TOPICAL REVIEW

Interspecies communication in the gut, from bacterial delivery to host-cell response

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Abstract Intestinal pathogens have a wide variety of strategies for communicating with host epithelial cells. This review highlights a few key examples of those strategies. Enteropathogenic *Escherichia coli* (EPEC) use a type III secretion system (T3SS) to alter host ion transport through both transcriptional and post-translational mechanisms. *Salmonella* use a similar T3SS to invade host cells and modify an intracellular vacuole, which also impacts host vesicle trafficking. *Helicobacter pylori* use host cell integrins to provide a conformational change which drives the type IV secretion system into the host cell for delivery of CagA. The novel type VI section systems are phage-like apparati that deliver VgrG-1, which causes actin cross-linking and fluid accumulation in a suckling mouse model. An entirely different delivery mechanism is the outer membrane vesicle (OMV) which is composed of bacterial outer membrane wrapped around contents of the periplamsic space. Enterotoxigenic *E. coli* use OMVs to deliver bundles of heat labile enterotoxin to host cells. Finally we discuss the host responses to these varied methods of communication.

Abbreviations CFTR, cystic fibrosis transmembrane conductance regulator; DRA, downregulated in adenoma; EHEC, enterohaemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; ENAC, epithelial sodium channel; IRSp53, insulin receptor substrate p53; LT, heat labile enterotoxin; LPS, lipopolysaccharide; NHE, sodium–hydrogen exchanger; OMP, outer membrane protein; OMV, outer membrane vesicle; QseC, quorum sensing enzyme C; SCV, *Salmonella* containing vacuole; SGLT-1, sodium glucose cotransport; SKIP, SifA and kinesin interacting protein; T3SS, type III secretion system; T4SS, type IV secretion system; VSVG, vesicular stomatitis virus G protein; ZO-1, zona occludens 1.

Gail Hecht (left) is Professor of Medicine and Microbiology/Immunology and Chief, Digestive Diseases and Nutrition at the University of Illinois at Chicago. She also serves as Editor-in-Chief of a new journal, *Gut Microbes*. The focus of her research is host–pathogen interactions with specific emphasis on enteropathogenic *Escherichia coli* and enterohaemorrhagic *E. coli*. Her goal is to determine how the bacterial effector proteins that are translocated into intestinal epithelial cells by these pathogens alter host cell physiology including tight junction barrier function, active transport processes, and the innate immune response. Her research is funded by both the NIH and the VA. **Kim Hodges** (right) is a microbiologist who trained at the University of Michigan in Ann Arbor and received her PhD at the University of Minnesota. She studied invasion of *Streptococcus pyogenes* under Dr Pat Cleary. Her current work focuses on the role of decreased absorption of ions in diarrhea caused by enteropathogenic *Escherichia coli* (EPEC).

Introduction

The interface of the host intestinal epithelial layer with the lumen provides a unique opportunity for inter-species communication. Bacteria constantly send and receive signals from the host epithelium and the host cells respond and initiate their own messages. For example, the hormones adrenaline and noradrenaline typically target adrenergic receptors and regulate smooth muscle contraction and thus intestinal motility. However, enterohaemorrhagic *Escherichia coli* are capable of intercepting those messages via the quorum sensing enzyme histidine kinase, QseC, and initiating the production of virulence factors such as the type III secretion system (Hughes *et al.* 2009), a molecular syringe that injects bacterial effector proteins into host cells. Intestinal pathogens communicate with the host in a variety of ways but in this review we focus on the delivery mechanisms of various toxins, effector proteins and even viable bacteria into the host cell and the response of the infected epithelium.

Secretion systems

Communication of bacteria with host cells often involves interaction with eukaryotic cell receptors; however, intestinal cells are polarized in such a way that most of the receptors that detect bacteria (i.e. Toll-like receptors) are positioned on the basolateral surface in order to avoid constant stimulation from the bacteria-filled lumen (Abreu *et al.* 2003). While there is some evidence that commensal bacteria interact with the host, they are primarily restricted to the mucus layer in part due to surveillance by host Paneth cells, which secrete anti-microbial peptides in a MyD88-dependent manner in response to encroaching bacteria (Ayabe *et al.* 2000; Vaishnava *et al.* 2008). In contrast, pathogens often have direct contact with the cell, and can become internalized or breach the epithelial barrier by passing though M-cells (Jones *et al.* 1994). As such, pathogens have become fluent in cellular communication and how to bypass host defences. Flagellum-, pilus- and phage-like structures have evolved into the type III, type IV and type VI secretion systems that deliver effector molecules to the host (Fig. 1) (Cascales & Christie, 2003). Type III secretion systems are the most abundant and are used by enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), *Salmonella* and *Shigella* for both extracellular and intracellular communication. EHEC and EPEC are closely related and both utilize the type III secretion system but in slightly different ways to recruit actin beneath bacterial microcolonies in a Tir-dependent process to form what is referred to as an attaching and effacing lesion. For EHEC, Tir is injected into the host cell where it is phosphorylated, which drives the recruitment of actin through an intracellular signalling process involving

