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Chihiro Sasakawa
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Molecular Mechanisms of Bacterial Infection via the Gut

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Preface

Our gut is colonized by numerous bacteria throughout our life, and the gut epithelium is constantly exposed to foreign microbes and antigens derived from digested foods. Thus, the gut epithelium acts as a physical barrier against microbial invaders and is equipped with various elements of the innate defense system. Resident commensal and foreign invading bacteria interact intimately with the gut epithelium and can impact host cellular and innate immune responses. From the perspective of many pathogenic bacteria, the gut epithelium serves as an infectious foothold and port of entry for dissemination into deeper tissues. In some instances, when the intestinal defense activity and host immune system become compromised, even commensal and opportunistic pathogenic bacteria can cross the barrier and initiate local and systematic infectious diseases. Conversely, some highly pathogenic bacteria, such as those highlighted in this book, are able to colonize or invade the intestinal epithelium despite the gut barrier function being intact. These pathogenic bacteria are capable of circumventing the gut defense barriers, leading to colonization within and beyond the gut. Some pathogenic bacteria can disseminate to distal tissues and cause severe enteric and systemic diseases. Therefore, the relationship between the defensive activity of the intestinal epithelium against microbes and the pathogenesis of infective microbes becomes the basis for maintaining a healthy life.

This book in the series *Current Topics of Microbiology and Immunology* entitled 'The Molecular Mechanism of Bacterial Infection of the Gut', begins with an overview of the structure and function of the gut epithelium, following which we highlight a series of current topics on major gastric and enteric pathogenic bacteria, including *Helicobacter pylori*, *Vibrio cholerae*, *Yersinia enterocolitica*, *Salmonella*, *Shigella,* and *Campyrobacter jejuni*. In the context of the title of this book, we originally wished to include an important chapter on enteropathogenic and enterohemorragic *Escherichia coli*, but unfortunately we were unable to do so due to its retraction by an author. Nevertheless, we are very satisfied with the state-of-the art reviews on each of the pathogens covered in this book, which I believe will offer readers an overview of the current topics related to major gastric and enteric pathogens, while highlighting their highly evolved host (human)-adapted infectious

processes. Clearly, an in-depth study of bacterial infectious strategies, as well as the host cellular and immune responses, presented in each chapter of this book will provide further insight into the critical roles of the host innate and adaptive immune systems and their importance in determining the severity of or completely preventing infectious diseases. Furthermore, under the continuous threat of emerging and re-emerging infectious diseases, the topic of gut-bacteria molecular interactions will provide various clues and ideas for the development of new therapeutic strategies.

Finally, I sincerely thank Prof. Tasuku Honjo at Kyoto University Graduate School of Medicine for inviting me to edit this book, all of the authors for providing current topics, and Anne Clauss for her help as the house editor of this book.

C. Sasakawa

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Functional Morphology of the Gastrointestinal Tract

Le Shen

Contents

Abstract The primary function of the gastrointestinal tract is water, electrolyte, and nutrient transport. To perform this function, the epithelium lining the gastrointestinal tract is in close contact with the gastrointestinal lumen. Because the lumen

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is connected to the external environment and, depending on the site, has a high bacterial and antigen load, the epithelium must also prevent pathogenic agents within the gastrointestinal lumen from gaining access to internal tissues. This creates a unique challenge for the gastrointestinal tract to balance the requirements of forming a barrier to separate the intestinal lumen from underlying tissue while simultaneously setting up a system for moving water, electrolytes, and nutrients across the barrier. In the face of this, the epithelial cells of the gastrointestinal tract form a selectively permeable barrier that is tightly regulated. In addition, the intestinal mucosa actively participates in host defense by engaging the mucosal immune system. Complex tissue organization and diverse cellular composition are necessary to achieve such a broad range of functions. In this chapter, the structure and function of the gastrointestinal tract and their relevance to infectious diseases are discussed.

Abbreviations

MLC	Myosin regulatory light chain
MLCK	myosin light chain kinase
CVB	Coxsackie virus B
M cells	microfold cells
ZO	zonula occludens

1 Introduction

The primary function of the gastrointestinal tract is water, electrolyte, and nutrient transport. To perform this function, the epithelium lining the gastrointestinal tract is in close contact with the gastrointestinal lumen. Because the lumen is connected to the external environment and, depending on the site, has a high bacterial and antigen load, the epithelium must also prevent pathogenic agents within the gastrointestinal lumen from gaining access to internal tissues. This creates a unique challenge for the gastrointestinal tract to balance the requirements of forming a barrier to separate the intestinal lumen from underlying tissue while simultaneously setting up a system for moving water, electrolytes, and nutrients across the barrier. In the face of this, the epithelial cells of the gastrointestinal tract form a selectively permeable barrier that is tightly regulated. In addition, the intestinal mucosa actively participates in host defense by engaging the mucosal immune system. Complex tissue organization and diverse cellular composition are necessary to achieve such a broad range of functions. In this chapter, the structure and function of the gastrointestinal tract and their relevance to infectious diseases are discussed.

Subsequent chapters will address how specific pathogens exploit or evade normal cellular processes to promote disease development.

2 Organization of the Gastrointestinal Tract Wall

The gastrointestinal tract is composed of four layers: the mucosa is the innermost layer; the submucosa is a layer of connective tissue that supports the mucosa; the muscularis externa is the muscle wall surrounding the submucosa; and the adventitia or serosa is the outmost layer of the gastrointestinal tube (Fig. 1).

The mucosa is composed of an innermost layer of epithelial cells, a layer of supporting loose connective tissue directly beneath the epithelium, termed the lamina propria, and a thin layer of smooth muscle cells, the muscularis mucosae, that forms the boundary between the mucosa and the submucosa (Fig. 2).

To increase the surface area for absorption, the mucosal and submucosal layers of the small intestine and colon are organized into regular ridges, known as plicae circulares, which increase the absorptive surface area by approximately three-fold. In addition, the villi, small finger-like projections of the mucosa that extend into the lumen, increase the absorptive surface area by another ten-fold (Fig. 2a). Within the core of each villus, arterioles rise to the tip without branching, and once at the tip, the arteriole splits to form a network of capillaries that subsequently course down along the sides of the villus in a fountain-like pattern in close apposition to the basement membrane and epithelial cells. The intimate association between ascending central arterioles and descending capillaries allows for countercurrent exchange of solutes, comparable to that occurring in the renal medulla. Such exchange results in the hypertonic lamina propria in the villus tip during active nutrient absorption.

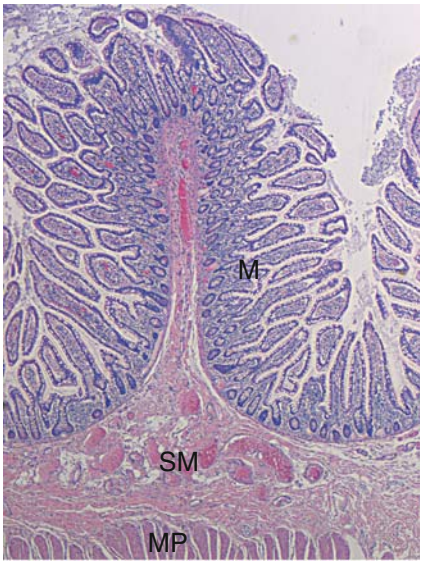

Fig. 1 Human jejunum. The mucosa (*M*) and submucosa (*SM*) are organized into series of ridges, or plica circularis, to increase the surface area for digestion and absorption. The small intestinal mucosa is organized into crypts and villi to further increase the surface area. The inner circular layer of the muscularis propria (*MP*) is shown at the bottom of the image

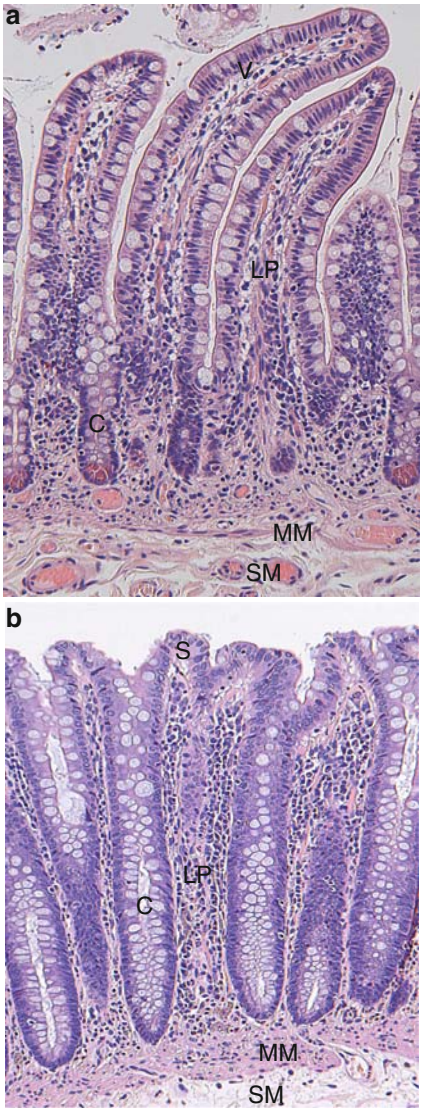

Fig. 2 Human intestinal mucosa. (**a**) The small intestinal mucosa is organized into crypts (*C*) and villi (*V*). Epithelial cell proliferation is limited to crypts. Enterocytes migrate to the top of the villi, becoming more differentiated with higher levels of brush border digestive enzyme and transporter expression. The lamina propria (*LP*) is composed of connective tissue, blood vessels, and immune cells. The muscularis mucosae (*MM*) separates the mucosa from the submucosa (*SM*). (**b**) The colonic epithelium invaginates to form crypts (*C*) along with flat surface epithelium (*S*)

Under ischemic conditions, countercurrent oxygen exchange can also occur, resulting in lower oxygen tension in the villus and greater damage to the villus epithelium, with relative preservation of the crypt epithelium. The protection of the crypt zone at the expense of the villus preserves intestinal stem cells located in the crypt and allows future mucosal repair after the acute damage has resolved.

In addition to vasculature, lymphatic drainage is also critical for villus function. The villus lymphatic duct, called a lacteal, is responsible for chylomicron absorption and reabsorption of plasma proteins that have leaked into the lamina propria

from the microvasculature. The importance of lymphatic drainage is demonstrated in Whipple's disease. During *Tropheryma whipplei* infection, bacterial laden macrophages within the lamina propria compress and obstruct the lacteal, leading to malabsorption, malnutrition, and vitamin deficiencies.

In contrast to the small intestine, the colonic mucosa contains a flat layer of surface epithelium with invaginating crypts, without the presence of villi (Fig. 2b). Within the colon, submucosal arterioles branch after penetrating the muscularis mucosae and form a chain of capillaries. These capillaries ascend along the colonic crypts and form a network around the crypt openings just beneath the surface epithelium, which allows countercurrent exchange. In addition, the lacteals are not present in the colonic mucosa, which may contribute to limited metastatic potential of colon cancers limited to the mucosa.

3 Regional Specification of the Gastrointestinal Tract

To facilitate nutrient absorption, the mucosa of the gastrointestinal tract is highly specialized. The gastric mucosa supports digestion by secreting hydrochloric acid and digestive enzymes, the microvilli present in the small intestine increase surface area and support massive transcellular transport, while the colon is specialized for maintaining ion and water balances. Although structurally similar, the small intestine can be anatomically divided into three functionally distinct regions: the duodenum, the jejunum, and the ileum. Brush border digestive enzymes are highly expressed in duodenal and jejunal epithelium, and 90% of absorption occurs within the first 1 m of the small intestine. The jejunum is the major site for monosaccharide, amino acid, and free fatty acid absorption. The duodenum and jejunum are also the primary sites for absorption of water-soluble vitamins, iron, and calcium. In contrast, the ileum is the primary site for bile salt and vitamin B_{12} absorption, and the colon is primarily responsible for Na^+, Cl^-, H_2O absorption and HCO_3^- and K^+ secretion.

In addition to functional specification, distribution of differentiated cell types also differs in distinct parts of the gastrointestinal tract. For example, Paneth cells are abundant in the small intestine but are absent in the distal colon, resulting in a higher bacterial load in the latter region. Such regional specialization within the intestines is developmentally determined, as embryonic mouse small intestinal grafts implanted into subcutaneous tissue ultimately express features specific to the region from which they originated (Rubin et al. 1992).

Along with distinctive functions, there is a gradient of endogenous bacterial flora throughout the intestines. The luminal contents of the stomach, the duodenum and the jejunum contain about 100 bacterial organisms per milliliter of luminal content. The numbers of bacteria present in the lumen increase progressively, from about 100 microorganisms per ml in the proximal ileum, to 10^{12} organisms per ml in the colon. Such different bacterial loads present distinct challenges for local mucosal immune cells.

4 Specialization Along the Vertical Axis: From Crypt to Villus

In addition to regional compartmentalization, functional specification also exists along the crypt to the villus, or vertical, axis. In the small intestine, the crypt primarily contains stem cells, undifferentiated secretory cells, enteroendocrine cells, and Paneth cells (Fig. 4), while the villus is populated with absorptive enterocytes and goblet cells (Fig. 3). This distribution of distinct cell types creates functionally distinct compartments: the crypt is critical for cell renewal, ion and water secretion, endocrine/paracrine secretions to the lamina propria and

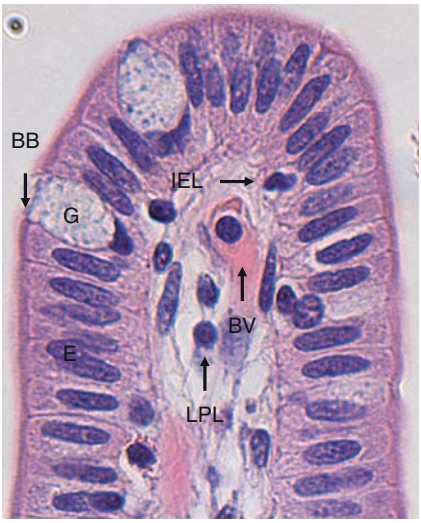

Fig. 3 Human jejunal villus tip. The villus tip is covered by the epithelium containing absorptive enterocytes (*E*) and goblet cells (*G*). The brush border (*BB*) locates at the free (apical) surface of the epithelial cells and has a fuzzy appearance. Intraepithelial lymphocytes (*IEL*) intersperse within the epithelium and lamina propria lymphocytes (*LPL*) are located in the lamina propria, below the epithelium. Blood vessels (*BV*) present within the lamina propria

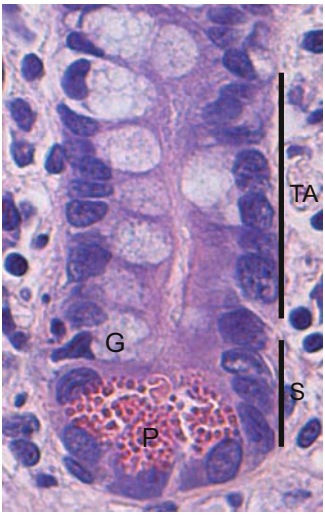

Fig. 4 Human jejunal crypt. Paneth cells (*P*) populate the bottom of the crypts, with prominent eosinophilic granules in the apical cytoplasm. Above the Paneth cells is the stem cell zone (*S*). The proliferating undifferentiated crypt enterocytes are located within the transit-amplifying zone (*TA*). Goblet cells (*G*) also present in the crypts

capillaries, and exocrine secretion of macromolecules, such as defensins, into the crypt lumen. The villus is responsible for nutrient absorption and mucin secretion to the epithelial surface.

Regardless of their ultimate fate, crypt and villus cells are all derived from intestinal stem cells. The stem cells first give rise to transit-amplifying undifferentiated secretory cells, which then differentiate into other cell types of the epithelium. While Paneth cells move downward to the crypt base, the other cell types move upward toward the villus tip. As enterocytes migrate up the crypt–villus axis, they progressively differentiate and produce increasing amounts of brush border digestive enzymes and nutrient transporters (Hwang et al. 1991; Mariadason et al. 2005). After epithelial cells reach the villus, they are eventually ejected from the epithelium. However, it is not clear if apoptosis or programmed cell death is the trigger or consequence of cell extrusion (Gavrieli et al. 1992; Hall et al. 1994; Watson et al. 2005). The extrusion process has been characterized morphologically as an orderly shedding of cells with maintenance of cell-cell junctions and barrier function (Madara 1990). Recent in vitro and in vivo studies suggest that during cell extrusion, actomyosin contraction occurs in cells surrounding the extruded cell, allowing these cells to form a contractile purse string to push shed cells out of the epithelium without significant loss of barrier integrity (Bullen et al. 2006; Rosenblatt et al. 2001). Interestingly, it has been suggested that cell shedding does not always lead to sealing of the epithelium. In some cases, epithelial cell shedding leads to gap formation in the villus without compromising the epithelial barrier (Kiesslich et al. 2007; Watson et al. 2005). The physiological significance of these gaps remains to be determined.

5 Stem Cells, Intestinal Proliferation, and Differentiation

In the small intestine, actively dividing cells are located only in the crypt. It has long been known that pluripotent stem cells, localized several cells above the base of the crypt, are the source of such proliferation (Bjerknes and Cheng 2006; Marshman et al. 2002). Approximately six independent stem cells reside within each crypt, only some of which are actively dividing (Nicolas et al. 2007; Taylor et al. 2003). In most cases, each cycling stem cell gives rise to one new stem cell and one transit-amplifying cell that is destined for differentiation. However, rare events can occur where a stem cell gives rise to two transit-amplifying cells or stem cells (Yatabe et al. 2001). Studies using cell lineage tracing techniques have shown that crypts are initially polyclonal but become monoclonal over time, while each villus receives epithelial cells from multiple crypts, and is, therefore, oligoclonal (Fuller et al. 1990; Hermiston et al. 1993; Novelli et al. 1996, 2003; Schmidt et al. 1988).

