimately adhere to the surface of infected

cells and form the A/E lesion (Wong et al. 2011). Due to this central role, Tir has been studied extensively and is arguably the most studied of the LEE effectors. Translocated Tir inserts into the host plasma membrane (PM) in a hairpin-loop topology and acts as the receptor for the bacterial outer membrane adhesin intimin (Kenny et al. 1997; Hartland et al. 1999). Binding to Tir by intimin induces Tir clustering, leading to downstream signalling events that result in the formation of actin-rich 'pedestal'-like structures underneath adherent bacteria (Campellone et al. 2004). However, despite its central role, there are differences in the mechanism by which Tir from different *E. coli* pathotypes acts. Typically, EPEC Tir relies on the phosphorylation of a tyrosine residue, Y_{474}, to recruit the host adaptor protein Nck and subsequently neural Wiskott–Aldrich syndrome protein (N-WASP) and the actin-related protein 2/3 (Arp2/3) complex, triggering actin polymerisation beneath attached bacteria (Wong et al. 2011). On the other hand, EHEC Tir lacks a Y_{474} equivalent and instead promotes Nck-independent actin polymerisation via a conserved Asn-Pro-Tyr motif (NPY_{458}, which is also found in EPEC Tir as NPY_{454}) to recruit N-WASP and Arp2/3 (Brady et al. 2007). Tir only produces weak actin polymerisation in vitro through the NPY_{458} pathway (Campellone and Leong 2005), and in order to overcome this and induce robust actin polymerisation, EHEC Tir cooperates with the non-LEE-encoded effector TccP/$EspF_U$. TccP interacts with Tir indirectly via the adapter proteins IRSp53 and insulin receptor tyrosine kinase substrate (IRTKS) (Weiss et al. 2009; Vingadassalom et al. 2009). Together, EHEC Tir and TccP/$EspF_U$ strongly activate Arp2/3, resulting in similar actin polymerisation to that seen during EPEC infection (Garmendia et al. 2004). Unusually, EPEC-2 strains can use both the Nck and TccP2 pathways to promote actin recruitment at the bacterial attachment site (Whale et al. 2006).

Intriguingly, whilst Tir is indispensable for A/E lesion formation in vivo, none of the pathways discussed above are necessary for the recruitment of N-WASP or A/E lesion formation at mucosal surfaces (Crepin et al. 2010). Therefore, other, as yet unknown, host factors must play a role in Tir-mediated actin polymerisation and A/E lesion formation in vivo. A growing body of work implicates Tir-mediated signalling in regulation of the lipid content of the host PM at the bacterial attachment site which may play a role in A/E lesion formation in vivo (Smith et al. 2010; Sason et al. 2009). Recently an EPEC strain with all T3SS effectors deleted, excepting Tir, was found to form pedestals on cultured cells but not A/E lesions on mucosal tissue (Cepeda-Molero et al. 2017). When all NLE effectors were absent, only marginal A/E lesions could be observed indicating that at least one NLE effector contributes to Tir-mediated A/E lesion formation. One example of this is the ubiquitination of JNK by NleL, which has been demonstrated to contribute to bacterial attachment and A/E lesion formation by EHEC, but is not present in EPEC (Sheng et al. 2017). Therefore whilst Tir is central to actin polymerisation and A/E lesion formation, it does not act alone in these processes.

4 Modulation of Host Small GTPases

After the translocation of Tir, the T3SS translocates additional effectors, detailed in Table 1. T3SS effectors function to repurpose host cell processes, creating a favourable environment in the gut for the replication and onward transmission of the infecting pathogen. Whilst EPEC/EHEC effectors manipulate a wide variety of host cell processes, subversion of host small GTPases is a recurring theme in effector function and an important paradigm in host–pathogen interaction more broadly. Due to the involvement of small GTPases in almost all essential cellular processes, a variety of pathogenic organisms have evolved to target small GTPases as a means to manipulate host cell function, with EPEC/EHEC being no exception.

Small GTPases are evolutionarily conserved hydrolase enzymes that function as molecular switches to control, amongst other things, protein recycling and actin dynamics at the plasma membrane (Donaldson et al. 2016; Heasman and Ridley 2008). Six major subfamilies make up the small GTPases: Ras, Rho, Rab, Ran, ARF and MIRO. These subfamilies are grouped together based on amino acid sequence, structure and cellular roles (Reis et al. 2009; Goitre et al. 2014), but common to all small GTPases is the ability to bind guanosine triphosphate (GTP) and hydrolyse GTP to guanosine diphosphate (GDP), via the universal 20 kDa 'G-domain' (Vetter and Wittinghofer 2001).

In order to efficiently cycle between the GTP-bound active state and the GDP-bound inactive form at specific times and in specific locations within the cell, small GTPases interact with a number of accessory proteins, known as Guanine-nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs) and in the case of the Ras, Rho and Rab subfamilies (all of which are prenylated at their C-termini) Guanine Dissociation Inhibitors (GDIs) (Cherfils and Zeghouf 2013). Together, these accessory proteins allow small GTPases to exert fine spatiotemporal control over a range of different cellular events.

GEFs activate small GTPases, and whilst many structurally distinct eukaryotic GEF families exist, the mechanistic basis for their activation of small GTPases is conserved. In the cytosol, GEFs form a transient complex with the GDP-bound form of their cognate GTPase, promoting the dissociation of GDP and the recruitment of GTP to the nucleotide-free GEF/GTPase complex. GTP binding displaces the GEF, creating the GTP-bound, active form of the GTPase (Bos et al. 2007). All small GTPases undergo conformational changes when GTP-bound to allow the recruitment of effector proteins and the stimulation of downstream signalling cascades. For the Ras, Rho, Rab, ARF and MIRO GTPases activation results in both effector recruitment and insertion into cellular membranes, consistent with the roles of these GTPases in directing membrane traffic, cytoskeletal rearrangements and organelle movement (Cherfils and Zeghouf 2013; Tang 2015).

Acting in opposition to GEFs, GAPs promote the inactivation of small GTPases by catalysing GTP to GDP hydrolysis. Classically, nucleotide hydrolysis occurs through the provision of an arginine by the GAP (the conserved 'arginine finger' motif) to the GTPase. This results in a conformational change leading to

GTP-hydrolysis (Bos et al. 2007). In the case of the prenylated GTPases (Ras, Rho, Rab), GAPs act in concert with GDIs, a third family of regulatory proteins that aid in the extraction of these GTPases from membranes. Once a prenylated GTPase has been extracted from a membrane, the GDI remains associated with the GDP-bound GTPase, maintaining it in a soluble state (Cherfils and Zeghouf 2013).

Subversion of small GTPases by EPEC/EHEC effectors is frequently achieved through mimicry of these accessory proteins, often, but not exclusively, to modulate Rho family GTPases.

4.1 Rho GTPases

The Rho GTPases were the second family of small GTPases to be described in humans, following their initial description in *Aplysia* spp. (sea slugs) (Madaule and Axel 1985). The 20 Rho GTPases are subdivided into 'classically activated' and 'atypical' proteins. Classically activated Rho GTPases are regulated by GEFs and GAPs, as described above, and are subdivided into four families based on amino acid sequence: the Rho subfamily (RhoA, RhoB, RhoC); the Rac subfamily (Rac1, Rac2, Rac3, RhoG); the Cdc42 subfamily (Cdc42, RhoQ and RhoJ); and the RhoF/RhoD subfamily.

The Rho subfamily (RhoA, RhoB and RhoC) are involved in the regulation of the actomyosin cytoskeleton and contractile stress fibre formation. Activation of RhoA and/or RhoC (which share 92% amino acid identity (Heasman and Ridley 2008)) in response to extracellular stimuli recruits the Rho-associated coiled-coil containing kinase (ROCK), a serine/threonine kinase involved in the formation of focal adhesions and actin stress fibres, acting in opposition to lamellipodia formation and cell migration (Ridley 2015). RhoB, which shares 84% amino acid identity with RhoA (Heasman and Ridley 2008), is involved in the regulation of endocytic trafficking and may play a role in the regulation of epithelial cell–cell contacts (Vega et al. 2015).

The Rac proteins act in opposition to the Rho subfamily and serve, in conjunction with other small GTPase families, to promote cell migration, membrane ruffling and lamellipodia formation by promoting cortical actin polymerisation (Ridley 2015; Santy et al. 2005). This occurs primarily through the activation of N-WASP and subsequent recruitment of the WASP family veroprolin-homologue (WAVE) regulatory complex (WRC), leading to Arp2/3-mediated actin polymerisation.

The Cdc42 family coordinates the actin cytoskeleton and apical–basolateral polarity in many eukaryotes, through regulation of filopodia and recruitment of the Par complex. Cdc42 promotes actin polymerisation, the bundling of F-actin into filopodia and the membrane curvature necessary for cell protrusions to form (Heasman and Ridley 2008) whilst also recruiting the Par complex (Martin-Belmonte et al. 2007) to the apical–lateral border of epithelial cells (Heasman and Ridley 2008). Localisation of the Par complex at the apical domain allows the polarised trafficking of proteins and the formation of polarised cell–cell barriers, such as tight junctions.

4.2 Map, EspT and EspM Mimic Host Rho GEFs

Rho GTPase function is manipulated by both intracellular and extracellular pathogens during infection, including *Shigella* spp., *Salmonella enterica* sv. Typhimurium (STm), *Pseudomonas aeruginosa, Yersinia* spp., EPEC and EHEC to facilitate bacterial invasion, and, in the case of extracellular pathogens, avoid unwanted internalisation into host cells. The WxxxE family of proteins are a major group of effectors found across Gram-negative pathogens. These effectors act as Rho GEFs, mimicking host proteins to activate endogenous Rho GTPases (Alto et al. 2006; Huang et al. 2009; Klink et al. 2010). The EPEC/EHEC effectors Map (Huang et al. 2009), EspT (Bulgin et al. 2009b) and EspM (Tobe et al. 2006; Arbeloa et al. 2008) are members of this family.

The LEE-encoded effector Map acts as a Dbl family GEF mimic, activating Cdc42 (Huang et al. 2009) to induce the formation of transient filopodia around infecting bacteria (Kenny et al. 2002) before re-localising to the mitochondria via an N-terminal mitochondrial targeting sequence (Kenny et al. 2002; Kenny and Jepson 2000). Whilst the contribution of filopodia formation to the infectious process of A/E pathogens remains unclear, a Δ*map* mutant of *C. rodentium* is attenuated in vivo and shows a significant colonisation defect (Mundy et al. 2004). Intriguingly, the induction of filopodia by Map has been shown to be dependent on Map's binding to ERM-binding phosphoprotein 50 (EBP50) at the plasma membrane (Alto et al. 2006; Simpson et al. 2006). However, the molecular details of Map's dependence on this interaction also remain unclear. In addition, Tir regulates Map-induced filopodia formation (Berger et al. 2009; Kenny et al. 2002) and together Tir and Map have been implicated in both the effacement of microvilli during infection and the rapid loss of function of the host sodium-glucose transporter SGLT-1 (Dean et al. 2006). This may have relevance for understanding the rapid-onset watery diarrhoea induced by EPEC/EHEC and provides a possible explanation as to why this watery diarrhoea is refractive to oral rehydration therapy in severe cases of infection (Dean et al. 2006). In addition, the interaction between Map and EBP50 has been shown to contribute to the development of diarrhoea during infection (Simpson et al. 2006), possibly through modulation of intestinal barrier function (Dean and Kenny 2004). The multiple phenotypes attributed to Map reflect the diverse roles of Cdc42 as a modulator of actin dynamics, cell cycle progression, cell polarity and membrane trafficking (Etienne-Manneville 2004).

EspT is a WxxxE effector found in a small subset of EPEC strains (Arbeloa et al. 2009). EspT-carrying strains are capable of invading non-phagocytic cells and forming intracellular actin pedestals (Bulgin et al. 2009b). Like the other WxxxE effectors, EspT is a GEF mimic, activating the host Rho GTPases Rac1 and Cdc42 (Bulgin et al. 2009a), resulting in the formation of lamellipodia and membrane ruffles on the surface of infected cells and subsequently the intracellular phenotype described above. The intracellular pathogens *Shigella flexneri* and STm also make use of this method of cell invasion (Cossart and Sansonetti 2004). Interestingly, despite the small number of EPEC strains that carry EspT, the causative strain of an

unusual outbreak of EPEC in Finland in the winter of 1987, in which adults as well as children were affected, was found to carry EspT (Viljanen et al. 1990). It is tempting to speculate that the presence of EspT may have been responsible for the expanded host range of this particular strain.

The homologous WxxxE effectors EspM1 and EspM2 were initially identified in EHEC O157:H7 Sakai using a bioinformatics screen for homologues of known T3SS effectors (Tobe et al. 2006) and share significant sequence identify with the EPEC B171 effector TrcA, another WxxxE effector (Arbeloa et al. 2008). EspM effectors are RhoA GEFs (Arbeloa et al. 2010) and EPEC/EHEC strains that carry EspM effectors are associated with severe human infections (Arbeloa et al. 2009).

Whilst all EspM effectors induce the formation of actin stress fibres within infected cells (Arbeloa et al. 2008; Simovitch et al. 2010), the phenotypes displayed by the EspM effectors are subtly different. For example, whilst both EspM1 from EHEC O157:H7 Sakai and TrcA from EPEC B171 induce the formation of parallel stress fibres that are confined to the bacterial infection site, EspM2 from EHEC O157:H7 Sakai induces parallel stress fibre formation throughout infected cells, which are linked to the plasma membrane through focal adhesions (Arbeloa et al. 2008). Stress fibre formation is a consequence of RhoA activation and downstream signalling mediated by the recruitment of the RhoA effector ROCK (Arbeloa et al. 2008, 2010).

In addition to promoting stress fibre formation, EspM1 and EspM2 disrupt the architecture of a polarised cell monolayer when translocated into epithelial cells during EHEC infection. This phenotype is dependent on RhoA (Simovitch et al. 2010) and is likely a consequence of the mislocalisation of both tight junction proteins and the basolateral protein β1-integrin that is induced by EspM, although it is important to note that EspM does not seem to decrease the barrier function of tight junctions (Simovitch et al. 2010). In conjunction with their effect on stress fibre formation, the ability of the EspM effectors to disrupt the integrity of the cell monolayer is indicative of the pleotropic consequences of aberrant RhoA activation. This is particularly noteworthy, as RhoA GTP-GDP exchange does not disrupt the RhoA/EspM complex, suggesting that RhoA is activated irreversibly upon binding of EspM (Arbeloa et al. 2010). On this point, it is interesting to note that the EHEC T3SS effectors EspO1 and EspO2 have been shown to interact directly with EspM2 to counteract stress fibre formation and prevent cell detachment caused by excessive RhoA activation (Morita-Ishihara et al. 2013). Finally, both EspM1 and EspM2 have been reported to modulate pedestal formation, supressing this process early during EHEC infection. However, the relevance of this accessory role in pedestal formation during infection is unclear (Simovitch et al. 2010). Taken together, the diverse phenotypes attributed to EspM demonstrate the multifaceted nature of RhoA's regulation of the actin cytoskeleton and cell–cell junctions.

4.3 EspH Inactivation of Host GEFs

Operating in conjunction with the EPEC/EHEC WxxxE effectors (Map, EspT and EspM), the LEE-encoded effector EspH inactivates multiple host Rho GEFs through binding to their Dbl-homology and pleckstrin homology (DH-PH) domain, preventing Rho GTPase activation (Dong et al. 2010). In this way, EspH represses filopodia formation and enhances pedestal formation beneath adherent bacteria (Tu et al. 2003), acting in concert with Tir to recruit N-WASP to the bacterial attachment site (Wong et al. 2012a). In addition, EspH in isolation promotes cell detachment and caspase-3 activation through the disassembly of focal adhesions (Wong et al. 2012b) and is able to prevent phagocytosis of EPEC by macrophages (Dong et al. 2010). Ingeniously, EspH does not affect the WxxxE effectors, allowing EPEC/EHEC to replace the endogenous Rho GEFs with bacterial mimics to exclusively control Rho GTPase activation in infected cells (Wong et al. 2012b). In fact, EspT and EspM (but not Map) counteract the focal adhesion disassembly induced by EspH, suggesting that EspH acts in collaboration with NLE WxxxE effectors that are also present in the infecting strain to negate its negative consequences and completely control host Rho GTPase function in infected cells (Wong et al. 2012b).

4.4 EspW Targets Microtubules and Control of Cell Shape

First identified in the same bioinformatics screen which identified EspM1 and EspM2 (Tobe et al. 2006), EspW has subsequently been found throughout the sequenced EHEC O157:H7 strains as well as in a number of non-O157:H7 EHEC strains, EPEC O111:H9 and a range of EPEC clinical isolates (Sandu et al. 2017). A truncated version of EspW, $EspW_{1\text{-}206}$ has also been observed in the EHEC O157:H7 progenitor strain EPEC O55:H7 (Sandu et al. 2017; Feng et al. 1998), although the function of this truncated protein remains unknown.

Unlike Map, EspT and EspM, EspW does not possess the WxxxE motif. Instead, full-length EspW promotes Rac-1-dependent actin remodelling during infection via interaction with the C-terminus of the host microtubule motor Kinesin-12 (Kif15) (Sandu et al. 2017), a homotetrameric protein (Drechsler et al. 2014) capable of forming parallel bundles of microtubules in vitro (Drechsler and McAinsh 2016). Ectopically expressed EspW promotes the formation of large flower-shaped actin ruffles on the surface of cells and, during EHEC infection, Kif15 is recruited to the actin pedestal where it localises with EspW. However, EspW is not required for the recruitment of Kif15 to the site of EHEC attachment, and instead, Kif15 may serve to restrict EspW to the pedestal (Sandu et al. 2017). Instead, the absence of *espW* results in significant cell shrinkage and rounding during EHEC infection, a phenotype that can be rescued by chemical activation of Rac1 (Sandu et al. 2017).

Therefore, it appears that EspW plays a role in maintaining cell shape during infection, via Rac1-mediated actin rearrangement, although the putative link between EspW's interacting partner, Kif15, and Rac1 remains to be elucidated.

4.5 EspG Manipulates Cell–Cell Contacts and the Host Cell Surface

Like Map and EspH, EspG is a LEE-encoded effector and thus is highly conserved across EPEC and EHEC strains. In fact, EspG is one of the most conserved effectors across the A/E pathogens (Hazen et al. 2013). However, unlike the effectors discussed above, EspG does not target host Rho GTPases, but rather ARF and Rab GTPases (Fig. 2).

EspG is able to bind to ARF GTPases, likely promoting the recruitment of a specific subset of ARF effectors (Selyunin et al. 2011, 2014) whilst simultaneously acting as a TBC domain-like Rab GAP (Dong et al. 2012). Thus, EspG has been

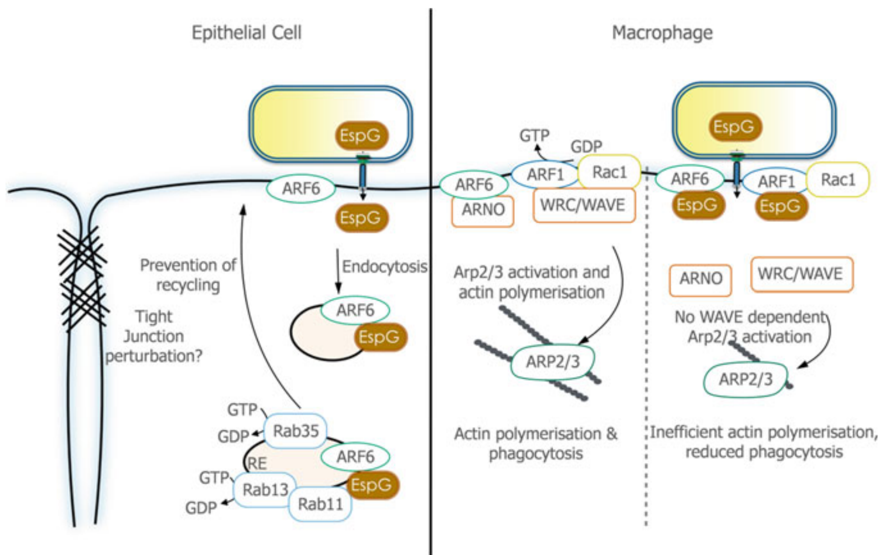

Fig. 2 Host cell targets of the LEE-encoded effector EspG. During infection of host epithelial cells, EspG binds to ARF6 whilst also interacting with Rab35. In this way, EspG leads to the formation of enlarged 'stalled recycling structures' (Furniss et al. 2016) and prevents the recycling of endocytosed host proteins back to the PM (Clements and Furniss 2014). EspG also alters paracellular permeability via modulation of tight junctions, although the mechanism of this is unclear at present (Glotfelty et al. 2014; Matsuzawa et al. 2004). In macrophages, EspG prevents phagocytosis of attached bacteria by binding to ARF6 and ARF1, ultimately preventing the WAVE-dependent Arp2/3-mediated actin polymerisation necessary for efficient uptake of the bacteria into the macrophage (Humphreys et al. 2016)

referred to as a ‘catalytic scaffold’. During infection of epithelial cells, EspG binds to the GTP-bound, active form of the ARF GTPase ARF6 (Dong et al. 2012; Selyunin et al. 2011; Furniss et al. 2016) whilst simultaneously directing its Rab GAP activity towards the Rab GTPase Rab35 (Furniss et al. 2016), disrupting this important signalling axis (Donaldson et al. 2016; Clements and Furniss 2014). In epithelial cells, EspG has been implicated in the modulation of host tight junctions (Glotfelty et al. 2014; Tomson et al. 2005; Morampudi et al. 2017) as well as in the removal of a range of host cell surface proteins from the plasma membrane (Clements and Furniss 2014; Morampudi et al. 2017; Gill et al. 2007; Guttman et al. 2007). The ability of EspG to affect a range of cell surface and cell–cell junction proteins likely arises from its ability to prevent correct recycling of host cell surface proteins, likely via the formation of ‘stalled recycling structures’ (Furniss et al. 2016), a phenotype that is dependent on EspG’s ability to function as a Rab GAP (Clements and Furniss 2014). However, the breadth of EspG’s effect on the host cell surface is yet to be determined.

With regard to EspG’s promotion of the GTP-bound, active form of ARF6, elegant in vitro work in which membrane-associated actin polymerisation was reconstituted using phospholipid-coated beads has revealed that EspG is able to block recruitment of the ARF6 effector ARNO, preventing formation of the WRC, an important mediator of macropinocytosis and phagocytosis. In this way, EspG plays a role in resisting phagocytosis by macrophages (Humphreys et al. 2016). Whether this role for EspG-stabilised ARF6 is restricted to phagocytic cells, or plays a role during infection of epithelial cells, remains to be determined.

4.6 Conclusion

Modulation of host small GTPases is central to the infection strategy of EPEC and EHEC and represents a fundamental strategy employed by pathogenic organisms. Of the six LEE-encoded effectors, three (Map, EspH and EspG) are known to modulate one or more host small GTPases either directly (Map, EspG) or indirectly (EspH), whilst Tir also possesses a putative GAP domain (Kenny et al. 2002). A number of NLE effectors also target small GTPases. The WxxxE effectors EspT and EspM act as Rho GEF mimics in order to modulate the host cytoskeleton, whilst EspW activates Rac1 through binding to the microtubule motor Kif15. The glutamine deamidase Cif reduces RhoA protein levels and increases stress fibres, which exemplifies yet another strategy of manipulating small GTPase function. Many effectors that target small GTPases do so in order to allow manipulation of the actin cytoskeleton, a hallmark of A/E pathogen infection. The mechanism by which these effectors cooperate with and/or antagonise each other and Tir, and how the spatial and, temporal control necessary for these complex processes to occur is achieved remain to be determined.

In addition, whilst much progress has been made towards understanding the molecular details of GTPase subversion by individual EPEC/EHEC effectors,

a number of unanswered questions remain, particularly regarding the effectors EspG and EspW. Simultaneously, bacterial effectors that manipulate host GTPase function are excellent tools for the study of fundamental eukaryotic cell biology.

5 NF-κB and Cell Death Signalling in Host Cells

Mammalian innate immunity relies on the detection of microbes by families of pattern recognition receptors such as the Toll-like receptors (TLRs), NOD and leucine-rich repeat domain-containing proteins (NLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs) amongst others (Kawai and Akira 2011; Broz and Dixit 2016; Newton and Dixit 2012). Proteins from most of these families induce transcriptional upregulation of cytokines that are responsible for inflammation, which if left unchecked can lead to tissue damage. Amongst the most proinflammatory cytokines are Tumour necrosis factor (TNF) and Interleukin-1 (IL-1) family proteins, which also upregulate their own expression in a feedforward mechanism (Newton and Dixit 2012). Thus, cytokine production amplifies inflammation and immunity, and as we discuss in the following sections, EPEC and EHEC have evolved several mechanisms to potently suppress or evade inflammatory and cell death signalling (summarised in Figs. 3 and 4).

Detection of microbial molecules through the TLRs results in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent transcriptional upregulation of proinflammatory cytokines. The TLRs and IL-1 receptor-1 (IL1R1) share sequence similarity in their intracellular region called the Toll-IL-1 receptor domain (TIR), and signalling by these families of receptors is similar (Newton and Dixit 2012). Intestinal epithelial cells (IECs) express several pattern recognition receptors and cytokine receptors, including TLR4, TLR5, TNF receptor, IL-1 receptor and IL-18 receptor (related to IL1R1), and are therefore capable of initiating as well as responding to inflammation.

TIR domains recruit the adaptor myeloid differentiation primary response 88 (MyD88) to activate mitogen-activated protein kinase (MAPK) and NF-κB pathways through the recruitment of a number of protein kinases, ubiquitin E3 ligases and ubiquitin-binding proteins, amongst others. Some TLRs, such as TLR4 and TLR3, also signal via the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) which also has a TIR. MyD88 recruits and activates IL1R-associated kinase (IRAK) family kinases that in turn recruit the TNFR-associated factor (TRAF) family E3 ligases TRAF6 and TRAF3. Ubiquitylation of the receptor complex recruits the transforming growth factor beta-activated kinase 1 (TAK1; also called MAP3K7) via the ubiquitin-binding proteins TAK1-binding proteins 2 and 3 (TAB2 and TAB3). TAB2/3 bind polyubiquitin chains and recruit a TAK1-TAB1 complex, resulting in TAK1 K63 ubiquitylation by TRAFs at the receptor. TAK1 is a central regulator of NF-κB and MAPK and is involved in a range of innate immune pathways. Activation of NF-κB via TAK1 is called canonical NF-κB signalling, which is common to TLRs, IL-1

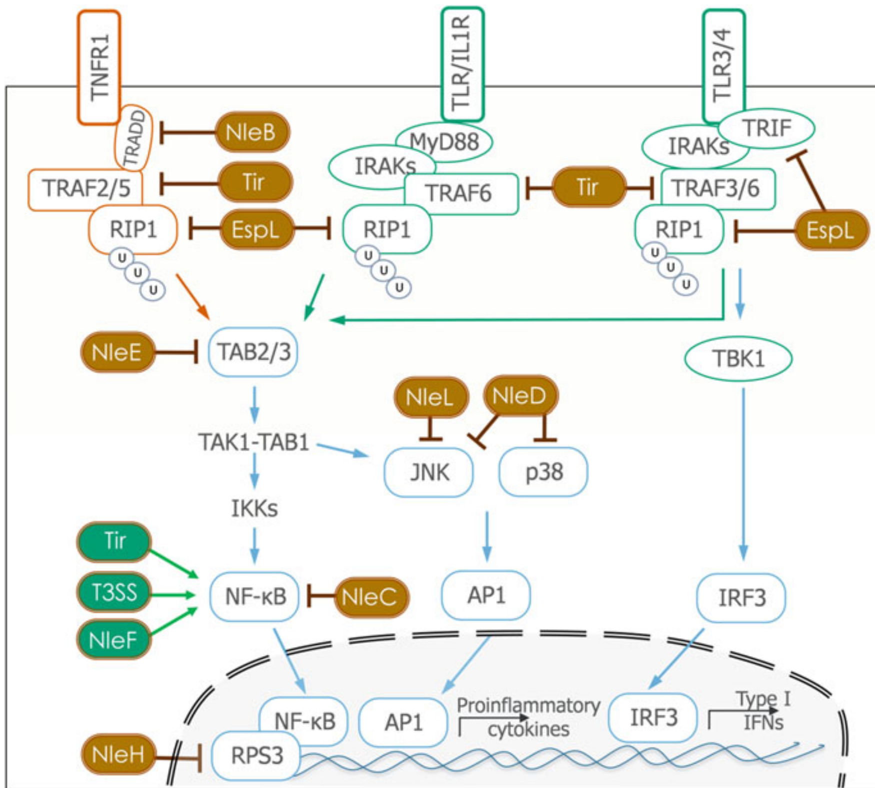

Fig. 3 Manipulation of TLR, TNFR1 and IL1R signalling by EPEC/EHEC. Schematics show signalling by TNFR1 via TRADD, TLR/IL-1-like receptors via MyD88 and TLR3/4 signalling via TRIF. Inhibitory effectors (EspL, NleB, NleC, NleD, NleE, NleL and Tir) are shown in brown and activating effectors (NleF, a functional T3SS and Tir) are shown in green. TRADD, TRAF6 and RIPK1 are prominent receptor-proximal proteins targets, whereas JNK, p38 and NF-κB exemplify downstream signalling targets in the host

receptors and TNFR1 signalling (Newton and Dixit 2012; Ajibade et al. 2013; Skaug et al. 2009). In contrast, TLR3/4 signalling via TRIF induces type I interferons (IFNs) via the interferon stimulatory factor (IRF) family of transcription factors.

Active TAK1 phosphorylates and activates IκB kinases (IKKs) and MAPKs. The IKKs phosphorylate the inhibitor of NF-κB inhibitory proteins (IκB), resulting in its proteasomal degradation, nuclear translocation of NF-κB and subsequently transcriptional activation of NF-κB-regulated genes. The three MAPK branches; Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAPK, phosphorylate and induce the nuclear translocation of the activator protein 1 (AP1) transcription factor. The synergistic action of NF-κB and AP1 enhances gene transcription, inflammation and antimicrobial responses.

TNF signalling activates canonical NF-κB via TAK1 via markedly different receptor-proximal mechanisms (Skaug et al. 2009; Chan et al. 2015). TNF family

receptors have death domains (DDs) in their intracellular regions that recruit DD-containing adaptors instead of TIR-containing adaptors. The TNFR1 adaptor TNFR1-associated DD-containing protein (TRADD) recruits receptor-interacting serine/threonine kinase 1 (RIPK1) and a different set of TRAF E3 ligases, including TRAF2, TRAF5 and the cellular inhibitors of apoptosis (cIAP) proteins. Ubiquitylation of RIPK1, by both K63 and linear chains, recruits TAK1 via TAB2/TAB3. Downstream induction of NF-κB and MAPK-dependent genes sustains inflammation.

Another unique aspect of DD-containing receptors is their ability to activate apoptosis or necroptosis by forming distinct receptor complexes. These complexes differ depending on the context (e.g. differential ubiquitylation) and cell type (e.g. myeloid versus epithelial cells). For example, TNF does not induce death in most cells but increases NF-κB-dependent proinflammatory cytokine production and inflammation. NF-κB activation by TNF results in the upregulation of several NF-κB-dependent genes that prevent cell death, including cellular inhibitor of apoptosis (cIAPs), B cell lymphoma 2 (BCL2) proteins, the caspase-8-inhibitor cellular FLICE-like inhibitory protein (cFLIP) and the ubiquitin-editing enzyme A20 amongst others. Altered signalling by TNF can, however, induce apoptosis via a process called receptor-induced apoptosis signalling, also known as the cell-extrinsic signalling pathway (Chan et al. 2015).

The outcome of TNF signalling is fine-tuned by ubiquitylation and caspase-8 activity. Reduced ubiquitylation of the TNFR1-complex, for example, by deubiquitylating enzymes or reduced activity of cIAPs E3 ligases, results in the formation of an apoptosis-inducing cytosolic complex that contains a related adaptor Fas-associated protein with death domain (FADD), RIPK1 and caspase-8 (Chan et al. 2015). Catalytic activation of caspase-8 into its p18/p10 processed form leads to cleavage of the BH3 interacting domain death agonist (BID) protein that induces mitochondrial damage, activation of caspases-9, -3, -6 and -7 and apoptosis. In some cases, such as LPS-induced sepsis or hepatic toxicity induced by TNF, BID-independent apoptosis proceeds via a mechanism that relies on JNK and reactive oxygen species (ROS) (Chen et al. 2007; Ni et al. 2009; Wu et al. 2007).

TNF-induced apoptosis is also prevented by cFLIP which prevents full caspase-8 activation but promotes cell survival by facilitating caspase-8-mediated cleavage and inactivation of RIPK1. An alternative scenario emerges when the proteolytic activity of caspase-8 is impaired or protein kinase activity of RIPK1 is increased, both of which can lead to necroptosis via the activation of RIPK3 and mixed lineage kinase domain-like pseudokinase (MLKL) (Chan et al. 2015). Just as in the TNF pathway, reduced caspase-8 activity during TLR4 or TLR3 signalling also induces necroptotic cell death.

In the case of the TNF-like molecules First Apoptosis Signal receptor Ligand (FASL) and TNF-related apoptosis-inducing ligand (TRAIL), which signal via TNFR-like receptors FAS and Death Receptor 5 (DR5), respectively, FADD directly recruits caspase-8 to the receptor complex to trigger apoptosis. FADD is thus critical for apoptosis and/or necroptosis by TNFR family receptors. Although traditionally thought to be the initiator caspase in the extrinsic apoptosis pathway, the cell survival role of caspase-8 is now better understood, including in IECs.

The nucleotide-binding oligomerization domain (NOD) and leucine-rich repeat domain (LRR) containing (NLR) proteins NOD1 and NOD2 can also activate NF-κB and MAPK pathways. These two cytosolic receptors of peptidoglycan components require RIPK2 to activate TAK1 (Caruso et al. 2014).

5.1 EPEC/EHEC-Induced NF-κB Activation

As flagellated Gram-negative bacteria, EPEC/EHEC molecules such as LPS and flagellin, amongst others, are likely to serve as potent triggers of inflammation by activating TLR pathways. Subsequent release of TLR/NLR-induced proinflammatory cytokines could further amplify inflammation during infection. A large body of work exists on the redundancy of effectors used by EPEC to block NF-κB activation. This has provided credence to the idea that EPEC actively subverts innate immune detection and stealthily limits intestinal inflammation to cause disease. The ablation of various LEE and non-LEE effectors in EPEC (e.g. loss of *nleB, nleC, nleD* and *nleE;* see below for a description on their roles) or additional removal of flagellin (*fliC*) revealed that these strains strongly induce NF-κB activation and IL-8 secretion in epithelial cells (Litvak et al. 2017). However, the host signalling pathway(s) responsible for NF-κB activation remained a mystery. By reconstituting the EPEC LEE locus encoding the T3SS and related effectors in *E. coli* K12, it was established that a functional T3SS system, but not effectors, was essential for NF-κB activation in infected epithelial cells. Further, whilst host cell contact-induced T3SS triggering induced NF-κB activation, ectopic expression of individual T3SS structural protein in host cells did not. Loss of *Myd88, Traf6* and *Ripk2* in the host did not affect NF-κB activation, ruling out TLR/IL1R-MyD88 or NOD1/NOD2-RIPK2 signalling as the underlying NF-κB-activating pathways. However, other possibilities remain to be tested. As *nleB, nleC* and *nleE* were essential to inhibit T3SS-dependent NF-κB activity, the as yet unknown pathway must still converge on the canonical NF-κB pathway which is targeted by these effectors. For example, the involvement of TRIF-dependent NF-κB signalling, autocrine activation by cytokines such as TNF (via TNFR1-TRADD), oligosaccharide sensing by C-type lectin receptors or cytosolic RNA sensing by RIG-I-like helicases has not been ruled out (Litvak et al. 2017).

5.2 Tir-Mediated Modulation of TLR and TNF Signalling

EPEC Tir induces actin pedestals via the phosphorylation of Tyr_{474} and Tyr_{454} residues. The *C. rodentium* model of in vivo infection allowed an investigation into the role of Tir phosphorylation and inflammation. Notably, translocation of Tir with both tyrosines intact led to higher CXCL1 and CXCL2 production by purified enterocytes from infected mice (Crepin et al. 2015). Mutation of both tyrosine

residues (Y451A/Y471A in *C. rodentium* Tir) reduced CXLC1 and CXCL2 production. Intriguingly, whilst actin-rich pedestals were absent on enterocytes during in vivo during infection with *C. rodentium* Y451A/Y471A Tir, colonisation and formation of A/E lesions were comparable to WT *C. rodentium* Tir. Importantly, loss of both tyrosine residues and actin polymerisation correlated with reduced colonic neutrophil influx at day 14 post-infection, which is the initial stage of pathogen clearance. Whilst further investigation is required, these findings suggest a positive regulation of NF-κB signalling by Tir in a manner that requires both Tyr471 and Tyr451 (Crepin et al. 2015).

Tir can also inhibit NF-κB activation (Ruchaud-Sparagano et al. 2011; Yan et al. 2013; Yan et al. 2012). The tyrosine phosphorylation motifs around Tyr483 or Tyr511 residues in EPEC Tir are similar to immune tyrosine-based inhibitory motifs (ITIMs). In the host, tyrosine phosphatases recruited via ITIMs dephosphorylate various signalling proteins and dampen signal transduction. Recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP1; also called PTPN6) to tyrosine phosphorylated EPEC Tir enhanced interaction between SHP1 and TRAF6 leading to reduced TRAF6 ubiquitylation. This impaired the production of proinflammatory TNF and IL-6 from EPEC-infected macrophages. Mutational inactivation of the tyrosine residue in individual ITIMs increased inflammatory cytokine production and mutation of both ITIMs increased it further, pointing to an additive role of the two motifs. Similar effects are reported via the recruitment of the related SHP2 phosphatase (Yan et al. 2013). Another study reported that EPEC infection of epithelial cells inhibited their response to exogenous TNF in a Tir-dependent manner. They found Tir-dependent proteasomal degradation of TRAF2 was responsible for reduced TNF-induced IL-8 production in HeLa and polarised Caco2 cells. Interestingly, these effects could be recapitulated by delivery of Tir by an almost effectorless strain of *Yersinia,* which pointed to a non-essential role of Tir in suppressing NF-κB (Ruchaud-Sparagano et al. 2011).