another effector protein, EspF$_u$, a host linker protein called IRSp53, and finally the actin nucleator N-WASP (Vingadassalom *et al.* 2009). In contrast, EPEC tyrosine 474 is phosphorylated and binds directly to the host cell protein Nck, which recruits actin via N-WASP and Arp2/3 (Campellone *et al.* 2002). In addition, some 25 putative EPEC effector proteins have been identified with additional phage-localized proteins being expressed by EHEC. Among these effectors are EspF, Map and EspG, which have been shown to have effects on both ion transport and tight junctions (Fig. 2) (McNamara *et al.* 2001; Dean & Kenny, 2004; Tomson *et al.* 2005; Dean *et al.* 2006; Gill *et al.* 2007; Hodges *et al.* 2008). EspF was first identified for its role in tight junction disruption, which alters paracellular permeability by interfering with the localization of occludin and ZO-1 in intestinal epithelium (McNamara *et al.* 2001). More recently it was found that EspF is also responsible for a reduction in sodium uptake through NHE3 (Hodges *et al.* 2008). EspF and Map have been implicated in reducing SGLT-1 activity through a brief process involving reduced glucose uptake or a more prolonged process caused by microvillus effacement (Dean *et al.* 2006). Finally EspG1 and EspG2 block Cl$^-$ uptake via Slc26a3/DRA (solute carrier 26a3/downregulated in adenoma) by breaking down microtubules, thus preventing its surface localization (Gill *et al.* 2007). Therefore, EPEC type III secretion effectors block the three major sodium and chloride transport pathways and alter tight junction function resulting in perturbations of the osmotic gradient which aids in salt and water absorption.
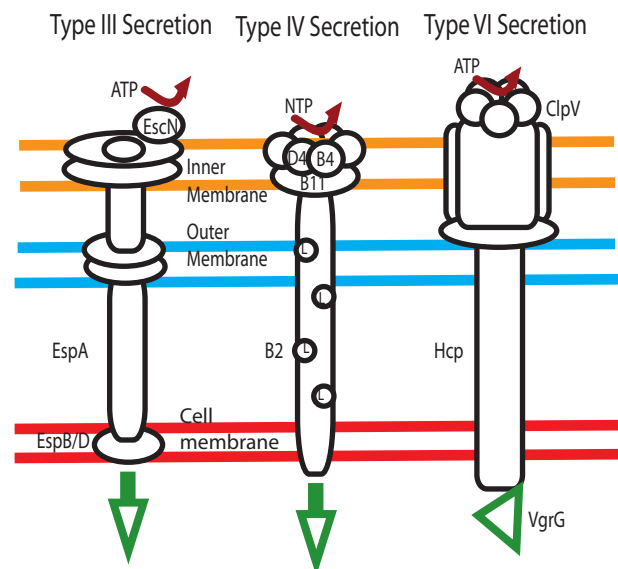