The balance of stem cell self-renewal and differentiation is likely maintained by a microenvironment that is in close contact with stem cells, called the stem cell niche. Intestinal stem cells have a free apical surface and a basolateral surface, allowing them to receive signals from adjacent epithelial cells, basement

membrane, and underlying mesenchymal cells, including subepithelial myofibroblasts (Mills and Gordon 2001). Within the stem cell niche, the Wnt signaling pathway is one of the major driving forces for stem cell proliferation (Pinto et al. 2003; van de Wetering et al. 2002). Upon Wnt dependent activation of the β-catenin/Tcf transcription pathway (Bienz and Clevers 2000; Korinek et al. 1998), multiple genes, including c-myc, are activated to promote cell cycle progression (He et al. 1998; Kosinski et al. 2007; Muncan et al. 2006; Stappenbeck et al. 2003). In addition to proliferation signals, various mesenchyme-derived factors also limit crypt proliferation, as genetic deletion of forkhead homolog 6, homeodomain transcription factor Nkx2-3, and bone morphogenetic factor all lead to increased crypt cell proliferation (Kaestner et al. 1997; Pabst et al. 1999; Haramis et al. 2004; He et al. 2004). The niche function can be regulated, as a recent report has shown that lack of MyD88, a gene critical to the innate immune response to bacterial products, leads to abnormal positioning of stromal cells in the colon and decreased crypt proliferation during epithelial injury (Brown et al. 2007).

Once stem cells divide, they give rise to a much larger number of transit-amplifying cells. Morphologically, the transit-amplifying cells present as undifferentiated crypt enterocytes and differentiation of these cells gives rise to all the mature epithelial cell types discussed above. The Notch pathway plays a central role in cell fate determination in the intestine. Mouse studies show inhibition of Notch signaling lead to loss of all absorptive cells and conversion of all epithelial cells to goblet cells (van Es et al. 2005b; Wong et al. 2004). Conversely, activation of Notch signaling results in loss of all secretory cell types (Fre et al. 2005; Stanger et al. 2005). Once Notch signaling is activated, it induces the expression of a transcription repressor, Hes1. Lack of Hes1 results in increased goblet, Paneth, and enteroendocrine cells and decreased absorptive enterocytes, suggesting that Hes1 prevents development of secretory lineages (Jensen et al. 2000; Suzuki et al. 2005). As Hes1 can directly inhibit the transcription factor Math1, which is critical in differentiation of secretory cells (Jensen et al. 2000; Yang et al. 2001), the development of absorptive versus secretory lineages of intestinal epithelial cells is mutually exclusive. Within the secretory lineage, a transcription activator–repressor network further ensures the separation of goblet, Paneth, and enteroendocrine cells (Jenny et al. 2002; Shroyer et al. 2005). This tightly-controlled transcriptional network ensures normal epithelial cell differentiation and maintenance of intestinal functions.

6 Specialized Epithelial Cells of the Gastrointestinal Tract

6.1 Absorptive Enterocytes

Villus enterocytes, the major cell type in the villus epithelium, are tall columnar cells whose primary function is nutrient absorption. The importance of this cell type is demonstrated by diseases that result in villus atrophy or blunting, such as celiac disease. In these diseases, intestinal epithelial cells do not differentiate fully

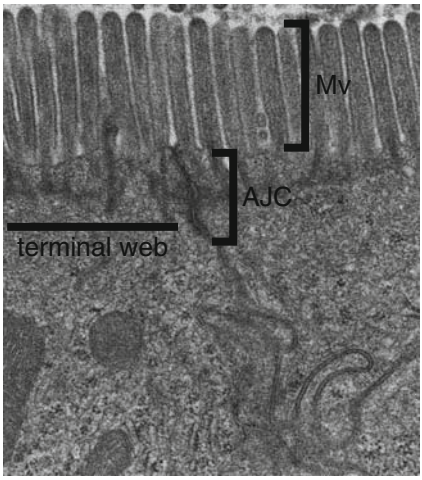

Fig. 5 Enterocyte brush border. This intermediate-magnification electron micrograph shows the apical portion of absorptive enterocytes. Well-developed microvilli (*Mv*) exist on the apical surface. The microvillus actin bundles extend downward to intersect with electron-dense terminal web. The apical junctional complex (*AJC*) is specialized to mediate adhesion between adjacent enterocytes

into absorptive enterocytes, resulting in decreased microvillus membrane enzyme activity, malabsorption, and diarrhea.

To accomplish their functions, absorptive enterocytes are structurally specialized, with their plasma membrane divided into distinct apical and basolateral domains. At the apical surface, villus enterocytes are covered by a dense brush border composed of microvilli, microscopic extrusions with a length of ~1 μm (Fig. 5). The structural integrity of microvilli is maintained by a cytoskeletal core composed of bundled actin filaments and associated proteins such as myosin I, villin, and fimbrin. As the cytoskeletal cores enter the cell body, they intersect with a dense network of microfilaments, termed the terminal web, that anchors the microvilli. The microvilli increase the membrane surface area as much as 20-fold, thereby greatly increasing the amount of brush border digestive enzymes, such as disaccharidases and peptidases, and transmembrane transporter proteins present on the apical membrane. Studies have shown that microvillus membranes are enriched in intramembranous protein particles and have a very high protein to lipid ratio, suggesting high levels of digestive enzyme and transporter expression. Congenital defects in microvillus membrane trafficking caused by mutations in myosin 5B or Rab8 GTPase result in accumulation of microvillus membrane inside the cell, and those affected suffer from chronic malabsorption (Muller et al. 2008; Sato et al. 2007), underscoring the functional significance of microvilli in nutrient absorption.

In contrast to the microvillus membrane, the basolateral plasma membrane of absorptive enterocytes is contoured and shaped by its close contact with the basolateral membranes of adjacent enterocytes. As a result, lateral intercellular space dimensions are plastic and can increase markedly during active nutrient absorption. The lipid and protein composition of the basolateral membrane domain also differs significantly from that of the apical membrane. Digestive enzymes and Na^+-coupled nutrient transporters that are highly expressed in the microvillus

membrane are absent from the basolateral membrane. However, the Na^+-K^+ ATPase, the facilitated glucose transporter GLUT2, and Na^+-independent amino acid transporters are highly enriched in the basolateral membrane.

The structurally and functionally distinct apical and basolateral membranes set up an efficient system for nutrient transport through the transcellular pathway. For example, using the extracellular to intracellular Na^+ gradient as energy source, brush border dissacharidase-released free glucose and galactose are transported by the apically located intestinal Na^+-glucose transporter, SGLT-1 (Ikeda et al. 1989). Intracellular Na^+ is pumped out at the basolateral membrane by the Na^+-K^+ ATPase, and glucose exits the cell through the glucose transporter GLUT2 on the basolateral surface of absorptive enterocytes along its concentration gradient. This process moves glucose from the intestinal lumen to the lamina propria. Analogous transport systems also exist for various classes of amino acids.

Such transcellular transport systems require polarized distribution of apical and basolateral membrane proteins. Without proper membrane partitioning, vectorial transport, both absorptive and secretory, would be impossible. A complex vesicular trafficking system exists to ensure correct post-synthetic delivery of transport proteins to their appropriate membrane domains, and additional mechanisms exist to remove mistargeted proteins selectively from the plasma membrane and redeliver these proteins to their correct membrane domains. Once delivered to the plasma membrane, mixing of apical and basolateral proteins is prevented by the tight junction at the interface between apical and basolateral membrane domains. As the tight junction forms an intramembranous fence, it is critical in maintaining proper epithelial polarization. In addition, the tight junction obstructs the space between adjacent cells, limiting the free passage of water, ions, and uncharged molecules across the epithelium through the paracellular pathway.

6.2 Undifferentiated Crypt Enterocytes

Undifferentiated crypt epithelial cells derived from the epithelial stem cells continue to divide within the crypt region. In contrast to villus absorptive enterocytes, undifferentiated crypt cells have shorter and sparser microvilli on their apical membranes with very low expression of digestive enzymes and nutrient transporters, and thus are inefficient at nutrient absorption. However, these cells have well-developed mechanisms for Cl^- secretion. The polarized distribution of different transporters and channels on apical and basolateral membranes is required for this to occur. Cl^- from the lamina propria enters the epithelial cell across the basolateral membrane via NKCC1, the Na^+-K^+-$2Cl^-$ transporter, by using the transmembrane Na^+ gradient generated by the basolateral Na^+-K^+ ATPase. Cl^- exits the cell apically, predominantly via the apical CFTR Cl^- channel. Excess K^+ leaves the cell through basolateral K^+ channels. Water and Na^+ follow secreted Cl^- passively through the paracellular route that exists between adjacent epithelial cells, resulting in secretory diarrhea.

The paracellular pathway is very different in villus and crypt enterocytes. Because undifferentiated crypt enterocytes have a smaller apical membrane area than absorptive enterocytes, the tight junction density is greater and the surface area for paracellular transport is increased in the crypt. Proteins that control paracellular permeability are also differentially expressed in villus absorptive and undifferentiated crypt enterocytes (Holmes et al. 2006). These have significant functional effects, as mathematic modeling suggests that the majority of epithelial conductance is determined by intestinal crypts, and studies using size-selective probes show the absorptive portion of the villus contains small pores with radii of less than 6 Å, while pores in the crypts have radii of 50–60 Å (Fihn et al. 2000; Marcial et al. 1984). The more porous paracellular pathway within the crypt epithelium may facilitate paracellular movement of Na^+ and water in response to transcellular Cl^- secretion, thus augmenting secretory responses.

6.3 Goblet Cells

Goblet cells are mucin-producing cells that present throughout the small intestine and colon; their numbers increase along the proximal–distal axis (Karam 1999). In contrast, although foveolar mucus cells are present in the gastric epithelium, goblet cells are normally absent in the stomach. In fact, the presence of goblet cells is a risk factor for gastric adenocarcinoma development (Morson et al. 1980).

The apical portion of the goblet cell cytoplasm is rounded and filled with secretory granules densely packed with dehydrated mucins. The cytoplasmic organelles are located beneath the mucous granules and the cell appears to narrow in this area. The nucleus is located below the Golgi complex, adjacent to the basal membrane, where the cell body is narrower. Under light microscopy, the wide apical portion containing secretory granules with a progressively constricted basal portion of the cell containing heavily stained nucleus enhance the wine goblet-like appearance of these cells. Unlike absorptive and undifferentiated enterocytes, goblet cells possess irregular microvilli that preferentially localize to the periphery of the apical membrane which express only small amounts of digestive enzymes and transporters.

The major function of goblet cells is mucin production. Multiple mucin genes exist in the human genome; some are secreted while others are membrane bound. Once synthesized, mucin proteins are heavily glycosylated in the endoplasmic reticulum and the Golgi then secreted to form a mucous layer on the apical surface of the epithelium. Constitutive mucin secretion occurs at a low rate through exocytosis of small vesicles, while stimulated secretion can occur through exocytosis of large vesicles containing densely packed mucin. Several secretagogous, such as acetylcholine, vasoactive intestinal pepetide, interleukin-1, and extracellular ATP are capable of inducing massive mucin release (Specian and Neutra 1980). Bacterial products, such as toxins secreted by *Escherichia coli* and *Vibrio cholerae,* can also induce mucin secretion (Leitch 1988; Moon et al. 1971). In fact,

mucin depletion in goblet cells is considered a histological feature of both infectious and idiopathic inflammatory diseases of the intestine. Based on these findings, it has been suggested that one of the major functions of mucin is to form a semipermeable gel-like layer on the apical surface of epithelial cells to prevent damage by abrasion and toxic agents. This idea is supported by studies showing that ablation of the Muc2 gene in mice causes spontaneous development of intestinal inflammation and gastrointestinal tumors (Heazlewood et al. 2008; Van der Sluis et al. 2006; Velcich et al. 2002), indicating protective role for mucins.

Mucins, which are rich in carbohydrates, can serve as binding sites for commensal bacteria and can be used by resident bacteria as energy sources. The normal microbiota compete with pathogenic bacteria for binding sites and promote mucin secretion, which may be one possible mechanism for beneficial effects of probiotics. The tight binding of microorganisms to mucin may lead to their eventual removal through mucous shedding, but pathogenic bacteria may overcome the presence of mucous layer by mechanisms that degrade mucus or decrease mucin expression. Alternatively, pathogenic bacteria can use mucin molecules as receptors to facilitate adhesion and promote pathogenesis.

6.4 Enteroendocrine Cells

Enteroendocrine, or neuroendocrine, cells secrete peptide hormones to coordinate gastrointestinal functions. Enteroendocrine cells are typically narrow at the apical side, and wide at the base, with relatively few microvilli at the apical surface. One of the prominent features of this cell type is the concentration of secretory granules in the basal end of the cytoplasm, below the nucleus. Based on the ultrastructure of these vesicles, their specific intestinal hormone, and unique marker gene expression, more than 10 subtypes of enteroendocrine cells have been defined (Schonhoff et al. 2004). The number and subtypes of enterendocrine cells vary along the proximal–distal axis, with gastrin-, secretin-, and cholecystokinin-expressing cells enriched in the gastric and duodenal regions, while peptide YY-, GLP-1-, and neurotensin-expressing cells are preferentially present in the ileum and colon. In contrast, somatostatin-, serotonin-, and substance P-producing cells are distributed throughout the gastrointestinal tract. A complex transcriptional network directs enteroendocrine cell differentiation, which controls development of enteroendocrine cell subtypes to specifically produce one or more peptide hormones. As the localization of secretory vesicles suggests, the contents of granules are secreted from the basolateral surface of enteroendocrine cells. The released hormones may function locally as paracrine signals or enter the blood stream to exert systematic effects. Although specific functions for each enteroendocrine cell type are not clear, they seem to be critical in intestinal function, as genetic mutation of neurogenin-3, a critical transcriptional factor for all subtypes of enteroendocrine cell development, leads to complete loss of enteroendocrine cells in human causing congenital malabsorptive diarrhea (Wang et al. 2006).

6.5 Paneth Cells

Paneth cells are long-lived secretory cells that normally reside at the crypt base of the small intestine, cecum, and ascending colon. Their normal distribution requires surface expression of EphB receptor tyrosine kinases and their ephrin B ligands, as genetic deletion of these genes in mice causes Paneth cells to lose their normal localization in the crypt base (Batlle et al. 2002; Cortina et al. 2007; van Es et al. 2005a). Paneth cells are pyramid-shaped columnar cells with basally localized nuclei; the apical cytoplasm is filled with eosinophilic and electron dense secretory granules. The exocytic granules contain a number of antimicrobial molecules including lysozyme, phospholipase A, α1-antitrypsin, and antimicrobial peptides such as defensins. With the ability to secrete such a wide array of antibacterial proteins, Paneth cells are thought to be important in innate immunity. When Paneth cells are depleted by specific toxins or genetic manipulation, animals are more susceptible to bacterial infection (Sherman et al. 2005; Wilson et al. 1999), while overexpression of antimicrobial peptides can help to limit intestinal infection (Salzman et al. 2003), underscoring the significance of this cell type. Although the molecular mechanisms for antimicrobial protein secretion are not well described, bacterial components can promote antimicrobial peptide secretion (Ayabe et al. 2000; Tanabe et al. 2005), at least partially though activation of membrane bound and intracellular pattern recognition receptors such as TLR9 and NOD2 (Kobayashi et al. 2005; Rumio et al. 2004; Voss et al. 2006; Wehkamp et al. 2004).

7 Basement Membrane and the Intestinal Epithelial Cells

The intestinal epithelial cells are anchored on the basement membrane, a thin layer of specialized extracellular matrix, which separates the epithelium from the lamina propria. The basement membrane is barely visible in H&E stained tissues and is more readily visualized by Periodic acid-Schiff and silver stains. The basement membrane is composed of a distinct array of extracellular matrix proteins, including the type IV collagen, laminins, heparan sulfate proteoglycans, nidogen/entactin, and fibronectin. The basement membrane components are secreted by both epithelial and mesenchymal cells: the type IV collagen is produced by mesenchymal cells, heparan sulfate proteoglycan is exclusively secreted by epithelial cells, while laminin is synthesized by both epithelial and mesenchymal cells. Epithelial cells are anchored on the basement membrane by β1 integrin-containing focal adhesions and β4 integrin-containing hemidesmosomes. In both focal adhesions and hemidesmosomes, the extracellular domains of transmembrane integrin molecules bind to basement membrane components including laminin, fibronectin, and collagen IV. At the cytoplasmic side, focal adhesions are stabilized by large protein complexes that are linked to the actin cytoskeleton, while hemidesmosomes are protein complexes anchored by intermediate

filaments. The focal adhesion contains multiple adaptor molecules and signaling molecules, which can transduce signals from the basement membrane to the inside of epithelial cells. In addition, intracellular signaling events can modulate integrin binding to the basement membrane components in an inside-out fashion, affecting the strength of epithelial–basement membrane interactions. It is well established that basement membrane components dictate epithelial proliferation, migration, and differentiation. Recent studies also show heparan sulfate and syndecan-1 knockout mice have increased protein leakage across the epithelium, suggesting critical roles of the basement membrane in maintaining the epithelial barrier (Bode et al. 2008). The basement membrane also functions as a physical barrier to prevent cell migration. However, this barrier can be breached under pathophysiological conditions by immune cell- and cancer cell-secreted proteases. In damaged epithelium, exposed basement membrane may also provide binding sites for bacterial attachment. Microbial pathogens may also secrete proteases to degrade basement components, facilitating their invasion and dissemination.

8 Maintenance and Regulation of the Epithelial Barrier

As discussed above, successful vectorial transcellular transport requires polarized distribution of transport proteins. Thus, it is important to separate apical and basolateral membranes. In addition, efficient transepithelial transport requires maintenance of ionic gradients across the epithelium. Free diffusion of ions between the cells would disrupt these gradients. Furthermore, the intercellular space between epithelial cells needs to be sealed to prevent luminal bacteria and antigens from accessing the lamina propria. All these requirements are met by a single structure, the tight junction.