5.3 NleE and Inhibition of TAB2 and TAB3

NleE was the first EPEC/EHEC effector demonstrated to have an NF-κB inhibitory activity (Nadler et al. 2010; Newton et al. 2010). Ectopic expression of NleE alone was sufficient to block NF-κB activation by exogenous TNF and IL-1β. This suggested that NleE acted at a common hub, the most upstream of which is TAK1. A subsequent study revealed that NleE methylates a critical zinc-coordinating cysteine residue in TAB2 (C673) and TAB3 (C692) and impairs their ability to bind polyubiquitin chains (Zhang et al. 2011). Methylated TAB2/3 fails to recruit TAB1-TAK1 to active TRAFs, which results in potent inhibition of NF-κB via both cytokines. Unusually, NleE uses S-adenosine L-methionine as a cofactor for methylation, and a C-terminal IDSY(M/I)K motif is essential for catalytic activity. The broad involvement of TAK1 in canonical NF-κB and MAPK activation in many cell types underscores the importance of NleE in A/E and other enteric

pathogens such as *Shigella flexneri* and *S. boydii* which express the related OspZ effector. The DBS 100 strain of *C. rodentium* lacking *nleE* is markedly impaired in colonisation in mice and thus causes less intestinal pathology (Wickham et al. 2007); in contrast, nleE-lacking ICC 169 *C. rodentium* have similar virulence as wildtype bacteria (Marches et al. 2005).

5.4 NleB Subverts FADD-Dependent Signalling

NleB has N-acetyl glucosamine transferase (GlcNAc) activity and adds a single GlcNAc to its target proteins TRADD, FADD and GAPDH that are involved in signalling via the TNFR family of receptors (Li et al. 2013; Pearson et al. 2013; Gao et al. 2013; El Qaidi et al. 2017; Scott et al. 2017). Thus, ectopic expression of NleB inhibits TNF-induced, but not IL-1β-induced, NF-κB activity. In addition, NleB also blocks apoptosis induced by FASL, which directly recruits FADD and triggers apoptosis (Newton et al. 2010). Whilst O-GlcNAc'ylation is known in host cells, and is reversible, N-GlcNAc'ylation is presumably irreversible, which helps explain the potency of NleB action. Mutational analyses of NleB indicated that catalytic activity requires the DXD motif, Tyr219 and Glu253. *C. rodentium* lacking *nleB* are highly defective for virulence and cannot colonise mice (Pearson et al. 2013; Gao et al. 2016; Kelly et al. 2006). Infection of FASL- or FAS-deficient mice with *C. rodentium* showed increased morbidity and delayed clearance of the pathogen. This suggested that inhibition of enterocyte apoptosis by A/E pathogens may promote colonisation and disease pathogenesis. *C. rodentium* NleB was also shown to GlcNAc'cylate GAPDH, which binds TRAF3 to promote its ubiquitylation (Gao et al. 2013). Ectopic expression of NleB reduced TRAF3 ubiquitylation and impaired type I interferon (IFN) production in response to stimulation of TLR4 or TLR3 with LPS or poly(I:C), respectively (Gao et al. 2016).

5.5 NleD and Proteolytic Inactivation of MAPKs

As an *nleB/nleE* double-mutant strain still blocked JNK activation by TNF, further studies based on this initial finding led to the identification of NleC and NleD as suppressive factors with protease activities (Baruch et al. 2011). NleD and NleC have related HExxH motifs for zinc coordination and metalloprotease activity. NleD cleaves the p38 and JNK kinases, but not ERK. Direct cleavage of JNK2 by NleD occurred within the protein kinase activation loop. UV irradiation induces JNK-dependent apoptosis that can be blocked by NleD. More recent biochemical studies have identified the molecular specificity of NleD; for example, Arg203 in NleD and NleD-like proteases are essential for cleavage of p38 but dispensable for JNK proteolysis (Creuzburg et al. 2017). NleD has also been reported to block RNAseL expression and production of type I IFNs in human Caco2 IECs infected

with EPEC. IFNs maintain the expression of tight junction proteins whose reduced expression accelerated barrier breakdown and increased trans-epithelial electrical resistance (Long et al. 2014).

5.6 NleC and Proteolytic Inactivation of NF-κB

NleC is a zinc metalloprotease that cleaves NF-κB family proteins, including p65, c-Rel, RelB and p50 (Baruch et al. 2011; Pearson et al. 2011; Yen et al. 2010; Muhlen et al. 2011; Sham et al. 2011). Like NleD, the protease activity relies on a HExxH motif that coordinates zinc ions. In addition, NleC also cleaves the acetyl-transferase p300, which is a positive regulator of NF-κB-dependent IL-8 production (Shames et al. 2011). Cleavage occurs within the DNA-binding domains of NF-κB subunits, which results in their inactivation (Li et al. 2014; Turco and Sousa 2014; Giogha et al. 2015; Hodgson et al. 2015). The conserved ^{22}EIIE^{25} and ^{177}PVLS^{180} motifs in p65 are involved in binding to NleC. Different homo- or hetero-dimers of NF-κB subunits have subtly different gene targets in cells, and by acting on multiple subunits, NleC has broadly suppressive effects on NF-κB-mediated transcription.

5.7 EspT and Activation of NF-κB

EspT (discussed in 'Map, EspT and EspM mimic host Rho GEFs') activates Rac1 and promotes invasion. However, EspT-mediated activation of Rac1 also induces NF-κB in a manner that is independent of bacterial invasion (Bulgin et al. 2009a, b; Arbeloa et al. 2009). The related STm effector SopE activates Rac1 and triggers NOD1-dependent NF-κB activation (Keestra et al. 2013). Whether EspT functions similarly remains to be tested.

5.8 NleF in Inhibiting Apoptosis and Activating NF-κB

NleF interacts with caspases-4, -8 and -9 (Blasche et al. 2013; Pallett et al. 2017) and the COP1 vesicle protein Tmp21 (Olsen et al. 2013). Recombinant NleF potently inhibits caspase-4 ($IC_{50} \sim 14$ nM), caspase-8 ($IC_{50} \sim 40$ nM) and caspase-9 ($IC_{50} \sim 80$ nM) (Blasche et al. 2013). Here we discuss the cellular effects of NleF-mediated inhibition of caspase-8/9; NleF-caspase-4 interactions are discussed in the section on inflammasomes. The cocrystal structure of caspase-9-NleF revealed that insertion of four C-terminal residues of NleF into the caspase-9 active site inhibits its proteolytic activity in a manner similar to the inhibitor peptide zEAD-Dcmbk. NleF, which partially localises to mitochondria, can block the intrinsic caspase-9-dependent apoptosis induced by staurosporine. As a direct inhibitor of caspase-8, ectopic expression of NleF could potently block

FASL- and TRAIL-induced receptor-mediated apoptosis. Whilst NleF and NleB both block apoptosis, they act at different steps in the pathway. By inhibiting a receptor-proximal step, NleB causes severe dampening of the immune response compared to NleF which acts 'downstream' on caspases (Blasche et al. 2013; Pallett et al. 2017; Pollock et al. 2017; Song et al. 2017). This is reflected by the relatively marked attenuation caused by *nleB* deletion as compared to *nleF* deletion in *C. rodentium* (Kelly et al. 2006; Pallett et al. 2017).

A second role of NleF is activation of NF-κB. EPEC increases NF-κB activity early during infection (1.5 h post-infection), dependent on the presence of NleF (Pallett et al. 2014). Consistent with this, EPEC infection-induced IL-8 production in IECs in vitro was NleF-dependent. The underlying mechanisms of NleF action on NF-κB were caspase-4, -8 and -9-independent.

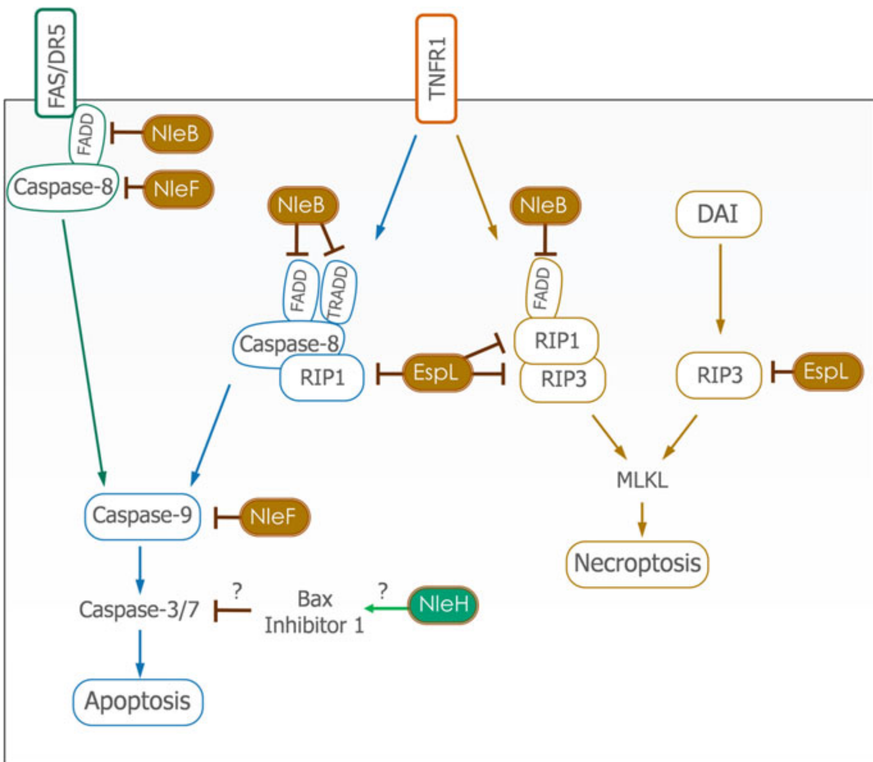

Fig. 4 Suppression of apoptosis and necroptosis by EPEC/EHEC. FAS and death receptor 5 (DR5) mediate FADD and caspase-8 dependent apoptosis. TNFR1 mediates apoptosis or necroptosis in a context-specific manner (see text). TNFR1-induced apoptosis via RIPK1 and caspase-8 typically requires inhibition of NF-κB activity and/or protein translation, and necroptosis requires inhibition of caspase-8 or reduced RIPK1 ubiquitylation. The cytosolic protein DAI directly induces necroptosis via RIPK3. The mechanisms of NleH-mediated inhibition of apoptosis via BAX-inhibitor 1 are poorly understood (depicted by ? marks)

5.9 NleH-Mediated Inhibition of NF-κB and Apoptosis

NleH1 and NleH2 reportedly inhibit NF-κB activity in cells via two different mechanisms. There is evidence that NleH1/2 binding to ribosomal protein S3 (RPS3), a subunit of NF-κB, prevents NF-κB nuclear translocation (Gao et al. 2009). Another study reported that transfection of EPEC NleH1/2 reduced IKK-β activity, IκB degradation and NF-kB activation in response to TNF stimulation (Royan et al. 2010). However, an alternative mechanism was suggested based on the finding that NleH1 inhibited IKK-β-mediated RPS3 phosphorylation on the key Ser209 residue required for its nuclear translocation (Wan et al. 2011; Pham et al. 2012). In contrast to NleH1, NleH2 inhibits NF-κB by blocking IκB degradation (Gao et al. 2013). Deletion of both NleH proteins in EPEC revealed a relatively weak inhibitory activity on TNF-induced NF-κB activation and KC-induction in vivo. *C. rodentium* encodes a single NleH, and its deletion reduced NF-κB reporter activity in the colonic mucosa in vivo. Complementation of *ΔnleH C. rodentium* suggested that NleH1 but not NleH2 has more anti-NF-κB activity (Gao et al. 2013). EHEC lacking NleH1 and NleH2 showed increased colonisation of calves; however, the mechanisms for this are unclear (Hemrajani et al. 2008). Thus, whilst several reports exist on NF-κB-suppressive roles for NleH proteins, the underlying mechanisms that have been proposed remain to be reconciled.

NleH proteins are related to Shigella OspG and have an atypical protein kinase structure and a C-terminal PSD-95/Disc Large/ZO-1 (PDZ) motif (Pham et al. 2012; Martinez et al. 2010; Halavaty et al. 2014). NleH1 is reported to undergo autophosphorylation but does not phosphorylate RPS3 or IKK-β. The v-Crk sarcoma virus CT10 oncogene-like protein (CRKL) protein was identified as an NleH1 substrate and implicated in inhibiting RPS3 phosphorylation (Pham et al. 2013). The crystal structure of NleH2 revealed that it might not require autophosphorylation for full activity due to the lack of the conserved Arg residue in the HRD motif typically found in other protein kinases (Halavaty et al. 2014). Furthermore, p38 and JNK inhibition independently of the kinase domain has also been reported for NleH proteins. The PDZ motif, also implicated in NF-κB inhibition, may also have additional subversive roles, for example, via binding to PDZ-containing proteins such as Na(+)/H(+) exchanger regulatory factor 2 (NHERF2) (Martinez et al. 2010).

In addition, EPEC NleH1 and NleH2 block pro-caspase-3 proteolysis and apoptosis induced by staurosporine, brefeldin A, tunicamycin or *Clostridium difficile* TcdB toxin (Hemrajani et al. 2010; Robinson et al. 2010). The ER-resident BAX-inhibitor protein 1 (BI-1) has been identified as an NleH1-binding partner and is required for NleH1-mediated inhibition of cellular apoptosis. BI-1 inhibits apoptosis, particularly when induced by ER-stress or Ca^{+2} elevation by blocking the action of BCL2 family protein BAX. Notably, the kinase activity of ectopically expressed NleH1 is dispensable for its anti-apoptotic roles. Consistent with this, *C. rodentium ΔnleH* induces reduced pro-caspase-3 cleavage in vivo (Hemrajani et al. 2010).

5.10 EspL Cleaves RHIM-Domain Proteins

The receptor-interacting protein (RIP) homotypic interaction motifs (RHIMs) present in signalling proteins such as TRIF, RIPK1, RIPK3, DAI (DNA-dependent activator of IFN-regulatory factors, also called ZBP1), amongst others, are essential for signal transduction by these proteins. An unconventional Cys-His-Asp catalytic triad and protease activity of EspL against these RHIM-containing proteins inactivates their respective signalling pathways (Pearson et al. 2017). For example, inactivation of RIPK1 by EspL redundantly inhibits TNF-dependent NF-κB activation in EspL expressing cells. Similarly, cleavage of TRIF abrogates TLR3- and TLR4-induced type I IFN production in EspL expressing cells. Importantly, cleavage of RIPK1 and RIPK3 also abrogates necroptosis induced by TNRF1 and DAI. During EPEC infection, levels of RIPK1 drop early post-infection in an EspL activity-dependent manner, and RIPK3 levels drop later during infection, suggesting a preference for RIPK1. Furthermore, EspL is only active against RIPK1 before it assembles into oligomeric amyloid-like fibrils that trigger necroptosis. *C. rodentium ΔespL* is cleared much faster during infection as compared to wildtype *C. rodentium* which points to the importance of EspL in vivo (Pearson et al. 2017).

5.11 NleL-Mediated Inhibition of JNK

NleL has ubiquitin E3-ligase activity that is biochemically similar to eukaryotic E6-AP Carboxyl Terminus (HECT) family proteins (Lin et al. 2011; Piscatelli et al. 2011); however, NleL is structurally unrelated to HECT ligases. NleL can monoubiquitylate JNK1 at Lys68 and reduce its interaction and phosphorylation by the upstream kinase MKK7 (Sheng et al. 2017). NleL can also target JNK2 and JNK3 and thus reduce AP1 activity in cells. JNK was also found to regulate EHEC Tir-mediated pedestal formation and bacterial attachment to cells, thus suggesting a role for NleL in this process.

5.12 EspJ and Non-receptor Tyrosine Kinases

EspJ is uniquely able to couple amidation and ADP-ribosylation (Young et al. 2014) and targets this biochemical activity to non-receptor tyrosine kinases (Pollard DJ et al. 2018). EspJ can ADP-ribosylate Src to inhibit complement receptor 3 (CR3) and FcγR-mediated phagocytosis (Young et al. 2014). Proteomic analysis of IECs isolated from mice infected with *C. rodentium* (WT, Δ*espJ* or Δ*espJ* complemented with a catalytically inactive EspJ) indicates a broad immunomodulatory effect of EspJ through regulation of multiple tyrosine kinases including Src, Abl, Csk, Tek and Syk families (Pollard et al. 2018).

6 Inflammasome Signalling Pathways

Inflammasomes are multimolecular scaffolds that activate caspase-1 (Broz and Dixit 2016; Eldridge and Shenoy 2015). Oligomeric inflammasome complexes form a single 'speck' or 'focus' ($\sim$1–3 μm in size) per cell upon their activation by microbial and environmental cues. The inflammatory outcomes of caspase-1 activation include the release of proinflammatory cytokines such as IL-1β and IL-18, alarmins such as IL-1α and HMGB1, and lytic cell death via pyroptosis. The inactive pro-caspase-1 zymogen undergoes autoproteolytic activation within inflammasome foci. Pro-IL-1β and pro-IL-18 are biologically inert precursors that require proteolytic maturation. Gasdermin-D (GSDMD) is also inert until it is processed, which releases the N-terminus fragment that inserts within membranes and forms pores. GSDMD pores cause release of ions leading to swelling and eventual cell lysis and may also facilitate the release of small proteins, including mature IL-1β and IL-18 from macrophages. IL-1α and HMGB1 release is also regulated by inflammasomes through mechanisms that are poorly understood; however, caspase-1 does not proteolytically process either protein (Broz and Dixit 2016; Eldridge and Shenoy 2015). Mature IL-1β and IL-1α have overlapping immune roles, for example, in elevating body temperature (causing fever), inducing proinflammatory cytokines and acute phase proteins, and as neutrophil attractants. IL-18 is especially important for neutrophil recruitment, the induction of type II interferon (IFNγ) from lymphocytes and tissue repair in the intestine. Inflammasomes thus orchestrate the early innate immune responses to infection and help launch effective adaptive immune responses. Differential expression of inflammasome sensors and substrates results in distinct outcomes following inflammasome activation in different cell types. Inflammasome signalling is best understood in macrophages and dendritic cells; however, intestinal epithelial cells also express several inflammasome signalling proteins and the pro-IL-18 substrate.

Inflammasomes sensors are modular proteins and share conserved domains. Examples include proteins from the NLR proteins with a PYD (pyrin domain; NLRPs) or NLRs with a CARD (caspase activation and recruitment domain; NLRCs), AIM2-like receptors (ALRs) and the non-NLR/ALR sensor called PYRIN. Caspase-1 has a CARD at its N-terminus which recruits it to inflammasome complexes, typically via the small adaptor protein PYCARD (protein with a PYD and CARD) also called ASC (apoptotic speck-associated protein containing a CARD). Whilst CARD-containing NLRs (e.g. NLRC4) can directly recruit caspase-1 for pyroptosis, for reasons not completely clear, ASC is required for optimal IL-1β and IL-18 processing by NLRC4 inflammasomes. EPEC and EHEC T3SS components, RNA and LPS have previously been suggested to activate inflammasomes in various cell types.

EPEC infection activates NLRC4 and NLRP3 inflammasomes, both of which rely on upstream receptors or molecules for their activation. The NLRC4 inflammasome requires proteins of a NLR subfamily called NAIPs (NLRs with apoptosis inhibitor repeat proteins) which contain N-terminal BIRs (baculovirus inhibitor of

apoptosis repeat domains). *NAIP* genes have diversified in the mouse, four of which have been characterised extensively: NAIP1 binds the T3SS needle proteins, NAIP2 binds the T3SS rods, and NAIP5 and NAIP6 bind flagellins. In contrast, a single human *NAIP* produces at least two isoforms that are capable of binding T3SS needle, rod and flagellin. Ligand-bound NAIPs stimulate NLRC4 oligomerization, ASC recruitment and caspase-1 activation (Vance 2015; Zhao and Shao 2015).

The NLRP3 inflammasome can be activated in two ways; by loss of cellular K^+ through bacterial pore-forming toxins or host proteins that can form pores ('canonical' signalling), or by cytosolic LPS ('non-canonical' signalling). Non-canonical NLRP3 activation requires upstream activation of caspase-4 (previously called caspase-11 in the mouse) or caspase-4 and caspase-5 in the human (Broz and Dixit 2016; Eldridge and Shenoy 2015). Caspase-4, -5 are cytosolic receptors for LPS and are directly activated by LPS binding. Therefore, unlike caspase-1 which is oligomerised within inflammasome scaffolds, current evidence suggests LPS binding is sufficient to induce oligomerisation and activation of caspase-4. Active caspase-4 can also proteolytically process GSDMD and cause pyroptosis in myeloid cells and IECs. The efflux of K^+ via GSDMD or pannexin-1 pores activates NLRP3-ASC-caspase-1 inflammasome which is essential for IL-1β and IL-18 processing in macrophages (Ruhl and Broz 2015; Kayagaki et al. 2015; Yang et al. 2015). Recombinant Shiga-like toxins Stx1 and Stx2 activate the NLRP3 inflammasome in THP1 macrophage-like cells in a manner that depended on their N-glycosidase activity (Lee et al. 2015). In contrast, Stx was dispensable during EHEC infection or mouse macrophages in which NLRP3 activation was reported via *E. coli* RNA:DNA hybrids that gain access to the host cytosol independently of the T3SS (Kailasan Vanaja et al. 2014). As discussed further below, EPEC LPS can activate caspase-4 in different cell types. The mouse *C. rodentium* infection model has highlighted the importance of inflammasomes in host defence against A/E pathogens. Loss of inflammasome genes, such as *Nlrp3, Nlrc4, Casp1/4, Il1b* and *Il18,* resulted in greater pathogen burdens at late stage of infection and higher morbidity (Gurung et al. 2012; Liu et al. 2012; Alipour et al. 2013; Song-Zhao et al. 2014; Nordlander et al. 2014). More recent work has highlighted the critical role of the microbiome in *C. rodentium* colonisation (Collins et al. 2014). Additional studies are required to dissect the contribution of the host genotype and the microbiome to the outcomes of *C. rodentium* infection in *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice to identify the relative contribution of these inflammasomes to host defence against A/E pathogens. In vivo studies have pointed to an important role for IEC-intrinsic inflammasome in innate immunity to *C. rodentium.* How EPEC proteins activate, suppress or evade detection by inflammasomes is discussed next and summarised in Fig. 5.

6.1 EPEC FliC, EscI, EscF and the NLRC4 Inflammasome

Due to its presence in diverse pathogenic bacteria, the T3SS is a major target for detection by the host immune system. The T3SS is evolutionarily related to the bacterial flagellar basal body, and the first studies on NLRC4 identified a role for it in detecting STm flagellin (Miao et al. 2006). The role of NLRC4 in detecting EPEC is evident from the lack of inflammasome response by *Nlrc4*$^{-/-}$ mouse macrophages (Zhao et al. 2011). During *C. rodentium* infection, *Nlrc4*$^{-/-}$ show marked weight loss between days 6–12 post-infection and increased pathogen burden (Nordlander et al. 2014). This was accompanied by increased intestinal

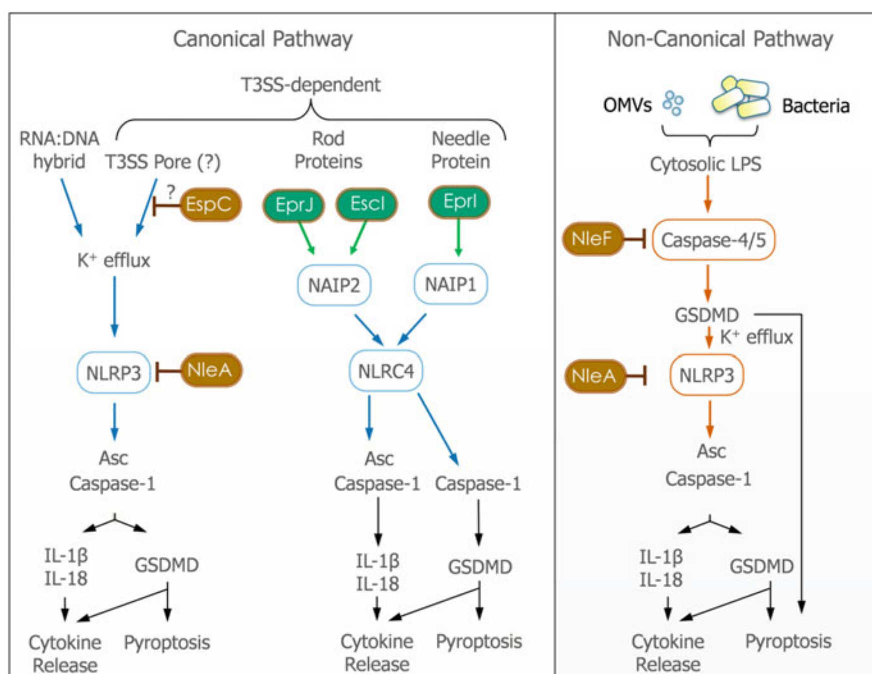

Fig. 5 Inflammasomes signalling during EPEC/EHEC infection. Schematics depict the canonical (left) and non-canonical (right) inflammasome activation pathways. T3SS structural proteins that activate inflammasomes are shown in green (EscI, EprJ and EprI), and effectors that suppress inflammasomes are shown in yellow (EspC, NleA and NleF). EHEC RNA:DNA hybrids gain access to the cytosol independently of the LEE T3SS. The EHEC ETT2 needle protein EprI is a ligand for human NAIP (not depicted) and mouse NAIP1. The LEE T3SS and ETT2 rod proteins are reported to activate murine NLRC4 via NAIP2. EspC blocks necrosis by regulating the T3SS pore, and whether this form of death is pyroptotic is not known (depicted by ?). EHEC bacteria and purified OMVs stimulate non-canonical signalling via caspase-4 in mouse macrophages. EPEC and EHEC NleF can block caspase-4 in IECs. The transcriptional upregulation of NLRP3 and pro-IL-1β require NF-κB activity which may be suppressed by the actions of other effectors that are not depicted (see text and Fig. 3)

inflammation and pathology. However, the production of antibodies, IFNγ and IL17A were higher in *Nlrc4*$^{-/-}$ animals, pointing to reduced early innate immune responses but a relatively competent adaptive immune response later during infection. Bone marrow chimera experiments indicated that NLRC4 signalling in the non-haemopoietic compartment was required for host defence. Consistent with this, NLRC4 expression was detected in intestinal crypts, which may respond by producing IL-18. However, as discussed next, EPEC has evolved mechanisms that largely allows it to escape detection by the NAIP-NLRC4 system, especially in human cells.

A novel cytosolic protein delivery approach using flagellin or T3SS proteins fused to the non-catalytic N-terminal regions of the anthrax lethal factor (LnF) plus protective antigen (PA) helped dissect NLRC4 signalling induced by individual bacterial molecules (Zhao et al. 2011). Flagellin proteins from STm (FliC) and *Legionella* (FlaA) interact with NAIP5 when delivered via LnF-PA. However, EPEC and EHEC flagella do not interact with NAIP5 or activate NLRC4 inflammasomes (Zhao et al. 2011). In agreement with this, a *ΔfliC* EPEC E2348/69 strain has unaltered inflammasome activation in mouse macrophages, which suggests that EPEC/EHEC flagellins evade detection by inflammasomes.

As a T3SS-deficient EPEC (Δ*escN*) fails to activate inflammasomes in macrophages, NLRC4 likely detects a T3SS component. Almost all strains of EHEC and EPEC also encode remnants of a second T3SS called ETT2 (Zhou et al. 2014). Frameshift inactivation and deletion of several genes within ETT2 point to a non-functional injectisome. EPEC strains have a deletion in ETT2 resulting in the loss of rod and needle genes; in contrast, EHEC strain Sakai encodes the complete ORFs for rod (EprJ) and needle (EprI). In a study that used transient transfection of the EPEC LEE T3SS rod protein EscI or the EHEC ETT2 rod EprJ in mouse macrophages, both proteins induced NLRC4-dependent pyroptosis (Miao et al. 2010). A subsequent study confirmed that EPEC EscI is a ligand for mouse NAIP2 and that a *ΔesfFΔfliC* EPEC strain fails to activate inflammasomes during infection of mouse macrophage (Zhao et al. 2011). Various T3SS rod proteins, including the *Salmonella* Pathogenicity Island 1 (SPI-1) rod protein PrgJ, activate the NAIP2-NLRC4 system. However, as the extensively used human macrophage cell lines (THP1 and U937) do not respond to flagellin or T3SS rods, the search for activators of human NLRC4 continued, leading to the identification of the T3SS needle CprI from *Chromobacterium violaceum* as a ligand for human NAIP and mouse NAIP1 (Zhao et al. 2011; Yang et al. 2013). Interestingly, the LEE T3SS needle protein EscF from EPEC and EHEC fails to bind to human or mouse NAIP and exemplifies another evasion mechanism (Zhao et al. 2011; Yang et al. 2013). In contrast, the EHEC ETT2 needle protein EprI, which is related in sequence to SPI-1 needle PrgI, readily activates human and mouse NAIP-NLRC4 (Miao et al. 2010). Whether EprI or EprJ are expressed and secreted by EHEC remains to be tested during infection.

6.2 EPEC EspC and Regulation of the T3SS Pore

The T3SS pore has the potential to activate NLRP3 by promoting K+ efflux (Guignot et al. 2015; Serapio-Palacios and Navarro-Garcia 2016). Whilst this has been suggested in the case of effectorless strains of *Yersinia*, whether this happens during EPEC infection has not been tested. However, in epithelial cells, the EPEC serine protease autotransporter of enterobacteriaceae (SPATE) family effector EspC targets EspA-EspD subunits of the T3SS translocon upon host cell contact. This effectively down-regulates pore formation and reduces EPEC-induced cytotoxicity. Therefore, a *ΔespC* strain is more cytotoxic to epithelial cells. However, the mechanisms of cytotoxicity and indeed whether this is inflammasome-dependent pyroptosis remains to be tested.

6.3 NleA and Suppression of NLRP3 Inflammasomes

The EPEC effector protein NleA (also called EspI) can inhibit NLRP3 inflammasomes in human THP1 cells (Yen et al. 2015). A screen of deletion mutants of non-LEE islands identified NleA in inhibiting NLRP3 activation by preventing its deubiquitylation. In naïve cells, ubiquitylation restrains NLRP3 activity, which is relieved by its deubiquitylation by BRCC3. NleA interaction with NLRP3 impaired its deubiquitylation, thus reducing oligomeric foci formation. How NleA affects NLRP3 ubiquitylation remains to be elucidated mechanistically. NleA was previously reported to block COPII vesicle trafficking and protein secretion by directly binding to host Sec24 (Kim et al. 2007). However, NleA-dependent reduced IL-1β secretion can be attributed to caspase-1 inhibition and not protein secretion inhibition as IL-1β (and IL-18) do not have signal peptides for ER-Golgi-mediated trafficking and secretion and are released via an unconventional secretion mechanism. Loss of *nleA* in *C. rodentium* severely reduces intestinal colonisation and inflammation, indicating its importance in vivo (Mundy et al. 2004).

6.4 NleE and Suppression of Inflammasome Priming

Inflammasome sensors such as NLRP3 and mouse caspase-4 require priming for post-translational licensing and increased expression (Yen et al. 2015). Importantly, pro-IL-1β expression is upregulated by NF-κB signalling in myeloid cells. As discussed previously, NF-κB inhibition by several EPEC effectors could potentially reduce mature IL-1β production in macrophages. A role for NleE in reducing pro-IL-1β expression was evident during infection of THP1 macrophages. The other NF-κB inhibitory effectors such as NleC and NleB are predicted to have similar suppressive roles on pro-IL-1β production.

6.5 EHEC OMVs/LPS and the Non-canonical Activation of Inflammasomes

EHEC, EPEC and *C. rodentium* were amongst the first Gram-negative pathogens shown to activate caspase-4 in macrophages in a TLR4- and TRIF-dependent manner (Gurung et al. 2012; Kayagaki et al. 2011; Rathinam et al. 2012). However, these studies used EPEC/EHEC/Cr that were grown in condition that does not induce T3SS, LEE or non-LEE effectors, presumably to avoid NLRC4 activation. How LPS from these bacteria gains access to cytosolic caspase-4 has remained elusive. Studies using outer membrane vesicles (OMVs) from non-LEE expressing EHEC or *E. coli* K12 showed that OMVs are endocytosed via processes that require Rab7 (Vanaja et al. 2016) and TLR4-TRIF (Gu et al. 2018; Santos et al. 2018). OMVs gain access to the cytosol via mechanisms that are not entirely clear but require the guanylate-binding proteins (GBPs), including GBP2 and GBP5 (Gu et al. 2018; Santos et al. 2018; Finethy et al. 2017). Mouse caspase-4 and GBPs are IFN-inducible genes, and their expression needs to be upregulated for optimal inflammasome activation (Rathinam et al. 2012). Thus, in naïve mouse macrophages, caspase-4 activation is delayed and can take up to 10 h. In contrast, both human caspase-4 and caspase-5 are constitutively expressed, further pointing to likely differences in human macrophages.

6.6 NleF and Suppression of Caspase-4 Activity

Human caspase-4 was reported to be inhibited ($IC_{50} \sim 5$ nM) by purified NleF in a manner that required its four C-terminal residues, which were also critical for NleF-caspase-4 interaction (Pallett et al. 2017). In addition, mouse caspase-4 was potently inhibited by *C. rodentium* NleF. Moreover, EPEC induced caspase-4-dependent pro-IL-18 processing in human Caco2 IECs. The direct processing of pro-IL-18 by caspase-4 in IECs, independently of NLRP3, was also reported during STm infection. EPEC *ΔnleF* affected markedly increased mature IL-18 production by IECs, pointing to a physiologically relevant subversive role for NleF during infection. In the *C. rodentium* infection model, higher IL-18 release was observed in colonic explants of mice infected with *ΔnleF C. rodentium* than the wildtype pathogen. Colonic explants from *C. rodentium* or *ΔnleF* infected *Casp4*$^{-/-}$ mice did not secrete detectable IL-18, which indicated that early IL-18 production was caspase-4-dependent in vivo. Importantly, reduced IL-18 levels correlated with lower colonic influx of neutrophils. Thus, NleF-mediated caspase-4-inhibtion blocked early neutrophil responses to infection (Pallett et al. 2017). Similarly, EHEC NleF is reported to block caspase-4-dependent pyroptosis and IL-1β conversion in HT29 IECs (PMCID: PMC5448047). Subversion of IEC caspase-4 by

A/E pathogens points to inflammasome-dependent antimicrobial host defence. An important outstanding question is how LPS from extracellular A/E pathogens is able to localise to the cytosol, and whether any effectors may be involved in the process.

7 Conclusion

Innate immune signalling leading to the transcriptional upregulation of proinflammatory cytokines and type I IFNs as well as removal of infected cells via programmed cell death are essential for effective antimicrobial immunity. A/E pathogens encode effectors with distinct biochemical activities to block multiple steps of signal transduction by a broad range of pattern recognition receptors and cytokine receptors. Transcriptional responses as well as rapid post-translational pathways, exemplified by TLRs and inflammasomes, respectively, are targeted by A/E pathogens for robust inhibition of host responses. Future studies should focus on the temporal nature of stimulatory and inhibitory actions of various effectors during infection.

8 Summary and Perspectives

A/E pathogens predominantly cause diarrheagenic disease in children but distinct pathotypes are increasingly associated with disease in adults. A number of LEE and NLE effectors are together responsible for damaging the intestinal lining, suppressing host responses and causing disease. Studies on T3SS-delivered effectors have led to the identification of exciting biochemical activities such as GEF mimics, arginine-GlcNac'lase, deamidase, combined deamidase and ADP-ribosylase, unconventional proteases, caspase inhibitors and ubiquitin ligases. Future studies should focus on the collective spatiotemporal nature of effector function and their contribution to intestinal disease.

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The 2011 German Enterohemorrhagic *Escherichia Coli* O104:H4 Outbreak—The Danger Is Still Out There

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Abstract Enterohemorrhagic *Escherichia coli* (EHEC) are Shiga toxin (Stx) producing bacteria causing a disease characterized by bloody (or non-bloody) diarrhea, which might progress to hemolytic uremic syndrome (HUS). EHEC O104:H4 caused the largest ever recorded EHEC outbreak in Germany in 2011, which in addition showed the so far highest incidence rate of EHEC-related HUS worldwide. The aggressive outbreak strain carries an unusual combination of virulence traits characteristic to both EHEC—a chromosomally integrated Stx-encoding bacteriophage, and enteroaggregative *Escherichia coli*—pAA plasmid-encoded aggregative adherence fimbriae mediating its tight adhesion to epithelia cells. There are currently still open questions regarding the 2011 EHEC outbreak, e.g., with respect to the exact molecular mechanisms resulting in the hypervirulence of the strain, the natural reservoir of EHEC O104:H4, and suitable therapeutic strategies. Nevertheless, our knowledge on these issues has substantially expanded since 2011. Here, we present an overview of the epidemiological, clinical, microbiological, and molecular biological data available on the 2011 German EHEC O104:H4 outbreak.