Figure 1. Schematic of the type III, IV and VI secretion systems
The EPEC type III secretion system uses EscN, an ATPase, to drive secretion through a flagellum-like apparatus. *H. pylori* uses several NTPases, D4, B4 and B11 to power its pilus-like system. Finally, the phage-like type VI secretion system uses the ATPase ClpV to deliver effectors like VgrG.
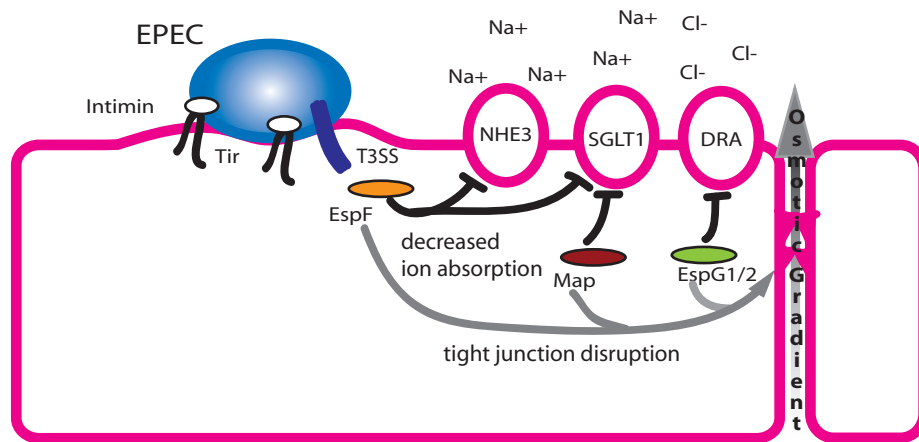
Figure 2. Enteropathogenic *E. coli* (EPEC) use a type III secretion system to deliver effector proteins including Tir, EspF, Map and EspG1 and EspG2
Injected Tir binds to Intimin on the bacterial surface and promotes attaching and effacing lesion formation. EspF decreases both sodium–hydrogen exchanger 3 (NHE3) and sodium–glucose cotransport 1 (SGLT1) activity in addition to disruption of tight junctions. Map acts along with EspF to decrease SGLT1 activity and tight junction function. EspG1 and EspG2 decrease Cl^-/OH^- exchange by DRA and also impair tight junctions. The combined activities of these proteins on ion transport alter the osmotic gradient leading to subsequent movement of water into the intestinal lumen.
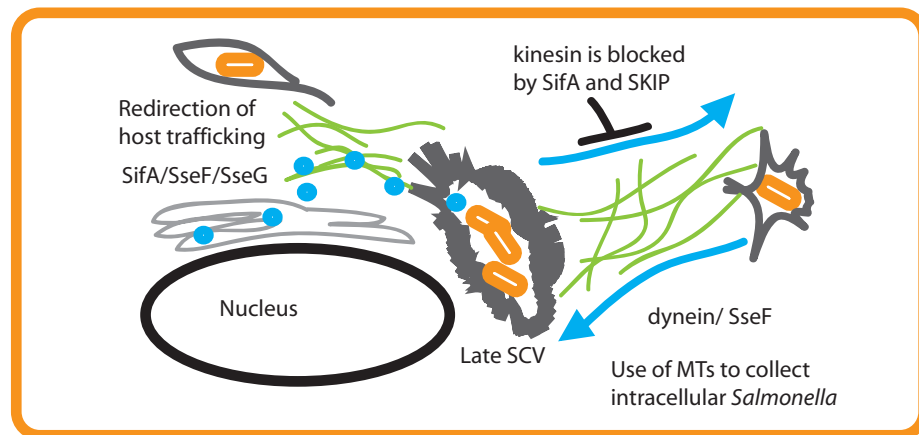
Figure 3. *Salmonella* containing vacuoles (SCVs) are located at the minus ends of microtubules due to trafficking via dynein, which is recruited to the SCV by the effector SseF
In contrast SifA through its interaction with SKIP inhibits the recruitment of kinesin, the plus end motor, preventing movement toward the cell periphery. Several effector proteins, SifA, SseF and SseG, also alter host cell trafficking toward the SCV.

Salmonella and *Shigella* also utilize type III secretion systems, initially for invasion and then subsequently to alter the host environment. In fact, invasion has been shown to be required for fluid accumulation in rabbit models of *Salmonella* infection (Giannella *et al.* 1973). *Salmonella* create specialized intracellular membrane bound compartments called *Salmonella* containing vacuoles (SCVs) (Fig. 3). The establishment of SCVs is critical to intracellular survival of *Salmonella* and also regulates host vesicle trafficking. These compartments are dependent on the host cell microtubule network for both formation and maintenance (Rajashekar & Hensel,

2011). Microtubule cargo transport is mediated by two opposing motor proteins, dynein and kinesin. The level of kinesin associated with SCVs is very tightly regulated by two proteins, SifA, which interacts with the host cell factor SKIP to exclude kinesin, and PipB2, which binds to it (Boucrot *et al.* 2005; Henry *et al.* 2006). In contrast, dynein, which is oriented toward the negative end of microtubules, is actively recruited to SCVs via the effector SseF where it plays an important role in the formation of bacterial microcolonies near the nucleus (Abrahams *et al.* 2006). Bacteria containing vacuoles can traffic on microtubules by utilizing dynein–SseF interaction, thus

allowing the bacteria to become clustered into a single SCV. SCVs are often found in association with micro-tubules and appear to redirect normal exocytic traffic to the SCV. Vesicular stomatitis virus G protein (VSVG) is often used to monitor trafficking and typically VSVG-GFP is randomly distributed; however, in infected cells there is a close association of vesicles with SCVs, suggesting that vesicle traffic is directed to the SCV (Kuhle *et al.* 2006). This recruitment was linked to three effector proteins, SifA, SseF and SseG, and was specifically inhibited by the microtubule disrupting agent nocodazole (Kuhle *et al.* 2006). Thus not only does *Salmonella* use type III secretion to create and position the intracellular SCV, it also redirects host vesicle traffic by controlling microtubule motor proteins.