The tight junction is located at the most apical site of epithelial cell contact and is a component of the apical junction complex (Fig. 6). It is a continuous structure that circumscribes the apical portion of the cell with a depth of 100–600 nm. The two major functions for this structure are to provide a gate between the cells to limit free passage of charged and uncharged molecules and to provide a fence to prevent diffusive mixing of apical and basolateral plasma membrane components. Transmission electron microscopy shows the tight junction to be a site where plasma membranes of adjacent epithelial cells are closely apposed. Freeze-fracture electron microscopy shows that the tight junction is composed of multiple continuous, anastomosing strands which consist of intramembranous particles. Such strands are thought to be responsible for limiting the free diffusion of molecules across the tight junction. Two models have been proposed to explain the molecular composition of the tight junction. The lipid model suggests that the outer leaflet of plasma membranes of adjacent cells fuse to form the strands observed by freeze-fracture microscopy. The protein model postulates that the tight junction strands are composed of protein particles which mediate adhesion between adjacent cells. With

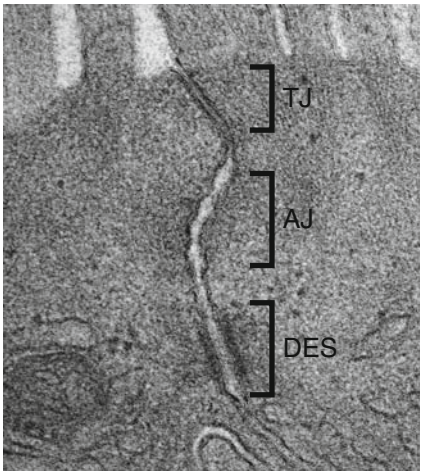

Fig. 6 The apical junctional complex. This high magnification electron micrograph shows the apical junctional complex between two adjacent enterocytes. The tight junction (*TJ*) is the most apical component within the complex, where plasma membranes of two cells are closely apposed. The adherens junction (*AJ*) and desmosome (*DES*) are located subapical to the tight junction

the identification of multiple tight junction proteins that can direct tight junction-like strand formation, the protein model has been widely accepted. However, specialized lipids such as cholesterol and sphingolipids are enriched within the tight junction (Francis et al. 1999; Nusrat et al. 2000b), suggesting that although they may not able to form tight junction strands by themselves, lipids also play a critical role in tight junction organization and function.

8.1 Molecular Organization of the Tight Junction

An expanding family of tight junction-associated proteins is being recognized. These proteins can be divided into integral membrane proteins and cytoplasmic plaque proteins. The integral membrane proteins may have one, three, or four transmembrane domains and are thought to define paracellular permeability, while tight junction plaque proteins localize at the cytoplasmic face of the tight junction, contain multiple domains for protein–protein interactions, and may cross-link and stabilize transmembrane proteins at the tight junction.

Occludin was the first transmembrane protein at the tight junction described (Furuse et al. 1993). It has four transmembrane domains, two extracellular loops, and N- and C- terminal cytoplasmic tails. It can interact with multiple proteins, including occludin itself, as well as plaque proteins ZO-1, -2, -3, and cingulin. It has also been shown to interact with a variety of signaling molecules such as PKCη, protein phosphotase 2A, and PI3 kinase (Cordenonsi et al. 1999; Furuse et al. 1994; Nusrat et al. 2000a; Seth et al. 2007; Suzuki et al. 2009), suggesting occludin plays a role in signal transduction. In addition, occludin can interact with the actin cytoskeleton both directly and indirectly (Fanning et al. 1998; Wittchen et al. 1999). In vitro studies have shown that when occludin is expressed in fibroblasts,

it can form tight junction-like strands (Furuse et al. 1996; Van Itallie and Anderson 1997). Furthermore, application of a synthetic peptide that corresponds to the sequence of second extracellular loop of occludin disrupts the tight junction, and siRNA knockdown of occludin leads to decreased epithelial barrier function, suggesting a role for occludin in maintaining the epithelial barrier (Furuse et al. 1996; Wong and Gumbiner 1997; Yu et al. 2005). However, occludin knockout mice are viable with normal tight junction organization and epithelial barrier function, demonstrating that occludin is not absolutely required for structural integrity of the tight junction (Saitou et al. 1998, 2000).

The lack of in vivo significance for occludin in tight junction formation prompted a search for additional transmembrane tight junction proteins. This search resulted in the identification of the claudin family of tight junction proteins (Furuse et al. 1998a). Over 20 claudin family members have been discovered in humans. Similar to occludin, they are tetraspanning transmembrane proteins with two extracellular loops and N- and C- terminal cytoplasmic tails; however, there is no sequence homology between occludin and the claudins. One common feature of the claudin proteins is that they have PDZ domain binding motifs at the very end of their C-termini, which can bind to the plaque proteins ZO-1, -2, and -3, and are important in targeting claudin to the tight junction (Itoh et al. 1999). When expressed in fibroblasts, claudins induce tight junction-like strand formation between adjacent cells (Furuse et al. 1998b). Claudins are the major determinants of tight junction charge selectivity. For example, claudin-16 forms pores that allow Mg^{2+} to cross the tight junction. Mutations in claudin-16 interrupt normal paracellular Mg^{2+} reabsorption in the renal tubule, resulting in a Mg^{2+}-wasting disease in humans (Simon et al. 1999). In vitro claudin-4 overexpression in renal epithelial cells causes decreased Na^{+} permeability without affecting Cl^{-} and uncharged molecule permeability across the tight junction (Van Itallie et al. 2001). When a negatively charged amino acid residue in claudin-4 is switched to a positively charged amino acid residue at position 65 within the first extracellular loop, Na^{+} permeability is increased. Similarly, when a claudin-2 mutant containing the first extracellular loop of claudin-4 is expressed in epithelial cells, charge selectivity is more similar to claudin-4 than native claudin-2 (Colegio et al. 2002, 2003). Therefore, the first extracellular loops of the claudins, which vary widely in number and position of charged amino acid residues, plays a critical role in determining tight junction charge selectivity. As multiple claudin proteins can be expressed by the same cell and they can interact heterogeneously, modulation of patterns of claudin protein expression can alter permeability properties to meet special functional requirements (Furuse et al. 1999; Holmes et al. 2006).

Zonula occludens (ZO)-1 is a representative plaque protein at the tight junction. Beginning at the N-terminus, it has three PDZ domains, an SH3 domain, a kinase-dead guanylate kinase domain, and an actin binding region. Additional domains in the C-terminal region of ZO-1 are poorly defined. The first PDZ domain for ZO-1 mediates its interaction with the C-terminal end of the claudins (Itoh et al. 1999), the second PDZ domain mediates its homotypic and heterotypic association with ZO-1 and its close relatives, ZO-2 and ZO-3 (Wittchen et al. 1999), the fragment

containing SH3 and guanylate kinase domains mediate ZO-1's association with occludin (Schmidt et al. 2004), and the actin binding region directly interacts with actin filaments (Fanning et al. 2002). Through these interactions, ZO-1 is thought to function as a cross-linker to stabilize trasmembrane proteins at the tight junction. Cell culture studies have shown that lack of ZO-1 expression, alone or in combination with loss of ZO-2, leads to defects in transmembrane protein targeting the tight junction (McNeil et al. 2006; Umeda et al. 2006), suggesting critical roles of ZO proteins in tight junction organization and function. In vivo genetic deletion studies have further shown that lack of ZO-1 or ZO-2 expression leads to embryonic lethality with aberrant tight junction organization, underscoring the functional importance of these proteins (Katsuno et al. 2008; Xu et al. 2008).

Based on the abundant interactions among tight junction proteins, it has been suggested that these proteins form a stable protein complex which maintains tight junction structure and function. However, a stable structure is incompatible with rapid and reversible regulation of the tight junction as discussed below. Indeed, a recent study demonstrated that the tight junction is a highly dynamic structure (Shen et al. 2008). In this study, the protein dynamics of occludin, claudin-1, and ZO-1 at the tight junction were determined by fluorescent recovery after photobleaching experiments (Fig. 7). These experiments showed that most of the occludin and ZO-1 molecules are highly dynamic at the tight junction. However, they do not exchange as a single complex, since occludin exchange is achieved through diffusion within the plasma membrane, while ZO-1 does not diffuse within the plasma membrane. Instead, tight junction-associated ZO-1 exchanges with a cytoplasmic pool. Unlike occludin and ZO-1, claudin-1 molecules are static and show only limited fluorescent recovery after photobleaching. This study demonstrates that each of the tight junction proteins studied has a unique

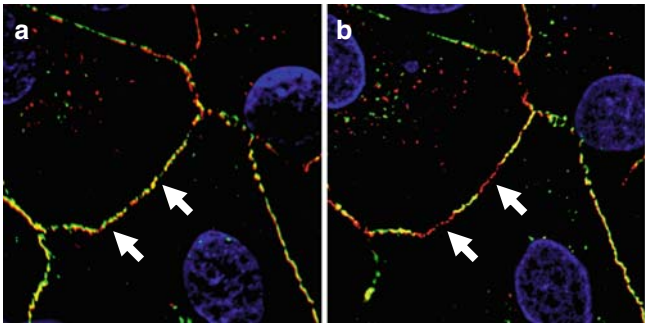

Fig. 7 Distinct tight junction proteins show unique dynamic behaviors. An epithelial monolayer expressing both green fluorescent protein-tagged claudin-1 and red fluorescent protein-tagged occludin was subjected to live-cell fluorescent recovery after photobleaching. Before photobleaching (**a**), the claudin-1 fluorescence (*green*) and occludin fluorescence (*red*) colocalize within the tight junction (shown in *yellow*). When indicated areas (*arrows*) were photobleached and allowed to fluorescent recover for 15 min (**b**), significant fluorescent recovery occurred for occludin, but not claudin-1, leading to red appearance of the bleached regions due to lack of claudin-1 fluorescence. Nuclei were stained with Hoescht 33342 (*blue*)

dynamic behavior, indicating that they do not reside in a single protein complex. These studies also show occludin and ZO-1 dynamics can be differentially regulated upon distinct stimuli that modify tight junction permeability, such as metabolic inhibition, cholesterol chelation, or reduced temperature, indicating that alteration of tight junction protein dynamics may provide a mechanism for rapid regulation of tight junction function.

8.2 *Physiological Regulation of the Tight Junction Through Cytoskeletal Contraction*

As mentioned above, the intestinal epithelial tight junction is functionally dynamic. One of the best examples is the increase in paracellular permeability that follows activation of Na^+-nutrient transporters, such as the Na^+-glucose co-transporter SGLT-1. The Na^+-glucose co-transporter induces a selective increase in the number of small pores in the villus, which results in increased permeability to ions and small non-charged molecules (Fihn et al. 2000; Madara and Pappenheimer 1987; Pappenheimer 1987). Such an increase in paracellular permeability has been suggested to complement transcellular glucose transport to maximize nutrient and water absorption (Meddings and Westergaard 1989; Pappenheimer 1993; Sadowski and Meddings 1993; Turner et al. 2000).

The pathways for tight junction regulation are under extensive investigation. One possible mechanism for such regulation is through transcriptional control of distinct claudin family members; however, such a mechanism cannot account for the rapid and reversible modulation of tight junction permeability by SGLT-1 activation. Another possibility is highlighted by electron microscopy studies showing that SGLT-1 activation induces perijunctional actomyosin ring condensation (Atisook et al. 1990; Madara and Pappenheimer 1987), suggesting actomyosin contraction maybe a mechanism for acute tight junction regulation under physiological conditions. Indeed, SGLT-1-dependent increases in tight junction permeability are characterized by local myosin regulatory light chain (MLC) phosphorylation (Berglund et al. 2001; Turner and Madara 1995; Turner et al. 1997), a marker for actomyosin contraction.

8.3 *Myosin Light Chain Kinase Regulates Actomyosin Contraction at the Tight Junction*

Although multiple signaling pathways such as activation of Rho kinase or inactivation of myosin light chain phosphatase MYPT can result in increased MLC phosphorylation, subsequent studies have shown that SGLT-1 activation-induced MLC phosphorylation is directly through the activity of MLC kinase (MLCK).

Pharmacological inhibition of MLCK blocks both SGLT-1-induced MLC phosphorylation and increased tight junction permeability, indicating that MLCK activity is necessary for acute tight junction regulation by SGLT-1 (Turner and Madara 1995; Turner et al. 1997). MLCK activity is also important in pathophysiological regulation of the tight junction. For example, proinflammatory cytokines such as TNFα and LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells) induce tight junction dysfunction in an MLCK-dependent manner in both cultured cells and mice (Clayburgh et al. 2005, 2006; Schwarz et al. 2007; Wang et al. 2005). Studies further show MLCK activity alone is sufficient to regulate the tight junction (Shen et al. 2006). When a constitutively-active form of MLCK is expressed in Caco-2 intestinal epithelial cell monolayers, it causes decreased epithelial barrier function and altered tight junction organization (Shen et al. 2006). Pathophysiological activation of MLCK activation also participates in human disease, as studies using patient samples show MLC phosphorylation and MLCK expression are highly upregulated during active inflammatory bowel diseases (Blair et al. 2006), suggesting MLCK-mediated epithelial barrier loss may participate in inflammatory diseases of the intestine (Clayburgh et al. 2004). This notion is supported by a mouse model of epithelial barrier dysfunction by tissue-specific expression of the constitutively-active MLCK in small intestinal and colonic epithelial cells. Similar to the tissue culture model, the constitutively-active MLCK increased small intestinal and colonic permeability (Su et al. 2009). Although these mice do not have histologically apparent disease, they show increased intestinal cytokine expression and increased susceptibility to immune cell-mediated experimental inflammatory bowel disease, suggesting a role for MLCK and tight junction dysfunction in contributing to intestinal inflammation (Su et al. 2009).

8.4 Endocytic Pathways and Tight Junction Regulation

It is clear that MLCK-mediated MLC phosphorylation is a major pathway for tight junction regulation; however, the mechanism through which perijunctional actomyosin ring contraction regulates tight junction structure and function is poorly defined. Because an intact actin cytoskeleton is critical in maintaining tight junction organization and function (Bentzel et al. 1976), it is conceivable that actomyosin contraction leads to tight junction reorganization.

A prominent feature of cytoskeletal-dependent tight junction disruption is tight junction protein internalization. Live cell microscopy coupled with simultaneous electrophysiological analysis of tight junction barrier function showed that the first morphological change induced by actin depolymerization is internalization of occludin. No significant change in ZO-1 and claudin-1 distribution occurs until well after barrier function is lost (Shen and Turner 2005). Morphological and functional studies show such internalization is through a caveolin-mediated pathway, and inhibition of caveolae-mediated endocytosis blocks actin depolymerization-

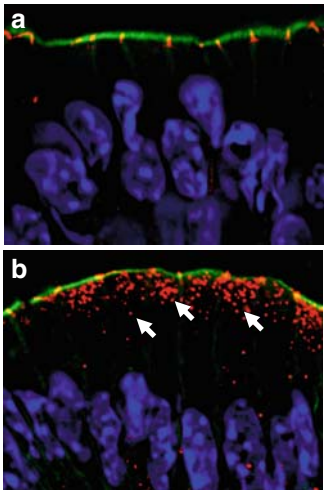

Fig. 8 TNFα induces occludin internalization. Frozen sections of jejunum taken from control (**a**) and TNFα-injected (**b**) mice were stained for occludin (*red*) and F-actin (*green*). Under control condition, occludin concentrates at the tight junction and colocalizes with the perijunctional actomyosin ring. In TNFα-treated mice, occludin is removed from the tight junction and exists in intracellular vesicles (*arrows*). Nuclei were stained with Hoescht 33342 (*blue*)

induced tight junction dysfunction, suggesting caveolae-mediated endocytosis is a critical pathway for tight junction regulation. Such occludin internalization also takes place during cytokine-induced barrier dysfunction (Fig. 8), as both cell culture and whole animal studies have shown that upon TNFα and LIGHT treatment epithelial occludin staining decreases at the tight junction and appears in intracellular vesicles, with limited changes of other tight junction proteins (Schwarz et al. 2007; Wang et al. 2005). Both pharmacological and genetic inhibition of MLCK activity results in decreased occludin internalization, suggesting that disruption of the tight junction, as represented by occludin internalization, is a downstream event following actomyosin contraction (Clayburgh et al. 2005, 2006; Schwarz et al. 2007; Wang et al. 2005). Although occludin internalization correlates with tight junction dysfunction, the role of occludin itself in tight junction regulation is not clear and is complicated by the absence of an obvious tight junction phenotype in occludin knockout mice (Saitou et al. 1998,, 2000). Nevertheless, occludin endocytosis is an excellent morphological marker for tight junction dysfunction (Fig. 9).

8.5 Infectious Agents and Tight Junction Dysfunction

Because the tight junction is critical in maintaining the epithelial barrier, it is targeted by multiple infectious agents. Some viral pathogens, such as reovirus, Coxsackie virus B (CVB), and hepatitis virus C, use tight junction proteins as receptors to enter eukaryotic cells (Barton et al. 2001; Coyne and Bergelson 2006; Evans et al. 2007). Of these, the mechanism for CVB entry through the tight junction is the best characterized. CVB first binds to an apical surface receptor,

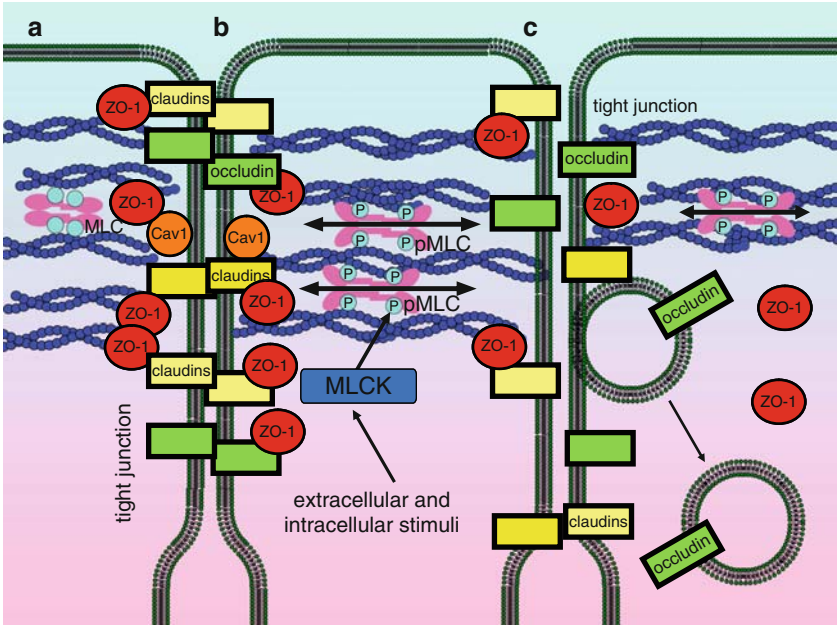

Fig. 9 Schematic presentation of MLCK-mediated tight junction disruption. Under resting conditions (**a**), only a small fraction of cellular MLC is phosphorylated causing low levels of perijunctional actomyosin contraction. When MLCK is activated through extracellular and intracellular stimuli (**b**), a large fraction of MLC is phosphorylated, leading to perijunctional actomyosin contraction. Such contraction leads to endocytic removal of occludin from the tight junction (**c**) and disruption of the epithelial barrier

the decay accelerating factor, which direct CVB to the tight junction and binding to the second receptor, the coxsackie and adenovirus receptor (CAR), within the tight junction. Once at the tight junction, CVB disrupts the tight junction and enters the epithelial cells through a macropinocytosis-like pathway which depends on occludin expression (Coyne and Bergelson 2006; Coyne et al. 2007). In addition to viral pathogens, the *Clostridium perfringens* enterotoxin exert its cytotoxic effect through binding to a selective number of claudins, including claudin-3 and -4, and formation of large protein complexes which contains occludin, thereby causing tight junction disruption (Katahira et al. 1997; Singh et al. 2000; Sonoda et al. 1999).