1 Introduction

Commensal *Escherichia coli* (*E. coli*) are part of the human gut microbiota (Human Microbiome Project 2012). Pathogenic *E. coli* strains, however, can cause various intestinal and extraintestinal diseases in humans (Kaper et al. 2004). Among them are the enterohemorrhagic *E. coli* (EHEC), which causes diarrhea, hemorrhagic colitis (bloody diarrhea), and hemolytic uremic syndrome (HUS, characterized by hemolytic anemia, thrombocytopenia, and acute kidney injury) (Karch et al. 2005). The hallmark of EHEC pathogenesis is the production of Shiga toxins (Stx), which irreversibly inhibit host cell protein synthesis and lead to cell death (Karpman et al. 1998; Tarr et al. 2005). The majority of EHEC infections and EHEC-associated HUS have been attributed to the serotype O157:H7 (Karch et al. 2005). Strains belonging to serogroups other than O157, however, have been also recognized as clinically important (Johnson et al. 2006; Mellmann et al. 2008b). For example, almost one-third of the 524 EHEC isolates from HUS patients that were used to generate the German HUS-associated *E. coli* (HUSEC) reference strain collection belonged to non-O157 serotypes (Mellmann et al. 2008b).

The largest EHEC outbreak ever recorded in Germany took place from May to July 2011. Nearly 4000 EHEC gastroenteritis and more than 850 HUS cases were reported, leading to 54 deaths (Robert-Koch-Institut 2011). This was also the largest incidence of EHEC-associated HUS worldwide. Moreover, the infections were characterized by an unusually high rate of progression to HUS (Frank et al. 2011b), further suggesting that the strain responsible for it is highly virulent. EHEC of the rare serotype O104:H4 (EHEC O104:H4) was identified as the causative agent for the outbreak. Interestingly, with respect to virulence gene content the outbreak strain is a hybrid of EHEC and enteroaggregative *E. coli* (EAEC) (Brzuszkiewicz et al. 2011;

Mellmann et al. 2011; Rasko et al. 2011). EAEC is another type of intestinal pathogenic *E. coli* associated with acute and persistent diarrhea (Nataro et al. 1998). Besides having a chromosomally integrated Stx bacteriophage, EHEC O104:H4 carries a pAA plasmid-encoded aggregative adherence fimbriae (a characteristic feature of EAEC), mediating its tight adherence to cultured epithelial cells (Bielaszewska et al. 2011). Up to date, only a few sporadic or small outbreak-related cases of infections associated with other EHEC strains displaying an aggregative adherence phenotype have been reported (Morabito et al. 1998; Mellmann et al. 2008b; Jourdan-da Silva et al. 2012). Therefore, it remains unclear if the severity and dimensions of the 2011 EHEC O104:H4 outbreak were due to a particularly virulent strain or favorable outbreak settings or both.

In this chapter, we aimed to provide an overview of the current knowledge on the 2011 EHEC O104:H4 outbreak and its highly pathogenic causative agent. We summarized the epidemiological and clinical data on the outbreak. Furthermore, we described the genomic organization of EHEC O104:H4, as well as the factors and mechanisms, which were shown to contribute to its virulence. Last, but not least, we reviewed the treatment approaches used during the outbreak and other still experimental therapeutic strategies.

2 The 2011 EHEC O104:H4 Outbreak

2.1 Time Course and Epidemiology of the Outbreak

On May 1, 2011, first symptoms of EHEC infection attributed to the outbreak appeared in patients. Two days later the first patient developed HUS. On May 19, 2011, the Robert-Koch-Institut, Germany's national public health authority, was informed about a cluster of three cases of HUS in children admitted on the same day to the university hospital in the city of Hamburg (Frank et al. 2011b). On 22 May, the peak of the outbreak was reached. After this time point, the numbers of new EHEC infections and associated HUS cases decreased. In the mid of June, only single cases but no disease clusters were reported. The last recognized infection was recorded on 4 July. Three weeks after no additional case was reported, the end of the outbreak was declared on July 26, 2011 (Robert-Koch-Institut 2011) (see also Fig. 1).

In total, over 4000 cases of gastroenteritis, 852 HUS cases, and 54 deaths attributed to the outbreak were reported. Though in every part of Germany cases occurred, the four northern federal states (Hamburg, Bremen, Lower Saxony, and Schleswig-Holstein) reported more than 50% of all cases, with an incidence rate of 10/100,000 (Robert-Koch-Institut 2011). Cases linked to this outbreak were also communicated from other European countries: On May 25, 2011, Sweden reported nine cases of HUS of whom four had traveled to northern Germany from 8 to 10 May. Denmark reported four cases of gastroenteritis, two of them progressed to HUS. Here, all cases had a recent travel history to northern Germany. Another two HUS cases with travel history to northern Germany in the relevant period were

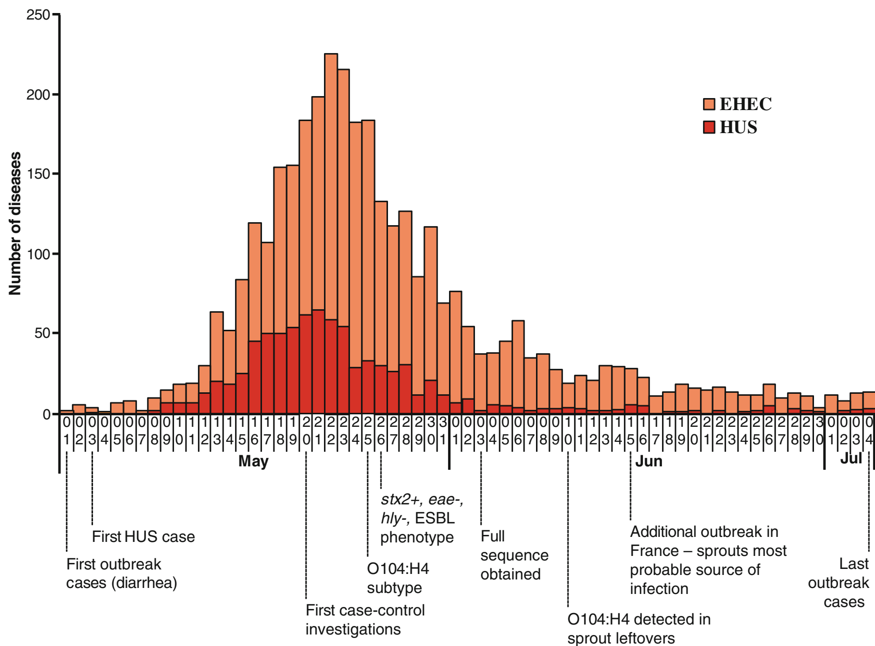

Fig. 1 Time course and epidemiology of the EHEC O104:H4 outbreak. The diagram shows the epidemiological course of the outbreak including 809 HUS and 2717 EHEC cases with known onset of diarrhea, reaching its peak on 22nd May 2011(Robert-Koch-Institut 2011). The bottom line indicates analyses and diagnostic pathways during the outbreak (Karch et al. 2012)

communicated, one by the Netherlands and other by the UK (Frank et al. 2011a). German cases could also be linked to 15 cases of an O104:H4 outbreak in Bordeaux, France, since the pulsed-field gel electrophoresis pattern of the French isolates was identical but different from pre-outbreaks O104-reference strains (Mariani-Kurkdjian et al. 2011).

2.2 *Diagnostic of the Outbreak Strain*

After the HUS clusters were reported to the Robert-Koch-Institute on May 19, 2011, *E. coli* strains were examined in the national reference laboratory for bacterial enteritis. On 23 May, conventional PCRs on two cultured isolates revealed that the outbreak was caused by an stx_1- and *eae*-negative and stx_2-positive EHEC strain (Robert-Koch-Institut 2011). Simultaneous molecular subtyping via partial *gnd*-sequencing, *fliC*-RFLP-typing and multilocus-sequence typing (MLST), performed in the German national consulting laboratory for HUS revealed an O104:H4 serotype of MLST sequence type (ST) 678 (Bielaszewska et al. 2011; Mellmann et al. 2011). On 26 May, an additional ESBL phenotype with resistances against

third-generation cephalosporines was confirmed and the Stx2a subtype was identified (Bielaszewska et al. 2011; Karch et al. 2012) (see also Fig. 1).

A multiplex PCR of the rfb_{O104} (gene from the O104 biosynthetic cluster), $fliC_{H4}$ (gene from the H4 antigen biosynthetic cluster), stx_2 and *terD* (gene from the tellurite resistance cluster) was initially used to identify outbreak isolates (Bielaszewska et al. 2011). A real-time multiplex PCR targeting stx_2, wzy_{O104}, *and* $fliC_{H4}$ was additionally developed to allow for the rapid and sensitive detection of EHEC O104:H4 in human stools (Zhang et al. 2012). To further increase diagnostic specificity, a high throughput alignment-free strategy based on whole genome sequencing data was developed to design PCR primers which could discriminate between the 2011 outbreak strain and the closely related HUSEC041 (Pritchard et al. 2012), which is another *stx2* positive O104:H4 strain isolated from a single HUS patient in 2001 in Germany (Mellmann et al. 2008b).

2.3 Origin, Reservoir, Transmission, and Shedding of the O104:H4 Outbreak Strain

The identification of the outbreak source was initiated while the outbreak was still ongoing. In a case-control study including 26 cases and 87 control patients the source of illness was found to be significantly associated with sprout consumption in a univariable analysis and with sprout and cucumber consumption in a multivariable analysis (Buchholz et al. 2011). Later a study investigating outbreak cases related to a community center event could show that the consumption of fenugreek sprouts was significantly associated with the development of symptoms (King et al. 2012). Fenugreek sprouts origin could be traced back to a common import of seeds from Egypt that arrived in Rotterdam and was distributed to Germany, and then partly redistributed to the UK from where a portion finally made its way to France (Karch et al. 2012). After the indirect identification of fenugreek sprouts as the most probable infection vehicle and establishing a sales stop for this food product at the beginning of June 2011, no clusters of diseases occurred. Nevertheless, the outbreak strain was not detected on any of the sprout samples analyzed and no contamination was found on investigated farms (European-Food-Safety-Authority 2011). The question when and where the potential contamination occurred is not clarified as well. A recent study did not show any exceptional or prolonged survival of culturable EHEC O104:H4 on dry fenugreek seeds. This indicated that the contamination might have not initially occurred in Egypt, but rather at later stages of the seed processing (Knodler et al. 2016).

Initial investigations conducted during the outbreak, revealed cattle is unlikely to be a reservoir of EHEC of serotype O104:H4, different from what was shown for other EHEC variants like O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 (Wieler et al. 2011; Auvray et al. 2012; Pierard et al. 2012). However, recent investigations based on experimental inoculation of the outbreak strain in calves showed that cattle can at least transiently carry and therefore be a reservoir for

O104:H4 (Hamm et al. 2016). Infections via contaminated sprouts were considered to be more likely, thus classifying O104:H4-associated infections as a foodborne disease (Buchholz et al. 2011; King et al. 2012) even though it remained unclear which was the main mode of transmission during the outbreak. Two studies described that smear infections/secondary transmission can easily occur within household settings, in particular, as shedding of O104:H4 begin prior to development of hemorrhagic colitis or HUS (Aldabe et al. 2011; Kuijper et al. 2011; Diercke et al. 2014). In contrast, trials observing 14 households containing 20 carriers of EHEC O104:H4 could not detect any household transmission in a prospective follow-up-study (Sin et al. 2013). The overall duration of shedding of the outbreak strain after the occurrence of symptoms was found to be 14–15 days in adults and 35–41 days in children (Vonberg et al. 2013).

2.4 Clinical Characteristics and Outcome of Infected Patients

Prior to 2011, EHEC was described to mainly affect young children and the elderly equally in both sexes (Rangel et al. 2005; Tarr et al. 2005). During the 2011 outbreak, however, 90% of the cases were recorded in adults (older than 17 years) (Frank et al. 2011b). Studies performed in 13 pediatric departments reveal that median age among children suffering from infections with the outbreak strain was 11.5 (Loos et al. 2012). Interestingly, 58% of the gastroenteritis and 68% of the HUS cases were recorded in female patients (Frank et al. 2011b).

EAEC infections are mostly characterized by watery diarrhea, low fever, and little or no vomiting. However, cases of bloody stools and persistent diarrhea (longer than 14 days) are also recorded (Nataro et al. 1998). Typical symptoms of an EHEC infection include bloody or non-bloody diarrhea, vomiting, and fever. Patients infected with EHEC O104:H4 developed these symptoms after a median incubation time of 8.5 days (Werber et al. 2013). This incubation period is considerably longer when compared to the onset of disease upon infections with O157:H7 (3–4 days) or EAEC 042 (8–18 h) (Nataro et al. 1995; Tarr et al. 2005) (see also Fig. 2). EHEC-associated illness is self-limiting and the majority of patients exhibit spontaneous recovery. The percentage of HUS cases among infected individuals during the 2011 outbreak (22%) was considerably higher than the estimated HUS rate in O157:H7 outbreaks (Gould et al. 2009), suggesting that EHEC O104:H4 was exceptionally virulent. Surprisingly, the development of severe symptoms as hemorrhagic diarrhea and HUS during the 2011 outbreak was shown to steadily increase with age (King et al. 2012; Menne et al. 2012; Kielstein et al. 2013; Soon et al. 2013; Werber et al. 2013).

Long- and short-term follow-up studies investigated outcome of patients after EHEC O104:H4 infection. In pediatric departments, more than two-thirds of patients suffered from renal complications and received different forms of dialysis therapy (hemodialysis, hemofiltration, peritoneal dialysis). Severe neurological

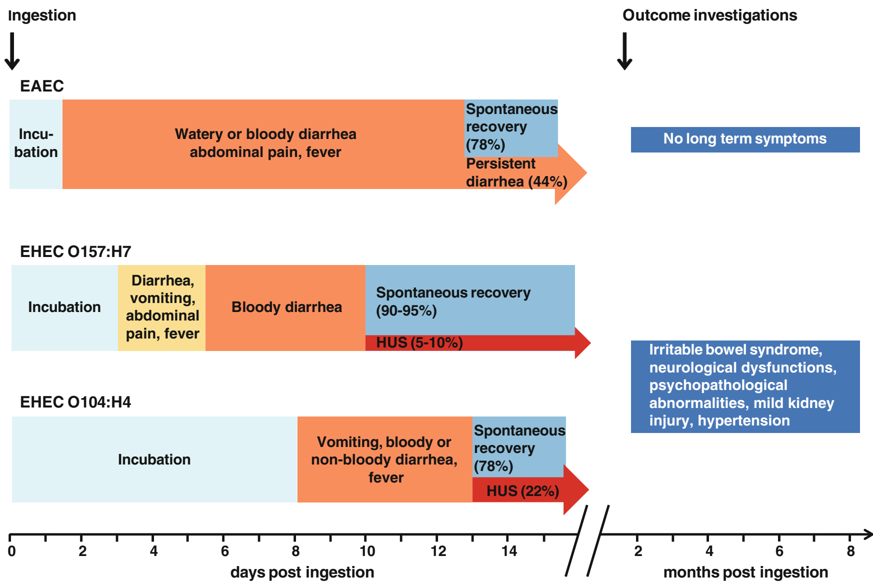

Fig. 2 Clinical course of EAEC, EHEC O157:H7 and EHEC O104:H4 outbreak strain-infections. Short-term manifestations and long-term outcome of patients after EAEC, EHEC O157:H7 and EHEC O104:H4 outbreak strain infections are shown. The clinical course of EAEC is based on numerous strains and studies (Nataro et al. 1998). In contrast to EAEC 042 (Nataro et al. 1995) and EHEC O157:H7 (Tarr et al. 2005), after infection with the O104:H4 outbreak strain (Frank et al. 2011b) incubation time is comparably long and results in HUS in more than 1/5 of all infected patients. Long-term outcome of patients is comparable to disorders observed in patients after EHEC O157:H7 infections (Jandhyala et al. 2013)

complications occurred in 26%. Short-term outcome after 4 months showed a recovery of renal function in 94% and neurological symptoms in 18 out of 23 patients (Loos et al. 2012). Other studies performed in adult patients report that 48% of patients with a severe O104:H4-infection displayed neurological symptoms as disorientation, reduced attention, restlessness, prominent, nervousness, amnestic deficits, aphasia, epileptic seizure, oculomotor disturbance, myoclonus, and headache. Eight months after the outbreak only 3/217 patients still suffered from neurological symptoms (Magnus et al. 2012). Half of the patients with neurological symptoms showed abnormalities within MRI imaging, performed within 20 days after onset of diarrhea. 81% of these are resolved on follow-up investigations (Lobel et al. 2014). Most frequent psychopathological abnormalities in follow-up studies after EHEC O104:H4 infections were feelings of anxiety, formal disorders of thought, disturbances of attention and memory, disturbances of effect, panic attacks, and disorders of drive and psychomotility. Occurrence of these abnormalities was increased with age, family history of heart disease, and higher levels of C-reactive protein (Kleimann et al. 2014). Prospective follow-up of six patients with HUS due to O104:H4 did not show end-stage renal diseases but milder forms of kidney injury including proteinuria (27%), increased serum creatinine (4.4%),

increased cystatin C (47%), and reduced GFR (47%). In 9 out of 36 patients without previous hypertension de novo hypertension occurred (Derad et al. 2016). Additionally, numbers of post-infectious irritable bowel syndromes increased from 9.8% to 23.6% after six months and to 25.3% 12 months after EHEC infection. Incidence of new irritable bowel syndrome was 16.9% (Andresen et al. 2016). In summary, outcome of patients suffering from EHEC O104:H4 infections is similar to patients suffering from O157:H7 diseases (Fig. 2), who presented renal insufficiency, hypertension, psychopathological and neurological disorders and long-term gastrointestinal complications in outcome investigations (Siegler 1994; Siegler and Oakes 2005).

2.5 Predictive and Prognostic Factors

Until recently, clinical trials that address predictive and prognostic factors in EHEC O104:H4 infected patients are rare. Initial investigations show a correlation of higher levels of microRNAs circulating in serum of HUS patients with neurological impairment and thrombocytopenia (Lorenzen et al. 2012). Further studies evaluated CD55 and CD59 expression on peripheral blood cells in EHEC O104:H4 infected patients concerning HUS evolvement. Here, data did not support a role for CD55 and CD59 in HUS development (Dammermann et al. 2013). Parameters reflecting renal perfusion during ultrasound examinations, however, correlated with severity of acute kidney injury in patients after EHEC O104:H4 infection and might have prognostic value within clinical settings (Reising et al. 2016). Further studies in which levels of angiopoietin-2, an antagonistic receptor ligand known to be involved in the development of endothelial dysfunction in HUS, were quantified, showed a predictive relevance for complicated clinical courses in case of early presence of this protein (Lukasz et al. 2015). Moreover, the same group found neutrophil gelatinase-associated lipocalin, a biomarker indicating degree of acute kidney injury, to be significantly increased in patients developing HUS and requiring renal replacement therapy after EHEC O104:H4 infection (Lukasz et al. 2014).

Since patients will continue to have severe disease and complications from EHEC O104:H4 infections, further (prospective) research is necessary to clarify predictive values of mentioned parameters. In particular, studies are needed that evaluate parameters predicting development of HUS and severe long-term disorders.

3 The "Patchwork" Genome Structure of EHEC O104:H4

Early PCR-based genotyping analysis revealed that with respect to virulence gene content the 2011 outbreak strain is a hybrid of EHEC and EAEC (Bielaszewska et al. 2011; Scheutz et al. 2011). Shortly afterward, several next-generation sequencing-based studies further elucidated the "patchwork" genome structure of

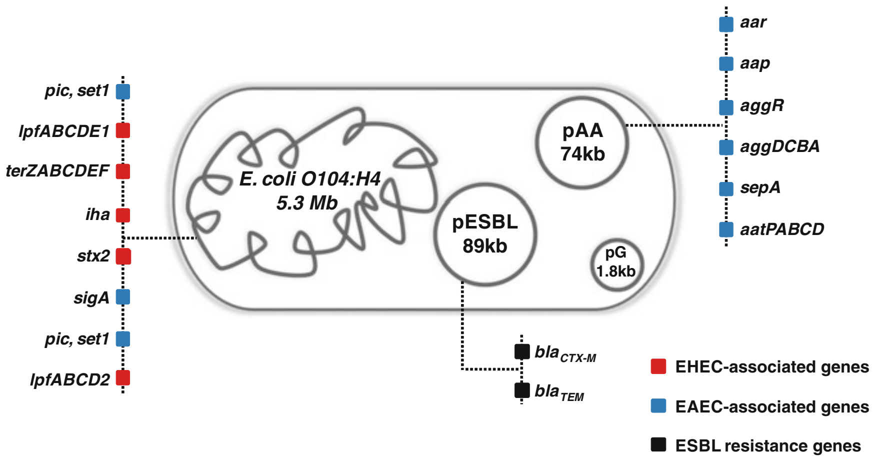

Fig. 3 The virulence-associated loci in the "patchwork" genome of EHEC O104:H4. The EHEC O104:H4 outbreak strain contains both EHEC- (red) and EAEC-associated virulence loci (blue). The additional genetic elements located on the pESBL plasmid (black) mediate the phenotypic resistance against third-generation cephalosporines (Mellmann et al. 2011; Rasko et al. 2011)

EHEC O104:H4 (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Rohde et al. 2011) (Fig. 3), which consists of a chromosome (5.3 Mb) and the plasmids pAA (74 kb), pESBL (89 kb) and pG (1.5 kb small cryptic plasmid).

3.1 The Chromosome

Comparative genomics and phylogenic analysis revealed that the EHEC O104:H4 chromosome has closest sequence relationship to the one of the EAEC strain 55989 (also of serotype O104:H4) and is only distantly related to one of the commonly isolated EHEC strains (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Rohde et al. 2011). However, the 2011 EHEC O104:H4 outbreak strain also harbors a chromosomally integrated bacteriophage encoding Stx, which is not present in the majority of EAEC strains (including the EAEC strain 55989) and is the primary virulence factor involved in EHEC pathogenesis. Stxs are classified as Stx1 or Stx2 type, and further divided into subtypes (Stx1a,c,d and Stx2a–g) (Scheutz et al. 2012), with Stx2a being the one most often associated with the severity of illness and development of HUS (Friedrich et al. 2002). The Stx2a phage in EHEC O104:H4 was found to be closely related to the one in the EHEC O157:H7 strains EDL933 and Sakai and also integrated into the *wrbA* site (Mellmann et al. 2011; Rohde et al. 2011). In contrast to the majority of EHEC strains associated with HUS (Karch et al. 2005), the chromosome of the 2011 outbreak strain does not encode the LEE (locus of enterocyte effacement)

pathogenicity island, which is responsible for the intimate bacterial attachment to the intestinal mucosa and the formation of the characteristic attaching and effacing lesions (Donnenberg et al. 1993; McDaniel et al. 1995).

Other chromosomally encoded EHEC O104:H4 loci linked to EHEC virulence are the operons encoding the long polar fimbriae (Lpf) 1 and 2. The *lpf* operons were extensively characterized in EHEC O157:H7 and reported to contribute to the intestinal adherence and colonization in vivo (Jordan et al. 2004; Torres et al. 2004). In addition, EHEC O104:H4 carries a gene coding for the IrgA homologue adhesin (Iha), which was shown to confer adherence to non-adherent *E. coli* and thus proposed to function as a novel adhesin in EHEC O157:H7 (Tarr et al. 2000). The *lpf* and *iha* loci, however, are also present in the closely related EAEC 55989 strain.

The chromosome of EHEC O104:H4 also carries several EAEC (and *Shigella*) – specific virulence traits. Similar to EAEC strain 55989, the 2011 outbreak strain has two chromosomal copies of *pic*, which encode a SPATE (serine protease autotransporters of *Enterobacteriaceae*) with mucinase activity described to be involved in EAEC pathogenesis by promoting intestinal colonization and mucus hypersecretion (Henderson et al. 1999; Harrington et al. 2009; Navarro-Garcia et al. 2010). Interestingly, *set1AB* coding for the two subunits of Shigella enterotoxin 1 (ShET1) is found on the opposite strand within the *pic* coding region. The *Shigella* ShET1 homologue is able to elicit an immune response (Fasano et al. 1995), but its role in EAEC virulence still remains to be elucidated. Moreover, EHEC O104:H4 encodes SigA, another SPATE which is highly prevalent among EAEC strains (Boisen et al. 2009) and characterized in *S. flexneri* to be cytotoxic to cultured human epithelial cells (Al-Hasani et al. 2009).

The whole genome sequencing revealed the presence of several chromosomal resistance markers in EHEC O104:H4. The 2011 outbreak strain displays tellurite resistance encoded by the gene cluster *terZABCDEF*, which is typically found in EHEC O157:H7 strains but missing in the EAEC strain 55989 (Bielaszewska et al. 2005; Mellmann et al. 2011). The *ter* operon is located in close proximity to *iha* on the TAI (tellurite resistance and adherence conferring island) (Tarr et al. 2000). In addition, the outbreak strain carries also genomic island-encoded resistance determinants to mercury (the *mer* operon), ethidium bromide, sulfonamides, beta-lactams, and tetracyclines (Brzuszkiewicz et al. 2011; Grad et al. 2013).

3.2 The PAA Plasmid

The 2011 EHEC O104:H4 outbreak strain carries a 74 kb pAA virulence plasmid—another characteristic feature of EAEC strains. Different pAA-encoded aggregative adherence fimbriae variants (AAF/I to V) confer the distinct “stacked-brick” adherence of EAEC to cultured human epithelial cells (Nataro et al. 1992; Czeczulin et al. 1997; Bernier et al. 2002; Boisen et al. 2008; Jonsson et al. 2015). Both ex vivo and in vivo experiments suggest that the AAF-mediated adherence is a key step in EAEC pathogenesis (Tzipori et al. 1992; Hicks et al. 1996). The tight

aggregative adherence of the 2011 outbreak strain to cultured cells is conferred by the *aggDCBA* cluster coding for AAF/I (Bielaszewska et al. 2011). In contrast to EHEC O104:H4, the EAEC strain 55989 carries a different pAA plasmid encoding another AAF variant, the AAF/III. Even though there is considerable sequence heterogeneity among the pAA plasmids of these strains, the majority of their virulence-associated features are found conserved (Rasko et al. 2011).

The pAA of EHEC O104:H4 harbors several other EAEC-specific virulence loci. The *aap* gene is coding for the surface protein dispersin, which facilitates proper fimbrial extension from the bacterial surface and thus contributes to EAEC adhesion and intestinal colonization (Sheikh et al. 2002; Velarde et al. 2007). The Aat secretion system, encoded by the *aatPABCD* operon, was characterized as the ABC (ATP-binding cassette) transporter responsible for dispersin secretion out of the bacterial cell (Nishi et al. 2003). The *sepA* locus, which is not shared between EHEC O104:H4 and the EAEC strain 55989, encodes the serine protease SepA. This SPATE is the major extracellular protein of *S. flexneri* and a *sepA* deletion was associated with reduced mucosal inflammation in vivo (Benjelloun-Touimi et al. 1995). The *aggR* gene is coding for the EAEC master virulence gene regulator AggR. AggR is an AraC-type transcriptional activator and regulates the expression of AAF/I, dispersin, Aat and SepA, as well as other pAA- and chromosomally encoded loci (Nataro et al. 1994; Sheikh et al. 2002; Nishi et al. 2003; Morin et al. 2013; Berger et al. 2016). Recently, it was shown that pAA of EAEC strain 042 also encodes Aar (AggR-activated regulator), which negatively regulates AggR expression (Santiago et al. 2014). The *aar* locus is also found transcribed in the pAA of EHEC O104:H4 (Berger et al. 2016).

3.3 The pESBL Plasmid

The extended-spectrum beta-lactamase (ESBL) phenotype of the EHEC O104:H4 outbreak strain is conferred by the presence of an 89 kb plasmid (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Rohde et al. 2011). pESBL is a conjugative IncI1 (incompatibility group I1) plasmid with high sequence similarity to the pEC_Bactec plasmid isolated from a horse with arthritis (Smet et al. 2010). pESBL carries the genes $bla_{\text{CTX-M-15}}$ and bla_{TEM} coding for the beta-lactamases CTX-M-15 and TEM-1, which hydrolyze and confer resistance to penicillins and extended-spectrum cephalosporins (Shaikh et al. 2015).

3.4 Evolution of EHEC O104:H4

Phylogenetic analyses and comparative genomics based on whole genome sequencing data led to the formulation of two hypotheses about the evolution of the EHEC O104:H4 outbreak strain. Due to its high genome sequence similarity to the

EAEC strain 55989 and the missing LEE pathogenicity island, which is characteristic to some EHEC strains, it was hypothesized that the 2011 outbreak strain has originated from an EAEC progenitor that acquired an Stx2 phage (Brzuszkiewicz et al. 2011). Since the loss of Stx-encoding genes has been described as a frequent event both in vitro and in vivo (Bielaszewska et al. 2007a, b), another evolutionary model was proposed, in which the 2011 outbreak strain and the EAEC 55989 strain have evolved from a common Stx producing ancestor with an EAEC genotype (Mellmann et al. 2011). Whole genome phylogenetic comparison of 53 *E. coli* and Shigella strains revealed that the 2011 outbreak strain was present within the distinct clade formed by the analyzed EAEC strains of O104:H4 serotype and thus further supported the evolutionary model of Stx phage acquisition by an EAEC ancestor (Rasko et al. 2011). Similarly, the EHEC strain EHEC O157:H7 strain is believed to have evolved from enteropathogenic *E. coli* (another diarrheagenic *E. coli* pathotype) of serotype O55:H7 through a series of horizontal gene transfer events including the acquisition of Stx1- and Stx2-encoding bacteriophages (Feng et al. 1998).

There are several reports describing that Stx phages can lysogenize *stx*-negative *E. coli* strains (Schmidt et al. 1999; Toth et al. 2003; Mellmann et al. 2008a). Recently, it was shown that regions characteristics to the Stx-encoding phage isolated from a 2011 outbreak strain were also present in several bovine EHEC isolates. Moreover, the phage isolated from the outbreak strain and one from the bovine EHECs were able to form lysogens on an *stx*-negative EAEC O104:H4 strain by integrating into its *wrbA* locus. This led to the conclusion that the 2011 EHEC O104:H4 outbreak strain could have evolved by acquisition of an Stx phage from a bovine origin (Beutin et al. 2013). However, only transient lysogens were observed in a study in which strains belonging to different diarrheagenic *E. coli* pathotypes, among them EAEC, were infected by a panel of Stx2 bacteriophages suggesting that the event of a stable Stx phage acquisition is rather uncommon (Tozzoli et al. 2014).

The evolutionary path of the 2011 *E. coli* outbreak strain from either a hypothetical EAEC strain 55989 progenitor or Stx producing O104:H4 progenitor is assumed to involve several other horizontal gene transfer events, such as the exchange of an AAF/III-encoding plasmid for the pAA plasmid coding for AAF/I and the acquisition of pESBL (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011). Interestingly, the ESBL phenotype is a characteristic feature only of the 2011 EHEC O104:H4 outbreak strain and not present in other sequenced *stx*-positive O104:H4 isolates, suggesting that the plasmid might have recently been acquired by the outbreak strain or have been unstable in the other genetic backgrounds (Rasko et al. 2011; Ahmed et al. 2012; Grad et al. 2013).

4 Virulence Factors and Mechanisms of EHEC O104:H4

4.1 Shiga Toxin—The Cardinal Virulence Factor of EHEC

The link between Stx production and the development of hemorrhagic colitis and *E. coli*-associated HUS was established in the 80 s (Karmali et al. 1983; Riley et al. 1983). Stxs are AB_5 cytotoxins composed of a 32 kDa enzymatically active A subunit noncovalently associated with five identical 7.7 kDa B subunits. The A subunit is a N-glycosidase, while the B pentamer mediates the binding of the Stx holotoxin to a eukaryotic membrane glycosphingolipid receptor globotriaosylceramide (Gb3Cer) (Donohue-Rolfe et al. 1991). The amount of Gb3Cer present on the host cell surface appears to correlate with the clinical complications of EHEC infections. The highest Gb3Cer content is found in the microvascular endothelium of the kidney, as well as in the colonic microvascular endothelia and the endothelial vasculature of the cerebellum (Müthing et al. 2009; Bauwens et al. 2013), and is thus consistent with the observed renal pathology, hemorrhagic colitis and neurologic symptoms, respectively (Jacewicz et al. 1999; Ren et al. 1999; O'Loughlin and Robins-Browne 2001). A recently published report suggests that Stx is capable of direct injury of erythrocytes at certain developmental stages of erythropoiesis (Betz et al. 2016). Stx-mediated damage of erythrocyte progenitor cells may therefore contribute to anemia observed in EHEC-caused extraintestinal complications and furthermore explains the huge demand for blood transfusion during onset of HUS. External Stx is not cytotoxic to macrophages, but it is stimulating the release of pro-inflammatory cytokines (Tesh et al. 1994; van Setten et al. 1996), which increase the susceptibility of endothelial cells to Stx by enhancing Gb3 synthesis and expression on the membrane (Louise and Obrig 1991; van de Kar et al. 1992). However, both cell-free Stx and Stx produced by ingested bacteria are cytotoxic to the phagocytic single-celled protozoan *Tetrahymena thermophila* (Lainhart et al. 2009; Stolfa and Koudelka 2012). Bacterial Stx production was shown to function as a defense mechanism against predators and to confer a survival advantage over those bacteria that do not encode Stx (Lainhart et al. 2009), which suggests that Stx toxicity to humans may have evolved accidentally.

Upon binding to the Gb3Cer receptor, Stx is internalized in a clathrin-dependent or independent manner and transported by the retrograde pathway from the endosomes via the Golgi apparatus to the endoplasmic reticulum (ER) (Sandvig et al. 1992; Romer et al. 2007; Sandvig et al. 2010). The Stx holotoxin dissociates in the reducing environment of the ER and the enzymatically active A subunit is translocated to the cytosol, where the A1 portion depurinates an adenine of the 28S rRNA (Obrig et al. 1987; Endo et al. 1988; Lee et al. 2016) and thus irreversibly inhibits protein synthesis and induces cell death, inflammatory response or activation of the ribotoxic stress response (Obrig et al. 1988; Thorpe et al. 2001). From the cytosol, Stx can reach the nucleus and a body of evidence has been provided that Stx (like other ribosome-inactivating proteins) is able to remove adenine moieties not only from rRNA in the cytosol, but can also efficiently depurinate

DNA in the nucleus. This effect leads to DNA damage observed in cell cultures and is likely to result from direct DNA-damaging activities and/or indirect DNA repair inhibition (Brigotti et al. 2002; Sestili et al. 2005) indicating the existence of more than one retrograde pathway.

Animal experiments have been successfully employed to study EHEC O104:H4 pathogenesis in vivo. Infection of germ-free mice with the 2011 outbreak strain resulted in the development of acute renal tubular necrosis [ATN; (Al Safadi et al. 2012)]. Such renal damage was previously described in EHEC O157:H7 infection experiments of streptomycin-treated mice and mainly attributed to Stx2 production (Wadolkowski et al. 1990). Interestingly, mice infected with EHEC O104:H4 were characterized by a delayed development of ATN in comparison to the ones infected with EHEC O157:H7 (at 13–15 days post-infection vs. 5 days (Wadolkowski et al. 1990; Al Safadi et al. 2012), which is consistent with the observed longer incubation time during the 2011 German outbreak (Frank et al. 2011b). Another study with EHEC O104:H4 and *stx*-negative variants demonstrated that the 2011 outbreak strain causes weight loss and mortality in ampicillin-treated mice and that Stx2 is the key virulence factor responsible for the observed pathogenesis (Zangari et al. 2013). Moreover, similar observation linking Stx2 production of EHEC O104:H4 to disease progression was made in two rabbit models (Zangari et al. 2013; Munera et al. 2014).

The *stx2* operon is located downstream of the phage late genes and its expression is solely dependent on phage induction and the resulting transcription from the phage late promoter (Karch et al. 1999; Wagner et al. 2001). Therefore, the release of free Stx2 from the bacteria cells is mainly attributed to phage-mediated lysis (Waldor and Friedman 2005). In addition, Stx2 is detected together with other virulence factors in outer membrane vesicles (OMVs) shed by EHEC (Kolling and Matthews 1999; Kunsmann et al. 2015; Bielaszewska et al. 2017). EHEC OMVs were shown to bind to, get internalized by and be cytotoxic to human intestinal epithelial cells (EHEC O104:H4 and O157 OMVs) and brain and renal microvascular endothelial cells (*E. coli* O157 OMVs; (Kunsmann et al. 2015; Bielaszewska et al. 2017). Moreover, the Stx2 was found to be the main factor for the observed OMV cytotoxicity in EHEC O104:H4 (Kunsmann et al. 2015). Thus, OMVs provide an alternative means for bacterial Stx2 release. Even though this mechanism would allow for a Gb3 independent cellular uptake of Stx2, it was recently shown that similar to free Stx2, after liberation from OMVs interaction of the OMV-delivered Stx2 with the Gb3 receptor is essential for its retrograde transport and cytotoxicity (Bielaszewska et al. 2017).

Interestingly, the EHEC O104:H4 outbreak strain was shown to produce in culture and in cell culture infection experiments significantly less Stx2 than the prototypical EHEC O157:H7 strains EDL933 and Sakai. (Laing et al. 2012). Moreover, the Stx translocation rates across an epithelial monolayer during microaerobic human colonic infection were found significantly lower in O104:H4 than that of O157:H7 (Tran et al. 2014). Thus, one could argue that the enhanced virulence of the EHEC O104:H4 in comparison to typical EHEC strains could not be accredited to increased Stx2 expression and transcytosis. However, upon induction with mitomycin C, the 2011 outbreak strain was shown to produce in

culture significantly more Stx2 than both EHEC O157:H7 strains EDL933 and Sakai (Laing et al. 2012). It remains to be further elucidated if the conditions in the human gut could induce a similar response in EHEC O104:H4 to that obtained with mitomycin C in vitro.

