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Gad Frankel · Eliora Z. Ron *Editors*

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
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Shigella and Enteroinvasive *Escherichia Coli*

Ilia Belotserkovsky and Philippe J. Sansonetti

Contents

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