Intestinal pathogens also disrupt tight junctions through regulating the actin cytoskeleton. The *Clostridium difficile* toxins inactivate Rho, a small GTPase that regulates the actin cytoskeleton, by glucosylation, ultimately causing gross disruption of the tight junction and inducing occludin internalization through a caveolae-mediated pathway (Hecht et al. 1988; Nusrat et al. 2001). In contrast, the *E. coli* toxin cytotoxic necrotizing factor-1 activates Rho via deamidation, leading to tight junction disruption. Instead of regulating small GTPase activities, both enteropathogenic *E. coli* and *Helicobacter pylori* increase MLC phosphorylation

through an MLCK-mediated pathway to induce occludin intenalization (Fedwick et al. 2005; Shifflett et al. 2005; Simonovic et al. 2000; Wroblewski et al. 2009; Yuhan et al. 1997; Zolotarevsky et al. 2002), suggesting that the pathway for physiologically relevant MLC phosphorylation and occludin internalization can be hijacked by bacterial pathogens to induce barrier dysfunction. Such tight junction disruption and increased paracellular permeability contribute to bacteria-induced diarrhea and may facilitate bacterial invasion into deeper tissues.

9 Gastrointestinal Immune System

The interface between the intestinal mucosa and the lumen is a challenging environment. The inner surface of the intestine covers about 100 m^2. Along this surface, the intestinal epithelial cells are continuously exposed to commensal bacteria that reside in the intestinal lumen, as well as dietary and environmental antigens. Such interactions provide constant immunological stimulation. Indeed, a large number of immune cells, distribute throughout the gastrointestinal tract. Despite the harsh environment, under normal conditions the mucosal immune system in the gut is maintained to be hyporesponsive to environmental stimuli. However, the intestinal immune system is capable of rapidly mounting an immune response against pathogens. The balance between immune tolerance and immune activation at the mucosa is maintained by a complex network of immune cells and intestinal epithelial cells, and breakdown of this balance will lead to intestinal disease.

9.1 Intestinal Lymphoid Tissues

Lymphoid aggregates are frequently found in the intestines. They can be well differentiated and developmentally determined, or they can form following stimulation. The best recognized intestinal lymphoid tissue is the Peyer's patch, a secondary lymphoid tissue located in the ileum. The Peyer's patch has distinct T cell and B cell zones, with prominent B cell follicles containing germinal centers (Fig. 10). One unique feature of Peyer's patches is that they do not contain afferent lymphatics, instead they receive antigenic signals from follicle-associated epithelia, which contains antigen-sampling M (microfold) cells, and is a major route for bacterial invasion. Another type of intestinal lymphoid tissue is the isolated lymphoid follicles found in the small intestine (Hamada et al. 2002; Moghaddami et al. 1998). Similar to the Peyer's patches, fully developed isolated lymphoid follicles have B cell follicles with overlying follicle-associated epithelia and M cells. However, isolated lymphoid follicles do not have T cell zones, their formation is not developmentally controlled, and their number can be altered in response to changed intestinal microflora (Hamada et al. 2002; Lorenz et al. 2003; Pabst et al.

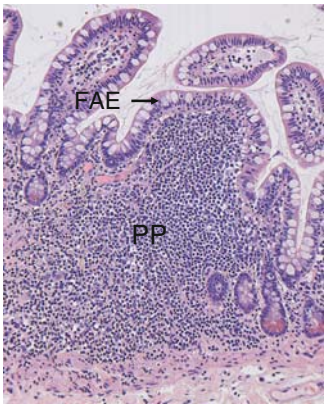

Fig. 10 Human Peyer's patch. Peyer's patches (*PP*) are located within ileal mucosa and are covered by follicle associated epithelium (*FAE*). M cells reside within the follicle-associated epithelium and are specialized for antigen transport

2006). Furthermore, upon stimulation, tertiary lymphoid tissues can form at random sites by lymphoid neogenesis (Kratz et al. 1996). These lymphoid tissues can be well organized, containing germinal centers, lymphatics, and high epithelial venules, or can be less organized with no apparent structure. Although the mechanisms for development of these lymphoid structures are distinct, lymphotoxin expressed by hematopoitic cells and lymphotoxin β receptors on stromal cells seems to play a critical role. Indeed, lack of lymphotoxin and lymphotoxin β receptor results in decreased intestinal lymphoid tissues (Honda et al. 2001; Lorenz et al. 2003; Rennert et al. 1996), while overexpression of lymphotoxin induces tertiary lymphoid tissue formation (Drayton et al. 2003; Kratz et al. 1996).

Like other lymphoid tissues, intestinal lymphoid tissue is important for mounting immune responses. Peyer's patches are major sites of antigen sampling through their interaction with M cells and unique antigen-presenting dendritic cells. Such interactions promote lymphocyte homing to the gut and mounting of immune responses (Lugering et al. 2005; Mora et al. 2003; Salazar-Gonzalez et al. 2006; Shikina et al. 2004; Yamamoto et al. 2004), indicating that intestinal lymphoid tissue plays unique and critical roles in intestinal immune responses.

9.2 Intraepithelial Lymphocytes and Lamina Propria Lymphocytes

Once lymphocytes receive antigen stimulation in lymphoid tissues, they can migrate to effector sites within the intestine. Based on their location, they can be divided into intraepithelial lymphocytes, which localize within the epithelium, and lamina propria lymphocytes, which are found below the basement membrane.

The frequencies for intraepithelial lymphocytes vary, ranging from one intraepithelial lymphocyte for every 4–10 epithelial cells in the small intestine, to one for

every 30–50 epithelial cells in the colon (Beagley et al. 1995). Almost all intraepithelial lymphocytes are T cells with memory characteristics, which may contain some unique surface makers, such as homodimeric form of CD8α and γδ form of T cell receptors. Based on their surface makers, intraepithelial lymphocytes can be divided to two different groups. Type a intraepithelial lymphocytes possess αβTCRs with CD4 or CD8αβ, which recognize non-self-antigens in classical MHC restricted manner, similar to conventional T cells. In contrast, type b intraepithelial cells are non-conventional, and are αβTCR CD8αα, γδTCR CD8αα, or double negative γδTCR lymphocytes. The activation of type b cells is less MHC dependent; rather, they may recognize nonpolymorphic surface molecules such as CD1 and MICA/B. The development of these cells is also different, as some type b cells still arise in athymic conditions, may recognize self-antigens, and are positively selected in the periphery. The number of type a and type b cells varies along the proximal–distal axis: type b cells make up ~50% of all intraepithelial lymphocytes in the human small intestine; however, this type is very rare in the colon. The specific function for intraepithelial lymphocytes is not clear, but it has been suggested they may play a variety of roles, such as immunoregulation and promoting epithelial damage repair (Boismenu and Havran 1994; Komano et al. 1995; Poussier et al. 2002).

Unlike intraepithelial lymphocytes, a large number of B cells reside in the lamina propria. These B cells are derived from naïve cells in the intestinal lymphoid tissue or the lamina propria, with most of them will differentiate into IgA-secreting plasma cells after activation. After secretion, IgA binds to the polymeric IgA receptor on the basolateral surface of the intestinal epithelial cells, translocates across epithelial cells, and forms secretory IgA complexes. The secreted IgA generates immune protection in a noninflammatory manner: it can promote immune exclusion by trapping bacteria and dietary antigens in the mucus layer, thereby downregulating proinflammatory epitopes on commensal bacteria, blocking bacterial binding to the epithelial surface, mediating intraepithelial neutralization of pathogens, and facilitating antigen sampling. Lamina propria T cells express αβTCR with CD4 to CD8 ratios similar to blood lymphocytes, and express memory markers. These cells may exert regulatory functions by producing a variety of cytokines, such as interferon-γ, IL-4, IL-10, and TGF-β, to maintain immune homeostasis in the lamina propria.

9.3 Antigen Sampling at the Intestinal Mucosa: M Cells and Dendritic Cells

To sample luminal antigens and to condition the immune system, specialized cells are present in the intestinal mucosa. These cells include M cells and mucosa-associated dendritic cells.

M (microfold) cells are specialized epithelial cells for endocytic sampling of luminal contents. As briefly mentioned above, M cells are located within the follicle-associated epithelium that lies immediately above Peyer's patches in the ileum and with isolated lymphoid follicles throughout the gastrointestinal tract. In addition, isolated M cells are also present in the intestinal villus (Jang et al. 2004). At the apical surface, M cells have short and irregular microvilli which are relatively sparse, with very low digestive and nutrient transport protein expression. The mucous layer is very thin on the M cell surface, facilitating endocytosis of luminal bacteria and antigens. At the basal surface, the plasma membrane of M cells detaches from the basement membrane to form intraepithelial invaginations, creating clefts between the M cell plasma membrane and the basement membrane. As their location and structure suggest, the major function of M cells is transepithelial antigen transport. Bacteria, bacterial products, and other antigens are endocytosed at the apical membrane, transported to the endosomal compartment, sorted, and finally exocytosed at the basal membrane. Within the space between the M cell basal plasma membrane and the basement membrane, dendritic cells and macrophages receive antigens released from M cell exocytic vesicles to activate downstream immune responses. Experiments have shown a complete endocytosis–exocytosis sequence can take only 10 min (Neutra and Kraehenbuhl 1992), suggesting that M cell-mediated transcytosis can be very rapid and efficient. During bacterial infection, such transcytosis pathways can be utilized by intestinal pathogens to cross the epithelium and promote bacterial infection.

Intestinal dendritic cells are critical within the intestinal mucosa to present antigens to lymphocytes and secret various cytokines to direct lymphocyte development. Under normal conditions, these cells generate regulatory signals for lymphocytes to maintain a low level of immune response to normal microbiota. In the presence of intestinal pathogens, they can stimulate lymphocytes to mount immune responses. As dendritic cells distribute throughout the gut, they can bind to antigens transported across the epithelium through various routes. Within lymphoid tissue, dendritic cells can receive antigens transcytosed by M cells, and at the lamina propria, they can bind to antigens transported across epithelium through an Fcγ receptor dependent mechanism, or bind to antigens leaked into the lamina propria through the paracellular pathway (Jang et al. 2004; Yoshida et al. 2004). Lamina propria dendritic cells can also extend dendrites between intestinal epithelial cells and across tight junctions, thus directly sensing luminal bacteria. The dendritic extensions do not disrupt the epithelial barrier, as these dendritic cells express tight junction proteins, and form tight junction-like structures with adjacent epithelial cells. Formation of these dendrites depends on Toll-like receptor signaling and CX3CR1 expression, and the presence of invasive bacteria increases the number of such extensions (Chieppa et al. 2006; Niess et al. 2005; Rescigno et al. 2001).

The dendritic cells in the intestine are not a homogenous population: they can be located in both secondary lymphoid tissues and the lamina propria; and they can

be either migratory, moving from lamina propria to mesenteric lymph nodes to present antigens, or lymphoid resident, presenting antigens at the site of antigen binding within intestinal lymphoid tissues. Furthermore, these dendritic cells can be divided into multiple subsets based on their surface marker expression. The different subsets of dendritic cells at distinct locations have unique functions. For example, activated Peyer's patch dendritic cells produce higher levels of IL-10, which promotes naïve CD4 cells differentiation into IL-4 and IL-10 producers (Iwasaki and Kelsall 1999), while the CD11b+ dendritic cells within the Peyer's patch produce IL-10 and promote Th2 responses. CD8a+ and CD11b-CD8a- dendritic cells produce IL-12 and drive T cell production of interferon-γ (Iwasaki and Kelsall 2001).

9.4 Immune Functions of Intestinal Epithelial Cells

In addition to absorptive and secretory functions, intestinal epithelial cells are also critical in controlling immune responses in the gut. They form a physical barrier to prevent bacterial invasion of deep tissue, and, by interacting with luminal bacteria and antigens, they regulate immune cell function. Intestinal epithelial cells express a variety of pattern recognition receptors, such as plasma membrane-bound Toll-like receptors and cytoplasmic NOD family receptors. Upon binding to bacterial products, these receptors activate the NFκB signaling pathway, resulting in cytokine and chemokine production. Under nondiseased conditions, NFκB activation appears to play a key regulatory role, as intestinal epithelial specific deletion of upstream activators of the NFκB pathway leads to aberrant inflammation in the gut (Chen et al. 2003; Greten et al. 2004; Nenci et al. 2007; Zaph et al. 2007). Intestinal epithelial cells can secrete various factors, such as CXC chemokines, IP-10, thymic stromal lymphopoietin, and retinoic acid, to regulate dendritic cell and lymphocyte functions. In addition, intestinal epithelial cells express classical and nonclassical MHC molecules, such as MHC class I and II, CD1d, MICA/MICB, and machinery necessary for antigen processing; thus, they can directly present antigens to T cells. As intestinal epithelial cell normally do not express some of the co-stimulatory molecules, they are unable to activate naïve T cells, and most of them direct memory T cells to induce immunoregulatory responses. However, under inflamed conditions, intestinal epithelial cells can be induced to express co-stimulatory molecules, thus promoting T cell activation and tissue inflammation (Nakazawa et al. 1999, 2004).

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Vibrio cholerae Interactions with the Gastrointestinal Tract: Lessons from Animal Studies

Jennifer M. Ritchie and Matthew K. Waldor

Contents

Abstract *Vibrio cholerae* is a curved Gram-negative rod that causes the diarrheal disease cholera. One hundred and twenty five years of study of *V. cholerae* microbiology have made this lethal pathogen arguably the most well-understood non-invasive mucosal pathogen. Over the past 25 years, modern molecular techniques have permitted the identification of many genes and cellular processes that are critical for *V. cholerae* colonization of the gastrointestinal tract. Review of the literature reveals that there are two classes of genes that influence *V. cholerae* colonization of the suckling mouse intestine, the most commonly used animal model to study *V. cholerae* pathogenesis. Inactivation of one class of genes results

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in profound attenuation of *V. cholerae* intestinal colonization, whereas inactivation of the other class of genes results in only moderate colonization defects. The latter class of genes suggests that *V. cholerae* may colonize several intestinal niches that impose distinct requirements and biological challenges, thus raising the possibility that there is physiologic heterogeneity among the infecting population. Efficient *V. cholerae* intestinal colonization and subsequent dissemination to the environment appears to require temporally ordered expression of sets of genes during the course of infection. Key challenges for future investigations of *V. cholerae* pathogenicity will be to assess the degree of heterogeneity in the infecting population, whether such heterogeneity has functional significance, and if stochastic processes contribute to generation of heterogeneity in vivo.

1 Introduction

Cholera is a severe and sometimes lethal diarrheal disease that is caused by the curved Gram-negative rod *V. cholerae*. Cholera patients can become rapidly dehydrated from the severe watery diarrhea (known as 'rice-water stool') that is characteristic of this illness. Without adequate re-hydration, patients can die less than a day after infection. This disease has likely afflicted humans on the Indian subcontinent since ancient times (Pollitzer 1959). During the past two centuries, cholera has spread beyond the Indian subcontinent and given rise to seven pandemics. Cholera also sometimes appears in explosive epidemics and, over the years, millions have lost their lives to this disease (Pollitzer 1959). Studies of cholera have influenced many scientific fields (Greenough 2004; Kavic et al. 1999). Most prominently, John Snow's observations linking the spread of cholera in London in 1854 to the water supply are usually cited as the beginning of modern epidemiology. Robert Koch is generally credited with isolating *V. cholerae* ("comma bacilli") as the cause of cholera in 1883. However, scientists before Koch, including the Italian anatomist Filippo Pacini in 1854, appear to have detected the comma-shaped *V. cholerae* bacillus in autopsy specimens from the intestines of cholera victims (see Howard-Jones 1984).

2 *V. cholerae* Classification, Genomics and Evolution

V. cholerae can be classified into more than 200 different serogroups based on a scheme developed by Shimada and colleagues (Yamai et al. 1997). Variability in the composition of the O side chains of lipopolysaccharide (LPS) molecules accounts for the distinct antigenicity associated with each serogroup. Only *V. cholerae* O1 serogroup strains were associated with epidemic cholera up until 1992, when *V. cholerae* serogroup O139 emerged as a cause of epidemic disease in India and Bangladesh (Cholera working group 1993). *V. cholerae* O1 are divided

into two biotypes, classical and El Tor, based on several phenotypic assays (Kaper et al. 1995). The classical biotype of *V. cholerae* is thought to have caused the first six cholera pandemics, whereas the ongoing seventh pandemic, which began in 1961, is caused by the El Tor biotype of *V. cholerae* O1.

Phylogenetic analyses place *V. cholerae* within the γ-proteobacteria, and there is significant similarity in the gene content of *V. cholerae* and other γ-proteobacteria such as *Escherichia coli*. However, unlike most γ-proteobacteria, the *V. cholerae* genome does not consist of a single circular chromosome; instead, the *V. cholerae* genome is divided unequally between two circular chromosomes (Trucksis et al. 1998). In fact, the presence of two circular chromosomes is a defining feature of all *Vibrio* species (Okada et al. 2005; Tagomori et al. 2002; Yamaichi et al. 1999). The smaller second *V. cholerae* chromosome has many fewer essential genes than chromosome I (Heidelberg et al. 2000). It seems plausible that the second *V. cholerae* chromosome was originally acquired by an ancestral proto-*Vibrio* species as a megaplasmid; subsequently, the second chromosome must have acquired essential genes and become indispensable.