4.2 The Importance of the PAA Plasmid to EHEC O104:H4 Virulence

EHEC O104:H4 expresses pAA-encoded AAF/I and displays tight "stacked-brick" adherence both to cultured Hep2 epithelial cell and cecal mucosa in germ-free mice (Bielaszewska et al. 2011; Al Safadi et al. 2012). This adherence pattern is characteristic for EAEC strains (Nataro et al. 1992; Tzipori et al. 1992) but unusual for EHEC, which often colonize in single layers displaying LEE-mediated intimate attachment to the epithelia (Donnenberg et al. 1993). Thus, the increased virulence of the 2011 outbreak strain was hypothesized to be attributed to the AAF/I-mediated intestinal adherence, which could facilitate the absorption of Stx from the gut to the systemic circulation (Bielaszewska et al. 2011).

EHEC O104:H4 can sporadically lose the pAA plasmid during the course of the disease. Interestingly, pAA loss was correlated with a significantly reduced HUS progression in patients, which speaks for an attenuated virulence of the pAA-negative isolates (Zhang et al. 2013). In contrast, the pAA plasmid was found not to be essential for the colonization and intestinal pathology in a rabbit model (Munera et al. 2014). Nevertheless, it was shown that the AAF/I indeed contribute not only to the tight adherence of the outbreak strain but also to translocation of the Stx2 across an epithelial cell monolayer, further suggesting that the pAA plasmid has a crucial importance to EHEC O104:H4 virulence (Boisen et al. 2014). Mutant analysis revealed that disruption of the actin cytoskeleton and the reduction of trans-epithelial resistance, which accompany EHEC O104:H4 infection of polarized T84 cells, depends on AggR and AggA (the major AAF/I subunit) but not on Stx2. On the other hand, the prototype EHEC O157:H7 failed to disrupt the polarized T84 cell monolayer and did not lead to significant levels of Stx2 transport from the apical to the basolateral side of the cells. Moreover, the expression of AAF/I alone in an *E. coli* K12 strain was found to enhance the translocation of exogenous Stx2 across the epithelial monolayer and thus demonstrated a direct effect of the fimbriae on epithelial permeability. Interestingly, the inflammatory response to the outbreak strain in the T84 system was dependent on both Stx production and the EAEC virulence factors AggR, AggA, and SepA (Boisen et al. 2014).

A recent study sheds light on the EHEC O104:H4 pAA transcriptional organization and gene regulation (Berger et al. 2016) (Fig. 4). The pAA transcriptome was analyzed using differential RNA-seq that allows for the high throughput mapping of transcription start sites (TSS; 5′-PPP ends of primary transcripts) and processing sites (PS, 5′-P ends of processed transcripts) (Sharma and Vogel 2014) (Fig. 4, Track I and II). TSS were detected for the majority of pAA-encoded

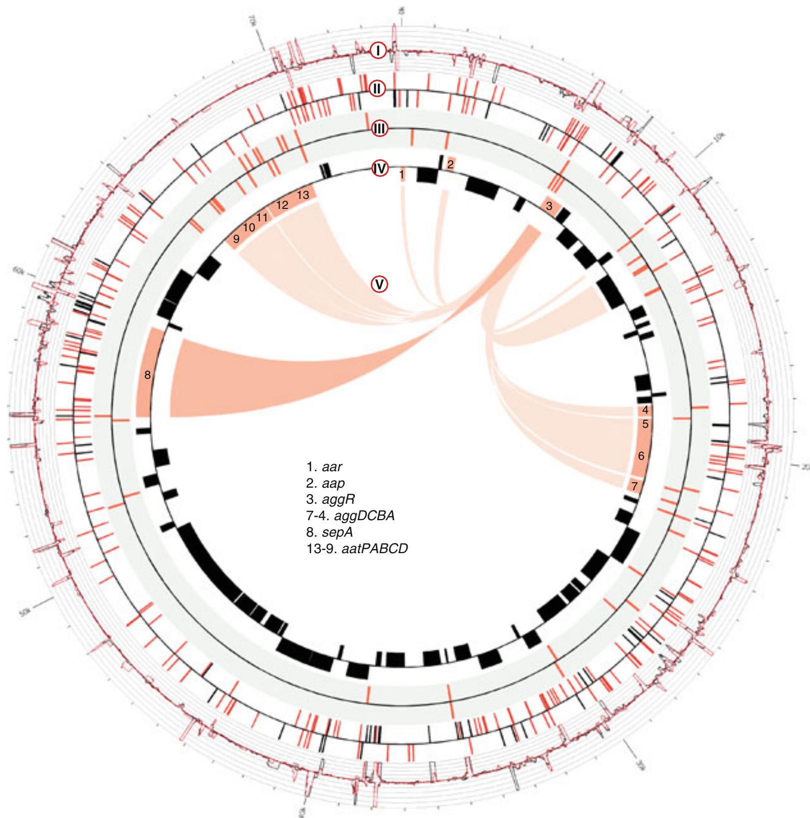

Fig. 4 The transcriptional organization and gene regulation in the pAA plasmid of EHEC O104:H4. Track I: Differential RNA-seq data on the pAA plasmid. The graphs represent the normalized number of pAA reads mapped per nucleotide in terminator exonuclease (TEX) + (red) and TEX- (black) libraries (y-axis = abundance relative score, max. 100). Track II. TSS (red) and PS (black) candidates annotated by dRNA-seq. Track III. Computationally predicted AggR (red) binding sites. Track IV. Annotated ORFs in pAA. Virulence-associated genes are colored in orange and a gene legend is given in the middle of the circle. Track V. AggR regulon. The AggR regulon is based on Morin et al. 2013 (light orange) and Berger et al. 2016 (dark orange). The figure was modified from Berger et al. 2016

virulence genes, suggesting that they were expressed at least on the mRNA level in EHEC O104:H4 (Berger et al. 2016). Interestingly, operon-internal TSS were detected within the AAF/I gene cluster, which could allow for the transcriptional uncoupling of the secreted AAF/I protein subunits AggA and AggB from the outer membrane usher protein AggC and periplasmic chaperone AggD. Moreover, numerous antisense RNA candidates mapped in this analysis were found to be associated with virulence genes, suggesting that also post-transcriptional regulation may be important for their appropriate expression. In addition, a computational-based screen for AggR binding sites followed by experimental

validation revealed that the EHEC O104:H4 pAA-encoded serine protease SepA is a new member of the AggR regulon (Berger et al. 2016), which was previously characterized in the EAEC strain 042 (Morin et al. 2013) (Fig. 4, Track III and IV). The AggR-dependent regulation coordinates SepA expression with other important EAEC virulence factors and may be a hint for a role of the serine protease in EHEC O104:H4 pathogenicity (Berger et al. 2016).

4.3 *Other Factors of Importance to EHEC O104:H4 Virulence*

Along with Stx2 and pAA-encoded factors, several other determinants have been suggested to play an important role in the virulence of the 2011 outbreak strain. EHEC O104:H4 encodes the serine proteases Pic, SigA, and SepA—a number and combination of SPATEs which has been rarely reported in EAEC strains (Boisen et al. 2009). The chromosomally–encoded Pic and SigA, but not the pAA plasmid, were found to be critical for the EHEC O104:H4 colonization and disease severity in an infant rabbit model. Surprisingly, SigA release rather than its protease activity contributed to EHEC O104:H4 pathogenicity (Munera et al. 2014).

Lpf1 and Lpf2 were shown to be important virulence factors in EHEC (Jordan et al. 2004; Farfan et al. 2013). Therefore, it was also relevant to address their role in the adhesion and colonization of EHEC O104:H4. An *lpf2* deletion mutant was characterized only by a significant reduction in its ability to colonize polarized cells. The loss of functional Lpf1, however, resulted in a reduced ability of O104:H4 to adhere to both polarized and non-polarized cells, as well as to form a stable biofilm. In addition, the *lpf1* mutant showed a reduced capacity to colonize the cecum and large intestines in a murine model, thus suggesting that even in the presence of AAF/I, Lpf1 is an important colonization factor of EHEC O104:H4 (Ross et al. 2015).

In contrast to EHEC O157:H7, the outbreak strain is a strong biofilm producer in vitro and in vivo. Its extensive in vivo biofilm formation was found to correlate with an enhanced *stx2* and other virulence gene expression and increased kidney damage in a germ-free mouse model (Al Safadi et al. 2012). The second messenger c-di-GMP stimulates the production and secretion of the biofilm-associated polysaccharide PGA, as well as activates the expression of the positive transcriptional biofilm regulator CsgD, which in turn is regulating the expression of the extracellular matrix component curli (Hengge 2009). Curli fibers are involved in adhesion and biofilm formation, and their expression is more pronounced below 30 °C (Barnhart and Chapman 2006). Typical for EAEC genotypes, the 2011 outbreak strain was shown to produces high levels of c-di-GMP. Moreover, EHEC O104:H4 and the EAEC strain 55989 displayed high CsgD expression levels and strong curli production not only at 28 °C but also at 37 °C (Richter et al. 2014). In contrast to EAEC 55989, however, the outbreak strain produced no cellulose (Richter et al. 2014), which is known to counteract the adhesive and pro-inflammatory properties of curli fibers (Wang et al. 2006). Interestingly, the

closely related HUSEC041 strain (Mellmann et al. 2008b) was characterized by low CsgD and curli synthesis at 37 °C and high cellulose production. Thus, the EHEC O104:H4 unique biofilm-related properties have been proposed to additionally contribute to its enhanced virulence (Richter et al. 2014).

5 Treatment

In general, one has to differentiate between treatment of EHEC infections and HUS, even though both lack a causative therapy. Current guidelines recommend measures preventing EHEC infected persons from developing HUS after the onset of diarrhea. During HUS conventional supportive treatment (see below) is state of the art (Wurzner et al. 2014). Evaluation of treatment strategies in HUS patients infected with O104:H4, however, revealed results contrary to the current guidelines in particular by calling into question benefits from plasmapheresis and harmful effects of antibiotic treatment (Menne et al. 2012).

5.1 Supportive and Symptomatic Therapy

Symptomatic treatment options during EHEC triggered HUS have diversely been discussed. Trials evaluating fresh frozen plasma transfusion (Loirat et al. 1988; Rizzoni et al. 1988), heparin (Vitacco et al. 1973) with or without urokinase (Loirat et al. 1984) or dipyridamole (Van Damme-Lombaerts et al. 1988) and steroids as anti-inflammatory substances (Perez et al. 1998) in young children with post-diarrheal HUS did not show an outcome superior to classical supportive therapy.

Recent recommendations deal with supportive therapy as state of the art including fluid management, treatment of hypertension, renal replacement and ventilatory support (Bitzan et al. 2010; Wurzner et al. 2014). In this context, early volume expansions can have positive effects on both, short- and long-term disease outcomes (Ardissino et al. 2016). Early recognition of and parenteral volume expansion during EHEC O157:H7 infections have been associated with attenuated renal injury failure (Ake et al. 2005). In an observational cohort study, Hickey et al. determined that intravenous fluid therapy during the pre-HUS phase prevents oligoanuric HUS significantly (Hickey et al. 2011). Renal replacement therapy, however, is recommended to be performed according to clinical manifestations of HUS as oligo- or anuria. Peritoneal dialysis and hemodialysis are equivalent options in these cases, preferring peritoneal dialysis in infants <4 years and hemodialysis in older children and patients receiving higher amounts of blood products as platelets or PRBC (Bitzan et al. 2010). Best clinical practices involve rapid and accurate clinical and microbiological identification of infected patients, volume expansion, and support of the intestinal and extraintestinal complications that can ensue during acute enteric infection and associated HUS (Tarr 2009).

5.2 Antibiotics

The use of antibiotics during EHEC infections is currently not recommended in most countries, as it is believed to increase the risk for HUS. However, the majority of studies leading to that assumption are of retrospective nature and limited to a surprisingly few antibiotics. In addition, none of them systematically excludes antibiotics, that are known to induce Stx production in vitro, e.g. trimethoprim-sulfamethoxazole (Karch et al. 1986; Proulx et al. 1992; Wong et al. 2000, 2012). Moreover, a meta-analysis of nine high-quality studies did not show a higher probability of HUS development upon administration of antibiotics (Safdar et al. 2002). A recent meta-analysis of seventeen reports also showed no significantly increased risk of developing HUS associated with antibiotic administration. However, including only the studies (n = 5) which were with low risk of bias and meeting an acceptable definition of HUS, a significant association was reached (Freedman et al. 2016). Notably, also these five studies did not omit antibiotics which induce Stx production in vitro.

The experience with the hypervirulent strain EHEC O104:H4 outbreak strain made it very clear that a rational approach on the question which antibiotics may be beneficial in EHEC infections is of utmost importance, especially as the capacities for symptomatic treatment may become rapidly limited, if a future outbreak is just an order of magnitude larger. Therefore, in order to finally obtain an unbiased picture of the usefulness of antibiotics in the therapy of EHEC infections (i) the effects of inhibitory and sub-inhibitory concentrations of antimicrobial substances on Stx production should be determined in vitro and (ii) only those antimicrobial substances that do not stimulate Stx production in vitro should be afterward tested in vivo, ideally in prospective, randomized, placebo-controlled studies.

In vitro data for the effects of antibiotics on Stx production is already available, even though it would be desirable to systemically include more clinically relevant bacterial genetic backgrounds in the future (Karch et al. 1986; Kimmitt et al. 2000; McGannon et al. 2010; Bielaszewska et al. 2012; Corogeanu et al. 2012). According to these studies, antibiotics that inhibit bacterial transcription and translation appear to be very promising candidates for therapy. In addition, the translational inhibitor azithromycin was shown to be effective in reducing the elevated Stx levels detected in presence of phage sensitive *E. coli*, which may play an underestimated role in overall toxin production and the individual course of illness during EHEC infections (McGannon et al. 2010). Even though not routinely recommended by the authorities, antibiotic therapy administered during the 2011 outbreak also proved to be beneficial. A retrospective case-controlled study on the German EHEC O104:H4 outbreak showed that an aggressive antibiotic therapy (meropenem + ciprofloxacin ± rifaximin) applied once the disease had progressed to HUS was associated with significantly lower mortality rate, duration of shedding and seizure frequency (Menne et al. 2012). Moreover, azithromycin treatment resulted in a lower frequency of long-term carriage of the outbreak strain in patients (Nitschke et al. 2012).

5.3 Eculizumab

The use of a group of monoclonal antibodies, namely eculizumab, targeting the complement component 5 (C5) was controversially discussed. Some studies found eculizumab to have beneficial effects on the recovery from O104:H4-associated HUS during the French and German outbreak (Greinacher et al. 2011; Delmas et al. 2014). Due to this treatment, neurological disorders and renal function but also laboratory parameters as hemoglobin, platelets, lactate hydrogenase could be rapidly improved in HUS patients infected with the O104:H4 outbreak strain or O157:H7 (Greinacher et al. 2011; Lapeyraque et al. 2011; Delmas et al. 2014; Saini et al. 2015). On the other hand, different studies could not prove any benefit of eculizumab therapy compared to conventionally performed therapeutic regimens like supportive care, therapeutic plasma exchange, hemodialysis or antibiotic treatment (Kielstein et al. 2012; Menne et al. 2012; Ullrich et al. 2013), calling into question this new therapeutic approach. Even though, short- and long-term outcome in some critically ill patients with eculizumab could be improved, demonstrating no obvious side effects after application, further randomized controlled trials are needed before a beneficial effect can be assigned to this therapeutic agent.

5.4 Probiotics

Although probiotics do not provide therapeutic options in the acute phase of disease, they might have a relevant preventive function. Multiple studies have been performed verifying the beneficial effect of probiotics in vitro and in experimental animal models. Promising candidate as *Lactobacillus* and *Bifidobacterium* spp. showed protecting effects and decreasing cytotoxic activity after co-incubation with EHEC O157:H7 in vitro (Mogna et al. 2012; Kakisu et al. 2013) and in vivo (Asahara et al. 2004; Eaton et al. 2011; Chen et al. 2013), most likely mediated by lactic acid production, which directly correlates to bacteriostatic/bactericidal effects (Ogawa et al. 2001) and level of *stx2a* expression in EHEC O157:H7 (Carey et al. 2008). Recent studies concentrate on different *E. coli* strains mediating protective activity, namely *E. coli* 1307 (Reissbrodt et al. 2009) and *E. coli* strain Nissle 1917 (EcN). EcN, first used in 1917, is one of the most investigated probiotics, known to significantly improve various dysfunctions within the intestinal tract as e.g. ulcerative colitis and inflammatory bowel disease (Kruis 2004). Antagonistic effects of EcN could be proved for the mouse intestine colonized by EHEC O157:H7 (Leatham et al. 2009). In addition, in an investigation including two EHEC O104: H4 isolates derived from the German outbreak EcN showed a very efficient antagonistic activity regarding adherence of these pathogenic strains to human gut epithelial cells, their growth, and their Stx2 production in vitro (Rund et al. 2013), which confirms that commensal *E. coli* strains can provide a barrier to infection by intestinal pathogenic *E. coli* including the O104:H4 outbreak strain.

5.5 Stx Receptor Analogs and Stx Neutralizing Molecules

Different agents, which imitate Stx receptor properties, can reduce the amount of cellular bound Stx. Receptor analogs consisting of or harboring the Gb3 trisaccharide were shown to bind Stx in the circulation, to exert neutralizing effects in vitro and to significantly reduce brain damage in animal models after application of a fatal dose of EHEC O157:H7 (Kitov et al. 2000; Nishikawa et al. 2002; Mulvey et al. 2003; Watanabe et al. 2004; Nishikawa et al. 2005). Synthetic Stx receptors were effective in vitro but could not prove this promising effect in a multicentre randomized placebo-controlled clinical trial in children aged 6 months to 18 years with diarrhea-associated HUS (Trachtman et al. 2003). Other strategies concentrated on constructing a recombinant bacterium that displayed a Stx receptor mimic on its surface. High efficiency in adsorption and neutralizing Stx were shown in vitro and mice were completely protected from consequences of Stx producing *E. coli* infections (Paton et al. 2000, 2001). There are several experimental approaches concentrating on partially cell-permeable agents neutralizing Stx. MMA-tet protected mice from fatal doses of EHEC O157:H7 after oral application and did not affect vesicular transport mechanisms (Tsutsuki et al. 2013). Intravenous administration of the cell-permeable peptide TVP in animals resulted in the absence of acute kidney injury and reduction of thrombocytopenia, but did not alter anemia (Stearns-Kurosawa et al. 2011). Two peptides TF-1 and WA-8, which specifically block the binding of Stx2 to target cells, protected mice from toxicity by significantly decreasing the concentration of Stx2 in the bloodstream (Li et al. 2016). Small molecules, inhibiting retrograde toxin trafficking from the early endosomes to the trans-Golgi network, showed first promising protecting effects in vitro (Stechmann et al. 2010; Noel et al. 2013).

5.6 Phages

Another therapeutic concept concentrates on controlling Stx producing *E. coli* via lytic phages, specifically reducing their absolute number. Several phages have been investigated up to now, showing promising results in reduction of EHEC O157:H7 and other pathogenic serogroups in vitro, on surfaces, fruits, vegetables, beef, and in milk (Abuladze et al. 2008; Niu et al. 2009; Sharma et al. 2009; Alam et al. 2011; Patel et al. 2011; Viazis et al. 2011; Carter et al. 2012; Ferguson et al. 2013; Hudson et al. 2013; McLean et al. 2013; Liu et al. 2015). In vivo experiments in animal models could mostly confirm these findings showing less disease complications and reduced shedding after phage therapy (Tanji et al. 2005; Raya et al. 2006; Sheng et al. 2006; Rozema et al. 2009; Rivas et al. 2010; Coffey et al. 2011). These effects could be observed even in intestinal pathogenic *E. coli* showing a high profile of antibiotic resistances (Viscardi et al. 2008). Candidate therapeutic phages

efficiently lysing the EHEC O104:H4 outbreak strain could be identified by Merabishvili et al. (Merabishvili et al. 2012). Nevertheless, in vivo studies are still pending until these phages can be considered a therapeutic option in the future.

6 Conclusion

In 2011 EHEC O104:H4 caused the largest EHEC outbreak in German history and the highest incidence rate of EHEC-related HUS ever recorded worldwide. The highly aggressive strain carries virulence loci characteristic to both EHEC and EAEC and showed therefore not only Stx production and but also an aggregative adherence phenotype. The dimensions and severity of the 2011 outbreak demonstrated the catastrophic potential of this rare combination of pathogenic traits. In addition, not knowing the natural reservoir of the strain makes it harder to minimize the risk of future exposure to EHEC O104:H4. The major challenges remain improving diagnostic speed and treatment. The latter is of utmost importance, as EHEC infections are one of the few bacterial infections for which still no causative, but only a few symptomatic therapies exist. Moreover, there is the danger that new strains of similar or even greater pathogenic potential may arise in the future, which could cause even larger outbreaks or higher incidence rates of HUS. The handling of an EHEC outbreak larger than the one in 2011 in Germany solely on the basis of currently available therapies will become problematic even in developed countries with state of the art health care system.

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Extraintestinal Pathogenic *Escherichia coli*

Dvora Biran and Eliora Z. Ron

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Abstract Extraintestinal pathogenic *E. coli* (ExPEC) present a major clinical problem that has emerged in the past years. Most of the infections are hospital or community-acquired and involve patients with a compromised immune system. The infective agents belong to a large number of strains of different serotypes that do not cross react. The seriousness of the infection is due to the fact that most of the infecting bacteria are highly antibiotic resistant. Here, we discuss the bacterial factors responsible for pathogenesis and potential means to combat the infections.

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1 Introduction

Although most strains of *Escherichia coli* are commensals and abundant, many strains are virulent. In addition to the well-established role of *E. coli* as the causative agent of intestinal infections, many virulent strains cause extraintestinal infections.

The importance of ExPEC is increasing rapidly because they are abundant and are highly resistant to antibiotics. Many of the ExPEC infections are associated with immunodeficiency due to very young age (neonatal), old age, chemotherapy, or diseases that weaken the immune system, such as HIV. Thus, as a human pathogen, ExPEC are the leading causative agents in hospital- and community-acquired infections (healthcare-associated infections). According to the WHO (Healthcare-associated infections FACT SHEET), "hundreds of millions of patients are affected by health care-associated infections worldwide each year, leading to significant mortality and financial losses for health systems. Of every 100 hospitalized patients at any given time, 7 in developed and 10 in developing countries will acquire at least one health care-associated infection." The estimated cost of treating healthcare-associated infections is about 20 billion US$ a year.

ExPEC bacteria are involved in infections of humans and farm animals. They are often classified as APEC (avian pathogenic *E. coli*), UPEC (*E. coli* causing urinary tract infections = UTI), NMEC (neonatal meningitis-causing *E. coli*), or septicemic. However, although this classification is sometimes convenient, it is actually meaningless because there is much overlap between the groups (Ron 2006). Several examples include APEC strains, such as *E. coli* serotype O2 which are a frequent cause of UTI; *E. coli* serotype O18 that is involved in avian colisepticemia and human newborn meningitis (Ewers et al. 2007; Krishnan et al. 2015; Nicholson et al. 2016; Tivendale et al. 2010) and UPEC strains often become septicemic. The similarities between the human and animal strains can also be characterized at the genomic level (Bauchart et al. 2010; Maluta et al. 2014; Zhu Ge et al. 2014) and multilocus sequence typing (MLST) of *E. coli* O78 strains indicate that several isolates from newborn meningitis cluster with avian septicemic isolates (Adiri et al. 2003). The similarity between ExPEC strains involved in animal infections and human infections raises the possibility of zoonosis. This possibility is difficult to prove, but it should certainly be considered especially for the transfer of antimicrobial-resistant ExPEC through contaminated food (Manges 2016).

In a few cases where host specificity was documented, it appears to involve specificity of adherence. Such specificity can be shown in clinical isolates of *E. coli* serogroup O78—human intestinal strains produce the human-specific adherence fimbria CFA/I that bind specifically to intestinal epithelia (Buhler et al. 1991; Cheney and Boedeker 1983), isolates from septicaemia of lambs produce the P, S, and F1C adhesins (Dozois et al. 1997) or the K99 fimbriae (E. Z. Ron. Unpublished), and some O78 isolates from avian colispeticemia code for avian-specific fimbriae (AC/I pili, belonging to the group of S-fimbriae) (Babai et al. 1997, 2000; Dobrindt et al. 2001; Yerushalmi et al. 1990).

Here, we will discuss ExPEC strains and the genetic and physiological factors that promote the virulence.

2 Infections Involving ExPEC

2.1 Avian Colisepticemia

This is an important disease in poultry leading to losses of millions each year to the poultry industry. This disease is characteristic for birds under stress—high temperature, high humidity, or mild viral infections, even due to vaccinations. The disease starts from the upper respiratory tract and the bacteria enter the bloodstream, are dispersed in the body and infect vital organs. This infection involves high morbidity and mortality. The majority of infections (about 80%) are caused by *E. coli* serotypes O1, O2, and O78 but many additional serotypes were shown to be involved (Cordoni et al. 2016; Dho-Moulin and Fairbrother 1999; Dziva et al. 2013; Huja et al. 2015; Mangiamele et al. 2013; Mellata et al. 2009; Nicholson et al. 2016; Rodriguez-Siek et al. 2005; Sola-Gines et al. 2015).

2.2 Veterinary Infections

ExPEC are the cause of several diseases of calves and lambs. The bacteria infect the newborns and cause a lethal septicemia (Ansari et al. 1978; Duff and Hunt, 1989; Kjelstrup et al. 2013). Apparently, these diseases are not of major veterinary impact.

2.3 Neonatal Meningitis

NMEC (Neonatal meningitis-causing *E. coli*) are the major Gram-negative pathogens associated with meningitis in newborn infants (Czirok et al. 1977; Milch et al. 1977; Wijetunge et al. 2015a, b). This group includes several serotypes such as O1, O18 (Wijetunge et al. 2015a, b), and O78 (Czirok et al. 1977; Milch et al. 1977). Although quite rare (1 per 1000 births in developing countries and 1 per 10,000 in developed countries) it is severe, as it involves a very high mortality rate.

2.4 Urinary Tract Infections (UTI)

UTI is the most common ExPEC infection (Ejrnaes 2011; Ena et al. 2006; Foxman 2010, 2014; Jacobsen et al. 2008; Marrs et al. 2005; Zhang and Foxman 2003). In

2007, there were in the US about 10 million ambulatory visits and about 2 million admissions to hospital emergency departments (Foxman 2010, 2014). It is very common in young women, where the infection can become recurrent, and in older patients following catheterization. UTIs can get complicated and cause kidney failure and quite often, especially in the elderly, lead to bloodstream infections such as sepsis.

2.5 Blood Stream Infections/Septicemia/Sepsis

This ExPEC infection is the most serious one in terms of severity as well as an economic burden. Every year there are more than a million cases of sepsis in the US and the estimate is that about 30% of them die. This number is higher than deaths in the US due to prostate cancer, breast cancer, and HIV combined (Sepsis Fact Sheet, CDC, 2016). In 2011, the US spent $20.3 on hospital care for sepsis patients—about 55 million US$ a day and the cost per patient can be as high as 56,000 US$. Sepsis is clearly an emerging disease as the number of cases per year increases rapidly. There are several reasons for this escalation such as the increased longevity of people, the broader use of invasive procedures, immunosuppressors, and chemotherapy. But probably the most important reason for the current situation is the fast spread of antibiotic-resistant *E. coli*, the major cause of sepsis.

3 Virulence Factors

A general feature of ExPEC is that production of exotoxins is not a major factor in their virulence, in contrast to many intestinal strains. There is evidence for production of cytotoxin by ExPEC, but it is not clear if they are important for pathogenicity (De Rycke and Oswald 2001; Peres et al. 1997; Taieb et al. 2016). The virulence of ExPEC strains appears to depend on their ability to survive in host tissues, especially in serum. Many of the genes involved in virulence are present on large plasmids, most frequently on a ColV plasmid (Huja et al. 2015; Milch et al. 1984; Waters and Crosa, 1991; Wijetunge et al. 2014). The ColV plasmids are a family of related plasmids that encode a broad spectrum of iron uptake systems and genes for increased serum survival.

In general, there is an extensive variability in virulence-associated genes of ExPEC (Mokady et al. 2005a, b; Ron 2006, 2010). There appears to be a large "pool" of such genes and much overlap between them. For example—there are several genetic systems for iron acquisition and an ExPEC strain can carry one or more of them, the same for genes coding for fimbriae or adherence factors, etc. It is clear that many of the virulence factors were obtained by lateral gene transfer, such as the gene coding for Yersiniabactin, the Yersinia iron uptake system (Gophna et al. 2001; Huja et al. 2015). However, all the ExPEC strains carry at least one

adherence system and septicemic strains carry at least one efficient iron-binding system and genes for serum survival (ISS—increased serum survival).

3.1 Adherence

Adherence to host cells is the initial step of an *E. coli* infection and is essential for invasion and infection. Adherence also influences host specificity and even tissue specificity. Thus, intestinal pathogens adhere preferentially to gut epithelium while bacteria involved in UTI adhere to bladder epithelium (Kalita et al. 2014).

Adherence depends mainly by specific organelles—pili, or fimbriae—that recognize specific ligands on the epithel. Infections of mammalian farm animals (cattle, sheep, pigs, etc.) begin by intestinal colonization of newborn and often involve K99 and K88 pili and AC/I pili were found only in APEC and show specificity to chicken tracheal epithelium (Babai et al. 2000; Yerushalmi et al. 1990). The most common fimbriae in strains involved in UTI/sepsis are the P-fimbriae that bind glycolipids containing a-D-Gal-1,4-b-D-Gal (Korhonen et al. 1982; Lane and Mobley 2007; Lund et al. 1988; Stromberg et al. 1990), F1C fimbriae, which bind b-GalNac-1,4-bGal (Khan et al. 2000; van Die et al. 1991) and fimbriae of the S-family. The S-family includes the SfaI, SfaII, Foc, and AC/I fimbriae. The Sfa fimbrial adhesins are produced by strains involved in sepsis and newborn meningitis and interact with glycoproteins containing sialic acid (Babai et al. 2000; Bauchart et al. 2010; Dobrindt et al. 2001; Hacker et al. 1985; Moch et al. 1987; Parkkinen et al. 1986). The group of S-fimbriae is interesting as there is evidence for horizontal gene transfer and combinatorial gene shuffling resulting in pili with different adherence specificities that are related to the clinical symptoms or the host. Thus, the *sfaAII* gens (from a NBM strain) is homologous to the *facA* gene of AC/I pili (APEC) while the *sfaIIS* gene—coding for the adhesion—is homologous to this of the *sfaI* cluster from a human sepsis strain (Babai et al. 2000). The combinatorial shuffling of fimbrial genes is probably of ecological and functional importance as it increases the fimrial diversity to improve adaptation to different hosts and resistance to the immune system of the host. Moreover, many of the ExPEC strains express more than one type of fimbriae and the expression of fimbrial genes appears to be coordinated, also important for diversity and increase the probability of survival under changing environmental conditions (Holden and Gally 2004).

3.2 Type Three Secretion Systems (TTSS)

Type three secretion systems are needle-like structures used to secrete effector proteins into host cells. The TTSS of intestinal pathogenic *E. coli*, especially the LEE system, have been well characterized. ExPEC strains do not have an LEE system but do have a homologous gene cluster—ETT2 = *E. coli* Type Three secretion system 2,

similar to the SPI1 pathogenicity island of Salmonella. It is present in the majority of ExPEC strain from humans and animal farms (Cheng et al. 2012; Hartleib et al. 2003; Ren et al. 2004; Wang et al. 2016b). However, the ETT2 gene clusters carry a large number of mutations and deletions and it is not even clear how many of the strains express the ETT2 genes (Ideses et al. 2005; Ren et al. 2004). So far, there is no evidence that the ETT2 system is a secretion system, as no secreted proteins have been detected (Hu et al. 2017). Yet, in *E. coli* O157:H7 it encodes regulators that affect expression of genes in the LEE gene cluster (Zhang et al. 2004), and in avian *E. coli* O78, the ETT2 system affects motility (Wang et al. 2016a). The ETT2 system of *E. coli* O78-9 is degenerate, as it carries a large deletion and several point mutations. Yet, it is critical for virulence and for serum resistance (Huja et al. 2015; Ideses et al. 2005; Wang et al. 2016a). Recently it was shown that ETT2 has a global effect on the cells surface and is involved in secretion of flagella and fimbriae, in production of outer membrane vesicles and multicellular behaviour (Shulman et al. 2018).

4 Avoiding the Immune Response

ExPEC strains are characterized by high resistance to serum, which contains antibodies and complement. The complement complex mediates direct killing by the formation of pores in the cell membrane. Pathogens evolved outer surface features that inhibit complement-dependent killing, such as lipopolysaccharides and capsules, which are the important factors involved in serum resistance (Phan et al. 2013)

4.1 Lipopolysaccharides—LPS

Complete lipopolysaccharides are essential for serum survival and pathogenicity of ExPEC (Hammond 1992; Kusecek et al. 1984). However, because a very large number of LPS serotypes are involved in septicemia, there does not appear to be an advantage for specific serotypes. An important factor is the length of the O-antigen chain, which also influences the level of serum resistance (Grozdanov et al. 2002)

4.2 Capsules

The capsules produced by *E. coli* strains are divided into four groups according to their composition and biosynthesis (Whitfield and Roberts 1999). Capsules of group 1, 2, and 3 have been extensively studied, they are acidic polysaccharides composed of oligosaccharide repeating units and their role in virulence is well established. (Buckles et al. 2009; Goller and Seed 2010; Hafez et al. 2009; Kim et al. 2003; Sarkar et al. 2014). Capsules belonging to group 4—also called

"O-antigen capsules" have only recently been studied and shown to contribute to enteropathogenic *E. coli* resistance to human alpha-defensin 5 (Thomassin et al. 2013) to shield intimin and the type three secretion system of intestinal pathogenic *E. coli* (Shifrin et al. 2008) and to facilitate spreading of *Shigella sonnei* to peripheral organs (Caboni et al. 2015). Its essential role for virulence was shown in avian ExPEC strain serotype O78 when a transposition that abolished capsule synthesis resulted in reduced virulence (Dziva et al. 2013). Moreover, a precise deletion of the *etp* gene involved in the biosynthesis of the group 4 capsule resulted in serum sensitivity (Biran and Ron 2017). Thus, it is clear that O-antigen/group 4 capsule is also a critical virulence factor for the spread of bacteria in the bloodstream and for septicemia.

4.3 ISS—Increased Serum Survival

Studies in avian pathogenic *E. coli* indicated that a gene present in the ColV plasmid confers serum resistance (Binns et al. 1979). This gene—called *iss* for increased serum survival—encodes a small membrane protein (Binns et al. 1982; Horne et al. 2000; Nolan et al. 2002, 2003). This gene is homologous to the *bor* gene of *E. coli* K-12 that originated from bacteriophage λ (Johnson et al. 2008; Lynne et al. 2007). It is clear that the *iss* gene is a major factor in serum survival (Binns et al. 1982; Huja et al. 2015; Nolan et al. 2002, 2003). Yet, the molecular basis for its role in serum survival is not clear. Moreover, a deletion of the *iss* gene from the ColV plasmid results in serum sensitivity and is not complemented by the chromosomal *iss* (*bor*) gene (Huja et al. 2015). This finding is difficult to explain, as the chromosomal gene codes for the homologous protein as the plasmid gene.

5 Avoiding Metabolic Immunity

As already noted, to survive in serum, bacteria must overcome the innate immunity of the host, mainly the effect of the complement system. However, another obstacle is the nutritional immunity of the serum caused by the fact that nutrients are bound in storage molecules and are unavailable to the bacteria (Weinberg 2009). Most significant is the limitation in iron, which is bound in the blood to human proteins (such as ferritin, hemosiderin) Therefore, most of the ExPEC strains contain genes involved in iron sequestering and it is clear that iron acquisition systems and receptors play a pivotal role in the virulence of septicemic pathogens. Indeed, systems-wide analyses of the response of septicemic bacteria to serum show an induction of the genes involved in iron metabolism and controlled by the iron homeostasis regulator Fur (Huja et al. 2014). It appears that the presence of multiple iron acquisition systems is essential, but just as important is their precise regulation upon exposure to serum. Thus, the nonpathogenic *E. coli* K-12 grows poorly even

in serum in which the complement system has been heat inactivated, and its iron metabolism is not induced upon exposure to serum (Otto et al. 2016). Furthermore, these bacteria grow much better in the presence of serum (inactivate) upon introduction of the *fur* gene from septicemic strains (Otto et al. 2016).

Functional genomic analyses indicate that exposure to serum changes the expression of a large number of genes, most of which are induced even in the absence of active complement (Huja et al. 2014). Therefore, it is clear that overcoming the nutritional immunity is an essential step for surviving serum and establishing a bloodstream infection.

6 Concluding remarks and future perspectives

ExPEC—Extraintestinal Pathogenic *E. coli* constitute a clinical problem of increasing importance. Yet, our understanding of the pathogenesis of these bacteria is quite limited. As they do not appear to produce potent secreted toxins, their ability to cause infection depends on their ability to survive and multiply in the host. In order to overcome hostile environments, such as the urinary tract or even blood where they are exposed to innate immunity and nutritional immunity, a whole series of functions and regulatory mechanisms were evolved. The role of most of these functions and regulations in infection is not clear yet, but it is evident that the majority of these is important for overcoming the nutritional immunity and not only the innate immunity.