While type III secretion systems are prevalent, another gastrointestinal pathogen, *H. pylori*, utilizes a type IV secretion system (T4SS) to deliver its primary effector protein, CagA, into host cells. Type III and type IV secretion systems have similar functions but differ in their origin. Type III systems evolved from flagella while type IV systems more closely resemble conjugative pili (Cascales & Christie, 2003). It has been shown recently that β_1 integrins play a critical role in T4SS effector translocation by *H. pylori*. Kwok *et al.* (2007) proposed that CagL located on the exterior of the T4SS pilus binds to $\alpha_5\beta_1$ integrin via an RGD motif similar to the typical interaction for the endogenous ligand fibronectin. An independent group, however, suggest that the RGD motif is irrelevant and instead propose that multiple T4SS proteins including CagL, CagY, CagI and even CagA bind to the β_1 chain of integrins in a novel manner (Jimenez-Soto *et al.* 2009). These interactions were identified initially by yeast two-hybrid screening using the extracellular portion of the β_1 chain as bait (Jimenez-Soto *et al.* 2009). Bead-coupled $\alpha_1\beta_1$ and $\alpha_5\beta_1$ integrins bound to CagA but not CagL or CagY. Subsequent studies suggested that CagL and CagY require an intact T4SS for integrin binding while CagA binds independently of the T4SS. In both studies, interaction of the T4SS with β_1 integrin is required for translocation of CagA (Kwok *et al.* 2007; Jimenez-Soto *et al.* 2009) (Fig 4). CagA translocation did not occur in integrin-deficient cells (GE11) or β_1 knock-out cells (GD25); however β_1 complementation rescued CagA translocation (Jimenez-Soto *et al.* 2009). Clustering of integrins also appears to be a requirement as blocking integrin reorganization in the lipid bilayer using calpeptin prevented CagA translocation. The authors theorize that the conformational changes in integrins after binding help position the T4SS closer to the membrane, thus allowing for translocation (Jimenez-Soto *et al.* 2009). Once injected into the cell, CagA binds to upwards of 20 proteins, including host kinases and SH2 domain containing proteins, and precipitates a number of phenotypes including activation of Map kinases, NF-κB,

and disruption of the tight junction protein ZO-1 (Amieva *et al.* 2003; Lamb *et al.* 2009; Selbach *et al.* 2009; Backert *et al.* 2010). CagA induces a specific alteration in cellular morphology called the hummingbird phenotype, which causes elongation of cells with pointed 'beak-like' extensions (Segal *et al.* 1999). Perhaps the most interesting aspect of CagA is its ability to mimic the substrate of the host cell kinase Par1b, which is involved in establishing and maintaining host cell polarity (Zeaiter *et al.* 2008; Nesic *et al.* 2010). This is thought to contribute to both the disruption of apical junctions and the establishment of the hummingbird phenotype (Zeaiter *et al.* 2008; Lu *et al.* 2009).

Type VI secretions systems were only recently identified and appear to be limited to only a few pathogens. Interestingly, they target both eukaryotic and prokaryotic hosts (Russell *et al.* 2011). The type VI secretion system is derived from bacteriophage tail proteins and delivers effector proteins that are fused to the spike protein VgrG (Bonemann *et al.* 2010). The type VI system exists in *Vibrio cholerae*, *Salmonella enterica*, and a variety of non-intestinal pathogens including *Pseudomonas aeruginosa* and *Legionella pneumophila*. In *V. cholerae*, the cap protein VgrG breaks away from the tip of the type VI apparatus after insertion into the host cell resulting in an alternative form of effector delivery (Pukatzki *et al.* 2006; Pukatzki *et al.* 2007). There are several variants of VgrG which are designated by a number,
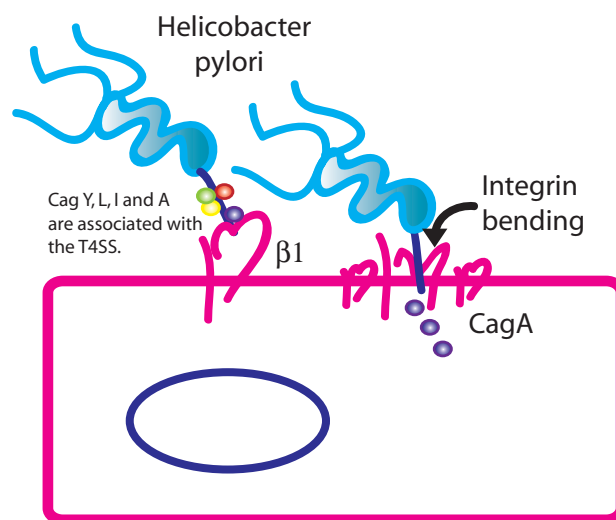