Horizontal gene transfer has been instrumental in the evolution of pathogenic *V. cholerae*. The two most critical *V. cholerae* virulence factors, TCP, a type IV pilus that is an essential *V. cholerae* intestinal colonization factor (Herrington et al. 1988; Taylor et al. 1987), and cholera toxin (CT), an A-B type exotoxin that accounts for much of the secretory diarrhea characteristic of cholera (see Sanchez and Holmgren 2008 for a recent review of CT action), were both likely acquired via lateral gene transfer. TCP is encoded by a pathogenicity island (Karaolis et al. 1999; Kovach et al. 1996) and the mechanism of its acquisition by *V. cholerae* has yet to be determined. CT is encoded within the CTX prophage (Waldor and Mekalanos 1996). CTXφ is a filamentous phage that utilizes TCP as a receptor to infect *V. cholerae*. Thus, it seems likely that there were two key sequential steps in the evolution of pathogenic *V. cholerae*. First, a precursor strain acquired the TCP pathogenicity island and thereby became capable of colonizing the human small bowel and being infected by CTXφ. Second, the TCP^+ CT^- *V. cholerae* strain was infected by CTXφ and thereby became able to produce CT and to cause prodigious diarrhea. Production of cholera stool, which is laden with up to 10^8 *V. cholerae* cells per ml, provides a potent means for the pathogen to disseminate into the environment and reach new hosts.

The primary aim of this chapter is to review the knowledge of *V. cholerae* pathogenicity that has been garnered from experimental animal models of *V. cholerae* infection. The reader is directed elsewhere for recent reviews of *V. cholerae* microbiology and evolution (Sawabe et al. 2007; Faruque and Mekalanos 2003; Kaper et al. 1995), virulence gene regulation (Matson et al. 2007; Butler and Camilli 2005; Peterson 2002), lifecycle (Pruzzo et al. 2008; Schild et al. 2008; Faruque et al. 2004), and vaccine development (Lopez et al. 2008).

3 Observations from Studies of Cholera Patients

Epidemiological studies suggest that disease caused by the *V. cholerae* El Tor biotype tends to be less severe than that caused by the classical biotype (e.g., see Woodward and Mosley 1972). However, the pathologic findings from patients with severe cholera (cholera gravis) caused by either biotype appear similar. These are described below to enable comparison of the attributes of infected humans and animals, which are crucial for assessing the validity of animal models of infection.

Biopsy studies from cholera patients have demonstrated that *V. cholerae* is a noninvasive pathogen and that disease pathology is largely limited to the small intestine (Fresh et al. 1964; Gangarosa et al. 1960); although bacteria pass through the remainder of the intestinal tract, they have a relatively limited effect upon it. Within the small bowel, there is marked congestion and dilation of blood vessels, degranulation of mucosal mast cells, and edema and accumulation of an amorphous proteinaceous precipitate in the lamina propria (Koshi et al. 2003; Chen et al. 1971; Gangarosa et al. 1960). Furthermore, goblet cells appeared to be either actively secreting mucus or empty, an observation that likely explains the abundant mucus particles (the 'rice' of rice-water stool) often present in cholera stool. Recently, detailed electron microscopy studies of the small intestine have found some ultrastructural abnormalities in the villi and in a subset of enterocytes. For example, a marked widening of the lateral intercellular space and distortion of apical junction complexes were observed in the upper portion of villi in the small intestine (Mathan et al. 1995). In addition, irregular blebbing of the microvillus border was observed in about 5–8% of enterocytes in the upper regions of the villi and in about 50% of enterocytes present in the crypts. The inflammatory response to *V. cholerae* is considered relatively mild and consists predominantly of mononuclear cells and lymphocytes (Sprinz et al. 1962; Gangarosa et al. 1960). However, infiltrations of neutrophils into the epithelium and lamina propria have been observed in some infected individuals (Qadri et al. 2004; Mathan et al. 1995). Consistent with the latter findings, neutrophils and lactoferrin have been detected in the stools of some cholera patients (Silva et al. 1996; Stoll et al. 1983).

Human volunteer studies have provided definitive proof that CT causes the secretory diarrhea that is characteristic of cholera. Levine and colleagues found that human volunteers administered as little as 5 ug of purified CT developed severe cholera-like diarrhea (Levine et al. 1983). Furthermore, ingestion by human volunteers of a *V. cholerae* mutant that lacks the toxin genes did not result in secretory diarrhea (Herrington et al. 1988). However, these strains still elicited mild diarrhea and abdominal cramps (referred to as 'reactogenicity'), suggesting that factors other than CT also contribute to pathogenicity. The factor(s) responsible for reactogenicity have not been identified.

4 Historical Perspective on Animal Models of *V. cholerae* Pathogenicity

Ultimately, studies of bacterial virulence in animal models should enable mechanistic explanations of the signs, symptoms and pathology of human infection. Investigations of cholera pathogenesis date back at least to Koch's studies in the late 1800s (e.g., see Howard-Jones 1984). Nicati and Rietsch were the first scientists to produce a cholera-like illness in experimental animals in 1884 (cited in Pollitzer 1959). They found that direct inoculation of *V. cholerae* into the duodenums of guinea pigs yielded a cholera-like illness, though these animals were relatively resistant to disease following oral inoculation of *V. cholerae*.

Ilya Metchnikoff postulated that the relative resistance of experimental animals to oral infection with *V. cholerae* was "in large part due to the influence of other microbes in the digestive tract" (Metchnikoff 1894, translation by L. Slamti). He proposed that the absence of significant intestinal flora in newborn animals, in particular in infant rabbits, would facilitate *V. cholerae* colonization. Indeed, he found that 1- to 4-day-old infant rabbits were susceptible to lethal diarrhea following oral infection of *V. cholerae,* though he observed significant variability in the disease course and mortality in these animals. Intriguingly, when he administered "3 favorizing microbes" (isolated from the human stomach) prior to *V. cholerae* inoculation, disease became more uniform. He found that the "ingested vibrios go through the stomach . . . and establish in the small intestine and cecum, where . . .they wait for a favorable condition to manifest their pathogenic action" (Metchnikoff 1894, translation by L. Slamti). He observed that the large quantities of serous mucus-laden liquid in the ceca of infected animals appeared identical to that of the 'rice-water' diarrheal fluid that is characteristic of severe cholera in humans, and found that the small intestines of the infected rabbits were filled with mucoid liquid that upon culture in a variety of media yielded only *V. cholerae*. Metchnikoff concluded from his studies that "we should consider the intestinal cholera process in young rabbits as intoxication by vibrios that grow in the intestinal content. . .[as] the vibrios do not generalize [beyond the intestine]. It is then a poisoning due to the infection of the digestive tract by the vibrio of Koch" (Metchnikoff 1894, translation by L. Slamti). Thus, Metchnikoff deduced the essence of our current thinking about the pathogenesis of cholera from his work with infant rabbits in 1894.

Both adult and infant rabbits have been used to elucidate key aspects of *V. cholerae* pathogenicity. In a classic study published in *Nature* in 1959, S.N. De demonstrated that an activity in a cell-free supernatant could account for *V. cholerae* enterotoxicity (De 1959). After inoculating cell-free supernatants into the lumen of ligated loops of the adult rabbit small intestine, he observed large accumulations of fluid that resembled rice-water stool in appearance and chemistry. Thus, De's work established that a factor (now known to be CT) released by *V. cholerae* in the intestine could cause a secretory response in the intestine. Subsequently, fluid accumulation in ligated ileal loops has been used as an indicator

of the enterotoxicity of several additional *V. cholerae*-derived factors (Trucksis et al. 1993; Ichinose et al. 1987).

Elegant scanning and transmission electron microscopy studies using ligated rabbit ileal loops or infant rabbits revealed that *V. cholerae* attached to a large fraction of the villous surface, where they were often observed piled several layers thick (Nelson et al. 1976). The distribution of organisms from the tops to the bases of the villi did not differ although there was a relative paucity of *V. cholerae* present at the villus tips. Nelson et al. also noted changes in the appearance of the microvilli during infection; the villi became elongated and gave off blebs, as has been observed in human biopsies, often with attached *V. cholerae*. To date, the activity of a specific *V. cholerae* factor(s) has not been linked to this phenotype. By 12 h after inoculation into ligated loops, Nelson and colleagues observed far fewer *V. cholerae* attached to the villi. They hypothesized that clearance of bacteria from the villous surface could be accounted for either by host mechanisms, such as mucus secretion, or by the actions of bacterial products (Nelson et al. 1976). It is not clear whether such detachment also occurs within infected humans.

During the past two decades, infant mice have become the predominant model host used to elucidate bacterial factors that enable *V. cholerae* to colonize and grow in the small intestine. Murine intestinal colonization has been shown to be dependent upon genes such as *tcpA*, which encodes the major subunit of TCP, and *toxR*, which encodes a key regulator of *tcpA* and other *V. cholerae* virulence genes (Taylor et al. 1987). These genes are critical for *V. cholerae* colonization of the human intestine as well (Herrington et al. 1988), suggesting that results from suckling mice are relevant for understanding human disease. Nonetheless, it should be noted that infant mice do not develop profuse watery diarrhea, and are of limited utility for understanding factors that promote this and other manifestations of disease induced by *V. cholerae*. Infant mice are typically 3–5 days old upon inoculation with *V. cholerae*; as mice age, they became resistant to oral infection (Ujiiye et al. 1968). Adult mice, like rabbits, are naturally resistant to gastrointestinal (GI) infection with *V. cholerae*. Adult mice raised in a germ-free environment or treated with streptomycin can be colonized with *V. cholerae* (Butterton et al. 1996); however, survival and growth of *V. cholerae* in their intestines does not require TCP (Olivier et al. 2007), and consequently the significance of observations garnered from such studies of colonization of adult mice is not clear.

5 *V. cholerae* Genes Important for Intestinal Colonization

Before the advent of modern genetic techniques, investigators (e.g., Freter and O'Brien 1981b; Baselski et al. 1979; Guentzel and Berry 1975) used undefined *V. cholerae* mutants to identify *V. cholerae* phenotypes that were associated with intestinal colonization. They found that strains that were either rough (defective LPS), purine auxotrophs, or deficient in toxin production or motility, had reductions in their recovery from intestinal homogenates. About 20 years ago, investigators

Table 1 Genes important for *Vibrio cholerae* colonization of the suckling mouse intestine

Gene (locus)[a]	Function	C.I.	Reference
I. Cell surface structures			
TCP biogenesis			
tcpA (VC0828)	Major pilin subunit	< 0.001	Taylor et al. (1987) and many others.
tcpB (VC0829)	Pilin subunit	< 0.02	Peterson and Mekalanos (1988)
tcpQ (VC0830)	Pilin biogenesis	<0.001	Kirn et al. (2003)
tcpC (VC0831)	Pilin biogenesis	<0.001	Kirn et al. (2003)
tcpR (VC0832)	Pilin biogenesis	<0.001	Kirn et al. (2003)
tcpD (VC0833)	Pilin biogenesis	<0.001	Kirn et al. (2003)
tcpS (VC0834)	Pilin biogenesis	<0.001	Kirn et al. (2003)
tcpT (VC0835)	Membrane associated ATPase	< 0.001	Chiang and Mekalanos (1998)
tcpE (VC0836)	Pilin biogenesis	< 0.11	Chiang and Mekalanos (1998)
tcpF (VC0837)	Secreted colonization factor	< 0.001	Kirn and Taylor (2005); Kirn et al. (2003); Chiang and Mekalanos (1998)
tcpH (VC0827)	Pilin biogenesis	0.05	Carroll et al. (1997)
LPS/capsule biogenesis			
rfbB (*wbfB*/*manB*) (VC0242)	O-antigen biosynthesis	< 0.001	Chiang and Mekalanos (1998, 1999); Iredell et al. (1998)
rfbA (VC0241)	O-antigen biosynthesis	0.003	Iredell et al. (1998)
rfbL (*wbeL*) (VC0249)	O-antigen biosynthesis	< 0.001[b]	Chiang and Mekalanos (1998)
waaL (VC0237)	O-antigen ligase	0.03	Nesper et al. (2002)
wbeW (VC0263)	Capsule biosynthesis	0.024	Nesper et al. (2001)
galEK (VCA0774/VC1595)	O-antigen and capsule biosynthesis	<0.001	Nesper et al. (2002)
galU (VC0395)	LPS core oligosaccharide synthesis	0.03	Nesper et al. (2001)
wavB (VC0224)	LPS core oligosaccharide synthesis	0.003	Nesper et al. (2002)
wbfF (*otnA*)[c]	O139 capsule biosynthesis	0.03	Nesper et al. (2002)
gmd (VC0243)	O139 capsule biosynthesis	<0.1	Waldor et al. (1994)
Other			
ompW (VCA0867)	Outer membrane protein	0.1	Nandi et al. (2005)
VCA1008	Outer membrane protein	0.025	Osorio et al. (2004)
yabN (VCA0578)	Putative transport protein	< 0.01	Chiang and Mekalanos (1998)
pilD (vcpD) (VC2426)	Type IV prepilin peptidase	~ 0.01	Fullner and Mekalanos (1999), Marsh and Taylor (1998)
Mfrha (VCA0447)	Mannose-fucose-resistant hemagglutinin	0.003	Franzon et al. (1993)

(*continued*)

Table 1 (continued)

Gene (locus)[a]	Function	C.I.	Reference
II. Transport			
tolC (VC2436)	Efflux	< 0.001	Bina and Mekalanos (2001)
vexBDK (VC0164, VC1757 and VC1673)	Efflux	0.02	Bina et al. (2008)
VC2705	Sodium/solute symporter	0.07	Osorio et al. (2005)
fhuC (VC0201)	Ferrichrome ABC transporter	0.05	Lombardo et al. (2007), Schild et al. (2007)
mgtE-1 (VC1655)	Magnesium transport	< 0.002[b]	Chiang and Mekalanos (1998)
III. Motility			
flaA (VC2188)	Flagellar subunit	0.07	Lauriano et al. (2004), Lee et al. (2001), Watnick et al. (2001)
motX (VC2601)	Flagellar motor	0.05	Lauriano et al. (2004)
motY (VC1008)	Flagella motor	0.09	Silva et al. (2006), Lee et al. (2001)
motAB (VC0892 / VC0893)	Flagellar motor	0.03	Lee et al. (2001)
flgP (VC2206)	OM lipoprotein	0.05	Morris et al. (2008)
IV. Metabolism			
purD (VC0275)	Phosphoribosylglycinamide synthetase	< 0.001	Chiang and Mekalanos (1998)
purH (VC0276)	Phosphoribosylaminoimidazole-carboxamide formyltransferase, IMP cyclohydralase	< 0.006	Chiang and Mekalanos (1998)
purK (VC0051)	Phosphoribosylaminoimidazole carboxylase	0.08	Chiang and Mekalanos (1998)
bioB (VC1112)	Biotin synthetase	0.06	Chiang and Mekalanos (1998)
ptfA (VCA0518)	Phosphotransferase	< 0.001	Chiang and Mekalanos (1998)
pta (VC1097)	Phosphotransacetylase	0.01[b]	Chiang and Mekalanos (1998)
als (VC1590)	Acetolactate synthase	0.09	Yoon and Mekalanos (2006)
glnA (VC2746)	Glutamine synthetase	0.02	Klose and Mekalanos (1998)
mrsA (VC0639)	Phosphoglucomutase	<0.01	Merrell et al. (2002a)
nqrA (VC2295)	NADH:ubiquinone subunit	0.01	Merrell et al. (2002a)
pnp (VC0647)	Polyribonucleotide nucleotidyltransferase	< 0.01	Merrell et al. (2002a)
cpdA (VC2433)	cAMP phosphodiesterase	~ 0.1	Merrell et al. (2002a)
V. Gene regulation			
toxR (VC0984)	Virulence gene activator	< 0.002	Waldor and Mekalanos (1994b), Taylor et al. (1987)

(*continued*)

Table 1 (continued)

Gene (locus)[a]	Function	C.I.	Reference
toxT/tcpN (VC0838)	Virulence gene activator	< 0.003	Chiang and Mekalanos (1998)
luxO (VC1201)	Quorum sensing regulator	< 0.001	Zhu et al. (2002)
rpoN (VC2529)	Alternative sigma factor	0.03	Klose and Mekalanos (1998)
fliA (VC2066)	Sigma 28	0.04	Liu et al. (2008)
hfq (VC0347)	sRNA chaperone	< 0.001[b]	Ding et al. (2004)
hepA (VC2506)	Transcription regulation	< 0.001	Merrell et al. (2002a)
rpoE (VC2467)	Sigma E	0.03	Kovacikova and Skorupski (2002)
fur (VC2106)	Ferric uptake regulator protein	~ 0.1	Mey et al. (2005)
rpoH (VC0150)	Alternative sigma factor	~ 0.001[b]	Slamti et al. (2007)
VI. Miscellaneous/Hypothetical			
gshB (VC0468)	Glutathione synthetase	< 0.001	Merrell et al. (2002a)
recO (VC2459)	DNA repair	< 0.001	Merrell et al. (2002a)
recA (VC0543)	Homologous recombination	0.07	Kumar et al. (1994)
acfA (VC0844)		0.06	Peterson and Mekalanos (1988)
acfB (VC0840)	Putative methyl-accepting chemotaxis protein	0.04	Peterson and Mekalanos (1988)
*acf*C (VC0841)		0.07	Peterson and Mekalanos (1988)
acfD (VC0845)	Lipoprotein	0.02	Peterson and Mekalanos (1988)
*rtxL*1 (VC1619.1)	RTX family	0.001	Chatterjee et al. (2008)
*rtxL*2 (VC1619.1.1)	RTX family	< 0.001	Chatterjee et al. (2008)
VC2487	Hypothetical ORF	0.05	Osorio et al. (2005)
VC0874	Hypothetical ORF	0.04	Osorio et al. (2005)
mop (VC0823)		0.011	Zhang et al. (2003)

[a] TIGR designation from sequenced *V. cholerae* N16961
[b] Mutant has in vitro growth defect in a competition assay of <0.2
[c] Gene not found in sequenced *V. cholerae* N16961

started to engineer defined *V. cholerae* mutants to study the importance of particular gene products in *V. cholerae* colonization of the suckling mouse intestine.