Why are ExPEC strains so difficult to combat? There are several major reasons, which are as follows:

1. The extraintestinal infections involve a very large number of serotypes that do not cross react. Therefore, simple vaccines comprising several strains are not feasible. In addition, if there is a vaccine—who should be vaccinated? As in most cases, the infection is opportunistic, often following a medical intervention, it is difficult to define the population at risk.
2. ExPEC carry a variety of genes coding for drug resistance, which are often on conjugative plasmids that easily spread in the whole bacterial population. Moreover, ExPEC are present in large number in the intestine, where they encounter bacteria, such as Klebsiella and Acinetobacter from which they can get resistance genes by horizontal gene transfer.
3. The search for new anti-ExPEC targets is a real challenge, as many of the genes involved in pathogenesis have overlapping activities, and inhibiting one of them will probably be insufficient to prevent the infection. For example—in order to overcome the deprivation of iron in serum, ExPEC strains code for several efficient iron binding systems, most of which were obtained by horizontal gene transfer. In order to prevent ExPEC from resisting serum, it should probably be necessary to inhibit all of these iron acquisition systems.

4. Once the bacteria enter the bloodstream the infections progress very quickly, with the bacteria getting to the vital organs and reaching high numbers. As *E. coli* contains the endotoxic cell envelope of lipopolysaccharides, the patients are exposed to critical danger even only from the endotoxin of dead bacteria.

In conclusion—it is essential to identify new targets for developing drugs or vaccines and, in parallel, to develop means that can constitute early warning systems, especially in hospital and community institutions.

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Pandemic Bacteremic *Escherichia Coli* Strains: Evolution and Emergence of Drug-Resistant Pathogens

Yael Yair and Uri Gophna

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Abstract In recent years, there have been several pandemics of *E. coli* strains which are highly virulent and antibiotics resistant. Here, we discuss one recent pandemic strain, ST131. These *E. coli* strains are members of the virulence-associated phylogenetic group B2 and exhibit extraintestinal virulence factors, including various adhesins, toxins, siderophores, and protectins. This group often also harbors a diverse range of antimicrobial resistance types and mechanisms and may have particular metabolic capacities that enable it to colonize many individuals asymptomatically, while out competing other *E. coli* strains. Here, we discuss this clonal group in the context of other pathogenic *E. coli* and focus on its specific characteristics in terms of resistance, virulence, and metabolism.

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1 Introduction: Pathogenic *Escherichia Coli* in Human Health and Disease

Since its discovery in 1885, *Escherichia coli* has been one of the best studied Gram-negative bacteria, and commonly used as a "workhorse" for molecular biology and biochemistry. *E. coli* strains of biological significance to humans are generally categorized as commensal strains, typically found in a healthy human gut microbiome (Eckburg 2005) and pathogenic strains. In humans, pathogenic *E. coli* strains are responsible for two types of infections: gastrointestinal infections and extraintestinal infections that include urinary tract infections (UTIs), bloodstream infections, and neonatal meningitis (Russo and Johnson 2000). The intestinal infections can be complicated by extraintestinal syndromes, such as in the case of Shiga toxin-producing intestinal strains that can cause hemolytic uremic syndrome (Rasko et al. 2011). Among the strains causing intestinal infections, there are six defined categories (also referred to as "pathotypes") of pathogenic strains of *E. coli*: enterotoxigenic (ETEC), Shiga toxin-producing/enterohemorrhagic (STEC/EHEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and diffusely adherent (DAEC) (Russo and Johnson 2000). The most common extraintestinal *E. coli* (ExPEC) infections occur at multiple anatomical sites such as the urinary tract (Kaper et al. 2004), the respiratory tract, the cerebral spinal fluid, meningitis (mostly in neonatal), and peritoneum (spontaneous bacterial 58 peritonitis) (Soriano et al. 1995; Russo 2003). The majority of UTIs in young healthy women are caused by ExPEC strains (85–95%), and along with Group B Streptococcus, ExPEC is considered to be a leading cause of neonatal meningitis worldwide (Russo 2003; Poolman and Wacker 2015).

ExPEC strains can often be found in a normal healthy human gut, without causing any clinical symptoms (Eckburg 2005). Thus, they can be asymptomatically carried by many, and later be transmitted via contact, infecting the urinary tract or blood, often of the same individual that has been hosting them for years. When bacteria are present in the blood in large numbers, this can lead to other infections and sometimes trigger a serious body-wide inflammatory response called sepsis, which can be life-threatening, since it may progress to one or more organ failure, that often leads to death (Nguyen et al. 2006). Once ExPEC strains enter the bloodstream, for example, as a result of advanced UTI or transrectal ultrasound-guided (TRUS) biopsy, they can cause bacteremia that can lead to sepsis (coliseptisemia) (Johnson and Russo 2002). In fact, although TRUS prostate biopsy is generally considered to be a relatively safe medical procedure, severe sepsis has been described in 0.1–3.5% of cases after TRUS biopsy, with ExPEC being the most common cause (Williamson 2012; Lange et al. 2009).

In the past decade, there has been a rapid increase in the rates of hospitalization and mortality due to ExPEC infections, mainly because of the spread of antibiotic resistance among clinical isolates (De Kraker et al. 2013). In 2010, the estimated economic burden of UTI-associated hospitalization in the USA alone was estimated

to be around 2.3 billion dollars and between 85 and 95% of the cases were *E. coli* related (Poolman and Wacker 2016). *E. coli* is also one of the most common causes of community-acquired bacteremia and sepsis (De Kraker et al. 2013). In seniors, *E. coli* is the most common source of community-acquired bacteremia (Jackson et al. 2005). In the USA, at 2001, it was estimated that about 40,000 deaths per year are caused by *E. coli*-associated sepsis (Russo 2003), and it is the most common bacterial species associated with septicemia (Elixhauser et al. 2006), with cost of nearly $15.4 billion in aggregate hospital costs. In Europe, similar trends have been observed with an increase of reported *E. coli* bacteremia cases from 20,151 reports in 2002 to 32,194 reports in 2008. Thus, ExPEC strains have a great impact on public health and represent an increasing economic burden on society.

2 *E. coli* Sequence Type 131: A Worldwide Pandemic Clone

In 2008, reports of a previously unknown *E. coli* clonal group emerged from three different continents, noticed by two research groups who were studying CTX extended-spectrum-β-lactamase (ESBL)-producing *E. coli* (Nicolas-Chanoine et al.

Fig. 1 Reported cases of ST131 worldwide, as of 2017 (Nicolas-Chanoine et al. 2014; Vignoli 2016; Chattaway et al. 2016; Eibach et al. 2016; Begum and Shamsuzzaman 2016; Ouedraogo et al. 2016; Yahiaoui et al. 2015; Ebrahimi et al. 2016; Hristea et al. 2015; Markovska et al. 2017; Rogers et al. 2011; Severin et al. 2010; Peirano et al. 2014). **Africa**: Algeria, Burkina Faso, Cameroon, Central African Republic, Egypt, Guinea-Bissau, Ghana, Kenya, Madagascar, Morocco, Nigeria, South Africa, Tanzania, and Tunisia. **Europe**: Belgium, Bulgaria, Croatia, Denmark, France, Germany, Hungary, Italy, Netherland, Norway, Portugal, Romania, Spain, Sweden, Switzerland, the Czech Republic, and the United Kingdom. **Asia**: Bangladesh, Cambodia, China, India, Israel, Jordan, Japan, Kuwait, Lebanon, the Philippines, South Korea, People's Democratic Republic of Laos, Pakistan, Russia, Turkey, Thailand, and United Arab Emirates. **North America**: Canada, the United States. **South America**: Argentina, Brazil, Colombia, Ecuador, Mexico, Panama, Puerto Rico, and Uruguay. **Oceania**: Australia and New Zealand

2008; Coque 2008). This clonal group, which later became known as sequence type 131 (ST131), caught the attention of the clinical research community due to several unique characteristics: increased occurrence of resistance to antimicrobial agents, enhanced virulence, and fast spread. The two initial studies showed that ST131 had emerged predominantly in the community, and was simultaneously identified in different parts of the world spanning three continents. Since then, ST131 has spread globally (Fig. 1).

The serotype most associated with ST131, O25:H4, was also identified among both intestinal and extraintestinal adherent-invasive *E. coli* (AIEC) strains (Martinez-Medina et al. 2009).

3 ST131—Characterization of a Novel Pandemic Lineage

The first step in identifying the mysterious clonal group was to examine basic characteristics—phylogenetic group, serotype, O antigen, and virulence genes, as well as multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE). Most ExPEC strains belong to group B2, while a small fraction belongs to group D and most commensal strains have been shown to belong to group A (Clermont et al. 2000). Phylogenetic analysis of the ST131 clone revealed that like other ExPEC strains it belonged to phylogenetic group B2 (Nicolas-Chanoine et al. 2008; Coque 2008; Clermont et al. 2008). The lineage was identified as belonging to ST131 (Nicolas-Chanoine et al. 2008; Coque 2008; Wirth et al. 2006) based on MLST, perhaps the most widely accepted bacterial typing method today. As for the three surface antigens O, H, and K (the specific part of the LPS, the flagella, and the capsule, respectively) (Ørskov and Ørskov 1984), most ST131 isolates exhibit serotype O25b:H4, except for a small subset of strains that exhibit serotype O16:H5 (Nicolas-Chanoine et al. 2008; Johnson et al. 2014), and some isolates that cannot yet be typed for either O (Dahbi et al. 2013) or H antigens (Suzuki et al. 2009).

Although all ST131 are coherent and homogenous when examined according to their MLST-determining genes, there is significant within-lineage genetic variations as shown by the PFGE method which is based on the specific digestion (using rare-cutting restriction enzymes) of DNA into fragments of varying sizes, followed by the separation of these DNA fragments by gel electrophoresis using a periodically changing electric field. In contrast to its otherwise clonal character ST131 presents highly variable PFGE profiles, and different pulsotypes can be observed depending on geographic location, time periods, and ecological niches (Johnson et al. 2012). Pulsotype comparison of 579 ST131 isolates resolved 170 distinct pulsotypes (Johnson et al. 2012), with a small number of dominant pulsotypes, including one pulsotype (968) that accounts for 24% of the general ST131 population. In summary, the ST131 lineage, while monolithic in terms of the sequence of housekeeping genes, is highly variable in terms of its genome content, and often contains different “flexible genome” elements, such as plasmids and prophages.

4 What Are the Reasons for the Worldwide Rapid Dissemination of ST131?

The initial consensus hypothesis for explaining the rapid worldwide dissemination of ST131 was that these bacteria are more virulent, combining the B2 group background with additional novel virulence traits (Banerjee and Johnson 2014; Johnson et al. 2010). Typical virulence-associated genes in ExPEC are adhesin-encoding genes (such as *papAH*, *papC*, *papEF*, *papG*, *sfa/focDE*), toxin-encoding genes (*such as hlyA*, *hlyF*, *cnf1*), siderophore-related genes (*iroN*, *fyuA*, *ireA* and *iutA*), protectin/invasin-encoding genes (*kpsM II*, *kpsMT III*, *iss,* etc.), pathogenicity island markers, and miscellaneous genes (*cvaC*, *usp*, *ompT*, *clbB*, etc.). These genes contribute to the pathogenic potential and are seldom found in non-pathogenic strains (Johnson and Stell 2000).

Indeed, 12 virulence genes were found to be significantly more prevalent among ST131 than among non-ST131 isolates: *iha* and *fimH* (adhesin–siderophore receptor and type I fimbriae, respectively), *sat* (a secreted toxin), *astA* (an enteroaggregative *E. coli*-associated toxin), *fyuA* and *iutA* (yersiniabactin and aerobactin receptors, respectively), *kpsM II-K2* and *kpsM II-K5* (group II capsular polysaccharide synthesis), *usp* (uropathogenic-specific protein), *traT* (surface exclusion, serum resistance-associated), *ompT* (outer membrane protease), and *malX* (a pathogenicity island marker). The overall virulence scores were significantly higher for ST131 isolates compared to most of the non-ST131 isolates (Johnson et al. 2010). Similarly, in ESBL-producing isolates, 11 virulence genes (*papG III*, *afaFM955459*, *cnf1*, sat, hlyA, kpsM II-K2, *kpsM II-K5*, *traT*, *ibeA*, *malX,* and *usp*) were significantly associated with ST131, whereas only *papG II* and *tsh* were significantly associated with non-ST131 strains (Coelho et al. 2011). Analysis of 130 clinical ST131 isolates revealed four distinct virotypes of ST131 (labeled arbitrarily as A, B, C, and D) (Blanco et al. 2013), with an additional virotype added later on (virotype E (Dahbi et al. 2013)), based on the presence or absence of four distinctive virulence genes, including *afa* FM955459 (specific for an ST131 clone encoding an *Afa/Dr* adhesin), *iroN*, *ibeA* (invasion of brain endothelium), and *sat*. The patterns were as follows (Table.1):

Table 1 Distribution of *afa* FM955459, *iroN*, *ibeA*, and *sat* genes among the different main virotypes of ST131

Virotype/gene	*Afa* FM955459	*IroN*	*IbeA*	*Sat*
A	+	–	–	∓
B	–	+	–	∓
C	–	–	–	+
D	–	∓	+	∓
E	–	–	–	

Table 2 Summary of selected studied pointing the major finding regarding ST131 virulence phenotype and genotypes in experimental, in vivo models, and epidemiological surveys

Year	Geographic location	Method	Non-ST131	ST131	Main findings	Reference
2011	Spain	Genotypic characterization (virulence factor genes profile analyses)	64	30	ST131 isolates have higher virulence scores than non-ST131 isolates	Coelho et al. (2011)
2012	USA and Canada	Animal model (mouse subcutaneous sepsis model)	34	27	ST131 isolates are not more virulent than other ExPEC isolates	Johnson et al. (2012)
2012	France and Spain	Animal models (*Caenorhabditis elegans* and zebrafish embryos)	5	3	No advantage detected for the ST131 isolates	Lavigne et al. (2012)
2017	Spain	Epidemiologic survey	56	33	ST131 isolates are more drug resistance but not more clinically virulent and no significant differences in risk factors or prognosis	Morales-Barroso (2017)
2010	USA	Genotypic characterization (virulence factor gene profile analyses)	73	54	ST131 isolates have higher virulence scores compared to non-ST131 isolates	Johnson et al. (2010)
2014	Spain	Mouse subcutaneous sepsis model	2	23	Broad virulence diversity among ST131. All ST131 isolates exhibited some lethality rate for mice	Mora et al. (2014)
2008	France, Tunis and Central African Republic	Mouse lethality assay, adhesion on human cells, biofilm production, and genomic characterization	4	4	ST131 isolates yielded 100% lethality rate for mice	Clermont et al. (2008)
2012	Portugal, Spain, United Kingdom, USA, France, Norway, Czech Republic, South Korea, Switzerland, and Croatia	Adherence in vitro to abiotic surfaces	1	32	None of the ST13 isolates was able to form biofilm in the tested conditions	Novais et al. (2012)

Virotype D isolates exhibited significantly higher virulence scores than did those of other virotypes and was significantly associated with younger patients and community acquisition (Blanco et al. 2013). In contrast, virotype B was associated with older patients and a lower likelihood of symptomatic UTI, but a higher likelihood of respiratory tract infection, while virotype C was significantly associated with a generally higher likelihood of symptomatic infections. Overall, ST131 isolates of the major serotype O25b:H4 exhibited higher virulence scores than ST131 isolates of the rarer serotype O16:H5 (Dahbi et al. 2013).

In contrast to molecular epidemiology, experimental studies in animal models do not clearly support the hypothesis that ST131 is more virulent than other *E. coli* strains. When ST131 isolates were compared to non-ST131 ExPEC isolates in a mouse subcutaneous sepsis model, no significant advantage for the ST131 was observed in terms of lethality and clinical illness (Johnson et al. 2012). These findings were also supported by studies in *Caenorhabditis elegans* and zebrafish embryos (Lavigne et al. 2012). Some evidence that ST131 is not necessarily hyper-virulent comes from a recent case–control study from 2017 that showed that the non-ESBL-producing ST131 strains did not cause a worse clinical outcome in human bacteremia (in terms of mortality, severe sepsis, hospitalization time, etc.) than non-ST131 isolates (Morales-Barroso 2017). Taken together, the studies contradict the assumption that the rapid emergence and global dominance of ST131 are due to enhanced virulence.

The findings concerning the virulence of ST131 in comparison to non-ST131 strains are summarized in Table 2.

From these experiments, it appears that ST131 strains are not significantly more virulent in vivo than the non-ST131 strains. Yet, it is highly likely that there are other factors, such as enhanced metabolic capacities, and capacity for asymptomatic carriage (discussed below), that have contributed to the success of ST131 as a global pathogen. Indeed, there is evidence that ST131 isolates have higher metabolic potential compared to non-ST131 isolates (Vimont et al. 2012; Gibreel 2012), in terms of catabolic enzyme repertoire but further study is required, such as in vivo colonization studies that involve competition between ST131 isolates and other strains, or ST131 mutant that lack these enzymatic functions.

5 Carriage of ST131 in the Community

A key factor that probably contributes to the global dissemination of ST131 is its carriage among healthy individuals. ST131 lineage is strongly associated with community-onset infections, and carriage rates in healthy subjects can range from 7% in independent healthy Parisians (Leflon-Guibout et al. 2008) up to over 35% in long-term care facilities (LTCF) for the elderly in Italy (Giufrè 2017). ST131 is associated with older age, intensive antibiotic treatment, and high prevalence among residents in nursing homes and LTCF (Banerjee et al. 2013), which may represent the largest human reservoir for ST131. Taken together with the potential

of ST131 for increased virulence, these findings indicate the opportunistic nature of this lineage. Indeed, several case reports of transmission of ST131 within household, resulting in severe or fatal extraintestinal infections (Morales-Barroso 2017; Mora et al. 2014), demonstrate its potential for causing deadly opportunistic disease. Since, like other ExPEC lineages, ST131 has the ability to colonize healthy individuals without causing any symptoms, health authorities should consider future measures in order to prevent its dissemination, or in the very least take steps to prevent infections in high-carriage communities.

6 ST131 in Companion and Non-companion Animals—Additional Natural Reservoirs

One of the intriguing aspects of the ST131 clonal group is its natural reservoirs. ST131 is found among drug-resistant *E. coli* isolates in companion and non-companion animals (Rogers et al. 2011)—dogs, cats (Ewers et al. 2010; Pomba et al. 2009; Johnson et al. 2009), poultry (Mora et al. 2010; Cortes et al. 2010), horses (Ewers et al. 2010), and pigs. In non-companion animals, ST131 was found in glaucous-winged gulls (Hernandez et al. 2010), seagulls (Simoes et al. 2010), and rats (Guenther et al. 2010). A European collection of 177 ESBL-producing *E. coli* isolates collected from eight countries, mainly obtained from companion animals with various clinical manifestations, revealed that 5.6% of the isolates were ST131 O25b (Ewers et al. 2010). Many clinical ST131 isolates from companion animals were found to have high resemblance to human clinical ST131 isolates based on their virulence genotype, resistance characteristics, and PFGE profiles. These findings suggest either recent or ongoing zoonotic transmission between humans and animals (Rogers et al. 2011), and have been corroborated by more recent genomic analysis showing that most, though not all, ST131 strains frequently cross-host species boundaries (McNally et al. 2016). A possible reason for the relative lack of ST131 case reports in animals might be the veterinary sector's relatively limited microbiological diagnosis and reporting systems, probably resulting in many unreported cases. Reports of ST131 carriage among non-companion animals are even more rare, since such animals are little studied. Although according to most data, the ST131 pandemic appears mostly a human-based phenomenon, the risk of inter-species transmission of these multi-resistant strains between humans and animals should be seriously considered (Table 3).

Table 3 A summary of all reported ST131 cases in companion and non-companion animals

Geographic location	Year	Total number of isolates	% of ST131	ST131 isolates	Total number of isolates	Species	Reference
Portugal	2007–2008	139	9%	4	Feces	Seagull (*L. fuscus, L. cachinnans*)	Simoes et al. (2010)
Spain	2004–2006	61	1.6%	1	Chronic cystitis	Dog	Pomba et al. (2009)
USA	2008	5	20%	1	Urine, feces	Dog and cats	Johnson et al. (2009)
Germany	2010	211	0.47%	1	Feces	Brown rat (*Rattus norvegicus*)	Guenther et al. (2010)
Spain	2009–2010	100	8%	8	Retail chicken	Poultry	Mora et al. (2010)
Spain	2007–2009	463	1.5%	7	Clinical	Chickens	
Spain, France, Belgium	1991–2001	1601	0.18%	3	Clinical	Turkeys, chicken	
Spain	2003	57	1.8%	1	Feces	Chickens	
Spain	2003	59	1.6%	1	floor	Poultry	Ortes et al. (2010)
Russia	2010	145	0.68%	1	Fecal or cloacal	Mainly Glaucous-winged gull (*L. glaucescens*), Tufted Puffin (*F. cirrhata*) and Black-headed gull (*L. ridibundus*)	Hernandez et al. (2010)

(continued)

Table 3 (continued)

Geographic location	Year	Total number of isolates	% of ST131	ST131 isolates	Total number of isolates	Species	Reference
Germany, Italy, the Netherlands, France, Spain, Denmark, Austria, Luxembourg	2008–2009	84	10.7%	9	Clinical	Dogs	Ewers et al. (2010)
		50	2%	1	Clinical	Horse	
		7	0%	0	Clinical	Cattle	
		2				Guinea pigs	
		1				Pygmy rabbit	
		1				Pig	
		1				American Kestrel	
		31				Cats	
Australia	2007–2009	120	6.6%	8	Clinical	Dogs	Platell et al. (2011)
		5	20%	1	Clinical	Cat	
Australia	2009	232	2.1%	5	Feces	Dogs	Guo et al. (2013)

7 Drug Resistance Among the ST131 Clonal Group

Extended-spectrum β-lactamase (ESBL)-producing bacteria are resistant to most beta-lactam antibiotics, including penicillins, cephalosporins, and monobactams. These enzymes cleave the amide bond in the β-lactam ring, and thus inactivate those antibiotics. The rapid dissemination of antibiotic resistance among bacteria is an alarming trend and considered to be one of the world's main health threats (Bonnet 2003). In recent years, CTX-M enzymes have become the predominant ESBLs encountered in the clinic. These enzymes have originated from *Kluyvera* spp. (Pitout et al. 2005) and are generally plasmid-associated. *Klebsiella pneumoniae* and *E. coli* are the major ESBL-producing organisms isolated worldwide. The ST131 clonal group initially caught the attention of researches because of its CTX-M-15 ESBL (Nicolas-Chanoine et al. 2008). Since then, ST131 isolates carrying CTX-M enzymes were reported in many countries worldwide (Rogers et al. 2011). In Canada, a multi-center study that included 209 clinical isolates revealed that 46% of ESBL isolates belonged to the ST131 clonal group, with the vast majority (91%) of these strains producing CTX-M-15 (Peirano et al. 2010).

It was previously reported that CTX-M-producing *E. coli* isolates often carry resistance to additional antibiotic classes, which can include co-trimoxazole, aminoglycosides, and fluoroquinolones (Pitout and Laupland 2008). Indeed, fluoroquinolone resistance is one of the most frequently reported resistances among ST131 strains, including ESBL-producing ones. There are several mechanisms that can lead to fluoroquinolone resistance in ST131, and they provide varying levels of resistance. High-level fluoroquinolone resistance in *E. coli* was reported to be caused by chromosomal mutations of genes coding the fluoroquinolone targets, which are *gyrA*, *gyrB*, *parC,* and *parE* (Rogers et al. 2011). The aminoglycoside-modifying enzyme AAC(6′)-Ib-cr also contributes to quinolone resistance via acetylation of selected fluoroquinolones. Low-level resistance can also be conferred by the presence of plasmid-mediated quinolone resistance genes, including *qnrA*, *qnrS,* and *qnrB*. Population analysis performed on historical and recent ST131 isolates found that fluoroquinolone resistance in the ST131 fimH30 sub-lineage is mostly due to *gyrA1AB* and *parC1aAB* mutations in genes encoding gyrase and topoisomerase IV, respectively (98% of FQ-R isolates) (Johnson et al. 2013).

Resistance to the carbapenemases among *E. coli* is yet another alarming trend worldwide (Nordmann and Poirel 2014). Three major carbapenemases have been reported: KPC (*Klebsiella pneumoniae* carbapenemases), NDM (New Delhi metallo-β-lactamase), and OXA-48 (for "oxacillinases"). An extended analysis of 116 carbapenemase-producing *E. coli* isolates found that 35% of the isolates belonged to ST131. 58% of ST 131 isolates were positive to the bla_{KPC}, 32% for $bla_{OXA\text{-}48\text{-}like}$, 7% for $bla_{NDM\text{-}1}$, and 2% for $bla_{IMP\text{-}14}$ (Peirano et al. 2014).

It is obvious that antimicrobial resistance is widespread among the ST131 clonal group, and it is safe to assume that this feature has a strong impact on the spread of ST131 in the community, helping this lineage to replace other, antibiotic-sensitive

strains in a world where antibiotic exposure is common. Thus, their antibiotic resistance is making ST131 more abundant as well as harder to eliminate (Banerjee and Johnson 2014).

8 "A Shark Among Sharks"—The H30 and H30-Rx Sub-clones of ST131

In order to better understand the genetic structure of the ST131 clone, an analysis of 350 historical and more recent ST131 isolates and over 700 non-ST131 *E. coli* isolates was performed in 2010, which utilized a variety of typing strategies, such as sequencing of selected genes (*fimH*, *gyrA,* and *parC*), MLST, and PFGE. This analysis identified 185 unique PFGE types and 7 distinct *fimH*-based putative clonal lineages of ST131: H15, H22, H27, H30, H35, H41 and H94, with H30 being the dominant allele (Johnson et al. 2013). The high genetic similarity of most H30 isolates to one another suggested that they originate from a single fimH30-carrying ancestor. Moreover, this H30 ST131 sub-clone was closely associated with fluoroquinolone resistance (FQ-R) and with ESBL production mediated by CTX-M-15. Since this lineage was rare among fluoroquinolone-susceptible ST131 isolates (<1%), it was suggested that FQ-R is associated almost exclusively with the ST131 H30 sub-clone, which originated from a single strain about 14 years ago (Johnson et al. 2013; Banerjee et al. 2013). The H30 ST131 clone was found to be associated with persistent infections, subsequent hospital admissions, and subsequent new infections (Johnson et al. 2016). A recent study from 2017 suggests that H30 isolates tend to be less frequently nosocomially acquired, and more frequently affect patients aged >65 years. Moreover, H30 isolates were also found to be more frequently resistant to ciprofloxacin and less frequently resistant to trimethoprim/sulfamethoxazole (Morales-Barroso 2017). In addition, the H30 lineage was found to be associated with virotype C (see above) and CTX-M-14 (Peirano et al. 2014).

An important sub-lineage within H30 is the single, highly virulent sub-clone, H30-Rx. This clone was identified by whole-genome single-nucleotide polymorphism (SNP) analysis performed on 105 ST131 isolates cultured from humans and animals between 1967 and 2011 (Price 2013). High-resolution phylogenetic analysis enabled the identification of a single-ancestral sub-clone within H30-R, the fluoroquinolone-resistant H30 sub-clone. Because of its more extensive resistance characteristics, this CTX-M-15-associated sub-clone was designated H30-Rx. Assessment of the demographic, geographic, and clinical prevalence of H30-Rx revealed that the relative prevalence of H30-Rx was highest among German Hospital isolates (where it even exceeded the prevalence of other H30-R isolates), intermediate among US-based hospital isolates, and lowest among the US outpatient isolates (Price 2013). In addition, a more recent study that analyzed a global collection of ESBL-producing *E. coli* isolates found that the majority of ST131 (92%) isolates belonged to the H30 lineage, and 82% H30 isolates belonged to the H30-Rx sub-lineage. The H30-Rx lineage was recovered from all 9 countries

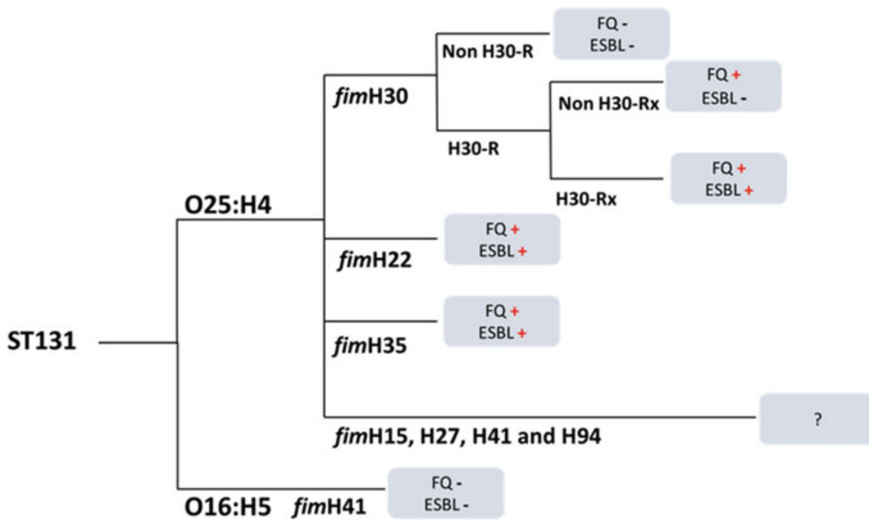

Fig. 2 General structure of the population of *E. coli* ST131. Presented are the two main serotypes (O25b:H4 and O16:H5) with lineages and sub-lineages producing or not producing ESBL enzymes and being resistant or sensitive against fluoroquinolones (FQ). Adapted from Schaufler (2017), Mathers et al. (2015)

examined in that study (spanning all five continents) and also showed strong association with drug resistance (having the $bla_{CTX\text{-}M\text{-}15}$, and *aac(6′)-lb-cr* genes) and virotype A (Peirano et al. 2014). Moreover, H30-Rx isolates were found to have higher resistance scores compared to non-H30-Rx ST131 isolates and were associated specifically with CTX-M-15. Three virulence genes (*iha*, *sat*, and *iutA*) were more prevalent among H30 than non-H30 ST131 isolates. Thus, the H30 and H30-Rx sub-clones are considered to be more drug-resistant and have virulence profiles that are distinct from those of non-H30 ST131 (Banerjee et al. 2013). Several studies were performed in order to determine the prevalence and distribution of ST131 sub-lineages worldwide. For instance, a population-based study performed in Minnesota, USA, revealed that 88% of all ST131 infections were due to the H30 sub-lineage. The H30 sub-lineage was most common among adults over 50 years old and its prevalence was positively correlated with age. However, among children under the age of 10, both H30 and non-H30 ST131 isolates were highly prevalent, suggesting that both the old and the young are more vulnerable to ST131 and its sub-lineage (Banerjee et al. 2013). Another population-based study from Canada revealed that 46% of FQ-R *E. coli* isolates were ST131, and 96% of these belonged to the H30 sub-lineage, with 32% belonging to the H30-Rx sub-lineage. The study identified the association of H30-Rx sub-lineage with the clinical features of primary sepsis, upper UTIs, and complication of prostate biopsies. Predictably, the H30-Rx sub-lineage was also associated with multidrug resistance, and with the presence of $bla_{CTX\text{-}M\text{-}15}$ and *aac(6′)-lb-cr* resistance genes (Peirano and Pitout 2014) (Fig. 2).

Taken together, its high prevalence in the young and old, its pathogenicity, and drug resistance make the H30 sub-lineage potentially the most important *E. coli* from a public health perspective.

9 Conclusions

The global spread of ST131 is probably a combination of different factors. Unlike epidemic strains that cause outbreaks that are eventually contained by the medical community, pandemic lineages such as ST131 require a constant reservoir to maintain their extended footprint. The high asymptomatic carriage rates of ST131 provide this reservoir within the human population, but how did they replace other *E. coli* lineages and have become so commonly carried remains unclear. While ST131 is not more infective than other *E. coli* strains, they appear to be stable for longer periods within their hosts (Giufrè 2017), implying that they are fitter either due to resistance to antibiotics, to which carriers are often exposed, especially in LTCF, to increased metabolic potential, or both. A key question is how these strains interact in the colon with many other intestinal bacteria, and also additional *E. coli* strains. Residents of LTCF have been shown to have a microbiota that is less diverse than community-dwelling elderly subjects (Claesson et al. 2012), and one may speculate that such microbiota may be more conductive to colonization with ST131. Furthermore, this less diverse microbiota was associated with higher levels of inflammatory markers raising the question of whether ST131 strains contribute more to chronic inflammation than other *E. coli* lineages.

It remains to be determined whether more responsible antibiotics usage worldwide will lead to a decrease in ST131 carriage or whether specific anti-ST131 measures such as vaccination or phage therapy (Pouillot et al. 2012; Green 2017) will have to be undertaken in order to reduce the burden of this lineage to healthcare systems and to human health.

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Current Trends in Antimicrobial Resistance of *Escherichia coli*

Yossi Paitan

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Abstract *Escherichia coli* is the most common Gram-negative bacterial pathogen, presenting both a clinical and an epidemiological challenge. In the last decade, several successful multidrug-resistant high-risk strains, such as strain *E. coli* ST131 have evolved, mainly due to the growing selective pressure of antimicrobial use. These strains present enhanced fitness and pathogenicity, effective transmission and colonization abilities, global distribution due to efficient dissemination, and resistance to various antimicrobial resistances. Here, we describe the emerging trends and epidemiology of resistant *E. coli,* including carbapenemase-producing *E. coli*, *E. coli* ST131 and colistin resistant *E. coli*.

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1 Introduction

The introduction of penicillin and other antibiotics to the clinical world, about 75 years ago, has led to a significant reduction of death from bacterial infections saving millions of lives. Consequently, it was assumed that the high mortality due to infectious diseases would be a thing of the past. Unfortunately, emergences of resistance have been published shortly after by Abraham and Chain (1940), describing penicillinase, which degrades benzylpenicillin, conferring resistance to penicillin. Since then, development of resistance has become an important factor in the fight against infectious diseases. De novo or transmitted antibiotic resistance mechanisms are extensively studied, involving detoxification of the antibiotic molecule or mutations in the designated target. Antibiotic molecules can be (i) removed by efflux pumps (ii) modified to be less efficient or (iii) destroyed. Antibiotic targets can be (i) enzymatically modified (ii) rendered insensitive by mutations, (iii) protected by a barrier and (iv) replaced.

The selective pressure of antimicrobial use, overuse and misuse in humans, animals and agriculture comprises the engine driving this process leading to a gradual increase in antibiotic resistance. Subsequently, once treatable bacteria are now either untreatable or require the last line of antibiotics (Ventola 2015). Multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan-drug-resistant (PDR) strains of *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* are now reported worldwide, becoming a critical global issue (Boucher et al. 2009; Shlaes et al. 2013). Interspecies and intra-species horizontal gene transfer is considered to be the dominant process for achieving multiresistant bacteria, both in the community and hospital settings (Tzouvelekis et al. 2012). Emerging antibiotic resistance is currently acknowledged as one of the most significant public health problems and mortality rates since MDR bacterial infections are high. It was estimated that in 2002, 1.7 million healthcare-associated infections occur each year in American hospitals and were associated with about 99,000 deaths (Klevens et al. 2007). The ECDC estimated that in the EU, Iceland and Norway about 37,000 patients die each year as a direct outcome of a hospital-acquired infection (HAI), an additional 111,000 die as an indirect outcome of the HAI (ECDC 2008), and about 25,000 patients die from infections with multidrug-resistant bacteria; two-thirds being due to Gram-negative bacteria (ECDC/EMEA Joint Working Group 2009).

Among the resistant bacteria, *E. coli* is the most common Gram-negative bacterial pathogen, causing a diverse range of clinical diseases that affect all age groups. *E. coli* primarily inhabits the lower intestinal tract of humans and other warm-blooded animals and is discharged to the environment through faeces and wastewater treatment plants (Berthe et al. 2013). It represents a diverse collection of bacteria which are usually commensals but also includes pathogens that cause a variety of human diseases, resulting in more than 2 million deaths each year (Kaper et al. 2004). Currently, six types of intestinal pathogenic *E. coli* strains are well studied and characterized. These are classified by different pathogenicity mechanisms and

virulence properties, causing gastrointestinal diseases such as diarrhoea (Kaper et al. 2004; Nataro and Kaper 1998). The intestinal strains include: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and Shiga toxin-producing *E. coli* (STEC) and enterohemorrhagic *E. coli* (EHEC). *E. coli* O157:H7, the most well-known serotype of EHEC, has caused many outbreaks of water- and food-borne diseases worldwide. In addition to the intestinal strains, several *E. coli* strains are involved in extraintestinal diseases. They are called extraintestinal pathogenic *E. coli* (ExPEC). ExPEC comprise a common cause of bacteremia and septicemia, a frequent cause of meningitis in neonates and the majority of urinary tract infections (UTIs) in young healthy women (Russo and Johnson 2003; Dale and Woodford 2015). Therefore, the worldwide emergence of the ExPEC MDR *E. coli* strains, such as sequence type (ST) O25b:ST131 clone, represents a major challenge for the prevention and management of *E. coli* infections (Mathers et al. 2015).

Recent surveillance data from the 2000s indicate that antibiotic resistance to all major antibiotic classes exists among *E. coli* strains. These include the production of extended-spectrum-beta-lactamases (ESBLs) (including TEM, SHV, CMY, and CTX-M types), production of carbapenemases (including KPC, NDM, VIM, OXA-48 and IMP types), resistance to fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole (Pitout 2012a), and recently also plasmid-mediated colistin resistance (Liu et al. 2016). Rapid dissemination of newly resistant ExPEC clones is known to lead to localized outbreaks of extraintestinal disease. Currently, CTX-M-15 is the most prevalent ESBL among ExPEC (Mathers et al. 2015). Moreover, the rise of *E. coli* ST131, which is frequently resistant to extended-spectrum cephalosporins and fluoroquinolones (FQs), has severely complicated the treatment of blood-borne and urinary tract infections in the United States (Johnson et al. 2010) and globally. A clinical antibiotic resistance survey reported that *E. coli* ST131 lineage is accountable for about 70% of clinical *E. coli* infections resistant to FQs, and 55% resistant to both FQs and trimethoprim-sulfamethoxazole (Johnson et al. 2010). Antibiotic-resistant *E. coli* strains are increasingly prevalent, in the US, 31.3% of *E. coli* isolates were FQ resistance (FQ-R) among hospitalized patients, during 2007–2010 (Edelsberg et al. 2014). In India, the prevalence of MDR *E. coli* isolates, among inpatients with UTIs, was 76% (Niranjan and Malini 2014).