Figure 4. *H. pylori* type IV secreted effector proteins Cag Y, L, I and A form an association with the type IV secretion system (T4SS) and bind to the β_1 chain of integrins
Injection of Cag A via the T4SS requires a conformational change in the β_1 chain in addition to integrin clustering. An antibody that locks the conformation of β_1 integrin was able to prevent CagA delivery. It is concluded therefore that the physical change of the integrin upon binding is needed for insertion of the T4SS tip and subsequent injection of effector proteins.

such as VgrG-1, which has an actin cross-linking domain. *V. cholerae* strains with a deletion in VgrG-1, VgrG-2 or VasK, part of the type VI secretion apparatus, induced less fluid accumulation in a suckling mouse model suggesting less diarrhea (Ma & Mekalanos, 2010). Type VI secretion systems are in the early stages of discovery and only a limited number of host effects have been described to date.

Outer membrane vesicles

Some bacterial toxins, including heat labile enterotoxin (LT) of enterotoxigenic *E. coli*, have been demonstrated to associate with outer membrane vesicles (OMVs) (Fig. 5). OMVs are composed of lipid and protein components of the outer membrane of bacteria such as lipopolysaccharide (LPS) and outer membrane proteins (OMPs) that surround a residual periplasmic space containing characteristic proteins such as alkaline phosphatase (Horstman & Kuehn, 2000). LT has been shown to reside both in the periplasm of these OMVs and in a LPS-associated fraction. Antibodies against the LT-receptor GM_1 blocked cell rounding caused by LT-containing OMVs, suggesting that vesicles and their contents are directly internalized in a process that depends on GM_1 (Kesty *et al.* 2004). A number of Gram negative bacteria produce OMVs which enhance toxin delivery, including *V. cholerae* and *H. pylori* (Parker *et al.* 2010; Chatterjee & Chaudhuri, 2011).

Understanding the host: mouse models

While a number of bacterial communication strategies with the host have been discussed, the host has its own repertoire of responses. To fully explore this response, animal models are needed. *Citrobacter rodentium* is a natural murine pathogen often used to mimic the human pathogen EPEC and EHEC infection as they all belong to the attaching and effacing (A/E) family of pathogens. However, the response of most mouse breeds to *C. rodentium* infection is intestinal crypt hyperplasia. A mouse model for diarrhoea has long been lacking. A specific inbred strain of mouse, FVB, derived from Swiss–Webster mice, is uniquely susceptible to *C. rodentium* infection. Specifically, mice appear ill at 6 days post-infection and begin to die at 9 days with only a 23% survival rate (Borenshtein *et al.* 2007). There is a correlation between death and weight loss, but inflammatory markers such as serum TNF-α are not increased and systemic infection, characterized by colonization of the spleen, does not occur. Instead, mice exhibit signs of dehydration including ruffled coat, decreased skin turgor and sunken eyes. Importantly, most of the infected animals exhibit severe diarrhoea, and fluid rehydration by subcutaneous injection prevented

mortality. Rehydration did not, however, alter bacterial counts suggesting that death was due to fluid loss.