A list of genes that have been shown to promote *V. cholerae* colonization of the suckling mouse intestine is shown in Table 1. To be included in this table, a mutant must have had at least a 10-fold defect in small bowel colonization in competition assays with the appropriate wild-type strain. In competition assays, equal numbers of wild-type and mutant cells are co-inoculated into the suckling mouse stomach. After ~24 h, the ratio of the two strains in intestinal homogenates is determined and the results are expressed as a competitive index (CI), the ratio of mutant/wild-type colony forming units (CFU) in intestinal homogenates divided by the ratio of mutant/wild-type CFU in the inoculum. One advantage of this assay format is that the wild-type strain serves as an internal control for each assay, thereby lowering the inter-assay variability. A potential caveat with competition assays is

the possibility of complementation in trans whereby the wild-type strain produces some factor within the intestine that masks a mutant's true attenuation. Setting the limit at a 10-fold reduction in CI for inclusion in this table is somewhat arbitrary; however, given the variability inherent in animal studies, setting the CI cut-off at this level provides confidence that the identified genes and pathways are truly important for intestinal colonization. In most cases, mutations in the genes listed in this table did not significantly compromise growth of the mutant strains in rich media, suggesting that the function of the gene in question is especially relevant for *V. cholerae* survival and growth in the intestine. Genes identified using signature-tagged mutagenesis (STM) screens were only included in this table if mutants identified in the original pools were subsequently confirmed using 1:1 competition assays with the wild-type strain. The genes listed in Table 1 were placed into six broadly defined functional categories, including one group of genes which includes hypothetical open reading frames of unclear function. The means by which some genes contribute to colonization are discussed briefly below.

5.1 Cell Surface Structures

The *V. cholerae* cell surface is the site where the organism directly confronts the host intestine, thus it is not surprising that surface exposed *V. cholerae* structures, including TCP, the LPS O-antigen, and an outer membrane protein, are important colonization factors. The mechanism(s) by which TCP promotes colonization are not clear. These pili likely promote the formation of *V. cholerae* microcolonies in vivo as they do in vitro (Kirn et al. 2000; Taylor et al. 1987), and they may also confer resistance to host bactericidal activities (Chiang et al. 1995; Parsot et al. 1991). It is also possible that TCP promotes *V. cholerae* adhesion to the intestinal epithelium. Besides encoding proteins required for TCP biogenesis, the TCP operon encodes TcpF, a soluble secreted protein that is essential for *V. cholerae* colonization of the mouse intestine (Kirn and Taylor 2005; Kirn et al. 2003). This discovery suggests that TCP acts as a secretion apparatus and uncovers yet another important role for TCP in *V. cholerae* pathogenesis.

The O-side chain and core oligosaccharide of the LPS of *V. cholerae* O1 is critical for this organism's growth in the intestine (Nesper et al. 2001; Angelichio et al. 1999; Chiang and Mekalanos 1998). Mutants lacking an O-antigen and/or a core oligosaccharide are sensitive to bactericidal activities present in the gut such as cationic antimicrobial peptides (Nesper et al. 2001). It would be interesting to engineer a *V. cholerae* O1 strain to express an O-antigen and/or core oligosaccharide derived from a serogroup not associated with cholera to explore whether there are specific properties of O1 LPS that promote colonization or whether any O-antigen could promote colonization. In part, this experiment has been carried out in nature as *V. cholerae* O139 arose via exchange of the genes coding for O1 O-antigen biosynthesis for the genes coding for O139 O-antigen biosynthesis (Stroeher et al. 1998; Bik et al. 1995; Waldor and Mekalanos 1994a). The *V. cholerae* O139 O-antigen forms a

capsular polysaccharide that is also important for the intestinal colonization of this epidemic causing serogroup (Nesper et al. 2002; Waldor et al. 1994).

5.2 Transport

Several types of membrane transport systems have been shown to promote *V. cholerae* intestinal colonization. Efflux systems can, like LPS, engender resistance to gut-derived antimicrobial agents such as bile and cationic peptides. *V. cholerae* encodes six RND family efflux pumps, for which TolC is thought to function as the outer member component. The *V. cholerae* RND efflux pumps appear to have some redundant functions, as single deletions of any of the six RND efflux pumps did not significantly attenuate *V. cholerae* intestinal colonization (Bina et al. 2008). However, a strain bearing deletions in three RND efflux systems had more than a 40-fold colonization defect while a strain deleted for all six RND efflux pumps was not recovered from the infant mouse intestine (Bina et al. 2008). Furthermore, a *tolC* mutant had more than a 1,000-fold colonization defect in the mouse intestine (Bina and Mekalanos 2001). At least part of the dramatic colonization defect of the RND-null strain can likely be attributed to its reduced production of TCP (Bina et al. 2008).

5.3 Motility

All *V. cholerae* are highly motile and bear a single sheathed polar flagellum, and motility was proposed to be important for *V. cholerae* intestinal colonization years before the generation of defined mutants (Richardson 1991; Freter and O'Brien 1981a; Freter et al. 1981; Baselski et al. 1979; Yancey et al. 1978; Guentzel and Berry 1975). Freter proposed that motility was important for *V. cholerae* to swim from the lumen of the intestine through the mucus gel overlying the intestinal epithelium, enabling the pathogen to contact and adhere to the epithelial surface (Freter and O'Brien 1981a). Since then, conflicting observations regarding the importance of motility in intestinal colonization have been reported (e.g., Lee et al. 2001; Klose and Mekalanos 1998; Gardel and Mekalanos 1996). Altered patterns of virulence gene expression in different types of motility mutants may help to explain these results. The inverse correlation between motility and virulence gene expression (Hase and Mekalanos 1999; Gardel and Mekalanos 1996) has led to the view that motility is required to localize *V. cholerae* to the crypts of the small intestine after which motility is reduced and virulence factor production increased. Deletions of genes important for the movement of the flagellum (such as *motY*) result in a similar reduction in intestinal colonization as deletion of *flaA*, the gene encoding the major subunit of the flagellum (Lee et al. 2001). These observations suggest that motility and not the flagellum per se promotes intestinal colonization.

5.4 Metabolism

Perhaps unexpectedly, relatively few metabolic pathways for the biosynthesis of cellular building blocks like amino acids, and for energy metabolism, have been confirmed to be critical for *V. cholerae* intestinal colonization. The STM screen carried out by Chiang and Mekalanos revealed that genes required for biotin and purine biosynthesis were important for intestinal colonization (Chiang and Mekalanos 1998). In this study, most of the auxotrophs identified in the mutant pools were not found to be attenuated for growth in vivo, suggesting that *V. cholerae* may be able to scavenge nutrients required for growth from the intestine, even though it is generally thought that the intestine is a nutrient-limited environment (Xu et al. 2003; Merrell et al. 2002a,b). The most comprehensive STM screen for genes important for intestinal colonization yielded many genes involved in the biosynthesis of amino acids, fatty acids, purines and co-factors as well as many genes involved in energy metabolism (Merrell et al. 2002a). However, these genes have not been included in Table 1 as these mutants were not re-tested in single strain competition assays versus the wild-type.

5.5 Regulation

Expression of TCP and other bonafide *V. cholerae* virulence factors is indirectly governed by several regulatory factors and by a variety of cellular processes, such as quorum sensing, that collectively determine the amount and activity of ToxT. This AraC family transcription factor controls expression of the genes required for TCP biogenesis (reviewed in Matson et al. 2007). Studies of strains bearing mutations in genes that result in decreased *toxT* expression, such as *toxR*, *toxT*, *luxO, tcpH, nqrA, aph*, *tcpP*, and *toxS,* lead to reductions in TCP expression and, where tested, dramatic reductions in intestinal colonization (see Table 1). Regulators that influence expression of genes that are not controlled by ToxT have also been implicated in *V. cholerae* intestinal colonization. For example, deletion of *rpoN*, which encodes an alternative sigma factor governing transcription of genes for *V. cholerae* flagellum and glutamine synthesis, also attenuates *V. cholerae* intestinal colonization (Klose and Mekalanos 1998).

Hfq is required for the activity of many small non-coding RNAs (sRNAs) (Brennan and Link 2007; Majdalani et al. 2005), and a *V. cholerae hfq* mutant is highly attenuated in intestinal colonization, suggesting that sRNAs govern expression of genes critical for growth and survival of *V. cholerae* in vivo (Ding et al. 2004). In part, Hfq is required for the activity of the *qrr* sRNAs, which indirectly block the repression of *toxT* expression that can occur as a consequence of quorum sensing (see Sect. 5.9) (Lenz et al. 2004). However, *hfq* mutants in which this repression does not occur (*hapR* mutants*)* still colonize poorly (Ding et al. 2004),

suggesting that sRNAs in addition to the *qrr* family are required for colonization by *V. cholerae*.

5.6 Miscellaneous/Hypothetical

A key challenge for future studies will be to decipher how genes of unknown function influence *V. cholerae* intestinal colonization. The two STM screens for *V. cholerae* genes involved in intestinal colonization yielded 17 hypothetical genes (Merrell et al. 2002a; Chiang and Mekalanos 1998), and other studies have implicated additional genes of unknown function in *V. cholerae* growth in vivo (Osorio et al. 2005). Furthermore, the mechanisms of many 'colonization' genes that have homologues in the database, for example *acfC*, remain obscure.

Without a doubt, the list of *V. cholerae* genes that promote intestinal colonization in Table 1 is incomplete. Future studies will show that many additional genes promote *V. cholerae* intestinal colonization. Also, it is important to keep in mind that Table 1 lists only those genes whose inactivation results in attenuated colonization. Efficient *V. cholerae* intestinal colonization not only requires the actions of many gene products but also the repression of the expression of certain genes. For example, Zhu and colleagues demonstrated that the *V. cholerae* mannose-sensitive hemagglutinin pilus is repressed during *V. cholerae* growth in the suckling mouse intestine; constitutive expression of this type IV pilus in the intestine resulted in a >10-fold colonization defect (Hsiao et al. 2006).

6 Classes of *V. cholerae* colonization mutants

It is interesting to note that there are two classes of colonization defective mutants listed in Table 1. One class of mutants has moderate reductions in intestinal colonization with CIs ranging from 0.1 to 0.01. The other class of mutants is severely attenuated in intestinal colonization with CIs <0.003. The latter set of 'severe' genes, such as the TCP biosynthesis cluster, encode functions that appear to be essential for colonization, whereas the former set of 'moderate' genes, such as *motX* or *glnA*, appear to promote high-level colonization but are clearly not essential for *V. cholerae* survival and growth in the intestine. Deletion of 'moderate' genes may simply result in uniformly slowed growth of the mutant strains in vivo; however, these results are also consistent with the possibility that there is more than one niche that *V. cholerae* can occupy in the intestine. For example, the observation that non-motile mutants have ~10- to 30-fold reductions in colonization may suggest that motility is important for *V. cholerae* to occupy a preferred intestinal niche but that the organism can survive and multiple

in alternative niches. Similarly, the finding that *glnA* mutants, which are glutamine auxotrophs, are ~50-fold reduced in colonization (Klose and Mekalanos 1998) may suggest that there are intestinal niches where some glutamine is available and others where it is not. The profound reduction in colonization observed in strains with mutations in severe genes may suggest that all intestinal niches require the functions of these genes; alternatively, the activities of the severe genes may be essential only in certain locations at specific times during *V. cholerae* survival and growth in the GI tract, such as during passage through high concentrations of bile in the duodenum.

7 Dynamics of *V. cholerae* Gene Expression within the Intestine

The competition assays described above are a useful tool for identification of genes that contribute to colonization; however, they provide little information concerning the role or expression of the required genes. To address temporal aspects of gene expression, Camilli and colleagues have used recombinase-based in vivo expression technology (RIVET), which uses recombinase expression as a reporter, to assess transcription activation in vivo (Lee et al. 1999). They found that expression of *ctxA* (which encodes the catalytic subunit of CT) occurred after and was dependent on *tcpA* expression in vivo, but not in vitro. Consequently, they proposed that *ctxA* expression is delayed during infection until a TCP-dependent signal is received, and that there is a requisite temporal order of *V. cholerae* virulence factor expression during infection. Presumably TCP production enables infecting *V. cholerae* to occupy a niche that provides the optimal inducing environment, potentially including host-derived signals, for induction of the *ctx* genes. However, the significance of the finding that *ctxA* expression is reduced in a *tcpA* mutant is difficult to assess since *tcpA* mutants are highly defect for colonization.

RIVET-based investigations of gene expression provide an elegant way to explore gene expression in vivo, but there are several technical limitations to the RIVET method that make interpreting these intriguing observations somewhat difficult. First, the RIVET reporter system does not have gradations; gene expression is scored as either on or off. Yet it is possible that there is significant cell-to-cell variation in the levels of gene expression during infection within the population. Second, the RIVET reporter system (described by Lee et al. 1999) was purposely designed to ignore the low-level expression of *ctxA* and *tcpA* that occur in vitro. It is possible that there is for example, low-level expression of *ctxA* in vivo which occurs with or even before expression of *tcpA*. Finally, RIVET cannot be used to monitor increased transcription of genes that show significant transcription in vitro.

Camilli and colleagues recently modified the RIVET protocol to enable detection of *V. cholerae* genes that are expressed relatively late after inoculation into suckling mice (Schild et al. 2007). Inactivation of many of the ‘late’ genes that were

identified in this study did not result in intestinal colonization defects (Schild et al. 2007). Instead, Schild et al. found that in vivo induction of late genes promoted *V. cholerae* survival in stool and pond water. Overall, the work of Camilli and colleagues suggests that there is a temporal program of *V. cholerae* gene expression during infection, perhaps akin to the programmed temporal patterns of gene expression observed during development in higher eukaryotes. Induction of early genes, such as the *tcp* operon, promotes the initial stages of survival and multiplication in vivo. Then, later in infection, induction of late genes prepares the organism for survival and growth outside the host, presumably in low osmolar, nutrient-poor freshwater ponds. It will be interesting to explore if *V. cholerae* gene products expressed relatively early in infection, such as CT, influence late gene induction. That is, does the pathogen elicit changes in the host, such as secretion of diarrheal fluid, that in turn trigger alterations in its pattern of gene expression that enhance its fitness to grow outside the host?

8 Importance of *V. cholerae* Chemotaxis for Intestinal Colonization

Using an in vivo RIVET-based screen, Lee et al. found that chemotaxis genes regulate the kinetics of *V. cholerae* virulence factor expression within the intestine (Lee et al. 2001). Strains bearing mutations in several key chemotaxis genes exhibited delayed induction in *toxT* and *ctxA* during infection. The authors proposed that *V. cholerae* uses chemotaxis to occupy an intestinal niche which is optimal for the induction of virulence factor production. However, subsequent observations from this group may argue against this hypothesis; epistasis analysis indicated that chemotaxis and TCP act independently in *V. cholerae* intestinal colonization (Butler and Camilli 2004). Perhaps unexpectedly, nonchemotactic mutants exhibited elevated intestinal colonization (Butler and Camilli 2004; Lee et al. 2001). The hyper-colonization phenotype of these mutants can partially be accounted for by aberrant distributions of the mutant within the small intestine (Lee et al. 2001). Currently, there is no clear explanation why *V. cholerae* chemotaxis would act to limit its capability to proliferate in the intestine.

There is some controversy regarding the importance of chemotaxis in *V. cholerae*'s exit from the host intestine. Although *V. cholerae* in rice-water stool are often highly motile, work from Camilli and colleagues suggest that rice-water stool *V. cholerae* have reduced expression of chemotaxis genes (Merrell et al. 2002b) and that these organisms are phenotypically nonchemotactic (Butler et al. 2006; Butler and Camilli 2004). These authors argue that the nonchemotactic physiologic state promotes the infectivity of *V. cholerae* (Butler et al. 2006). Contrasting results were obtained by Nielsen et al. who investigated *V. cholerae* physiology during the late phases of the infectious process using the rabbit ileal loop model (Nielsen et al. 2006). These authors present evidence that by 12 h after inoculation of ligated ileal loops, *V. cholerae* initiates an RpoS-dependent genetic program in which

chemotaxis and motility are activated to promote *V. cholerae* escape from the host mucosa, thus preparing the organism for survival in the environment. It is not clear how the results of this elegant study are influenced by the closed ileal loop system that these investigators used to explore *V. cholerae* intraintestinal physiology.

9 Is Quorum Sensing Regulation Important for Intestinal Colonization?

Much progress has been made deciphering the genetic pathways that mediate *V. cholerae* quorum sensing (Svenningsen et al. 2008; Higgins et al. 2007; Lenz et al. 2004; Miller et al. 2002). Unlike the case for several other pathogens, high population densities inhibit *V. cholerae* virulence factor production. At high cell densities, LuxO's repression of *hapR* is relieved, and elevated HapR levels then indirectly represses the transcription of *toxT*. Consistent with this model, Zhu et al. found that a *luxO* deletion mutant is highly defective in colonization of infant mice, presumably because HapR repression of *toxT* is constitutive in this background (Zhu et al. 2002). High cell densities could promote *V. cholerae* detachment from the intestinal mucosa, because HapR promotes production of HapA, a protease that is thought to aide *V. cholerae* detachment from the epithelium (Finkelstein et al. 1992). However, Nielsen and colleagues found that *V. cholerae* escape from the host mucosa in ligated ileal loops did not require HapA (Nielsen et al. 2006), and consequently the significance of high cell density inhibition of *V. cholerae* pathogenicity is not clear. A *hapR* mutant exhibits no colonization defect in the infant mouse (Zhu et al. 2002), and many clinical isolates have inactivating mutations in *hapR* or in other genes in the quorum sensing pathway (Joelsson et al. 2006), casting doubt on the contribution of quorum sensing to *V. cholerae* virulence. However, the true frequency of inactivating mutations in *hapR* should be explored in fresh clinical isolates, since our knowledge of the intactness of *V. cholerae* quorum sensing systems is largely based on strains that have been maintained in the laboratory for many years.

10 Concluding Remarks

How uniform and environmentally determined is the physiology of *V. cholerae* during growth in vivo?