The emerging resistant trends of *E. coli* and their origin and epidemiology are the scope of this chapter.

2 Antibiotics Used for Treating *E. coli* Infections

The resistance of *E. coli* and other Gram-negative bacteria to various antimicrobials is due to the development of many different mechanisms. These include antibiotic inactivation and modifying enzymes, β-lactamases, altered permeability and porin

mutations, efflux pumps, binding site and target mutations and 'bypass' of metabolic pathway. The study of resistance mechanisms and patterns is highly important to achieve good clinical outcome, an adequate administration of antimicrobials, and for the development of new antimicrobial compounds. Similar to the variety of infections caused by *E. coli*, there are also plentiful of treatment options against susceptible *E. coli* infections, including penicillins, cephalosporins, monobactams, β-lactam/β-lactamase inhibitor combinations (BLBLI), fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole (TMP-SMX), among others. However, the antibiotic treatment of MDR *E. coli* infections is significantly more limited. The emergence of fluoroquinolone resistance, the production of ESBLs or plasmid-mediated AmpC (pAmpC), carbapenem resistance and recently also colistin resistance have dramatically complicated the treatment options.

2.1 Treatment Against ESBL and PAmpC Producing E. coli

ESBLs are a rapidly evolving group of β-lactamases which share the ability to hydrolyse penicillins, first, second and third generation cephalosporins, aztreonam, cefamandole and cefoperazone. They do not confer resistance to cephamycins and carbapenems and are inhibited by inhibitors of β-lactamases, such as clavulanic acid, sulbactam or tazobactam. The ESBL enzymes constitute more than 300 enzymes and include three major groups, the TEM family comprises more than 160 members, the SHV family comprises more than 110 members and the CTX-M family comprises more than 80 members. Other ESBLs include the OXA-type β-lactamase group and a variety of other β-lactamases such as PER, VEB, GES, BES, TLA, SFO and IBC groups. Different from ESBLs, the pAmpC β-lactamases convey resistance to penicillins, first, second and third generation cephalosporins, cephamycins and aztreonam. They do not confer resistance to fourth generation cephalosporins or carbapenems and are not inhibited by clavulanic acid.

ESBL and pAmpC *E. coli* isolates are often resistant to penicillins, fluoroquinolones, some aminoglycosides and TMP-SMX. Carbapenems are often used as the drug of choice for the treatment of severe infections caused by pAmpC and ESBL-producing isolates, as their activity is unaffected by these enzymes and they are associated with better outcomes than cephalosporins or fluoroquinolones. However, the presence of ESBL and pAmpC in community isolates has led to the increase in the use of carbapenems and to the emergence of carbapenemase producers (Laxminarayan et al. 2013), leading to the search for alternatives to carbapenems for pAmpC and ESBL producers. ESBLs (but not AmpC) are inhibited by β-lactamase inhibitors, and thus BLBLI such as amoxicillin–clavulanic acid or piperacillin-tazobactam are active against some ESBL producers. However, their effectiveness in comparison to carbapenems is still questionable and a randomized trial is currently ongoing in Australia and New Zealand addressing this question (Harris et al. 2015). Temocillin is a revived β-lactam that is stable against both ESBL and pAmpC enzymes, displayed good results in several uncontrolled studies

(Balakrishnan et al. 2011), but it is commercialized only in few countries and should be farther investigated in a randomized trial. Additional possible medications include non-β-lactamase antibiotics if the specific isolate is susceptible. Finally, fosfomycin and nitrofuraintoin are frequently active against ESBL producers and are therefore another option. In addition, colistin can be used and is prescribed in rare cases when other medications have failed to stop the ESBL infection, as it is usually reserved for extensively drug-resistant isolates.

2.2 Treatment Against CPE or Colistin Resistant E. Coli

Treatment of carbapenemase-producing *E. coli* is dramatically limited and the potential therapeutic options depend on the specific type of carbapenemase produced. Aztreonam is usually active against metallo-β-lactamases (MBLs) such as New Delhi metallo-beta-lactamase (NDM), Verona integron-encoded metallo-beta-lactamase (VIM) and Imipenemase metallo-beta-lactamase (IMP), while cephalosporins are usually active against Oxacillinase-48 (OXA-48) producers. However, if an ESBL or AmpC are co-produced with one of these carbapenemases (which unfortunately occurs frequently), the use of aztreonam against MBLs and cephalosporins against OXA-48 is eliminated. In comparison to the MBLs and OXA-48 carbapenemases, the *K. pneumoniae* carbapenemase (KPC) producers are resistant to all β-lactams. In such cases of KPC producers or co-production of NDM, VIM, IMP or OXA-48 and ESBL or AmpC, the most frequent active options are colistin, polymyxin B, tigecycline, and in some cases fosfomycin or aminoglycosides (Tzouvelekis et al. 2012). In addition, the use of different combination therapies has been reported, in some cases even as being superior to monotherapy (Tumbarello et al. 2012), moreover, dual-carbapenem treatment course was reported, in some cases, as being a successful combination therapy against CPE (Zavascki et al. 2013). However, combination therapy is still controversial as reported by a meta-analysis of studies on carbapenem-resistant Gram-negative bacteria (Paul et al. 2014).

Different from carbapenemase producer *E. coli* strains, which are often considered to be MDR or XDR, currently most colistin resistant *E. coli* isolates are still susceptible to several specific antibiotics, which are case-dependent and cannot be generalized for correct treatment. In the rare cases of co-expression of carbapenemase and plasmid-mediated colistin resistance (MCR), often the therapeutic options are limited to fosfomycin and tigecycline (Karaiskos and Giamarellou 2014) or to a double-carbapenem regimen in the case of PDR isolates (Giamarellou et al. 2013). As the use of combination therapy controversial has several problems, clinical therapy should be on a specific individual basis considering available options, and infection source and severity.

Colistin, tigecycline, carbapenem and different combination therapy are the last-resort antibiotic therapies against infections caused by MDR, XDR and PDR Gram-negative pathogens. Recently, a new generation of β-lactamase inhibitors and

inhibitor combinations is being developed (Bush 2015; Wright et al. 2017). These include avibactam, relebactam, nacubactam, AAI101 and the novel boronic acid inhibitor vabrobactam, which are able to inhibit most class A and class C beta-lactamases, with selected inhibition of class D enzymes by avibactam. All these inhibitors are being developed in combinations, targeting primarily carbapenemase-producing Gram-negative pathogens. β-lactamase inhibitors combinations such as ceftolozane/tazobactam and ceftazidime/avibactam were recently approved by the US Food and Drug Administration (FDA), others, including ceftaroline fosamil/avibactam, aztreonam/avibactam, imipenem/cilastatin/relebactam, meropenem/vabrobactam and cefepime/AAI101, are in different stages of clinical trials (Bush 2015; Wright et al. 2017). Other antimicrobial agents, in different stages of development or clinical trials, against gram-negative bacteria include cefidercol, a novel siderophore cephalosporin active against class A, B, C and D β-lactamase producers, carbapenem-resistant *P. aeruginosa*, *A. baumannii* and *Stenotrophomonas maltophilia*, plazmoicin, a new aminoglycoside inhibiting protein synthesis by binding to the ribosomal 30S subunit of bacteria and eravacycline, a novel fluorocycline tetracycline, which binds the bacterial ribosome and inhibits bacterial protein synthesis.

Clinical management of MDR infections is becoming more and more challenging. Therapeutic decisions are important for the individual patient clinical outcome, eliminating recurrence of infections and for avoiding further selection pressure and resistant development. High-quality randomized clinical trials and observational studies are very important for clinical infection management, that due to the resistant nature of specific isolates should be taken on an individual basis.

3 Carbapenemase–Producing *E. coli*

3.1 Introduction to Carbapenemases

Carbapenem resistance in Enterobacteriaceae (CRE) is an increasing worldwide concern and is a clinical challenge with implications for both clinical practice and for public health (Tzouvelekis et al. 2012). Accordingly, in 2013, the US CDC reported that carbapenem-resistant Enterobacteriaceae (CRE) are one of the three most urgent antimicrobial resistant threats, and in 2017, the WHO included CRE in the critical group of WHO Priority Pathogen List for Research and Discovery of New Antibiotics (WHO 2017). Carbapenem resistance can result from several mechanisms including, porins coupled with ESBL production, membrane permeability changes via mutations in efflux pumps or by hydrolysis of the beta-lactam ring by dedicated carbapenemase enzymes. Carbapenemases are specific beta-lactamases, which hydrolyses carbapenems, ertapenem, meropenem, imipenem, and doripenem, the four most clinically used carbapenems. It is highly alarming that carbapenemases, once rare, are now being reported extensively across

the globe (Nordmann and Poirel 2014). Often, the genes encoding these carbapenemases are associated with other genes encoding resistance to non-beta-lactam antibiotics on highly mobile plasmids, resulting often in MDR bacteria and in a possible rapid transmission of broad-spectrum resistance (Nordmann et al. 2011a). Carbapenemases belong to Ambler class A or D serine beta-lactamases and ambler class B MBLs. The most common variants of carbapenemases, in carbapenemase-producing Enterobacteriaceae (CPE), include KPC (Class A), NDM (Class B), OXA-48 (Class D), VIM (Class B), and IMP (Class B) but several other enzymes are also present.

Risk factors for CPE infections include prior hospitalization, exposure to broad-spectrum antibiotics, indwelling catheters, mechanical ventilation, transplantation and previous colonization with CPE (Tumbarello et al. 2012; Zarkotou et al. 2011). Severe CPE infections include pneumonia and urinary tract, bloodstream, intra-abdominal, central venous catheter-related and surgical site infections, which were associated with high mortality rates of approximately 40% (Tumbarello et al. 2012; Zarkotou et al. 2011). Carbapenems resistance among *E. coli* is highly concerning as these agents are often the last line of effective therapy available for the treatment of patients with serious infections (Pitout 2012a). NDM and OXA-48 are the most common carbapenemases among *E. coli* worldwide (Nordmann and Poirel 2014).

3.2 The CPE Global Epidemic

The global spread of CPE is presently one of the main public health threats (Sherchan et al. 2015) as carbapenems comprise one of the last treatment options for infections caused by ESBLs. Carbapenemases were originally described as a rare phenomenon in *P. aeruginosa* and *A. baumannii* from Greece or South-East Asia and are now widely disseminated in *E. coli* and *K. pneumoniae* (Tzouvelekis et al. 2012). KPC, the most clinically significant carbapenemase, was initially reported from *K. pneumoniae* strains isolated in the US in the early 2000s (Yigit et al. 2001), but soon dispersed to other regions of the world (Tzouvelekis et al. 2012). These were the first steps of the global CPE epidemic, which started in *K. pneumoniae*, first observed in Greece and in the USA in the early 2000s. The Greek epidemic was related to VIM (Giakkoupi et al. 2003) whereas the US epidemic was related to KPC. Few years after, KPC replaced VIM in Greece and also disseminated to Israel and later to Asia, Italy and South America (Tzouvelekis et al. 2012). Several years later, the first report of NDM in India was reported (Yong et al. 2009; Kumarasamy et al. 2010), since then, it disseminated globally to a large number of species, including community-related pathogens such as *E. coli* (Nordmann et al. 2011b; Nordmann and Poirel 2014). Finally, the OXA-48-like carbapenemas emerged, with a comparable pattern of dissemination like NDM, although its spread in Europe was mostly associated with healthcare institutions (Nordmann and Poirel 2014). Although KPC is the most common carbapenemase, NDMs have been

shown to be more promiscuous (Kim et al. 2013). As of June 2017, 50 states in the USA reported the isolation of CRE arising from KPC, 30 NDM, 25 OXA-48, 12 IMP and 10 VIM (US CDC 2016a)

3.2.1 KPC-Beta-Lactamases

KPC-1 was discovered in a carbapenem-resistant *K. pneumoniae* isolated in NC, USA (Yigit et al. 2001). As of August 2017, 18 different KPC variants have been described, KPC-1/2 to KPC-19 (KPC-2 was shown to be identical to KPC-1). Unlike previously described Class A carbapenemases (SFC, NMC/IMI and SME), KPC is plasmid borne, which may facilitate its rapid spread among Enterobacteriaceae (Hossain et al. 2004). KPC soon disseminated globally, resulting in several nosocomial outbreaks reported in the USA, and several other South American, European, Asian and Middle Eastern countries. Consequently, KPC has become endemic in several parts of the world, such as North-Eastern USA, Israel, Greece, China, Colombia and Puerto Rico (Nordmann et al. 2009).

KPC confers resistance to the carbapenems, cephamycins, cephalosporins and monobactams. They are weakly inhibited by ‘classical’ beta-lactamase inhibitors such as clavulanic acid and tazobactam. KPCs have been described in several enterobacterial species, predominantly *K. pneumoniae* but also *E. coli, P. aeruginosa* and *Enterobacter cloacae* (Tzouvelekis et al. 2012). The genes encoding the production of KPC enzymes are located on a transferable plasmid associated with the mobile genetic element Tn4401, which is responsible for the effective spread among different Enterobacteriaceae (Naas et al. 2008). The plasmids encoding KPC enzymes are often carrying additional genes to other antibiotic resistance agents, such as aminoglycosides, quinolone, sulfonamides, trimethoprim and tetracyclines. Several studies reported the identification of KPC in *E. coli* ST131 strains. These include reports from the United States, Italy, France, Ireland and China, where they recently caused outbreaks (Kim et al. 2012; Johnson et al. 2015; Accogli et al. 2014; Naas et al. 2011a; Morris et al. 2011; Cai et al. 2014). These strains emphasize the alarming scenario of pan resistance in an *E. coli* clone that has already demonstrated its capacity to disseminate globally.

3.2.2 NDM-Beta-Lactamases

NDM-producing *E. coli* and *K. pneumoniae* were initially isolated from a Swedish patient, hospitalized in New Delhi, India. The NDM gene was carried on plasmids, which readily transferred between bacterial strains in vitro (Yong et al. 2009). As of August 2017, 16 different NDM variants have been described, on a variety of plasmid types, consistent with the diversity of Enterobacteriaceae species reported to express NDM. Following this isolation, it was reported that NDM-producing *E. coli* were prevalent in medical centres across all India and Pakistan (Kumarasamy et al. 2010). NDM-producing bacteria were also reported in some

UK patients that recently travelled to India for some medical procedures. NDM quickly disseminated among different Enterobacteriaceae in the Indian subcontinent, UK and Americas (Castanheira et al. 2011a, b; Lascols et al. 2011). In some Indian hospitals, the prevalence of *E. coli* NDM-1 was as high as 6% among *E. coli* isolated from hospitalized and outpatients between February 2010 and July 2010 in a hospital in Varanasi (Seema et al. 2011), 7% in a hospital in Mumbai (Deshpande et al. 2010) and 15% in Rawalpindi, Pakistan (Perry et al. 2011). Since then, NDM-1 positive bacteria disseminated globally, initially isolated from patients with an epidemiological link to the Indian subcontinent and then found in other patients in the Balkan states and the Middle East, which were considered also as secondary reservoirs for the spread of NDMs (Nordmann et al. 2011b). NDM positive *E. coli* and other Enterobacteriaceae have been isolated from a variety of clinical conditions including septicemia, peritonitis, pulmonary infections, soft tissue infections, device-associated infections and hospital and community-onset UTIs. This diversification of clinical settings reflects on the wide disease spectra of these bacteria (Nordmann et al. 2011b), which also include recovery from gut flora of patients and travellers returning from endemic countries (Leverstein-Van Hall et al. 2010) and from a variety of environmental samples (Walsh et al. 2011).

The NDM-1 gene has been detected on different plasmids, often on broad-host-range plasmids such as IncA/C plasmids (Walsh et al. 2011) but also on a IncFII plasmid (pGUE-NDM) in an *E. coli* ST131 isolate, isolated in France from a patient who was living in Darjeeling (India) (Poirel et al. 2010). It was also isolated from a patient admitted to a hospital in Chicago after hospitalization in New Delhi, India (Peirano et al. 2011). It should be noted that in the New Delhi *E. coli* ST131 strain, NDM-1 was carried by an IncF plasmid carrying the FIA replicon (Peirano et al. 2011). Sequence analysis of pGUE-NDM indicated that the NDM-1 gene was acquired by a plasmid resembling plasmids containing CTX-M-15. However, there is no evidence that NDM-producing *E. coli* are prone to be more virulent than other *E. coli* isolates. The reports that NDM genes are present in *E. coli* ST131 are of high concern as *E. coli* ST131 is described as a very successful strain responsible to the global dissemination of CTX-M-15. Moreover, several reports (Zong 2013; Accogli et al. 2014) indicated that *E. coli* ST131 might contain several plasmids, carrying different resistant genes and VF, at the same time, even of the same incompatibility group (IncF). A comprehensive study, of 38,266 Enterobacteriaceae isolates from 40 different countries, reported that the global incidence of MBL enzymes, between 2012 and 2014, was generally low (0.5%, 163/38,266), but having high dissemination rates (85%, 34/40 countries). However, among these MBLs, NDM-1 was the most prevalent gene in Enterobacteriaceae (Kazmierczak et al. 2015). In the Study for Monitoring Antimicrobial Resistance Trends (SMART), from 2008 to 2012, NDM-1 was identified as the most prevalent variant in NDM associated infections (96.3%, 130/135). In addition, the presence of ESBLs and NDM in the same isolate was high (78.5%, 106/135). However, the prevalence of dual carbapenemase, in the same isolate, was low (1/135 with VIM-1+NDM, 2/135 with OXA-181+NDM) (Biedenbach et al. 2015).

These findings of NDM positive *E. coli,* high prevalence of ESBLs+NDM and the co-expression of two carbapenemases in the same isolate highlight the worsening global threat of such bacteria.

3.2.3 OXA-48-like Beta-Lactamases

The OXA family of enzymes comprises more than 400 enzymes; OXA-48-like enzymes (including OXA-48, OXA-162, OXA-163, OXA-181, OXA-204, and OXA-232) represent one of the key families responsible for carbapenem resistance in *E. coli* and other Enterobacteriaceae. OXA-48 confers resistance to penicillins, weakly hydrolyses carbapenems and has nearly no activity against oxyimino–cephalosporins (e.g. cefotaxime, ceftriaxone and ceftazidime) and the monobactams (e.g. aztreonam) (Nordmann et al. 2011a). In some cases, the carbapenem MICs may be only slightly elevated (Nordmann et al. 2012), therefore, detection of OXA-48 can be challenging. This was demonstrated by the late detection of a large outbreak in Rotterdam, the Netherlands (Dautzenberg et al. 2014). As OXA-48 is frequently present in ESBLs producing strains, such strains are rarely susceptible to extended-spectrum cephalosporins (Nordmann et al. 2012). Of all the carbapenemases, the OXA-48-like carbapenemase is spreading rapidly in many European Countries, and is endemic in the Middle East and in Northern Africa. Considerable dissemination has been observed in Belgium, France, the UK, Germany and the Netherlands (Glasner et al. 2013). OXA-48 was initially reported in *K. pneumoniae* isolates in Turkey in 2001 (Poirel et al. 2004), and since then in many other Mediterranean countries (including Spain, France, Italy, Israel, Egypt, Morocco, Lebanon and Turkey) North Africa, Europe, and in the USA. OXA-48 is often detected in *K. pneumoniae*, but also in *E. coli* and other members of the Enterobacteriaceae family (Poriel et al. 2012a). The OXA-48 gene is typically located on conjugative plasmids belonging to the IncL/M incompatibility group (recently re-classified into IncL and IncM; Carattoli et al. 2015), principally accountable for its spreading among Enterobacteriaceae (Poirel et al. 2012b; Carattoli et al. 2015).

OXA-48-like enzymes have been detected in several *E. coli* clonal groups, including ST131 (Peirano et al. 2014; Morris et al. 2012; Dimou et al. 2012). OXA-48-like positive *E. coli* ST131was reported in many countries, including Turkey, Iran, Denmark, UK, US and many other countries indicating a possible global dissemination. Sporadically, OXA-48 has also been found in the *E. coli* chromosome (Beyrouthy et al. 2013, 2014). In both cases (plasmids and chromosomes), the gene is carried by Tn1999-like transposons, designated Tn1999.1–Tn1999.4 (Beyrouthy et al. 2014; Poirel et al. 2012b; Giani et al. 2012). Of the OXA-48 group, OXA-163 differs in its enzymatic abilities. OXA-163 has a superior capacity to hydrolyse extended-spectrum cephalosporins and inferior capacity to hydrolyse carbapenems compared to the other enzymes in the OXA-48 group (Poirel et al. 2011), and consequently, it may not be recognized in phenotypic detection. OXA-163 is often located on an IncN plasmid harbouring also multiple

other resistance mechanisms, this is alarming, as IncN plasmids have played a significant role in the dissemination of other problematic extended-spectrum beta-lactamases/carbapenemases in *E. coli* ST131 (Adler et al. 2015). OXA-163 was reported in Cairo, Egypt and is endemic in several hospitals in Buenos Aires, Argentina (Poirel et al. 2012a). It is possible that OXA-163 is more prevalent than previously estimated, as its phenotypic identification is more difficult.

3.2.4 VIM-Beta-Lactamases

The VIM-beta-lactamase is the most commonly found class B carbapenemases, which has been identified in all continents (Nordmann et al. 2011a; Vatopoulos 2008). It hydrolyses a wide variety of beta-lactams, such as penicillins, cephalosporins and carbapenems, but not the monobactams (i.e. aztreonam) and it is inhibited by metal chelators. VIM is often associated with class 1 integrons that contain various gene cassettes, often conferring resistant to various groups of other antimicrobial agents (Poirel et al. 2007).

VIM-enzymes (there are now more than 30 derivatives) were first described in *P. aeruginosa* from Verona, Italy (Lauretti et al. 1999) and then emerged in Enterobacteriaceae as well. Since then, several hospital outbreaks caused by VIM-1 like enzymes were described from various regions in Italy (Aschbacher et al. 2008, 2011), followed by reports of endemic situation in *K. pneumoniae* from Greece (which was eventually replace by KPC in Greece) (Tzouvelekis et al. 2012). VIM is still largely found in Italy and Greece (Pitout et al. 2015). It is common in the Balkans, in the Indian subcontinent in the late 2000s and early 2010s, and in some areas of the Middle East (Tzouvelekis et al. 2012), including France (Lartigue et al. 2004), Spain (Oteo et al. 2015), Morocco (Barguigua et al. 2013), Egypt (Poirel et al. 2013), Algeria (Robin et al. 2010), and Tunisia (Ktari et al. 2006). VIM-1 was reported in various species of Enterobacteriaceae, isolated from a hospital in Province of Bolzano (Aschbacher et al. 2011, 2013). In these isolates, the VIM-1 gene was located on various plasmids belonging to the incompatibility group IncN (Carattoli et al. 2010) and was found to be associated with *E. coli* ST131 (Aschbacher et al. 2011; March et al. 2014), subtyping of 2 VIM-1 *E. coli* ST131 isolates identified them as the subclone H30 (Accogli et al. 2014). VIM containing *E. coli* isolates were also reported in other parts of the world including the USA (Yaffee et al. 2016).

3.2.5 IMP-Beta-Lactamases

The IMP-beta-lactamase gene was initially isolated in Japan, from a *Serratia marcescens* isolate resistant to imipenem, which had a chromosomally encoded MBL gene. This MBL was subsequently designated Imipenemase-1 (IMP-1) metallo-beta-lactamase (Osano et al. 1994). The IMP-1 was consequently isolated also from *P. aeruginosa*, *K. pneumoniae*, and *Acinetobacter* in Japan and Singapore (Osano et al. 1994; Senda et al. 1996a, b; Koh et al. 1999). The first

plasmid-mediated dissemination of IMP was also found in a *S. marcescens* isolate in Japan in 1991 (Ito et al. 1995). A second IMP variant was isolated from Acinetobacter in Italy, and designated IMP-2 (Cornaglia et al. 1999). IMP-2 has 84.9% amino acid homology with IMP-1 (Riccio et al. 2000), followed by description of the isolation of IMP-3 from Shigella flexneri in Japan (Iyobe et al. 2000), IMP-4 from Hong Kong (Chu et al. 2001) and other IMP variants. IMP enzymes are prevalent in Japan, China, and Australia (Nordmann et al. 2011a). In Australia, IMP-4 was the most common carbapenemase (82.7%, 48/58) found in July 2009 to March 2014, most frequent (60.4%, 29/48) in *E. cloacae* on a broad-host-range conjugative plasmid, and in *E. coli* isolates. These findings possibly explain its spread in Sydney, Australia (Sidjabat et al. 2015a), and the cause of a simultaneous outbreak in Melbourne, Australia (Peleg et al. 2005). In addition, an interspecies transfer IMP-4 plasmid from *E. cloacae* into *E. coli* was reported in Australia (Sidjabat et al. 2014). IMP-4 outbreak-like situations were also reported due to contaminated equipment or environment (Kotsanas et al. 2013). In China, a paediatric patient had 7 isolates of Raoultella ornithinolytica, each with a closely related IMP-4, one of which produced both IMP-4 and KPC-2 (Zheng et al. 2015a).

Several reports described the presence of IMP in different *E. coli* isolates. *E. coli* containing IMP were isolated in Turkey, Taiwan (Wang et al. 2015), Hong Kong (Ho et al. 2016), Italy (Ortega et al. 2016) and several other countries. In Japan, recently, two different IMP positive *E. coli* strains were isolated, belonging to *E. coli* ST95 and ST4508 (Ohno et al. 2017). The emergence of IMP-4 in *E. coli* ST131 constitutes another worrisome step in the spread of carbapenemases in the world, as *E. coli* ST131 has already demonstrated its capacity to disseminate globally. IMP-8 containing *E. coli* ST131 was initially isolated in Taiwan (Yan et al. 2012). In this report, six IMP-8 producing isolates of *E. coli* were isolated, two belonging to B1 and D phylogroups and were typed as ST359 and ST457, respectively. The remaining four, belonged to the B2 phylogenetic group and were farther classified as *E. coli* ST131. It should be noted, that these four strains were isolated from four different non-hospitalized patients, which were admitted to the same hospital 1–4 months before, but with no evidence for any epidemiological link between them (Yan et al. 2012). In Australia, two IMP-4 *E. coli* ST131 isolates were obtained from patients from two hospitals (Sidjabat et al. 2015b), and an IMP *E. coli* ST131 subclone H30 in Shanghai, China (Zhang et al. 2015b). The emergence of IMP in *E. coli* ST131 and its isolation also from the community is of high concern, as antibacterial therapy of such bacteria is very limited and often restricted only to tigecycline or polymyxins.

3.2.6 Co-expression of Carbapenemases

Carbapenems resistance among Enterobacteriaceae in general, and in *E. coli* in particular, constitutes one of the major concerns among healthcare systems, mainly due to increasing morbidity and mortality among hospitalized patients carrying such bacteria. The increase in CPE is mostly linked to the extensive dissemination

of acquired carbapenemases. Carbapenemase-encoding genes are often located in mobile genetic elements, harbouring also other resistance determinants and resulting in MDR and XDR strains. The dissemination of such genes by horizontal gene transfer poses an alarming public health threat and a difficult challenge for treatment. Carbapenems are considered one of the last-line treatments of enterobacterial infections. Co-expression of different carbapenemase genes in one isolate possesses even a higher threat, possibly enabling faster dissemination and a greater clinical challenge. Reports regarding co-expression of such genes have started appearing few years after the initial description of these genes. Currently, there are reports describing almost all possible combination of two genes of the five main carbapenemase genes (KPC, NDM, OXA-48, VIM and IMP) in different bacteria, including *E. coli*. Moreover, reports regarding co-expression of three carbapenemase genes have also been published. A *Klebsiella oxytoca* isolate co-expressing NDM-1, IMP-4 and KPC-2 was isolated in 2013 from a urine specimen, obtained from a 74-year-old patient, in China (Wang et al. 2017a). These reports regarding co-expression of different carbapenemases emphasize the important role played by mobile genetic elements in their dissemination and as a present threat to the successful treatment of such infections. These reports highlight the critical need for early detection and active surveillance of these resistance elements, particularly given the opportunity of different carbapenemase genes to cross onto the same plasmid.

The emergence of CPE including in *E. coli* ST131, and the co-expression of two or three carbapenemases in a single isolate, often with other antibacterial resistant agents, poses a worldwide concern, consequently polymyxins and tigecycline are frequently the only effective treatment for such MDR/XDR isolates.

4 Extraintestinal Pathogenic *E. coli* ST131

4.1 Phylogenetic Groups

Extraintestinal pathogenic *E. coli* is a global human pathogen involved and frequently the cause of many systemic infections including UTI, nosocomial pneumonia, cholecystitis, peritonitis, cellulitis, osteomyelitis, neonates meningitis, and it is also the most common Gram-negative causative of bacteremia worldwide (Pitout 2012b). A variety of virulence factors (VFs) including invasins, toxins, adhesions, proteases, lipopolysaccharides and capsules enables ExPEC the means to become a successful human pathogen. However, the precise role of these VFs in ExPEC physiology and pathogenesis has not been well defined. It appears that these putative VFs contribute to fitness (e.g. iron uptake systems, bacteriocins, proteases and adhesins) of ExPEC and increase the competitiveness, adaptability and the ability to colonize the human body (Mokady et al. 2005). ExPEC include several variants: avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal

meningitis-causing *E. coli* (NMEC) and septicemic *E. coli* (Kaper et al. 2004). However, there is a considerable overlap between the groups (Ron 2006). Phylogenetic studies indicated that intestinal *E. coli* and ExPEC fall into four main phylogenetic groups, namely, A, B1, B2 and D (Herzer et al. 1990). ExPEC belongs mainly to group B2 and, to a lesser extent to group D, while intestinal commensal isolates tend to belong to groups A and B1.

Most of the ESBL-related *E. coli* infections are due to a globally disseminated, recently emerged ExPEC clone ST131 (Banerjee and Johnson 2014), mostly corresponds to serogroup O25b (Rogers et al. 2011) or O16 (Johnson et al. 2014) and to the phylogenetic group B2 (Nicolas-Chanoine et al. 2008). Among ESBL enzymes, CTX-M-15 is the most prevalent type of CTX-M enzyme, isolated from human clinical *E. coli* isolates (Pitout and Laupland 2008). ExPEC producing this enzyme frequently belong to ST131, and to a lesser extent to ST38, ST405, ST648 or other STs (Peirano and Pitout 2010), contributing to the global emergence of CTX-M-15 producing bacteria. There is increasing evidence that certain clonal lineages of these species, such as *E. coli* ST131 have more epidemic potential than other lineages within their species group.

E. coli ST131 was first described in 2008 (Nicolas-Chanoine et al. 2008) and since it was isolated and reported worldwide, both in healthcare settings and in the community. ST131 is the predominant *E. coli* lineage among ExPEC isolates, and is mostly associated with ESBL production and FQ-R (Nicolas-Chanoine et al. 2014; Rogers et al. 2011) therefore associated with limited treatment options, recurrence and frequent clinical failure. CTX-M-15 beta-lactamase is the dominant ESBL in ST131 but other CTX-Ms also occur in ST131, particularly CTX-M-14/14-like variants (Nicolas-Chanoine et al. 2014). The parallel identification of CTX-M in ST131 isolates from different countries and continents suggests repetitive acquisition vie multiple horizontal gene transfer events (Cantón et al. 2012). Accordingly, both CTX-M-15 and CTX-M-14/14-like variants occur on conjugative plasmids, manly IncFII type (Naseer and Sundsfjord 2011), but also can be inherited stably or integrated into the chromosome (Rodríguez et al. 2014).

E. coli ST131 belongs mostly to subgroup 1 of phylogenetic group B2. ST131 strains are mostly of serotype O25:H4, with a specific O25 type, O25b (Nicolas-Chanoine et al. 2008). However, *E. coli* ST131 isolates of O16:H5 have been identified in several different countries (Blanc et al. 2014). Phylogenetic studies demonstrated that the ST131 strains EC958, NA114 and JJ1886 cluster together in a clade discrete from ST131 strain SE15 and separate from any other *E. coli* phylogroups (Schembri et al. 2015). Population genetics and next-generation-sequencing (NGS) studies of ST131 global epidemiology demonstrated that ST131 consists of different clades: clade A, associated with *fimH*41, clade B, with *fimH*22, and clade C, with *fimH*30. The diversity of *fimH* alleles may represent different colonization abilities of different clades. Moreover, two different subclasses were identified within clade C, a FQ-R strain, defined as C1/H30R possessing the FQ-R alleles *gyrA*1AB and *parC*1aAB, and an ESBL CTX-M-15 producer strain defined as C2/H30-Rx (Petty et al. 2014; Price et al. 2013).

4.2 Evolution of E. Coli *ST131*

Historically, before the 1990s, most ExPEC strains were susceptible to FQ and cephalosporins (Pitout 2012a). Recently, the World Health Organization (WHO) reported that FQ-R is highly prevalent globally and that in more than half of the clinical cases FQs are ineffective (WHO 2014). Moreover, FQ-R is frequently associated also with resistance to the cephalosporins, primarily due to production of the ESBL enzyme CTX-M-15 (Nicolas-Chanoine et al. 2014). Consecutive acquisition of various VFs, antimicrobial resistance genes and certain genomic islands (GIs), starting during the 1960–1980s, played a central role in the emergence of clade C, and primed the successful dissemination of both subclades C1 and C2 in the 1990–2000s. Clade B is mostly FQ susceptible and rarely harbours CTX-M-15 ESBL plasmids, while clade C is mostly FQ-R and the C2 subclade is frequently associated with the presence of CTX-M-15 ESBL plasmids (Nicolas-Chanoine et al. 2014). Several recent NGS-based studies (Stoesser et al. 2016; Ben Zakour et al. 2016) and plasmid analysis studies (Johnson et al. 2016; Andersson and Hughes 2010) explored the origin and evolution of ST131 clade C, characterizing more than 500 global ST131 clinical, environmental and veterinary isolates. These studies describe a sequential evolution process, wherein, clade C evolved from clade B, most likely during the late 1980s in North American context, which is consistent with the widespread use and introduction of FQs and extended-spectrum cephalosporins (Stoesser et al. 2016; Ben Zakour et al. 2016). They identified several ST131 isolates that share both clade B and clade C characteristics, which were referred to as intermediate strains named B0 (closer to clade B) and C0 (closer to clade C). The transition of clade B to clade B0 (intermediate clade) started with the insertion of the Flag-2 locus at the 1950s, and by acquisition of prophages Phi 2, 3 and 4 in the 1960s to 1970s (Stoesser et al. 2016). The next step, occurred around 1980, was the insertion of GI-*pheV*, GI-*leuV* and Phi 1 and recombination of *parC1a* and of *fimH30* resulting in the intermediate clade C0 (*parC1a* does not confer FQ resistance). It should be noted that GI-*pheV* carries several VFs, including *sat*, the ferric aerobactin biosynthesis gene cluster (*iucABCD*), its cognate ferric siderophore receptor gene *iutA*, and the autotransporter genes *agn-43*. At the same time, occurred the introduction of an F2:A1 plasmid type (without CTX-M-15) into clade B0 or clade C0, resulting in clade C (Johnson et al. 2016; Andersson and Hughes 2010), which separated into subclades C1 and C2. The evolution of clade C to C1 and C2 occurred, at the late 1980s, in parallel to the introduction of FQs to clinical practice and the wide use of extended-spectrum cephalosporins following the acquisition of high-level FQ-R mutations in *parC (prC1aAB)* and *gyrA (GyrA1AB)* (Stoesser et al. 2016; Ben Zakour et al. 2016). An additional final step of subclades C1 and C2 evolution was acquiring different plasmids. Subclade C1 acquired the F1:A2:B2 plasmid, while the F2:A1:B plasmid in subclade C2 acquired antibiotic resistance cassettes containing CTX-M-15, *catB4*, OXA-1, *aac(6')Ib-cr* and *tetAR* through IS26-mediated events (Johnson et al. 2016; Andersson and Hughes 2010). It should be noted that,

in some C2 isolates, the antibiotic resistance cassettes on plasmid F2:A1:B were lost, and in other cases, the CTX-M-15 gene integrated into the chromosome.

Despite the predominance of these two ST131 subclades C1 and C2 globally, there are several different ST131 strains that emerged in different countries. In Japan, CTX-M-15 is rare, ST131 subclade C1 containing a CTX-M-14 was predominant before 2005, and has been replaced by a CTX-M-27 containing subclade C1 (Matsumura et al. 2015) and is considered to be responsible for a substantial increase of ESBL-producing ExPEC in Japan, since 2010. An NGS-based study (Matsumura et al. 2016) of 10 global ST131 and 43 Japanese ST131 isolates containing CTX-M-27, CTX-M-14 or CTX-M-15 examined the emergence of this ST131 with CTX-M-27 strain (Matsumura et al. 2016). The study demonstrated that a diverse ST131:O75:H30 strain formed a different cluster in the C1 subclade and was named C1-M27. C1-M27 has a unique Phi-like region (M27PP1), and was since isolated also in Thailand, Australia, Canada and the USA.