To gain further insight into the host response, microarray analysis was performed. Interestingly, a number of host intestinal transporter levels were modified to a greater extent than even inflammatory response genes. Aquaporin 8, which contributes to water absorption, was decreased 268-fold. In another *C. rodentium* model system, aquaporin 2 and 3 proteins were mislocalized (Guttman *et al.* 2007). In addition to changes in water absorption, there were major changes in the expression of ion transporters, thus altering the osmotic gradient and increasing luminal water. Both sodium and chloride absorptive pathways, but not secretion, were impaired by *C. rodentium* infection. Levels of epithelial sodium channel (ENaC/Scnn Ia), and sodium–hydrogen exchanger NHE2 (Slc9a2) and NHE3 (Slc9a3) transcripts were decreased between 2- and 13-fold leading to a decrease in serum sodium from 144 to 139 $mEq\,l^{-1}$ in FVB mice. The chloride/bicarbonate transporter down-regulated in adenoma (DRA) was impacted even more dramatically with a 1100-fold decrease in transcript after 9 days of infection reducing serum chloride levels from 103 to 92 $mEq\,l^{-1}$. DRA is also controlled at the post-translational level. DRA is known to recycle and was found to exist predominantly in subcellular pools as opposed to the apical surface in EPEC infected mouse colon (Gill *et al.* 2007). Of some interest the transcriptional changes in DRA can be counteracted by using microflora of C57/BL/6 mice which are resistant to the effects of *C. rodentium* (Ghosh *et al.* 2011). Interestingly,
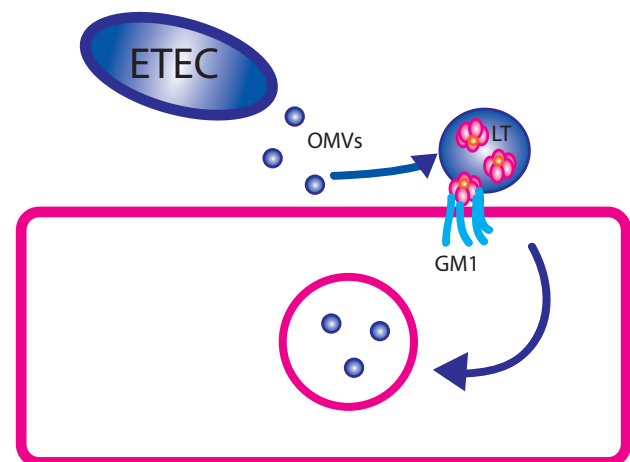

Figure 5. Enterotoxigenic *E. coli* (ETEC) form outer membrane vesicles (OMVs) from lipid and proteins constituents of the bacterial outer membrane
These vesicles contain a periplasmic space which carries heat labile enterotoxin (LT) in addition to other proteins such as alkaline phosphatase. LT in OMVs can interact with its receptor GM_1 and mediate delivery of OMVs into the host cell, thus enhancing toxin delivery.

CFTR levels remained unchanged; however A_{2B} adenosine receptor increased. A_{2B} interacts with free adenosine, generated during inflammation, thus increasing cAMP and activating CFTR. Therefore, infection by *C. rodentium* decreases the transcription of a number of intestinal absorptive transporters in the host that are directly involved in salt and water movement. A number of other transporters that indirectly alter salt transport by affecting H^+, HCO_3^- and Na^+ levels in the cell were also decreased. These include carbonic anhydrase I and IV, peptide transporters Pept1 and Petp2 and the Na^+/K^+-ATPase (Borenshtein *et al.* 2008). Thus the role of absorption, long overlooked, is key to diarrhoea caused by A/E, and probably other pathogens. In addition to these changes in ion transport, mice have been shown to respond to EPEC infection with decreased resistance, initially due to the bacterial effector EspF but with a subsequent EspF-independent effect at 5 days post-infection, which is thought to depend on TNF-α (Shifflett *et al.* 2005). Importantly, host inflammation differs between EPEC and *C. rodentium* in this case.

In summary, pathogens have devised a number of ways to manipulate intestinal epithelial cell function. Some pathogens initiate the infectious process by intercepting host signals to induce their own virulence via quorum sensing. The type III secretion system of EPEC alters surface levels of a number of intestinal transporters including NHE3, SGLT1 and DRA/Slc26a3 while the related pathogen *C. rodentium* dramatically decreases the transcriptional regulation of NHE2, NHE3 and DRA/Slc26a3. In *Salmonella* and *Shigella*, diarrhoea is dependent on invasion for which type III secretion is required. *H. pylori* uses a type IV secretion system in a manner that depends on host cell integrin clustering and conformational change to inject the primary virulence determinant CagA. The type VI secretion system injects a modified VgrG, which causes actin cross-linking and fluid accumulation. Finally, enterotoxigenic *E. coli* uses small vesicles made from their own outer membrane to carry LT to host cells. These examples illustrate that pathogens have acquired a wide variety of mechanisms for communicating with cells. Our exploration and understanding of these mechanisms stand to provide insight into the development of new therapeutic strategies for the treatment of infectious diarrhoea and other pathogen-mediated gastrointestinal diseases.

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