Two unstated and interrelated concepts underpin much of the thinking regarding *V. cholerae* survival and growth in vivo. One idea is that there is a high degree of uniformity in the physiology of organisms growing within the intestine. The second idea is that *V. cholerae* physiology in vivo is largely determined by environmental signals. Both of these concepts – uniformity and determinism – inform the idea of quorum sensing regulation of virulence and also permeate much of the thinking

Table 2 Questions for future investigation

1. Why does the susceptibility of experimental animals to cholera decline with age?
2. What is the role of the normal flora in resistance to cholera?
3. Do other microorganisms promote the infectivity of *V. cholerae* as Metchnikoff suggested?
4. What host mechanisms (particularly components of the GI tract's innate immune response) promote clearance of *V. cholerae* from the intestine?
5. What processes account for the changes in the small bowel microvilli observed during experimental cholera?
6. Are there any attributes of the O1 O-antigen that particularly promote intestinal colonization?
7. Does CT production influence production of 'late' gene products during infection?
8. Why does chemotaxis limit *V. cholerae* intestinal colonization?
9. What fraction of fresh clinical *V. cholerae* isolates contains an intact *hapR* gene and quorum sensing regulation of virulence?
10. Is there physiologically significant cell-to-cell variation in gene expression in vivo?
11. Are there functionally distinct *V. cholerae* subpopulations during intraintestinal growth?

about host–pathogen interactions in general. It is possible that the intraintestinal *V. cholerae* population does not 'behave' in a uniform fashion. There may be distinct subpopulations of organisms and such subpopulations could have distinct functional roles. For example, perhaps only a subset of cells, e.g., those that are in close apposition to the epithelium, produce significant amounts of CT and therefore trigger the host secretory response. How cells in the population 'decide' to enter one niche or another may be strictly determined by environmental signals; alternatively, such cell fate decisions may be made randomly. It is becoming increasingly clear that diverse cell fate choices are made stochastically (Losick and Desplan 2008). It is tempting to speculate that stochastic processes may at least in part explain some of the differences in the reports of the in vivo *V. cholerae* transciptome (Larocque et al. 2005; Bina et al. 2003; Merrell et al. 2002b) or in the variability of *V. cholerae* genes induced in human volunteers (Lombardo et al. 2007). In a recent RIVET-based study of *V. cholerae* gene expression in human volunteers, less than one-third of *V. cholerae* transcriptional units were induced in three or more volunteers (Lombardo et al. 2007). A formidable challenge for future studies of *V. cholerae* pathogenicity will be to quantitatively measure gene expression on the single cell level at different sites during infection. Some other important questions for future study are listed in Table 2.

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Interaction of *Yersinia* with the Gut: Mechanisms of Pathogenesis and Immune Evasion

Peter Dube

Contents

Abstract *Yersinia entercolitica* and *Yersinia pseudotuberculosis* are human food-borne pathogens that interact extensively with tissues of the gut and the host's immune system to cause disease. As part of their pathogenic strategies, the *Yersinia* have evolved numerous ways to invade host tissues, gain essential nutrients, and

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C. Sasakawa (ed.), *Molecular Mechanisms of Bacterial Infection via the Gut*,
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evade host immunity. Technological advances over the last 10 years have revolutionized our understanding of host–pathogen interactions. The application of these new technologies has also shown that even well-understood pathogens such as the *Yersinia* have many surprises waiting to be revealed. The complex interaction with the host has made *Yersinia* a paradigm for understanding bacterial pathogenesis and the host response to invasive bacterial infections. This review examines the mechanisms of immune evasion employed by the *Yersinia* and highlights recent advances in understanding the host–pathogen interaction.

1 Introduction

The genus *Yersinia* contains three species that are human pathogens, the two enteropathogenic *Yersinia* (*Y. enterocolitica and Y. pseudotuberculosis*), as well as *Yersinia pestis*, the causative agent of plague (Bottone 1997; Perry and Fetherston 1997). *Y. enterocolitica* and *Y. pseudotuberculosis* have served as paradigms of bacterial pathogenesis due to the ease with which these pathogens can be genetically manipulated and the ability of some serogroups to cause disease in mice that mimics human disease (Carter 1975a,b). Enteropathogenic *Yersinia* are well-suited human pathogens containing all of the machinery required to invade human tissues and modulate their environment to promote survival. The pathogenic strategies employed by the *Yersinia* center around modulating the host immune response to their advantage (Viboud and Bliska 2005). Recently, we have gained great insight into the intricate interactions of these remarkable pathogens with their hosts, and this chapter will focus on the interaction of *Yersinia* with the gut, virulence strategies, and specifically the interaction of these pathogens with the innate and adaptive immune systems.

1.1 Clinical Disease

Y. enterocolitica and *Y. pseudotuberculosis* are foodborne pathogens present in contaminated meat products, milk, and water (Bottone 1997). *Y. enterocolitica* strains are classified into biogroups based on biochemical properties and serogroups based on O-antigen immune reactivity (Brenner et al. 1976). Many serogroups of *Y. enterocolitica* can cause disease in humans, including biogroup 1B serogroups O:8, O:4, O:13a,13b, O:18, O:20, O:21, biogroup 2, O:9, O:5,27 biogroup 3, O:1,2,3, O:5,27 biogroup 4, O:3, and biogroup 5, O:2,3, with the most severe disease caused by biogroup 1B serogroup O:8 strains. *Y. pseudotuberculosis* can be classified into 21 serogroups, with most human disease being caused by strains in serogroups O:1 and O:3. The major reservoir for *Yersinia* leading to human disease is swine, but chocolate milk, tofu, and water have been recently reported as sources of contamination (Lynch et al. 2006). Outside of foodborne disease, there is

evidence that *Y. enterocolitica* can be transmitted via transfusion with contaminated blood products (Richards et al. 1992).

Infection of the gastrointestinal tract can lead to enterocolitis, terminal ileitis, mesenteric lymphadenitis, pseudoappendicitis, and in some cases, bacteremia. Clinically, children and young adults present more often than older individuals and, like many gastrointestinal infections, *Yersinia* infection is characterized by an acute inflammatory response that can include fever, bloody or watery diarrhea, and abdominal pain. These symptoms can be severe, but in otherwise healthy individuals GI manifestations of disease are usually self-limiting and resolve without medical intervention (Abdel-Haq et al. 2000).

Systemic infection with *Y. enterocolitica* or *Y. pseudotuberculosis* is a rare but serious manifestation of disease that may result from iron overload, immunodeficiency, or secondary to a transfusion with contaminated blood products (Richards et al. 1992). Septicemia due to *Yersinia* infection can have mortality rates as high as 50% due to the ability of the bacteria to infect most organ systems. Infections of the spleen, liver, kidney, heart, lung, eyes, bone, and central nervous system have been reported (reviewed in Bottone 1997).

Individuals may also develop chronic sequelae or autoimmune disease after *Yersinia* infection. Erythema nodosum and reactive arthritis are the most common chronic secondary sequelae. Reactive arthritis due to *Yersinia* infection is linked to a person's HLA type, with a strong correlation between HLA-B27 and *Yersinia*-induced reactive arthritis (Laitinen et al. 1977). Further, secondary sequelae may be dependent on the serogroup of the infecting strains with the majority of reported reactive arthritis being due to *Y. enterocolitica* O:3 infection (Laitenen et al. 1972). There are also strong correlations between *Y. enterocolitica* O:3 infection and autoimmune diseases of the thyroid such as Grave's disease (McIver and Morris 1998). Mechanistically, how *Y. enterocolitica* contributes to the pathogenesis of reactive arthritis and Grave's disease remains to be determined.

Although there is a correlation between infection with serogroup O:3 strains and chronic/auto-immune disease, multiple serogroups of *Y. enterocolitica* are capable of causing human disease. Some have speculated that chronic manifestations of disease may be related to nature of the *Yersinia* strain, with strains in the O:3 serogroup initially causing a milder disease that develops into chronic sequelae, whereas O:8 strains initially cause a more acute disease that does not develop into chronic disease (Lamps et al. 2006). This is an intriguing hypothesis but it needs to be formally tested. The contribution of *Y. enterocolitica* or *Y. pseudotuberculosis* infection to other chronic inflammatory diseases has been suggested in the literature. For example, recently, a number of clinical studies and individual case reports suggest a causal link between infection with enteropathogenic *Yersinia* and development of Crohn's disease or the exacerbation of Crohn's disease (Lamps 2003; Lamps et al. 2003). As we learn more about the pathogenesis of infection and detection methodologies improve, it is likely that *Y. enterocolitica* and *Y. pseudotuberculosis* will be linked to more chronic inflammatory human diseases.

Altogether, the enteropathogenic *Yersinia* are responsible for a wide spectrum of human diseases ranging for acute enterocolitis and life-threatening septicemia to

chronic sequelae and autoimmune disease. The full spectrum of human diseases influenced by the *Yersinia* are likely to increase as we become more aware of the role of infectious agents and pathogen-induced inflammation in chronic disease. Indeed, the overarching theme associated with diseases caused by the enteropathogenic *Yersinia* is inflammation.

2 Pathogenic Strategies

In order to cause disease, bacterial pathogens must be able to rapidly adapt to the mammalian host, attach to and invade host tissues, avoid host immune defenses, replicate, and gain access to the environment for further rounds of infection. *Y. enterocolitica* and *Y. pseudotuberculosis* both take similar strategies towards pathogenesis that will be discussed in detail below. As a brief synopsis, the bacteria are delivered to the body in contaminated food or water, and the subsequent changes in temperature and pH encountered in the host lead to changes in the expression of virulence genes (Pepe et al. 1994). Initially, urease genes are expressed to aid survival in the harsh environment of the stomach (Young et al. 1996). Once in the small intestine, the bacteria attach to and invade the specialized micro-fold epithelium, M cells, overlaying the Peyer's patches (Isberg and Barnes 2001). *Y. enterocolitica* and *Y. pseudotuberculosis* express a number of adhesins including Invasin, Ail, and YadA that mediate attachment and invasion of intestinal tissues (Miller and Pepe 1994). Once inside the Peyer's patches, the bacteria replicate, mainly extracellularly, and induce a robust inflammatory response (Autenrieth and Firsching 1996; Autenrieth et al. 1993a; Dube et al. 2001; Logsdon and Mecsas 2006). From the Peyer's patches, the *Yersinia* may disseminate to the mesenteric lymph nodes or other extra-intestinal tissues such as the spleen, liver, and/or lungs depending on the underlying health of the infected individual. Concurrently, the bacteria utilize a large array of virulence factors to protect themselves from the host's innate and adaptive immune responses (Cornelis and Wolf-Watz 1997; Revell and Miller 2001). The potent inflammatory response to infection leads to erosion of the lamina propria proximal to an infected Peyer's patch giving *Yersinia* access to the lumen of the small intestine for shedding into the environment (Dube et al. 2001). In an otherwise normal individual, CD4+ T-helper-1 (Th-1) type T cell immunity leads to the ultimate clearance of the infection (Autenrieth et al. 1993b).

3 Host Adaptation, Sensing, and Virulence Gene Expression

The pathogenic *Yersinia* all have multiple virulence genes located both on the chromosome and on a 70-kb virulence plasmid called pYV (Portnoy and Falkow 1982; Revell and Miller 2001). The last decade has seen a number of genetic approaches employed to further refine our understanding of virulence gene expression both in vivo

and in vitro (Darwin 2005). Transposon-based genetic screening and selections using in vivo expression technology (IVET) and signature-tagged mutagenesis (STM) allowed the identification of genes preferentially required for in vivo growth (Darwin and Miller 1999; Gort and Miller 2000; Young and Miller 1997). Recently, the genomes of *Y. enterocolitica* strain 8081 and *Y. pseudotuberculosis* strain PB-1 (unpublished, Genebank CP001048) have been sequenced and annotated, further facilitating the use of genetics to investigate host–pathogen interactions (Thomson et al. 2006). High throughput genetic screens have revolutionized how we genetically dissect the host-pathogen interaction and have led to the discovery of multiple new virulence genes and associated pathways some of which will be discussed below.

A remarkable aspect of the pathogenesis of the enteropathogenic *Yersinia* is the dynamic ability of these pathogens to rapidly change patterns of gene expression in response to a changing environment. The results of IVET and STM screens further validated this observation by identifying many transcriptional regulators as important virulence genes (Darwin and Miller 1999; Gort and Miller 2000; Young and Miller 1997). In addition, virulence gene products regulated by these regulators interact directly with the immune system and are key factors in the host–pathogen interaction as well as immune evasion (Dube et al. 2001, 2003; Revell and Miller 2000). Many of the conditions that trigger changes in gene expression are related to stresses encountered in the host, including changes in pH, calcium, magnesium, iron, salinity, temperature, and stress, which all induce differential gene expression that aids in bacterial survival (Pepe et al. 1994). Several of the better understood examples of environmental sensing and the corresponding virulence gene expression will be presented below, but it is important to note that many of the other regulatory networks involved in virulence gene regulation remain poorly understood.

3.1 Virulence Gene Regulation for Nutrient Acquisition

Iron is a key nutrient required for the growth of many bacterial species, and iron limitation is often one of the host's key innate immune strategies designed to limit bacterial replication (Finkelstein et al. 1983). In the mammalian body, there is not a sufficient concentration of free iron to support bacterial replication, and thus many bacterial species have developed high affinity iron scavengers called siderophores (Finkelstein et al. 1983). Strains of *Y. enterocolitica* and *Y. pseudotuberculosis* that cause severe disease in humans and rodents (high-pathogenicity strains) all contain the high-pathogenicity island (HPI) (Carniel 2001). The HPI is a 36–43-kb pathogenicity island containing the genes encoding for the iron acquisition system (yersiniabactin system) (Carniel et al. 1996). This system is positively regulated by the AraC-like regulator YbtA and negatively regulated by the iron-responsive negative regulator Fur (Carniel 2001). It is proposed that, upon entering the mammalian host, iron becomes limiting and Fur-mediated repression of the yersiniabactin system is relieved allowing for YbtA-mediated gene transcription. YbtA induces the expression of the siderophores and the structural genes required for iron uptake. The high affinity of the

siderophores for iron allows them to scavenge iron from the host. The ability to acquire iron in this fashion is required to cause disease, and the importance of this level of innate immune restriction is illustrated by the severe systemic *Yersinia* infections often observed in iron-overloaded individuals (Caplan et al. 1978; Chiu et al. 1986).

3.2 Virulence Gene Regulation for Adherence and Invasion

In addition to having to gain essential nutrients, *Yersinia* must be able to attach to and invade intestinal tissues. The major adhesin of the enteropathogenic *Yersinia* is called invasin (Inv), and its expression is regulated in vitro by temperature, pH, and growth phase (Isberg and Falkow 1985; Miller and Falkow 1988; Pepe et al. 1994). Optimal in vitro expression of Inv is observed at neutral pH and 26°C and under slightly acidic conditions (pH 5.5) at 37°C (Pepe et al. 1994). Inv expression is regulated at several levels including being positively regulated by the regulator of virulence A (RovA) and at the level of the promoter by *Yersinia*-modulating protein (YmoA) and histone-like nucleoid structuring protein (H-NS) (Ellison et al. 2003, 2004; Ellison and Miller 2006a,b; Nagel et al. 2001; Revell and Miller 2000). RovA is highly homologous to AraC-type regulators and specifically to the *Salmonella* SlyA protein involved the regulation of a variety of stress responses (Buchmeier et al. 1997). RovA is capable of modulating Inv expression both in vitro and in vivo and presumably directly enhances transcription through its interaction with RNA polymerase (Ellison et al. 2004; Revell and Miller 2000).

Interestingly, in addition to *inv* regulation, RovA plays a much more complex role in both virulence and immune modulation. *rovA* mutants are significantly attenuated via an oral route of infection whereas *inv* mutants show a delayed colonization phenotype but are as virulent as wild-type using LD-50 analysis (Ellison et al. 2004; Pepe and Miller 1993; Revell and Miller 2000). This suggests that RovA regulates genes in addition to *inv* that are required for virulence. When the entire RovA regulon was examined by micro-array analysis, 40 genes were activated and RovA repressed 23 genes. Included in the genes under the control of RovA were several other regulators, suggesting that RovA might be a master regulator of virulence gene expression (Cathelyn et al. 2007). In fact, we showed that RovA regulates a still unknown factor that is important for the induction of IL-1α in the gut and that the early induction of IL-1α is important for *Y. enterocolitica*-associated gut inflammation (Dube et al. 2001). The ability of RovA to induce gut-specific pathogenic mechanisms was illustrated by a study that compared the host response to oral and intraperitoneal (IP) routes of infection (Dube et al. 2003). The *rovA* mutant has a significant defect in virulence and inflammatory pathologies via the oral route of infection, but is nearly as virulent as the wild-type strain via intraperitoneal (IP) infection (Dube et al. 2003). Further, the *rovA* mutant causes significant inflammatory pathologies during IP infection but not during an oral infection. These data suggest that the RovA regulon includes the regulation of tissue specific virulence factor expression. This concept was bolstered by recent

studies that showed that the *Y. pestis* RovA regulon was significantly different from the *Y. enterocolitica* regulon, and that, in a mouse model, *Y. pestis* RovA was required for bubonic plague but not pneumonic plague (Cathelyn et al. 2006).

It is not completely understood how RovA is regulated or what are the in vivo signals leading RovA-dependent gene transcription, but a significant step forward was made with a recent study demonstrating a role for carbon storage regulator (Csr) non-coding small RNA in regulating RovA through RovM in *Y. pseudotuberculosis* (Heroven et al. 2008; Heroven and Dersch 2006). This same study implicated a two-component regulatory system in this regulation suggesting a possible sensory mechanism in the direct/indirect regulation of RovA. It remains unclear if similar regulatory networks regulate *Y. enterocolitica* RovA but it is likely. RovA's role in *inv* regulation suggest that temperature and pH are likely RovA-activating signals, but the intermediate proteins or additional regulatory RNAs involved in the process remain to be identified. Altogether, these studies nicely illustrate the ability of *Yersinia* to rapidly respond to specific host environments and the complexity of virulence-associated gene expression.

4 Adherence, Tissue Invasion, and Associated Aspects of Innate Immune Evasion

As a primary portal of entry into intestinal tissue, *Y. enterocolitica* and *Y. pseudotuberculosis* invade the intestinal epithelium to gain access to the lymphoid follicles of the small intestine. To facilitate this process, the enteropathogenic *Yersinia* utilize three adhesins/invasins: (1) invasin (Inv), the major invasin mentioned previously (Isberg and Falkow 1985; Miller and Falkow 1988); (2) the chromosomally-encoded attachment and invasion locus (Ail) (Miller and Falkow 1988); and (3) the virulence plasmid-encoded Yersinia Adhesin A (YadA) (Bliska et al. 1993). Once inside the Peyer's patches, *Yersinia* is predominantly an extracellular pathogen, and therefore vulnerable to opsonization and complement-mediated killing. *Yersinia* exploits both YadA and Ail to protect itself from complement and complement-mediated killing, effectively eliminating one of the first lines of innate immune defense (discussed in more detail below).