The widespread use of antibiotics in humans, animals and agriculture, in the last 50 years, has led to a growing selective pressure on bacteria, driving them to adapt accordingly and to develop resistance. *E. coli* ST131 is one of those bacteria that has developed resistance to both FQ (due to mutations in *parC* and *gyrA* and extended-spectrum cephalosporins (due to the production of the ESBL CTX-M-15). The global spreading of ST131 has led to a worldwide increase in ESBL-producing *E. coli* in both hospital and community settings. The success of ST131 clade C, partly driven by its sequential acquisition of VFs, FQ-R and ESBL production, probably resulted in a fitter ExPEC clone which better survives in its competitive environment. The accumulative studies regarding *E. coli* ST131 isolates and in particular the clade C indicate that they have all the important characteristics that define a high-risk clone. These include global distribution, effective transmission among hosts, ability to colonize and persist in its host, enhanced fitness and pathogenicity, various antimicrobial resistance including FQs and extended-spectrum cephalosporins and the ability to cause severe and recurrent infections. The likelihood of ST131 clade C to acquire additional antibiotic resistance genes including carbapenemases is, clearly, a global concern.

5 Colistin-Resistant *E. coli*

5.1 Introduction to Polymyxins

The polymyxins are a group of cyclic non-ribosomal cationic polypeptide antibiotics discovered in the 1960s, which soon after their discovery most of the agents displayed high toxicity to mammalian kidney and could not be used safely in humans. Exceptions were polymyxin E (colistin), and polymyxin B (PMB), which were considered to be safer and consequently brought to the market to fight against *Pseudomonas* spp. and other Gram-negative infections. However, following their

initial use in clinical practice, reports regarding their nephrotoxicity were published and they were ultimately shelved in favour of new and safer agents as systemic agents. For the treatment of human infections, they were restricted to ophthalmic and topical use; however, they have been used extensively for decades in veterinary medicine. The recent worldwide increase in Gram-negative MDR, XDR and PDR bacteria, has forced clinicians to reintroduce the systemic use of polymyxins, mostly colistin in its inactive prodrug form, colistin methane sulfonate (CMS), as a last-resort drug for infections with such bacteria (Levin et al. 1999). In Gram-negative bacteria, until 2015, resistance to polymyxin was attributed to chromosomal modification of the outer membrane lipopolysaccharide (LPS) biosynthesis pathway, or in some cases, to complete removal of the LPS part of the outer membrane (Trent 2004; Pelletier et al. 2013; Moffatt et al. 2010). These types of polymyxin resistance rate are still low in most countries but are increasing in some others such as Italy and Greece (ECDC 2015). However, few years after the reintroduction of polymyxins into systemic clinical practice, the emergence of colistin resistant (Col-R) strains was reported, predominantly in CRE isolates (Antoniadou et al. 2007; Kontopidou et al. 2011) and based on the known resistance mechanisms, was considered as chromosomally mediated and therefore not transmissible (Ah et al. 2004).

5.2 Plasmid-Mediated Colistin Resistance

At the end of 2015, the first report of plasmid-mediated colistin resistance (MCR) strains of Enterobacteriaceae was published; the resistance was attributed to a newly discovered MCR-1 gene (Liu et al. 2016). The MCR-1 enzyme belongs to the phosphoethanolamine transferase enzyme family and was initially described in China during a routine surveillance of food animals in 21% of healthy swine at slaughter, 15% of marketed pork and chicken meat, and in one case of a hospitalized patient (Liu et al. 2016). It is believed that this alarming report may have worsened the resistance situation at the global scale and indeed, consequently, reports from all continents have described the isolation of MCR-1-positive strains (Skov and Monnet 2016). Since the first report, MCR-1 has been detected in Enterobacteriaceae isolates from colonized and infected humans, from farm and wild animals, from food (meat and vegetables), from environmental samples and from aquatic environments. It should be noted that the emergence of the MCR enzymes is not considered to be recent, as MCR was recovered from chickens and veal calves samples traced back to the 1980s in China and in 2005 in France, respectively (Shen et al. 2016; Haenni et al. 2016). Like many other plasmid-mediated resistance mechanisms, MCR-1 positive strains can disseminate globally with their host, as shown in the identification of MCR-1 positive *E. coli* and *K. pneumoniae* in pilgrims attending the annual Hajj (the Muslim pilgrimage to Mecca) (Leangapichart et al. 2016), and in the case of an MCR-1 *E. coli* positive traveller returning home from India (Bernasconi et al. 2016). The resistance conferred by the MCR enzymes is generally low to moderate (from 4 to 16 mg/L).

Additionally, some cases of MCR-1 positive *E. coli* isolates were classified as susceptible according to the EUCAST breakpoint of 2 mg/L, as their resistance levels to polymyxins range from 0.25 to 2 mg/L, which also makes it difficult to diagnose (Kuo et al. 2016; Lentz et al. 2016). To date, five variants of MCR gene (MCR-1 to MCR-5) have been reported from various bacteria. A most important concern with MCR genes is their location on transferable plasmids such as pHNSHP45 (MCR-1 gene, IncI2; 64,105 bp) and pKP37-BE (MCR-2 gene, IncX4; 35,104 bp), which are able to propagate by conjugation among *E. coli* strains (Liu et al. 2016; Xavier et al. 2016). The majority of plasmids carrying MCR-1 gene belong to the IncI2, IncHI2 and IncX4 incompatibility groups, while others belong to the IncF, IncN, IncP, IncQ and IncX groups. Presently, there are several dozen studies describing the isolation of MCR positive *E. coli* strains from human and non-human samples; however, the rates have been considerably higher in livestock than in humans. In some cases, such as in Tunisian chicken, the rates were as high as 76% in *E. coli* isolates (Skov and Monnet 2016). This may point that currently, animals are the main reservoir with spillover to humans.

Since the first description of MCR-1 gene at the end of 2015, MCR strains were isolated from infected patients and asymptomatic human carriers including global travellers, from various types of meat and vegetables, from various food animals, and from environment and river water (Skov and Monnet 2016). This gene had spread to more than 35 countries, covering all continents (Giamarellou 2016) and was isolated primarily from *E. coli* and to a lesser extent from Salmonella, *K. pneumoniae* and Shigella sonnei. It is carried on several different plasmids (Skov and Monnet 2016) and it is transferable at a very high frequency of 10^{-1} to 10^{-3} cells per recipient (Liu et al. 2016). Until today (beginning of 2018), many of the *E. coli* strains carrying the MCR gene are still susceptible to cephalosporins and carbapenems (Nordmann and Poirel 2016). Dissemination of plasmids harbouring the MCR gene by horizontal gene transfer possesses a major worldwide concern, considering that polymyxins are often the last-resort antibiotics for treating infections due to CPE and other Gram-negative MDR infections. Moreover, the spread of such plasmids into a highly epidemical *E. coli* strain, such as *E. coli* ST131 or to CPE, and in particular to a carbapenemase-producing *E. coli* ST131, resulting in an XDR isolate which approaches true pan-drug resistance is truly worrying. A greater threat would be the co-location of the MCR gene along with an ESBL and carbapenemase genes on a single plasmid in an epidemic *E. coli* or *K. pneumoniae* strains. Reports regarding the co-occurrence of MCR and ESBL or carbapenemases in the same isolate are still limited but given the limited number of years since the discovery of the MCR gene, it is most likely that the number of such reports will only increase over time.

5.3 Co-occurrence of MCR, ESBL and CPE

Recently, several reports regarding the co-occurrence of MCR and ESBL in *E. coli* were published. Four *E. coli* strains harbouring both MCR-1 and different CTX-M

genes were isolated from hospitals sewage water in Beijing, China (Jin et al. 2018). These isolates belonged to different STs; including ST10 harbouring CTX-M-14, ST349 harbouring CTX-M-14, ST2016 harbouring CTX-M-15 and one untyped ST harbouring CTX-M-3-like genes. Similarly, six colistin resistant ESBL-producing *E. coli* isolates were isolated from cattle in Spain (Hernandez et al. 2017), five carried MCR-1 and one isolate carried both MCR-1 and MCR-3. Whole genome sequencing (WGS) of this isolate, designated ZTA15/01169-1EB1, indicated profile of serotype O9:H10, ST533 and that it contains a CTX-M-55 ESBL resistance gene among other resistance genes. The presence of plasmids was evaluated, revealing IncHI2 and IncI1 replicons. Both MCR-1 and MCR-3 genes were plasmidic, but colistin resistance was not conjugatable in standard conditions. The analysis indicated the possible co-location of both MCR-1 and MCR-3 on the same plasmid in this isolate (Hernandez et al. 2017). In the USA, an ESBL *E. coli* isolate, designated *E. coli* MRSN 388634, which harboured the MCR-1 gene and the ESBL CTX-M-55 gene on the same plasmid was isolated (McGann et al. 2016). In addition, co-location of the MCR-1 gene and the CTX-M-1 ESBL gene on a single IncHI2 type plasmid was described recently (Haenni et al. 2016). Many other reports, regarding the co-occurrence of the MCR gene with different CTX-M genes, in different *E. coli* ST isolates, are available in the database, indicating that the MCR gene has disseminated globally in different ESBL-producing *E. coli* strains.

Reports describing the occurrence of MCR genes in *E. coli* ST131 are still limited. The first report appeared few months after the first description of the MCR-1 gene. In this study, the MCR-1 gene was detected in five *E. coli* isolates obtained from chicken meat and in one *E. coli* isolate from a human bloodstream infection (Hasman et al. 2015). One of the chicken meat MCR-1 positive *E. coli* isolates belonged to ST131. Following this report, several other reports described the isolation of MCR positive *E. coli* ST131 in different countries. These strains were isolated from poultry in the United Arab Emirates and Germany (Sonnevend et al. 2016; Ewers et al. 2016) and from human clinical isolates in Taiwan, Spain, Denmark and China (Kuo et al. 2016; de la Tabla et al. 2017; Roer et al. 2017; Wang et al. 2017b). The reports regarding isolation of ST131 MCR-producing *E. coli* are of special concern since ST131 isolates have spread epidemically during the last decade and may consequently speed up the dissemination of MCR in hospital and community settings, as previously demonstrated for ESBLs and carbapenemases.

Few months after the first description of the MCR gene in China, reports regarding the co-occurrence of the MCR gene in different CPE isolates, predominantly *E. coli*, were published. The coexistence of MCR and one of the following carbapenemase genes, OXA-48, VIM-1, KPC-2, NDM-1 or NDM-5 has been detected in various clinical human samples, from different countries, mostly resulting in limited therapeutic options. In addition, the coexistence of MCR and NDM-5 or OXA-181 was detected in animal samples. It should be noted that in all the analysed cases, the MCR gene and the carbapenemase gene were expressed from two different plasmids within the isolate. Furthermore, the isolation of *E. coli* strains, co-expressing the MCR gene, an ESBL gene and NDM-1, NDM-5 or

NDM-9 gene were reported, and are highly concerning. In one report, an *E. coli* ST167 isolate recovered from a chicken wing sample purchased at a supermarket in Guangzhou, China, was resistant to all antimicrobial drugs tested except tigecycline and doxycycline (Yao et al. 2016). The recovery of this strain, designated THSJ02, coproducing MCR-1, NDM-9, CTX-M-65 and many other resistant determinants, from retail meat was suppressing and worrying, as such strains might colonize the human intestinal tract and transfer resistance plasmids to other Gram-negative pathogens, resulting in untreatable infections. A similar case was described in the isolation of two *E. coli* XDR isolates (STs: ST648 and ST156) from a single Muscovy duck, in a duck farm in Guangdong Province, China (Yang et al. 2016). Both isolates co-produced MCR-1, NDM-5 and CTX-M-55 among other resistant determinants; one was susceptible to tigecycline, aztreonam and amikacin and the second only to tigecycline (Yang et al. 2016). These reports describing the isolation of such multiresistant *E. coli* strains in fowls might raise a potential threat to human health via food chain transmission.

Although presently still rare, few reports have described the detection of such isolates from clinical human samples. The first report describes the isolation of an XDR *K. pneumoniae* isolate, from peritoneal fluid of a patient hospitalized in a tertiary hospital in Suzho, China, that co-expressed MCR-1, NDM-5 and CTX-M-1 on two different plasmids (Du et al. 2016). Following this report, an XDR *E. coli* ST19 belonging to serotype O100:H25 (which is related to human enteropathogenic *E. coli* (EPEC) strains) was isolated from a human faecal sample in Cumana, Venezuela (Delgado-Blas et al. 2016). This isolate, designated BB1290, co-expressed MCR-1, NDM-1 and CTX-M-15 among an array of other resistant genes conferring resistance to colistin, carbapenems, ESBL and beta-lactams, fluoroquinolones, sulphonamides, aminoglycosides, macrolides, phenicols, tetracycline and trimethoprim, in line with its XDR profile. Plasmid analysis detected the presence of the Incompatibility replicons IncHI2, IncHI2A, ColBS512, IncI2, and IncFII (Delgado-Blas et al. 2016). Two additional reports described the isolation of three *E. coli* strains, co-expressing MCR-1, NDM and ESBL genes resulting in an XDR *E. coli* isolates (Wang et al. 2017b; Zheng et al. 2017). The first report describes two *E. coli* strains isolated in China, co-expressing MCR-1, NDM-1 and different CTX-M genes. One strain, belonging to ST90, was isolated from an intra-abdominal fluid sample of an inpatient from Zhejiang, harbours the CTX-M-55 gene. The second strain, belonging to ST744, was isolated from a faecal sample of a healthy volunteer from Guangdong, and harbours both CTX-M-14 and CTX-M15 genes (Wang et al. 2017b). The second report describes an XDR *E. coli* ST206 strain susceptible only to tigecycline, which was isolated from a faecal sample of a hospitalized patient in Hangzhou, China, co-expressing MCR-1, NDM-5, and CTX-M-14 among many other resistant determinants (Zheng et al. 2017). However, in all the reported cases, analysing the co-expression of MCR and carbapenemases, these resistant genes were localized on two different plasmids and none of them in an *E. coli* ST131 strain. Analysis of the ability of the MCR-1-containing plasmid, pHNSHP45 (the first described plasmid harbouring MCR-1; Liu et al. 2016) to conjugate to an *E. coli* C600 strain indicated that it has

high in vitro transfer rate (10^{-1} to 10^{-3}) (Liu et al. 2016). Moreover, pHNSHP45 was successfully transformed into different epidemic Enterobacteriaceae strains, such as *E. coli* ST131 and *K. pneumoniae* ST11, as well as into *P. aeruginosa*, suggesting the possible spreads of MCR-1 into key epidemic human pathogens. Furthermore, it was also demonstrated that pHNSHP45 could be successfully transformed and was stable, in a KPC-2 positive *E. coli* ST131 (Liu et al. 2016). The co-expression of MCR, ESBL and NDM in one isolate, resulting in an XDR *E. coli* strain approaching a pan-drug-resistant phenotype, is alarming and worrying as it could represent a successful step towards true pan-resistance in Enterobacteriaceae. Recently, two studies have described two different isolates of truly pan-drug-resistant *K. pneumoniae*. Both were isolated from human samples, one in the United Arab Emirates (Zowawi et al. 2015) and in Reno, Nevada, USA (Chen et al. 2017) and were resistant to all available antimicrobial drugs, both carried carbapenemase (OXA-181 and NDM, respectively) but were negative to MCR-1. In the United Arab Emirates isolate, the colistin resistance was mediated by insertional inactivation of the chromosomal mgrB gene by an IS*Ecp1* transposon carrying OXA-181, conferring carbapenem resistance. This insertion disrupted and inactivated the mgrB gene (a *phoPQ* negative regulator) resulting in over expression of the *phoPQ* signalling system and of the *pmrHFIJKLM* operon which controls LPS modification, the target of polymyxin antibiotics (Groisman 2001). Fortunately, in both cases, no other such isolates were identified through appropriate infection control contact precautions and surveillances.

6 Concluding Remarks and Future Perspectives

Over the past 20 years, increasing antibiotic resistance among isolates of Enterobacteriaceae has become a main and global public health concern. In recent estimates of global antibiotic resistance, *E. coli* was named as one of the biggest concerns associated with human and animal health, farming and food industry and environment. The spread and emergence of antibiotic resistance in *E. coli* and specifically the recent resistant trends, their origin and epidemiology were discussed in this chapter. As described, intestinal pathogenic *E. coli* and ExPEC emergence and disseminated globally also as MDR clones. Among the ExPEC strains, *E. coli* ST131, first described in 2008, has evolved and became one of the most epidemiologically prominent strains which have successfully acquired VFs, an array of resistant determinants including ESBL, colonization and in host persistent abilities, enhanced fitness and pathogenicity, environmental adaptation skills and dissemination capabilities. Not long after the emergence of KPC, NDM, OXA-48, VIM, and IMP carbapenemases, and the discovery of the MCR gene, they have all been isolated from *E. coli* ST131 strains, resulting in more resistant strains, often as XDR isolates. Recently, non-ST131 isolates co-expressing different ESBLs, carbapenemases and the MCR gene have evolved. Unfortunately, it seems that the emergence of an *E. coli* ST131 strain co-expressing ESBL, MCR and a carbapenemase gene on

different plasmid, or in an even worse scenario on the same plasmid, is probably only a matter of time and opportunity. It is now the time to coordinate global programs for surveillance of such resistant markers producing bacteria, including proper infection control guidelines which are needed to be implemented worldwide. In addition, special attention should be pointed towards development and discovery of new antimicrobial drugs which could fight with MDR, XDR and PDR bacteria. One such effort was the launched of the 'bad bugs need drugs' campaign, by the Infectious Disease Society of America to promote the development of new antibiotics by 2020 (Boucher et al. 2009).

The persistent evolution of resistant determinants and their successful spread among bacteria, resulting in the loss of antibiotic effectiveness, is challenging both for clinical practice and public and animal health and therefore requires a global action. Consequently, the 'One Health' concept published by the US CDC (2016b) recognizes that the health of humans is connected to the health of animals and the environment. It is now recognized that understanding the significant role of the involvement and contribution of each of these three components is important in confronting global antimicrobial resistance.

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Vaccines Against *Escherichia coli*

Barbara Nesta and Mariagrazia Pizza

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Abstract *Escherichia coli* has a complex and versatile nature and continuously evolves from non-virulent isolates to highly pathogenic strains causing severe diseases and outbreaks. Broadly protective vaccines against pathogenic *E. coli* are not available and the rising in both, multi-drug resistant and hypervirulent isolates, raise concern for healthcare and require continuous efforts in epidemiologic surveillance and disease monitoring. The evolving knowledge on *E. coli* pathogenesis mechanisms and on the mediated immune response following infection or vaccination, together with advances in the "omics" technologies, is opening new perspectives toward the design and development of effective and innovative *E. coli* vaccines.

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1 Introduction

E. coli strains are classified into "pathotypes" (Kaper et al. 2004) and can be subtyped using a variety of criteria, including serotype, pulsotype, phage type, or biotype (Robins-Browne et al. 2016; Micenkova et al. 2016). *E. coli* can also be classified serologically on the basis of the O somatic antigen (Fratamico et al. 2016), K capsular polysaccharide surface antigen (Whitfield 2006; Kaczmarek et al. 2014), and H flagellar antigen (Geue et al. 2014; Chui et al. 2015). At the population level, *E. coli* can be phylogenetically assigned to five main groups: A, B1, B2, D, and E. Even if commensals mostly belong to the phylogroup A and B1, not all pathotypes group together, suggesting a disparate nature of pathogenic species (Leimbach et al. 2013).

The pathotypes of *E. coli* that are associated with intestinal disease are known collectively as intestinal pathogenic *E. coli* (IPEC) or diarrheagenic *E. coli* (DEC), while *E. coli* causing disease in tissues other than the intestinal tract are known collectively as extraintestinal pathogenic *E. coli* (ExPEC). ExPEC resides asymptomatically in the human intestinal tract of $\sim$20% of healthy individuals, sharing large genomic regions with nonpathogenic strains. In contrast to the facultative ExPEC pathogens which belong to the normal gut flora where they live as commensals, intestinal pathogenic *E. coli* (InPEC) are obligate pathogens epidemiologically and phylogenetically distinct from ExPEC and commensals.

Although all the classification schemes developed so far provide important information on the nature of the epidemiologically relevant strains, the whole genome analysis more accurately defines the differences in gene content and allelic variations among the different isolates allowing a more in-depth understanding of strain evolution and spreading.

The *E. coli* species undergo rapid genetic changes, referred to as microevolution, providing new traits, favoring the fitness and the adaptation to environmental changes (Brzuszkiewicz et al. 2009). Microevolutionary divergence is a common phenomenon in *E. coli*, as demonstrated by genomic studies on *E. coli* diversity (Moriel et al. 2012; Lo et al. 2015). Moreover, novel virulent isolates possessing hybrid features of different pathotypes are continuously causing emergent outbreaks worldwide. The 2011 outbreak in Germany was determined by an EAEC strain, which has acquired several mobile genetic elements including the phage-mediated Shiga Toxin Stx2a (Frank et al. 2011), opening new views on the designation of pathotypes (Brzuszkiewicz et al. 2011; Rasko et al. 2011). In addition, a growing number of studies are linking foodborne *E. coli* with uropathogenic strains. Thus, the term foodborne urinary tract infections (FUTIs) has been adopted to describe urinary tract infections (UTIs) with probable foodborne origins (Nordstrom et al. 2013).

E. coli is also rapidly evolving as multidrug-resistant bacterium, exacerbating the public health problems in the era of decline in antimicrobial drug discovery. The dangerousness of the prevalence of UPEC isolates resides in their resistance to the first-line oral antibiotic agents such as trimethoprim–sulfamethoxazole, ampicillin, and fluoroquinolones. In addition, the most common sources of infections consist of

fluoroquinolone-resistant strains colonizing the rectum and the urinary tract. In the recent years, the ST clonal group known as ST131, a virulent and epidemic antibiotic-resistant *E. coli*, caused severe hospital outbreaks with a strong potential for wide dissemination (Nicolas-Chanoine et al. 2014; Mathers et al. 2015). The increased extended-spectrum β-lactamase (ESBL)-producing *E. coli* by 300% is responsible for the growing burden and healthcare-related costs due to ExPEC and further highlights the urgent need for effective interventions (Blaak et al. 2014; Franz et al. 2015).

Today, the identification and tracking of multidrug-resistant microorganisms in hospitals and communities can be performed very rapidly by whole genome sequencing (Punina et al. 2015). The emerging and re-emerging infections and the spread of antibiotic resistance strains render the need for an effective vaccine able to prevent *E. coli* infection and disease a public health priority. Vaccination could represent a measure against antibiotic resistance spread by reducing the infection rate and as consequence, antimicrobial use (Lipsitch and Siber 2016).

1.1 ExPEC: Urinary Tract Infection, Neonatal Meningitis, and Sepsis

Extraintestinal pathogenic *E. coli* strains (ExPEC) include uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), septicemia-associated *E. coli* (SePEC), and avian pathogenic *E. coli* (APEC). UPEC infections account for >85% of cases of acute cystitis and pyelonephritis, >60% of recurrent cystitis. Infections that are not resolved with antibiotic prophylaxis could evolve in pyelonephritis, sepsis, or death. In the healthcare setting, catheter-associated UTIs (CAUTIs) represent the second most common cause of all nosocomial infections (1 million catheter-associated UTIs/year in the U.S.). UTIs are also a source of substantial morbidity in children under neonatal intensive care, where the risk of breakthrough UTIs (BUTIs) could occur during antibiotic treatments (Hidas et al. 2015; Lloyd et al. 2016).

E. coli is a leading cause of bacteremia worldwide (Laupland and Church 2014). The overall annual incidence of *E. coli* bacteremia in adults markedly increases with age, reaching 452 cases/100,000 person-years in individuals aged $\geq$ 85 years. Case fatality rates for bacteremia are between 13 and 19% but may be up to 60% in elderly persons with nosocomial infections (Roubaud Baudron et al. 2014) and neurological sequelae occur in 30–50% of cases (Logue et al. 2012). In addition, spread of *E. coli* bacteremia both in USA and in Europe is accompanied by a 30% annual increase in third-generation cephalosporin-resistant isolates (Carl et al. 2014; Basu 2015). Thus, *E. coli* bacteremia is a costly, potentially lethal, and increasingly frequent problem exacerbated by societal aging and increasing prevalence of antibiotic-resistant strains.

1.2 Intestinal E. coli *Infections*

The major intestinal pathotypes include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and adherent-invasive *E. coli* (AIEC).

ETEC produces both heat-labile (LT) and heat-stable (ST) enterotoxins and is the most important cause of watery diarrhea, with abdominal pains and vomiting, both in developing countries and in travelers endemic regions. ETEC is responsible for 280 million diarrheal episodes and more than 400 thousand death annually. In 2013, the World Health Organization (WHO) Child Health Epidemiology Reference Group estimated 42,000 (95% CI, 20,000–76,000) ETEC-associated deaths of children under five years of age (Lanata et al. 2013). Overall, ETEC causes approximately 10 million episodes of travelers' diarrhea each year, through Africa, Asia, and Latin America, including military personnel deployed to these areas. In addition, ETEC-associated travelers' diarrhea may go on to develop reactive arthritis, irritable bowel, and Guillain–Barré syndromes (Giddings et al. 2016).

Other intestinal *E. coli* pathotypes also contribute to diarrheal disease but can differ in terms of detection, diagnosis, epidemiology, public health, pathogenesis, and human disease. EPEC mainly affects small intestine of infant, causing diarrhea associated with fever, nausea, and vomiting, spreading an increased antibiotic-resistant strains in both developing and developed countries. EHEC affects large intestine causing severe abdominal pain, watery diarrhea followed by bloody diarrhea leading to hemolytic uremic syndrome. EIEC produces shigella-like diarrhea in large intestine and determines epithelial cells injury and tissue invasion. STEC is associated with a disease spectrum ranging from diarrhea and hemorrhagic colitis (HC) to the potentially fatal hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). EAEC, which affects small intestine, is responsible for endemic diarrhea of infants in both industrialized and developing countries.

2 Vaccines

Over the last two centuries, vaccination has been the most effective measure to save lives and improve public health. Conventional vaccinology, mainly based on the Pasteur's principles of inactivation of the disease agents and generation of killed or live attenuated vaccines, has experienced a deep renaissance, thanks to the understanding of virulence and immunity mechanisms and to the advent of new technologies of genetic engineering and of genomic sequencing and bioinformatics. *E. coli* vaccines proposed today are based on live attenuated strains rationally designed to be safe, with deletions in genes important for virulence and with improved immunogenicity, overexpressing selected antigens, or on whole inactivated strains and/or on new promising vaccine antigens discovered by proteomic and genomic approaches (Fig. 1).

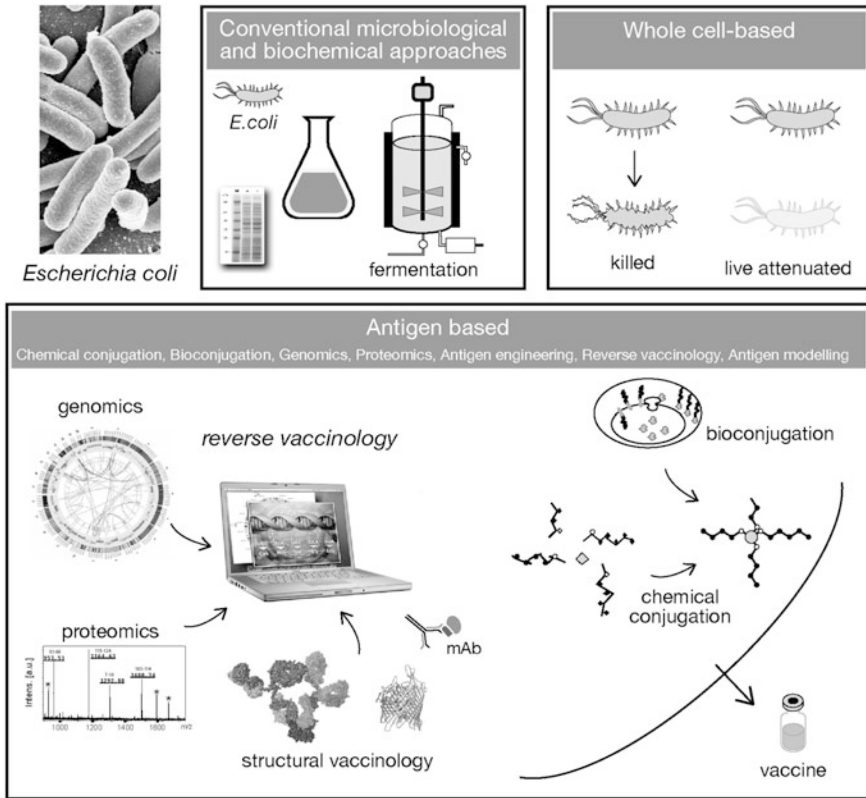

Fig. 1 Different strategies to vaccine discovery and development

2.1 *Vaccines Against Extraintestinal Pathotypes*

2.1.1 Conventional Vaccinology Against ExPEC

Conventional strategies applied to the development of an effective vaccine against ExPEC infections have been unsuccessful so far (Uehling and Wolf 1969; Kaijser et al. 1983a, b). In the 1990s, traditional vaccine strategies were based on single-purified virulence factors such as Hemolysin (O'Hanley et al. 1991) or on the O-specific polysaccharide (OPS) chain of the lipopolysaccharide (named O-antigen), conjugated to either *Pseudomonas aeruginosa* endotoxin A (TA) or cholera toxin (CT) as carrier proteins (Cryz et al. 1991; Cross 1994). Although the prevalence of capsular polysaccharide (K-antigen) and O-antigen is different among the different pathotypes, there is an association between K (K1, K5, 30 and 92) and O (O1, 2, 4, 6, 7, 8, 16, 16/72, 18, 25, 50 and 75) antigenic groups and uro-pathogenic strains (Brumbaugh and Mobley 2012). However, because of the high antigenic heterogeneity of the surface polysaccharides, the design of a

polysaccharide vaccine able to prevent ExPEC infections has been extremely challenging (Russo and Johnson 2006).

An O18-polysaccharide conjugated to either cholera toxin or to *P. aeruginosa* exoprotein A (EPA) was shown to be safe and able to induce antibodies with opsonophagocytic killing activity (OPK) in human volunteers. IgG purified from immunized individuals were protective in mice in an *E. coli* 018 challenge sepsis model (Cryz et al. 1991). When a 12-valent vaccine, based on O-antigen based on 12 serogroups of *E. coli* (O1, O2, O4, O6–O8, O12, O15, O16, O18, O25, O75) conjugated to EPA, was tested in a clinical trial, the functional immunoresponse induced by each O-antigen was different, underlying the difficulties of development of a cross-protective vaccine (Cross et al. 1994).

Vaccines based on whole or lysed fractions of inactivated *E. coli* have been evaluated in human clinical trials (Fig. 2) and have been so far the most effective in inducing some degree of protection in subjects undergoing recurrent urinary tract infections. The sublingual vaccine Uromune, an inactivated whole preparation of *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Enterococcus faecalis*, evaluated as prophylactic treatment in a multicenter retrospective observational study, demonstrated a certain degree of clinical benefit in terms of reduced recurrence rate in women with a history of recurrent UTI (Lorenzo-Gomez et al. 2013). The Solco Urovac vaccine, a vaginal suppository polymicrobial vaccine consisting of 10 inactivated uropathogenic bacteria, including 6 *E. coli* serotypes, *Proteus mirabilis*, *Morganella morganii*, *K. pneumoniae,* and *E. faecalis* strains, showed a minimal efficacy in Phase 1 and two Phase 2 trials in women suffering of recurrent UTIs (Uehling et al. 2001, 2003; Bauer et al. 2005). However, in two additional clinical studies, the vaginal mucosal vaccine given for a 14-week period increased the time to re-infection in UTI susceptible women, representing a valuable alternative to the antibiotic-based prophylactic regimens (Uehling et al. 2003; Hopkins et al. 2007). A post-marketing assessment further demonstrated the significant reduction of Solco-Urovac on recurrent UTIs when administered as standalone or in conjunction with standard antibacterial medications (Kochiashvili et al. 2014).

Human trials with a vaccine based on *E. coli* Extract (ECE) started in the 1980s (Frey et al. 1986; Tammen 1990; Magasi et al. 1994), and efficacy and safety of *E. coli* extract (ECE; Uro-Vaxom) were assessed in larger clinical trials a few years later (Bauer et al. 2005; Kim et al. 2010) leading to the recommendation of Uro-Vaxom for prophylactic treatment of patients with recurrent urinary tract infections. Additional studies, based on oral tablet, OM-89/Uro-Vaxom vaccine, demonstrated modest protection in women (Bauer et al. 2002). However, in a more recent trial on 451 female subjects, the lyophilized lysate of 18 *E. coli* strains, OM-89/Uro-Vaxom, manufactured using a modified lytic process, based on alkaline chemical lysis and autolysis, failed to show a preventive effect on recurrent uncomplicated UTIs (Wagenlehner et al. 2015).

Among the variety of strategies to develop a vaccine targeting ExPEC, those based on wild-type or genetically engineered inactivated uropathogens combination also resulted in weak success (Schmidhammer et al. 2002). Unfortunately, although

New antigens in Preclinical studies	Vaccines in Clinical studies
Antigens involved in iron acquisition FyuA IutA ChuA Iha IreA Hma IroN	**Uromune** Inactivated *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterococcus faecalis*
Highly conserved antigens SslE (YghJ) FdeC (EaeH)	**Solco-Urovac** Inactivated six *E. coli* serotypes, *Proteus mirabilis*, *Morganella morganii*, *Klebsiella pneumoniae* and *Enterococcus faecalis*
Fimbrial-based antigens MrpH-FimH	**OM89/Uro-Vaxom** Lyophilized lysate of 18 *E. coli* strains
	ExPEC-4V 4-valent "O" antigens conjugated to Exotoxin A from *Pseudomonas aeruginosa*

Fig. 2 Vaccines against Extraintestinal *E.coli* in clinical and preclinical studies

promising in preclinical studies, most of the vaccines based on these approaches failed to provide protection in clinical trials.

Regarding the use of single antigens, the most relevant that has been explored for its vaccine potential over the past 17 years is the Type 1 fimbrial adhesin FimH, which mediates UPEC adherence to bladder epithelial cells. The parenteral FimCH vaccine, composed of the FimH adhesin, the minor component of the Type 1 pili, in complex with its chaperone FimC, reduced bladder colonization in a mouse model, and induced protection from bladder infection and from inflammatory response in a monkey's model when used in combination with MF59 as systemic adjuvant (Langermann et al. 2000; Langermann and Ballou 2001). The data from the FimCH Phase II clinical trials have not been published yet and the level of efficacy of the FimCH vaccine is still unknown.

2.1.2 Emerging Approaches for Vaccines Against ExPEC

Chemically conjugated *E. coli* O-antigen vaccines are safe and immunogenic in humans; however, as discussed previously, production of multiple O-conjugates has been technically difficult. Very recently, an innovative technology, based on bio-conjugation, a process of in vivo synthesis, and conjugation of polysaccharide structures to carrier proteins in engineered bacterial cells, has been developed and shown to be a valuable approach for the production of multivalent conjugated vaccines. In the case of the ExPEC vaccine, the protein glycosylation machinery of

E. coli has been used to produce a conjugate vaccine based on a genetically detoxified form of *P. aeruginosa* exotoxin A (EPA) linked to the O1, O2, O6, and O25 *E. coli* serotype surface polysaccharide antigens. The immunogenicity and safety of the 4-valent "O" antigen bioconjugate *E. coli* vaccine (ExPEC-4V) have been evaluated in a phase 1b trial on healthy adult women with a history of recurrent UTIs. The vaccine was well tolerated and able to elicit functional antibody responses against all vaccine serotypes (Huttner et al. 2017). Phase II clinical trials are ongoing.

With the advent of the "omics" era, including genomic, proteomic, and transcriptomic, many new potential antigens have been identified and their protective properties tested in a variety of animal models (Fig. 2). UPEC strains survive in iron-limited conditions by upregulating the expression of iron acquisition systems. In 2010, putative UPEC-specific vaccine targets antigens involved in iron acquisition were identified: FyuA, IutA, ChuA, Hma, IhaA, IreA, and IroN (Durant et al. 2007; Alteri et al. 2009; Wieser et al. 2010; He et al. 2010). The newly identified antigens elicited a protective systemic and mucosal immune response in mice immunized intranasally being able to significantly reduce bladder and/or kidneys colonization (Alteri et al. 2009). These antigens have an important role in pathogenesis acting as siderophore receptors and meet all criteria for an antigen to be a potential vaccine candidate, including surface accessibility, recognition by the host immune system, in vitro expression in bacteria grown in human urine or in experimental conditions mimicking the urinary tract or the bladder environment, and in vivo expression in challenged mice and in women with UTI infections (Sivick and Mobley 2009; Vigil et al. 2011). Vaxign, a web-based vaccine design program, which contains prediction of vaccine targets for >70 genomes, has been used to predict new UPEC vaccine candidates based on the reverse vaccinology approach, successfully applied to the discovery of a new MenB vaccine (Pizza et al. 2000; Rappuoli 2001a, b). Vaxign predicts antigens on the basis of their subcellular localization, the presence of transmembrane helices, adhesin probability, low conservation to human and/or mouse proteins, the absence in genome(s) of nonpathogenic strain(s), and epitope binding to MHC class I and class II molecules (He et al. 2010). The selection criteria applied by H. Mobley's group in identifying the most promising candidates have been pivotal to reduce the number of potential vaccine antigens to be tested and allowed the selection of only six vaccine candidates for a single uropathogenic strain (Mobley and Alteri 2015). This approach highlights the importance of the basic knowledge in the virulence mechanisms as antibodies raised by the selected antigens are expected to interfere with the most critical steps of *E. coli* virulence and pathogenesis.

In 2010, a number of potential vaccine candidates against ExPEC were identified using the so-called "subtractive reverse vaccinology" approach, based on the genome comparison of three ExPEC strains (CFT073, 536, and IHE3034 to MG1655, DH10B, and W3110 nonpathogenic *E. coli* strains). By this approach, 230 potential antigens were identified and tested in a mouse model of sepsis, and nine of them were found to be protective (Moriel et al. 2010). Two of the newly identified antigens, ECOK1_0290 (FdeC, the Factor Adherence *E. coli*) and

ECOK1_3385 (SslE, Secreted and surface-associated lipoprotein from *E. coli*), were further analyzed for their protective ability in different animal models, functional and structural properties, in vivo expression during infection, and molecular epidemiological features. Mucosal immunization with the recombinant FdeC, deriving from an NMEC strain, using the cholera toxin (CT) as adjuvant, provided considerable protection in the ascending UTIs mouse model by challenge with two different UPEC disease isolates, supporting also its cross-protective ability. Of interest, FdeC conferred site-specific protection, as immunized mice were significantly protected from uropathogenic strains ascending toward the kidney, with a 1.5–2.5 log in median CFU/g range of reduction in kidney colonization. The high conservation of FdeC among strains belonging to different *E. coli* pathotypes, consisting of 99% gene presence in extraintestinal and 93–100% in intestinal pathotypes, and amino acid sequence identity >91% among all pathotypes, highlights the potential use of FdeC as a component of a broadly protective vaccine against extraintestinal and intestinal *E. coli* infections (Nesta et al. 2012).

Secondary structure prediction on FdeC, confirmed by the X-ray structure of the central domain, revealed an interesting structural similarity with the *Yersinia pseudotuberculosis* invasin and EPEC intimin. In agreement with the prediction, the recombinant FdeC protein demonstrated a strong affinity in binding to several epithelial cell lines in vitro and to specifically target different collagen types, including type V and VI, both widely expressed in the interstitial space of kidney and bladder. However, an intriguing FdeC peculiarity is that its expression on the bacterial surface is triggered upon interaction of an NMEC K1 strain with host cells in vitro. Interestingly, FdeC expression was detected in vivo, on UPEC strains closely associated with bladder tissues of mice following intraurethral challenge. In vivo competition experiments between UPEC wild-type and its derivative *fdeC* mutant revealed that the loss of FdeC caused a significant reduction in bacterial fitness (Nesta et al. 2012). In agreement, the ETEC *eaeH* gene, the homolog of ExPEC *fdeC*, was found significantly upregulated upon host cell contact (Kansal et al. 2013) and the expression of EaeH in ETEC was also demonstrated during pathogen–epithelial cell interaction in vitro (Sheikh et al. 2014). These findings are consistent with the hypothesis that this protein is activated by and participates in intimate interactions of both ETEC and ExPEC with the target epithelium. The indications on the structural and functional role of FdeC in bacterial pathogenesis and tissue adhesion may suggest that antibodies against FdeC could reduce colonization.

Ability of SslE to act as protective antigen against ExPEC infections has been confirmed in different animal models, using different clinical isolates as challenge strains (Moriel et al. 2010). In the UTI mouse model, intranasal immunization with SslE, using cholera toxin as mucosal adjuvant, led to a significant reduction of bacterial load in the kidneys and a more pronounced in the spleen with a 2.0 log reduction in median CFU/g following intraurethral challenge with the UPEC strain. In the sepsis model, SslE determined a significant protection from mortality (60% survival, $P < 0.0001$) against a SEPEC challenge strain, expressing a distant SslE variant. In addition, SslE was able to induce protection in terms of 1 log reduction

in bacterial load in the intestine of mice challenged orally with an ETEC strain, reinforcing the potential of this antigen as universal *E. coli* vaccine candidate (Moriel et al. 2010; Nesta et al. 2014).

By the functional point of view, SslE is the substrate of a T2SS (Type 2 Secretion System) and an outer membrane lipoprotein also known as YghJ in the case of ETEC (Yang et al. 2007; Iguchi et al. 2009). It has been associated with the M60-like extracellular zinc-metalloprotease subfamily, implicated in glycan recognition and processing. Functional activity of SslE has been controversial since it was originally shown to be involved in biofilm formation of an EPEC strain (Baldi et al. 2012), but this function was not confirmed in subsequent studies (Hernandes et al. 2013). More recently, the functional activity of SslE as a mucinase enzyme has been elucidated using a variety of in vitro methods (Nesta et al. 2014; Valeri et al. 2015). Of interest, an in vitro assay specifically set up to quantify the bacterial mucinase activity by counting the number of bacteria able to traverse an agar-based mucin matrix was used both to demonstrate SslE activity and, most importantly, to evaluate the ability of antibodies raised by immunization with SslE to inhibit mucinase activity. Interestingly, antibodies raised against an ExPEC SslE variant were able to specifically inhibit the mucinase activity of different *E. coli* pathotypes expressing distant SslE variants, including EPEC, SEPEC, ETEC, and the EAHEC strain responsible for the 2011 German outbreak, highlighting the potential role of this antigen as cross-protective against different pathotypes. On the basis of the functional role, it can be hypothesized that SslE may facilitate bacterial penetration of the mucosal surface and of the inner mucus layer, to allow *E. coli* to reach the underlying host epithelium.

Additional antigens have recently been shown to elicit protection in mouse model. Among them, the common pilus antigens EcpA and EcpD (*E. coli* common pilus, ECP) and iron uptake proteins IutA and IroN have been described as able to induce high levels of total IgG antibody of IgG1/IgG2a isotypes and to be protective in active and passive immunizations in a mouse model of sepsis (Mellata et al. 2016). Moreover, antibodies raised against a synthetic form of a conserved surface polysaccharide, β-(1-6)-linked poly-N-acetylglucosamine (dPNAG) containing nine monomers of (non-acetylated) glucosamine (9GlcNH2) conjugated to tetanus toxoid TT (9GlcNH2-TT) were shown to increase the efficacy of the passive immunization. These promising data represents an additional step toward the development of a broadly protective intervention against sepsis caused by *E. coli* (Mellata et al. 2016).

A recently proposed vaccine against UTIs is based on the immunogenic and protective MrpH-FimH fusion protein, made by MrpH from *P. mirabilis* and type 1 fimbrial FimH adhesin from a uropathogenic *E. coli* strain. Transurethral immunization of mice with the MrpH-FimH fusion induced a significant decrease in the number of bacteria recovered from bladder and kidney following challenge with UPEC or *P. mirabilis* strains, demonstrating the potential of MrpH-FimH as a promising vaccine candidate against UTIs caused by both UPEC and *P. mirabilis* (Habibi et al. 2016).

2.2 Vaccines Against Intestinal Pathotypes

2.2.1 Vaccines Against ETEC

Currently, there are no licensed vaccines against ETEC. Human challenge studies indicate that protective immunity against ETEC is induced after natural or experimental infection, suggesting that the development of an effective vaccine is feasible. Main efforts in developing vaccines against ETEC have been based on the induction of antitoxin and/or anti-colonization immunity (Fig. 3). Inhibition of ETEC adhesion to intestinal epithelial cells and neutralization of the toxic activity of the toxins should allow prevention of infection and disease. The only ETEC vaccine shown to provide some protection against diarrhea is the whole-cell vaccine containing the protective B subunit of the cholera toxin (CT-B), antigenically similar to the ETEC heat labile toxin (LT). The Dukoral vaccine, designed and licensed to prevent cholera, is in fact recommended to people visiting endemic regions, to prevent travelers' diarrhea.

ETEC bacteria use plasmid-encoded fimbrial colonization factors (CFs) or *E. coli* surface antigens (CS) to bind to enterocytes in the upper small intestine. Following preliminary colonization, the bacteria produce heat-stable (ST) and/or heat-labile (LT) enterotoxins that stimulate the release of fluid and electrolytes from the intestinal epithelium, resulting in the watery diarrheal illness. These plasmid-encoded antigens are known to be key virulence factors and have been proposed as vaccine components over the last three decades.

The LT enterotoxin is an ADP-ribosylating toxin, consisting of an enzymatically active A subunit non-covalently linked to a pentameric B subunit mediating the binding to host receptors, with strong immunogenic and adjuvant properties. Genetically detoxified derivatives of LT, devoid of toxicity but retaining the immunologic and adjuvant properties of the wild-type toxin, have been generated and extensively characterized in many animal models (Giuliani et al. 1998; Pizza et al. 2001; Norton et al. 2011). The majority of ETEC vaccine studies conducted so far only include LT-B as immunogen, but in more recent studies the A subunit is also included, based on the important contribution of LTA on the quality of the immune response in terms of IgG1/IgG2 balance and mucosal IgA and IL-17 secretion (Norton et al. 2012; Norton et al. 2015) and of the full toxin in inducing protective immunity (Giuliani et al. 1998). Genetically detoxified LT mutants are included in the newly proposed ETEC vaccine formulations (Zhang and Sack 2015).

Efficacy and safety of a skin patch vaccine containing the heat-labile toxin (LT) in travelers to Mexico and Guatemala have been assessed in phase 3 clinical trial. The transcutaneous LT-based ETEC vaccine failed in inducing protection against diarrhea in travelers, although the LT antigen was delivered effectively by skin immunization (Behrens et al. 2014). Because of these data, the use of the LT patches has no longer been considered a suitable approach for vaccination against ETEC (Riddle and Savarino 2014).

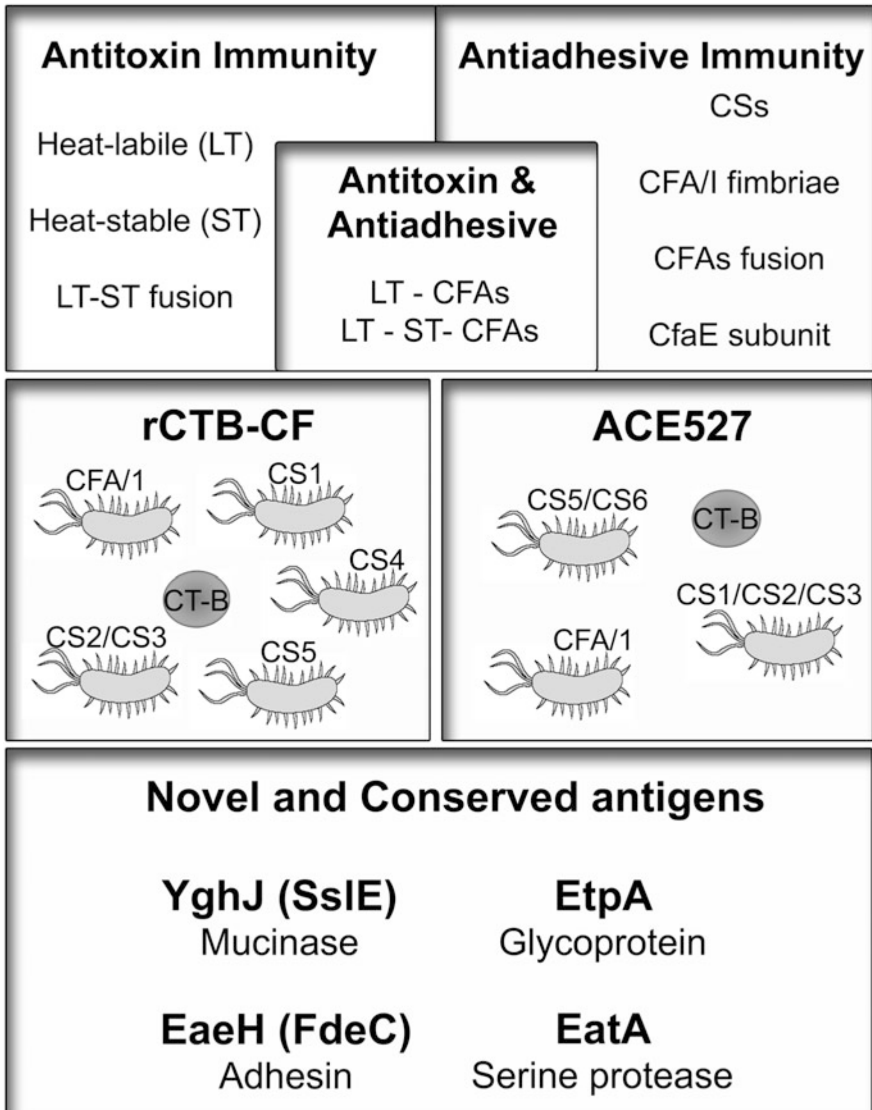

Fig. 3 New approaches to antigen-based vaccines against Enterotoxigenic *E.coli*

In contrast to LT, the reduced immunogenicity and the potent toxicity of STa has been an obstacle for many years to the development of toxoid-based vaccines against ETEC. ST consists of 18 (STp) or 19 (STh) amino acids and includes six cysteines that form three intramolecular disulfide linkages. Due to its small size, ST is nonimmunogenic in its natural form but becomes immunogenic when coupled to large-molecular-weight carrier, either by chemical conjugation or recombinant

fusion. Many genetic approaches have been explored to attenuate ST toxicity while enhancing its immunogenicity, considering that an ST toxoid-containing vaccine may cover potentially a broad range of ETEC infections (Taxt et al. 2010; Ruan et al. 2014).

ETEC colonization factors (CFs), categorized as colonization factor antigens (CFA) or *E. coli* surface antigens (CS), have been proposed as vaccine components, even if their distribution among ETEC is variable. CFA/I, CS3, CS5, and CS6 account for 50–80% of all CF-positive clinical ETEC isolates and some CF/CS antigens are immunologically related to the more prevalent CFs (i.e., CFA/I and CS14). In order to specifically abrogate the initial step of ETEC colonization, alternative approaches that target the CFA/I fimbriae or its CfaE tip-localized adhesin have been evaluated in preclinical animal models (Luiz et al. 2015). In addition, the ETEC fimbrial adhesin-based vaccine approach has been supported by further studies conducted both in mice and in nonhuman primates (Sincock et al. 2016). A novel multiepitope fusion antigen (MEFA) strategy has been recently used to construct ETEC fusion antigens starting from CFA adhesins, combined with or fused to an LT-STa toxoid fusion (Ruan et al. 2015).

In ETEC, there are more than 20 CFAs expressed in different combinations and in different geographic regions. However, a candidate vaccine formulated to cover CFA/I, CS3, and CS6 would only provide coverage for approximately 50–60% of the ETEC strains. To this end, inclusion of an LT toxoid in a CF-based vaccine may help to provide the potential vaccine strain coverage for LT-only strains that lack CF/CS selected antigens. Overall, optimal combination of toxin and CFAs for a specific target population may not always be easy. Consequently, additional antigens would need to be included to meet optimal vaccine coverage thresholds.

The rCTB-CF ETEC vaccine, composed of five formalin-killed *E. coli* strains expressing CFA/1, CS1, CS2/CS3, CS4, and CS5 adhesins, together with the recombinant B subunit of the cholera toxin, was tested in safety and immunogenicity in different trials, including adult volunteers from endemic areas such as Egypt and Israeli (Cohen et al. 2000), but did not reduce the overall rates of travelers' diarrhea (Sack et al. 2007). A phase 2b study of an oral, live attenuated, three-strain recombinant vaccine, ACE527, which expresses the colonization factors (CFs) CFA/I, CSI, CS2, CS3, CS5, and CS6 and heat-labile toxin B subunit (LT-B) induced clinically significant attenuation of diarrheal illness and reduced ETEC intestinal colonization in a stringent ETEC H10407 human challenge model (Darsley et al. 2012). A vaccine designed to specifically target ETEC, consisting of both killed whole cells and the recombinant CT-B, did not demonstrate clinically important benefits in two trials of 799 people traveling from the USA to Mexico or Guatemala, and from Austria to Latin America, Africa, or Asia, but was associated with increased vomiting (Ahmed et al. 2013c). A novel multicomponent oral inactivated whole-cell ETVAX vaccine, adjuvanted with an attenuated double-mutant form of LT (dmLT), was developed for both pediatric and traveler's indication. A phase I/II trial indicated that the addition of dmLT further enhanced mucosal immune responses to CF antigens present in low amounts in the vaccine as well as toxin-neutralizing antibody response to LT toxin (Lundgren et al. 2014).

In a recent study in which human volunteers were challenged or re-challenged with virulent ETEC strain H10407 serotype O78:H11, novel immunological benchmarks for the evaluation of ETEC vaccines were established as IgA responses to lipopolysaccharide (LPS), heat-labile toxin B subunit (LTB), and colonization factor antigen I (CFA/I) in lymphocyte supernatant (ALS), feces, lavage fluid, and saliva samples (Chakraborty et al. 2015). Overall, a limited number of ETEC vaccine trials conducted among younger age groups in endemic areas indicated that many questions still remain to be addressed to determine the vaccines impact against more severe or life-threatening ETEC disease (Das et al. 2013). Despite the significant effort in ETEC vaccine trials, these formulations have not been particularly effective in mediating cross-protective immunity.

Together with traditional approaches based on CFA and toxins, putative conserved pan-ETEC antigens have been also considered as promising vaccine candidates (Fig. 3).

Functional studies on the two-partner secretion system demonstrated that the secreted glycoprotein EtpA acted as a molecular bridge between ETEC flagellin and host cell receptors (Roy et al. 2009). Antibodies directed against either EtpA or the conserved regions of flagellin inhibited toxin delivery in vitro and prevented ETEC intestinal colonization in preclinical experiments. Of interest, mice intestinal colonization was significantly impaired using LT together with EtpA, suggesting the potential of EtpA as vaccine component (Roy et al. 2012).

The EaeH adhesin, the product of the *eaeH* gene first identified by subtractive hybridization of ETEC (Chen et al. 2006), is a conserved outer membrane protein that promotes bacterial engagement with host epithelial cell surfaces and ETEC colonization of the host's small intestine (Sheikh et al. 2014). The data on the EaeH adhesin, also known as FdeC in ExPEC, further support previous evidences on the crucial role of this antigen during bacterial colonization and highlight its potential as a component of a broadly protective vaccine against pathogenic *E. coli* (Nesta et al. 2012).

A chimeric vaccine containing the B subunit of heat labile toxin (LT-B) and the major subunit of CS3 was able to elicit high antibody titers in mice and to reduce ETEC adhesion to intestinal cells in vitro (Alerasol et al. 2014).

Proteins involved in mucin degradation were also proven as vaccine against ETEC in preclinical studies. EatA is a member of the serine protease autotransporter family of virulence proteins degrading MUC2, a major component of intestinal mucin. Of interest, antibodies against a secreted passenger domain of EatA were shown to impair the ETEC colonization of small intestine in mice (Luo et al. 2014). In agreement with evidences on SslE mucinase activity (Nesta et al. 2014), functional studies demonstrated that ETEC YghJ was specifically involved in degradation of mucin substrates, including Muc2 and Muc3 and required for efficient delivery of heat-labile toxin (Luo et al. 2014). In addition, SslE is present and conserved also among intestinal pathotypes, with an overall amino acid sequence identity ranged from 86 to 100%. The SslE heterologous and intrinsic protection against ETEC was assessed in a mouse model of intestinal colonization, resulting in a statistically significant 2.5 log reduction in the mean value of bacterial counts in

the caecum of immunized mice. In addition, protected mice developed anti-SslE antibodies belonging to both IgG and IgA isotypes, supporting the mucosal immunization as efficacious delivery, and reinforcing the potential of this antigen to broadly target pathogenic *E. coli* (Nesta et al. 2014).

Recently, antisera against a number of ETEC proteins that differed in their abundance in membrane protein preparations from wild-type versus a type II secretion mutant of ETEC, were tested in the ability to prevent ETEC adherence to cultured intestinal epithelial cells. Three of these antigens, ETEC_2479, Skp and MipA, were also able to provide a protective immunity in an intranasal mouse challenge model (Kumar et al. 2015).

2.2.2 Multivalent ETEC Vaccines

In the future, research will also be directed toward combining monovalent vaccines in a single complex vaccine to offer broad-spectrum coverage against different pathogens for the same target populations. The Global Enteric Multicenter Study (GEMS) revealed that *Shigella* and ETEC are among the top five major causes of moderate to severe diarrhea in children under 5 years of age in Africa and Asia (Kotloff et al. 2013). Among the many causes of diarrheal disease among travelers, military personnel visiting endemic areas, infants in developing countries, ETEC and *Shigella* are the two most important bacterial pathogens for which there are no currently licensed vaccines. Then, many attempts have been dedicated to achieve the goal of an immunogenic bivalent ETEC/*Shigella* vaccine. A potential attractive strategy is based on the use of attenuated strains of *Shigella* as live vectors for the expression of ETEC antigens, including CFs and mutant forms of LT (Ranallo et al. 2005; Barry et al. 2006). Even if an ETEC/*Shigella* is an evident option, other combinations may be considered. Since rotavirus and ETEC are of greatest threat to younger children, a combination vaccine against these may be an attractive approach. On the other hand, *Vibrio cholerae* and ETEC remain a massive burden in developing countries with increasing morbidity and mortality rates. Approaches aimed to target these two diarrhea-causing agents have been analyzed in preclinical studies. Immunization with a mixture of detoxified and enterotoxin-negative outer membrane vesicles (OMVs) derived from *V. cholerae* and ETEC induced a protective immune response against both pathogens (Leitner et al. 2015).

2.2.3 Vaccines Against Shiga Toxin-Producing *E. coli*

Shigatoxigenic *E. coli* (STEC), also referred to as verocytotoxin-producing *E. coli* (VTEC), are strains which produce Stx1 and Stx2 Shiga toxins, also known as verotoxins. Specific to their toxin-producing capabilities, VTEC and STEC *E. coli* nomenclature commonly refers to strains within the enterohaemorrhagic (EHEC) pathotype. The *E. coli* carrying both the Shiga toxin and intimin, the adhesive protein encoded by the *eae* gene and responsible for bacterial attaching to the

intestinal wall, commonly known as EHEC, elaborate potent Shiga toxins (Stx1 and/or Stx2) and are implicated in the development of hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS).

A fusion protein composed of the B subunits of the two types of Stx (named 2S protein) generated antibodies able to neutralize the cytotoxic activity of both Shiga toxins in vitro and to increase the survival of mice challenged with a lysate of *E. coli* O157:H7 (Gao et al. 2009). *Salmonella enterica* strains expressing the Stx2ΔAB toxoid colonized the mice gut and induced anti-Stx2B IgG. The anti-toxoid antibodies neutralized Stx2 toxic activity in vitro, but conferred only limited protection against the Stx2 challenge in vivo (Rojas et al. 2010). An S2 derivative fusion containing an enzymatically inactive Stx2A subunit instead of the Stx2B (Stx2Am-Stx1B) displayed enhanced immunogenicity compared to the S2 fusion. Stx2Am-Stx1B generated higher levels of Stx2 neutralizing antibodies and significantly higher level of protection against a lethal dose of an O157:H7 lysate (Cai et al. 2011). A vaccine containing a synthetic monomer of PNAG (9GlcNH2) conjugated to Shiga toxin 1b subunit was recently proposed to prevent intestinal infections caused by Shiga toxin (Stx)-producing *E. coli* (STEC) (Gould et al. 2013).

A number of EHEC vaccine approaches have been employed with different outcomes in animal models, including the use of recombinant proteins and virulence factors such as Stx1/2, intimin, EspA, fusion proteins of A and B Stx subunits, avirulent ghost cells of EHEC O157:H7, live attenuated bacteria expressing recombinant proteins, and recombinant fimbrial proteins (Rabinovitz et al. 2012; McNeilly et al. 2015). In order to prevent EHEC, vaccination of infected mice with a Stx2 toxoid resulted in decreased CFU detected in their feces, suggesting that active immunization leads to the generation of Stx2-neutralizing antibodies in the intestine (Mohawk et al. 2010). An EHEC multi-antigen vaccine consisted of the multivalent Stx2B-Tir-Stx1B-Zot protein, where Zot is used as an antigen delivery tool that binds a receptor in the intestinal epithelium affecting mucosal permeability. Mice immunized intranasally with this multivalent protein had reduced colonization and reduced amounts of EHEC detected in the stool (Zhang et al. 2011).

It is known that naturally occurring human EHEC O157:H7 infections induce antibodies against T3SS-related proteins, such as Tir, intimin, EspB, NleA. and EspA (Asper et al. 2011). This has led to the proposal of bacterial T3SS proteins (TTSPs) as vaccine candidates. Intestinal mucosa immune responses have also been targeted by intimin-, EspB-, and EspA-derived vaccines using different carrier strains. *Lactobacillus lactis* expressing EspA or EspB induced antigen-specific humoral IgG responses. Polyclonal anti-EspA antibodies were able to inhibit EHEC-induced actin rearrangements in vitro (Luan et al. 2010). Orally inoculated *S. enterica* serovar *Typhimurium* expressing intimin was able to colonize the mice Peyer's patches and spleen, producing specific serum IgG and fecal IgA antibodies and reducing EHEC shedding after challenge (Oliveira et al. 2012). In mice inoculated with EspB-expressing *L. lactis*, the antibody response consisted of not only IgG but also fecal IgA (Ahmed et al. 2013a). Recently, the immunogenicity of intranasal mice administration of a novel bivalent EHEC O157:H7 subunit vaccine,

made by antigen EspA-Tir-M, resulted in protection against EHEC O157:H7 colonization and infection at a rate of 90%. In contrast, subcutaneous immunization elicited a weak immune response and exhibited a low protection rate (Lin et al. 2017).

2.2.4 Vaccines Against Other Intestinal Pathotypes

Intestinal pathotypes differ in virulence factors and in their ability to cause a broad spectrum of diseases by different mechanisms. The enteropathogenic EPEC is defined as *E. coli* that produce a characteristic histopathology known as attaching and effacing (A/E) lesions on intestinal cells and that do not produce Shiga, Shiga-like, verocytotoxins. Typical EPEC (tEPEC) of human origin possess a virulence plasmid known as the EAF (EPEC adherence factor) plasmid that encodes localized adherence on cultured epithelial cells mediated by the bundle forming pilus (BFP), while atypical EPEC (aEPEC) does not possess this plasmid (Donnenberg and Finlay 2013). Animal pathogens and their corresponding native hosts, such as *Citrobacter rodentium* in mice and rabbit enteropathogenic *E. coli* (REPEC) in rabbits, have been widely used as model systems for EPEC infection studies. EPEC intimin has shown protection in the animal model, as immunized rabbits exhibited reduced fecal bacterial shedding, milder diarrheal symptoms, lower weight loss, and reduced colonization of REPEC in the cecum (Keller et al. 2010). In addition, an immunodominant domain of EPEC beta-intimin was protective in the REPEC challenge model (Ahmed et al. 2013b). Very recently, the immunogenic Dispersin virulence factor of EAEC, responsible for antiaggregation and bacterial penetration across the intestinal epithelium, has been proposed as vaccine antigen against EAEC infection (Asadi Karam et al. 2017).

A killed whole-cell vaccine based on a mixture of ETEC, EHEC, EIEC, EAEC, and EPEC diarrheagenic pathotypes combined with the cholera toxin B subunit (CT-B) has been proposed as vaccine inducing humoral immune response and providing protection in a mouse model following systemic or oral bacterial challenges (Gohar et al. 2016).

3 Vaccines Against *E. coli* Infections in Animals

The increasing incidence of *E. coli* foodborne-disease outbreak (FBDO) worldwide raises the urgent need for additional intervention strategies to reduce the rate of *E. coli* spreading. Identifying the major sources of risk is key to designing effective control strategies. On the other hand, a valid alternative strategy to control *E. coli* dissemination, often resulting in serious sequelae that include fatality, may consist in targeting the main animal reservoir and the primary means of human contamination. Usually, the *E. coli* transmission pathway for virulent clones to disseminate globally often has a relationship with animals and agriculture. Animal pathogenic

E. coli mainly belong to the ETEC and EPEC diarrhoeagenic, Shigatoxin-producing, uropathogenic, septicemic *E. coli* (SePEC) pathotypes as well as the avian pathogenic *E. coli* (APEC) and the mammary pathogenic *E. coli* (MPEC). Since cattle are the most important reservoir of foodborne EHEC pathogen and the root of contamination, reducing *E. coli* O157:H7 at the farm level should decrease the risk of human infection. Prevention of the *E. coli* O157:H7 global pathogen in cattle could help tackle the main reservoir of virulence, predicting a 60% decrease in human cases associated with O157:H7 assuming a bovine vaccination effectiveness of 80% and an adoption rate of 100% (Matthews et al. 2013). The ST131 pandemic in humans and other multidrug-resistant and urovirulent *E. coli* strains were found also in companion animals, ruminants, in wastewater treatment plant effluent (Johnson et al. 2009; Amos et al. 2014). Overall, *E. coli* outbreaks in humans often occurred worldwide as a result of consuming contaminated food and water, mishandling, and/or undercooking of meats or contaminated vegetables (Sharapov et al. 2016; Honish et al. 2017). Commercial vaccines against *E. coli* O157:H7 have targeted TTSS-secreted proteins (Econiche®, designed to reduce cattle contamination by EHEC), a siderophore receptor and porin proteins (SRPs) (Epitopix®, licensed for use in beef cattle in the USA). Recombinant type III secretion system (T3SS)-associated proteins EspA, intimin, and Tir from EHEC O157:H7 were proposed for calves vaccination, resulting in a reduction in EHEC shedding and in the generation of antibodies potentially cross-protective against different EHEC serotypes (McNeilly et al. 2015). Reverse vaccinology also exploited available animal-source ETEC genomes as an effective approach toward the development of subunit vaccines for animals (Dubreuil et al. 2016). Commercial vaccines for cows contain killed *E. coli* F5 isolates and/or the F5 adhesin, while commercial vaccines for female pigs contain F4 (also designated K88), F5 (K99), F6 (987P), and/or F41 fimbriae, either purified or as inactivated bacteria expressing these fimbriae with or without the LT toxoid. ETEC porcine post-weaning diarrhea (PWD) is still causing significant economic losses to swine producers worldwide. In this respect, several maternal vaccines are available. However, at weaning, lactogenic protection disappears and vaccines to protect weaned pigs from diarrhea caused by ETEC are still needed (Takeyama et al. 2015; Srivastava et al. 2016). The commercially available modified-live *E. coli* Poulvac® vaccine help protect against both the colibacillosis and productivity loss in poultry. The *E. coli* mastitis vaccine, Enviracor J-5, provides a safe and effective way to control clinical mastitis.

The major goals of veterinary vaccines are to improve the health and welfare of companion animals, increase production of livestock in a cost-effective manner, and prevent animal-to-human transmission from both domestic animals and wildlife. Interventions that would prevent zoonotic pathogens in animals will reduce *E. coli* transmission reducing the risk of contamination and bacterial spreading.

4 Adjuvants, Delivery, and Route of Immunization

Mucosal pathogens would probably require intestinal immunity, and therefore the oral route would be the preferred one for vaccine administration. Oral delivery is expected to mimic the course of natural infection that is known to confer immunity against many diarrheal *E. coli* strains. However, only a few mucosal vaccines for oral administration have been licensed for human use (Czerkinsky and Holmgren 2010). Since *E. coli* have a mucosal portal of entry and infections are confined to the mucosal surfaces, an *E. coli* vaccine should be able to induce a specific secretory IgA antibody response at the intestinal mucosa level. However, the immune response is complex and may require combinations of several immune effectors, as protection against *E. coli* does not directly correlate with mucosal sIgA content in stool or intestinal washes. On the other side, prevention against bacteremia is likely to rely on circulating antibodies capable of binding to O-antigen and promoting opsonophagocytosis. Mechanisms of protection against UTI are less well understood and may differ, such as for simple uncomplicated UTI versus persistent or recurrent UTI or for UTI in individuals with indwelling catheters. It is not known whether protection of the urinary tract would be conveyed through vaccine-induced IgA or IgG or whether urinary tract antibody levels are crucial. Thus, it is conceivable that high serum antibody levels that transudate into the mucosal tissues may be needed to achieve protection against recurrent or complicated UTI (Poolman and Wacker 2016).

Many alternative routes of vaccine delivery are being explored. In this respect, non-oral routes of immunization, such as intranasal, intradermal, sublingual, and intramuscular, are becoming very attractive because of their potential to induce a systemic and mucosal immune response. Sublingual as well as transdermal routes of administration have been shown to induce a broadly disseminated mucosal and systemic immune responses.

However, a parenteral vaccine can also elicit a mucosal immune response in individuals who have been already primed through natural mucosal exposure to the pathogen. Although IgA is the main isotype in the mucosal secretions, IgGs are also present and may contribute to the adaptive immune defenses in the gut. IgG reaches luminal secretions mainly by transudation of systemic antibodies, although small amounts are also synthesized locally. Parenteral vaccination may in itself be useful for immunization against those mucosal infections in which the pathogen is taken up or penetrates across the epithelium. In addition, parenteral administration might be used in tandem with mucosal vaccines, whether the latter are given by oral, nasal, or sublingual route. Traditional oral immunization is able to induce a substantial antibody response in the small intestine and in the ascending colon after oral immunization. On the other hand, when an *E. coli* infection occurs in the uroepithelial mucosae, which is more permeable than the intestines to transudation by plasma antibodies, a parenteral route of vaccination may also be very effective (Czerkinsky and Holmgren 2010).

Understanding cross-talk between mucosal and systemic immunity should expedite the development of vaccines against diseases caused *E. coli*. However, the gut immune system can change depending on the dietary conditions, environmental antigens, exposure to pathogens, and microbiome composition.

Cholera toxin (CT) and heat-labile enterotoxin (LT) are known to be powerful adjuvants. CT has been shown to induce increased permeability of the intestinal epithelium leading to enhanced uptake of coadministered antigens and enhanced antigen presentation by various APCs. CT markedly promoted isotype differentiation in B cells leading to increased IgA formation and exert complex stimulatory and inhibitory effects on T-cell proliferation and cytokine production. Cholera toxin and heat-labile enterotoxin (LT) also evoked both systemic and intestinal antibody responses when coadministered with protein antigens by transcutaneous immunization. Genetically detoxified derivatives of LT and CT, nontoxic but retaining the immunological and adjuvant properties of the wild-type toxins are considered the most promising adjuvants to augment local and systemic immune responses to coadministered antigens.

Vaccines against *E. coli* should be safe, immunogenic, and provide high level of protection against diarrhea in the primary target populations of infants and young children in developing countries (0–5-year-age range), and travelers to endemic areas. Moreover, an *E. coli* vaccine should be immunogenic and protective also in elderly, who are at highest risk of ExPEC bacteremia.

5 Impact of Vaccines on Microbioma

The gut microbial community (microbiota) undergoes to evolution and mutual adaptation to the host. In the postnatal period, the germ-free neonate moves from the sterile environment of its mother's uterus into a gradual colonization of mucosal and skin surfaces. During the early postnatal period, the intestinal microbiota plays a crucial role in the development of both local and systemic immunity. Then, in the intestinal tract, cholic acid, radial oxygen gradient, and dietary components become the driving forces of microbiota assembly, composition, modulation, and activities. Alterations of the normal colonization process, such as the presence of pathogenic microorganisms and toxins, can affect the important symbiotic relationship that is necessary for immune homeostasis (Walker 2013; Wu and Wu 2012). Bacteria utilize cooperative pathways to help maintaining their niches and consequently the microbial group behavior is essential to host homeostasis. These microbial relationships can be antagonistic or mutualistic, depending on the nature of the species. Bacteria express highly potent bacteriocins, microcins, and colicins to fend off other species or pathogens invading their niche without causing collateral damage to eukaryotic cells (Ohland and Macnaughton 2010). Even if the majority of genes (99.1%) examined by metagenomic sequencing of the intestinal tract consists of bacterial origin (Qin et al. 2010), also viruses, fungi, and archaea are present and may influence both specific host response and intestinal homeostasis (Norman et al.

2014). Analysis of the human microbiome indicates that proteobacteria, including *E. coli*, represent less than 0.1% of the human flora overall (Eckburg et al. 2005). Thus, vaccination against a low number of serotypes (of >180) would be unlikely to have any substantial impact on gastrointestinal and/or urogenital flora or result in serotype replacement. Emerging studies deciphering the relationship between microbiome changes and immune responses will provide more insights into the impact of the gut microbiota on vaccine efficacy (Nguyen et al. 2016). On the other hand, the interest in maintaining a healthy microbiota in commensal bacterial species with remarkable protective effects is increasing. Probiotics represent the great promise for rebuilding microbiotas and restoring health (Gensollen et al. 2016). Recent studies have addressed the issue of the potential impact of using subunit vaccines consisting of antigens that are also encoded by commensal organisms. These studies investigated the effect of vaccination with *E. coli* antigens (MipA, Skp, and ETEC_2479) conserved also in the commensals, on the intestinal mouse microbiome. Interestingly, immunization did not cause any changes to mouse health, to mouse weight gain as a function of time, or to the diversity or richness of mouse intestinal microbiomes (Hays et al. 2016).

6 Conclusions

The vaccinology field is evolving very rapidly, and new technologies are today available to make the development of effective vaccines against *E. coli* feasible in the near future. A multitude of colonization factors, toxins, and virulence determinants are necessary to allow adaptation of *E. coli* to the different niches. The enormous amount of genomic, proteomic, and transcriptomic data and their analysis could guide the search for the ideal vaccine antigens, not shared with commensal strains and with limited antigenic diversity. One approach could be to target antigens encoded by the core genome that, being shared by different pathotypes, could be potentially more cross-protective. On the other hand, including accessory antigens may be important to prevent the emergence of new pathogenic lineages. In this perspective, the subtractive reverse vaccinology approach applied to *E. coli* has allowed the identification of very promising protective antigens conserved in phylogenetically and epidemiologically distinct *E. coli* pathotypes, and has opened the way toward a universal *E. coli* vaccine.

There are still many scientific questions that need to be addressed and that could effectively guide vaccine development, such as the identification of reliable and predictive animal models, the definition of correlates of protection, the definition of relative contribution of mucosal and systemic immune response in protection, and the influence of impact of vaccination on the host microbiome. Finally, science-based studies aimed to discover the role played by any new potential vaccine antigen in virulence and pathogenesis might have a huge impact on the evaluation of the ability of the antibodies induced by such antigens in neutralizing important bacterial functional activities.

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