4.1 Invasin

Invasin is the major tissue invasion protein of the enteropathogenic *Yersinia*. In fact, *inv* is sufficient to confer an invasive phenotype on non-invasive bacteria (Isberg and Falkow 1985; Miller and Falkow 1988). The crystal structure of the extracellular domain of *Y. pseudotuberculosis* Inv was solved, revealing that Invasin is a 101.5-kDa integral outer membrane protein that consists of five distinct

extracellular domains that include 4 immunoglobulin super-family type folds and a distal C-type lectin-like domain (Dersch and Isberg 2000; Hamburger et al. 1999). The overall topology of Inv and substrate specificity is similar to fibronectin, suggesting that *Y. enterocolitica* and *Y. pseudotuberculosis* evolved a mechanism to compete for fibronectin binding in the host. Inv binds to β_1-integrins expressed on the surface of M cells and has a preference for $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $a_v\beta_1$ integrins (Isberg and Leong 1990). Further, the affinity of Inv for β_1 integrins is significantly higher than that of fibronectin suggesting a mechanism for pathogen mediated receptor competition and binding in vivo (Hamburger et al. 1999).

As briefly mentioned above, *Y. enterocolitica inv* mutants are not attenuated for virulence when analyzed by LD-50. However, if the kinetics of organ colonization and bacterial burdens are examined then there are marked delays in organ colonization following infection with the *inv* mutant (Pepe and Miller 1993; Pepe et al. 1995). There are differences in the distribution of bacterial abscesses of the animals infected with the *inv* mutant compared to the wild type control as evidenced by essentially no abscess formation in the mesenteric lymph nodes (Pepe and Miller 1993; Pepe et al. 1995). The significance of this observation needs to be investigated further but it may reveal a role for Inv in tissue tropisms and is consistent with newer data that suggests that Inv is critical for dissemination from the lumen of the small intestine in animals that lack Peyer's patches (discussed below) (Barnes et al. 2006; Handley et al. 2005). The lack of a difference in LD-50 is probably due to the fact that later in infection bacterial burdens in the *inv* mutant infected animals are similar to those observed in the animals infected with the WT control suggesting the presence of additional mechanisms for tissue invasion (Miller and Falkow 1988).

4.2 Ail

In addition to Inv, *Yersinia* encode two other major adhesins; YadA and Ail. Ail is a 17 kDa outer membrane protein expressed by both *Y. enterocolitica and Y. pseudotuberculosis* that is sufficient to promote the attachment and invasion of non-invasive bacteria into tissue culture cells (Miller et al. 1990; Wachtel and Miller 1995). Like many *Yersinia* virulence proteins, Ail is optimally expressed at 37°C and under aerobic conditions suggesting that *ail* is expressed in vivo. This was confirmed in virulence studies where Ail protein could be detected by immunoblot in the Peyer's patches of mice 48 h post-infection (Wachtel and Miller 1995). These studies also demonstrated that an *ail* mutant had no appreciable defect in virulence when evaluated in the mouse. Presumably, this lack of a virulence phenotype is due to redundant functions shared between Ail, Inv, and YadA. Redundancy in function is indicative of a crucial role in the disease process, and mutations in *ail* or *yadA* on the *inv* mutant background showed more significant defects in virulence than either single mutation supporting this notion (Pepe et al. 1995).

4.3 YadA

YadA is a surface-exposed 45-kDa homotrimeric protein encoded on the pYV virulence plasmid that serves as a multi-functional virulence factor conferring the ability to adhere to the extracellular matrix proteins (Balligand et al. 1985; Heise and Dersch 2006; Martinez 1989). Unlike Inv and Ail, YadA is positively regulated under conditions that promote the expression of the Yersinia outer proteins (Yops) (Cornelis and Wolf-Watz 1997; Skurnik and Toivanen 1992). YadA impacts virulence in a species-dependent manner with *Y. enterocolitica yadA* mutants having a strong virulence defect and *Y. pseudotuberculosis yadA* mutants have a minor defect (Bolin and Wolf-Watz 1984; Han and Miller 1997; Pepe et al. 1995; Rosqvist et al. 1988). YadA confers an adhesive phenotype and, in the case of *Y. pseudotuberculosis*, it also promotes invasion of mammalian cells through a unique domain in its N-terminus (amino acids 53–83) that is absent in *Y. enterocolitica* YadA (Heise and Dersch 2006). To promote uptake of *Y. pseudotuberculosis* into epithelial cells, the N-terminal uptake region of YadA binds to fibronectin that is bound to $\alpha_5\beta_1$ integrin initiating internalization in a manner similar to Inv-mediated uptake (Heise and Dersch 2006). The ability of YadA to adhere to cells, and in the case of *Y. pseudotuberculosis* invade cells, is probably a property of its ability to bind to extracellular matrix proteins (Heise and Dersch 2006; Nummelin et al. 2004; Schulze-Koops et al. 1993; Tertti et al. 1992).

YadA binds to fibronectin, collagen I, II, and IV, and laminin in a species-dependent manner with *Y.enterocolitica* YadA binding collagen and laminin with higher affinity than *Y. pseudotuberculosis* YadA (Heise and Dersch 2006). In contrast, *Y. pseudotuberculosis* YadA binds fibronectin with higher affinity than *Y. enterocolitica* YadA. Species-specific differences in YadA substrate binding could explain the differences between the virulence phenotypes of the two proteins (Heise and Dersch 2006). The interaction of *Y. pseudotuberculosis* YadA with fibronectin bound to β_1 integrins suggests that YadA may also be able to induce inflammatory responses from epithelial cells. In fact, both *Y. enterocolitica and Y. pseudotuberculosis* YadA proteins are capable of inducing the expression of IL-8 from epithelial cells suggesting that, in addition to promoting binding and invasion of host cells, YadA contributes to the inflammation associated with infection (Eitel et al. 2005; Schmid et al. 2004).

The ability of YadA to interact with collagen may also contribute to chronic inflammatory sequelae associated with *Yersinia* infection. In particular, there is evidence that infection with *Y. enterocolitica* is a pre-disposing risk factor for development of reactive arthritis in humans (Eitel et al. 2005; Laitenen et al. 1972, 1977; Schmid et al. 2004). This hypothesis is supported by studies that demonstrated that, in a rat model of reactive arthritis, YadA is required to induce disease and, specifically, the ability of YadA to bind collagen is critical for disease development (Lahesmaa et al. 1992, 1993; Skurnik 1995). Although definitive evidence for a causal role of YadA in human reactive arthritis is limited,

experimental studies in model animals strongly support this link and suggest that YadA may impact both acute and chronic inflammatory responses.

5 Role of Ail and YadA in Complement Resistance and Immune Evasion

In addition to conferring an adhesive/invasive phenotype, Ail and YadA also provide complement resistance by protecting the bacteria from complement-mediated lysis and phagocytosis (Bliska and Falkow 1992; Pierson and Falkow 1993). The complement system is composed of 20 serum complement proteins that function through a complex series of protein–protein interactions and proteolytic cleavage events ultimately leading to pathogen opsonization/phagocytosis, lysis and the induction of inflammation (Haeney 1998). The complement system is divided into pathways based on activation mechanisms, including the alternative pathway, classical pathway, and lectin pathway, all of which are important aspects of the host's immune response to extracellular pathogens. The ability to evade, complement and/or to be resistant to all three complement pathways is an important factor in protecting *Yersinia* from the immune system. Ail and YadA subvert the alternative complement pathway through the binding of the regulator factor H (Biedzka-Sarek et al. 2005, 2008a,b; Kirjavainen et al. 2008). YadA and Ail also bind to the C4b-binding protein to subvert the classical and lectin pathways (Kirjavainen et al. 2008). Altogether, YadA and Ail effectively block all complement pathways and allow *Yersinia* to effectively avoid this important innate immune mechanism (Kirjavainen et al. 2008).

Similar to the ability to promote adherence to and invasion of mammalian cells, the ability to resist complement is a redundant feature shared between Ail and YadA. The Ail protein is predicted to contain eight transmembrane beta sheets and four surface-exposed loops (Miller et al. 2001). Mutational and biochemical analysis of the surface-exposed loops of the Ail protein suggest that the invasion and serum resistance phenotypes can be separated, and that both of these properties are linked to loops 2 and 3 with loops 1 and 4 not being directly involved in these phenotypes (Miller et al. 2001). The topology of the Ail protein indicates that the loops of the protein will be close to the cell membrane creating the potential for masking by other proteins and lipids on the cell surface. Recently, in support of this concept, Skurnik and colleagues demonstrated that the O-antigen and the outer core of *Y. enterocolitica* serogroup O:3 might partially mask Ail (Biedzka-Sarek et al. 2005).

YadA, on the other hand, is a homotrimeric "lollipop-shaped" protein that is predicted to form a capsule-like coat that extends from the surface of the bacteria such that it is not effectively masked by other proteins or lipids (El Tahir and Skurnik 2001; Hoiczyk et al. 2000; Roggenkamp et al. 2003). This suggests that

YadA is the major complement avoidance mechanism, and that under conditions where Ail is capable of binding factor H or C4b-binding protein it provides significant protection from complement.

In summary, tissue adherence and invasion is a critical step in the pathogenesis of a *Yersinia* infection. *Yersinia's* ability to exploit the Inv-β_1 integrin interaction facilitates the targeting and invasion of Peyer's patches and M cells by *Yersinia* and in the absence of Inv:Ail and YadA can mediate interaction with the host epithelium and promote invasion. Species-specific differences in the N-terminus of YadA determine the ability of this protein to mediate invasion of epithelial cells with *Y. pseudotuberculosis* YadA conferring a much stronger invasive phenotype than the *Y. enterocolitica* YadA. Enteropathogenic *Yersinia* are predominantly extracellular pathogens that are constantly challenged by complement and other aspects of innate and humoral immunity. To counteract this serious challenge, *Yersinia* has evolved mechanisms to evade complement that involves both the YadA and Ail proteins and their ability to bind regulators of the three complement pathways. Altogether, these two virulence proteins not only play a central role in host invasion but also in immune evasion.

6 Type Three Secretion

Yersinia uses specialized secretion systems called type three secretion systems (TTSS) to deliver virulence factors into the cytoplasm of target host cells (Cornelis 2002; Cornelis and Wolf-Watz 1997; Viboud and Bliska 2005). All pathogenic strains of *Yersinia* harbor an approximately 70-kDa plasmid (pYV) that encodes the structural, regulatory, and virulence-associated effector proteins required for type three protein secretion (Cornelis 2002; Cornelis and Wolf-Watz 1997; Portnoy and Falkow 1982; Viboud and Bliska 2005). The pYV-encoded TTSS is also known as the Ysc system to distinguish it from the other two TTSS encoded by *Y. enterocolitica*, including a flagellar TTSS and a chromosomally-encoded TTSS known as the Ysa system (Haller et al. 2000; Young et al. 1999). The Ysa and Ysc systems are contact-dependent TTSS whereas the flagellar system is part of the apparatus involved in flagellum assembly. The Ysc TTSS is one of the best-studied bacterial virulence machineries currently known, and *Yersinia* has served as a paradigm for understanding TTSS-mediated manipulation of host immune responses. A comprehensive review of the interactions between the Ysc TTSS and the host is well beyond the scope of this review, but a number of excellent reviews have been published in the last couple of years and readers are referred to these for a more in-depth analysis (Cornelis 2002; Pujol and Bliska 2005; Viboud and Bliska 2005). However, a brief introduction to the Ysc TTSS and its effector proteins is required to discuss the Ysa and flagellar TTSS as well as subsequent discussions of immune evasion.

6.1 Ysc TTSS

The Ysc TTSS is absolutely required for the virulence of all three species of pathogenic *Yersinia*. Like many virulence-associated properties of *Yersinia*, the TTSS and the associated effector proteins are regulated by temperature and ion concentration. A variety of effector proteins called *Yersinia* outer proteins (Yops) are secreted from the cytosol of the bacteria through the TTSS directly into the cytoplasm of the host cell (Viboud and Bliska 2005). The Yops are exotoxins that mimic the action of host cell enzymes such as phosphatases, proteases, GEFs, GAPs, kinases, and acetylases to modulate the cytoskeleton and immune signaling pathways (Cornelis 2002; Viboud and Bliska 2005). This ultimately leads to inhibition of phagocytosis, proinflammatory cytokine production, and in the case of macrophages, the induction of apoptosis and pyroptosis (Table 1). Although the pYV is required for the virulence of *Yersinia*, only some of the Yops are absolutely required to cause disease in mice suggesting that some of the Yops are redundant (Trulzsch et al. 2004).

6.2 Ysa TTSS

In addition to the Ysc TTSS, the flagellar and Ysa TTSS have been implicated in the virulence of *Y. enterocolitica* infection and other processes such as the formation of bio-films (Haller et al. 2000; Kim et al. 2008; Young et al. 1999). The regulation and role of these TTSS are not as well described as the Ysc system but recently more information has emerged.

The chromosomally-encoded Ysa TTSS was identified through homology to the Ysc system (Haller et al. 2000). In vitro, the Ysa TTSS is optimally expressed under high salt concentrations, 26°C, and at stationary growth phase (Haller et al. 2000; Mildiner-Earley et al. 2007; Walker and Miller 2004). An AraC-like regulator known as YsaE and the SycB chaperone that regulates the expression of the sycBypspBCDA operon mediates salt responsiveness of the Ysa TTSS (Walker and Miller 2004). This operon is further regulated by the YsrS/YsrR two-component regulatory system and the RscC-YojN-RcsB phospho-relay system (Venecia and Young 2005; Walker and Miller 2004). In support of a role in virulence, a mutant in the Ysa TTSS displayed a ten-fold difference in LD-50 relative to the wild-type strain (Haller et al. 2000). Subsequently, it was demonstrated that the Ysa TTSS is important for colonization of the small intestine (Venecia and Young 2005). The Ysa TTSS secretes 15 proteins known as Ysa proteins (Ysps) (Matsumoto and Young 2006) (Table 1). The role of many of the Ysps remains unknown at this time but, interestingly, it was recently shown that that some Ysps were actually Yops including YopE, YopN, and YopP suggesting that the Ysa TTSS, like the Ysc TTSS, might be involved in the modulation of host immune responses in vivo (Matsumoto and Young 2006). Other Ysps proteins are unrelated to the Yops,

Table 1 Yersinia secreted effector proteins involved in immune evasion[a]

Protein	Type three secretion system			Function	Immune evasion/ Pathogenesis	Reference[c]
	Ysc	Ysa	Flagellar[b]			
YopE	X	X		Rho-GAP	Yes	Viboud and Bliska (2005)
Yop H	X			Tyrosine Phosphatase	Yes	Viboud and Bliska (2005)
Yop K	X	X		Unknown	Yes	Viboud and Bliska (2005)
YopJ/P	X	X		Protein Acetylase/ Protease	Yes	Mukherjee et al. (2006)
YopO	X			Ser/Thr Kinase	Yes	Viboud and Bliska (2005)
YopT	X			Cysteine Protease	Yes	Viboud and Bliska (2005)
YplA	X	X	X	Phospholipase	Yes	Young and Young (2002)
YspA		X		Unknown	?	Foultier et al. (2002)
YspB		X		Structural[d]	?	Foultier et al. (2002)
YspC		X		Structural	?	Foultier et al. (2002)
YspD		X		Structural	?	Foultier et al. (2002)
YspE		X		Unknown	Yes	Matsumoto and Young (2006)
YspF		X		Unknown	Yes	Matsumoto and Young (2006)
YspI		X		Unknown	No	Matsumoto and Young (2006)
YspK		X		Ser/Thr Kinase	Yes	Matsumoto and Young (2006)
YspL		X		Unknown	Yes	Matsumoto and Young (2006)
YspP		X		Tyrosine Phosphatase	Yes	Matsumoto and Young (2006)
YspM		X		Lipase	?[e]	Witowski et al. (2008)
YspN		X		Structural	Yes	Matsumoto and Young (2006)
YspY		X		Unknown	Yes	Matsumoto and Young (2006)

[a]Effector proteins not discussed in the main text weren't included in the table i.e. (LcrV, YopM, various FOPs)

[b]Other FOPs have been detected but they are poorly described

[c]In the cases of the well known Yops the original description isn't cited but to put these proteins in the wider context of *Yersinia* infection recent reviews are cited. In the case of newly described proteins or major changes in the understanding of the protein, the original reference is provided.

[d]proteins that are homologous to translocons and other structural features of TTSS are identified as structural proteins

[e]YspM is cytotoxic when expressed in yeast cells but it is yet to be tested in animal cells or in the mouse model

for example, in some strains of *Y. enterocolitica* serogroup O:8, the Ysa TTSS secretes YspM a bacterial lipase that is secreted directly into host cells where it exerts a cyto-toxic effect (Witowski et al. 2008). In addition to the YspM lipase, the Ysa TTSS is capable of secreting the YplA phospholipase (Schmiel et al. 1998). YplA was originally identified as a *Y. enterocolitica* virulence factor and has subsequently been shown to be secreted by the Ysa, Ysc, and the flagellar TTSS (Schmiel et al. 1998; Young and Young 2002; Young et al. 1999). The specific role of lipases and phospholipases in the pathogenesis of *Y. enterocolitica* infection remains unknown, but the Ysa secretion system is capable of secreting several of these virulence factors suggesting membranes and lipids as potential targets of the Ysa TTSS.

Even though the evidence in support of the Ysa TTSS as an important virulence-associated apparatus is increasing, how the Ysa TTSS and its effector proteins interact with the host's immune system remains to be determined. It is not unusual for enteric pathogens to encode for multiple TTSS; *Salmonella* sp. utilizes several TTSS at different stages of the infectious process setting precedence for the specialization of TTSS during infection (Hansen-Wester and Hensel 2001).

6.3 Flagellar TTSS

In addition to the two contact dependent TTSS (Ysc and Ysa), *Y. enterocolitica* also encodes for a flagellar TTSS (Young et al. 1999). The flagellar TTSS is important for the secretion of flagellin during the biogenesis of flagella. Significantly, it was recently observed that the flagellar TTSS secreted a number of proteins not associated with the biogenesis of the flagella; these proteins were called Flagellar outer proteins (Fops) (Young et al. 1999) (Table 1). As discussed above, one of the Fops, YplA (*Yersinia* phospholipase A), had been recently identified as a virulence factor required for the colonization of the Peyer's Patches and mesenteric lymph nodes and for the inflammatory responses to infection in these tissues. Interestingly, when the expression and regulation of YplA was examined, reduced YplA expression was observed in *flhDC* and *fliA* mutants suggesting that YplA is part of the flagellar regulon but that *Y. enterocolitica* is non-motile at 37°C and does not express a functional flagellum under in vivo conditions (Schmiel et al. 2000).

To summarize, the enteropathogenic *Yersinia* encode for multiple TTSS that are important for virulence. The plasmid-encoded Ysc TTSS has been extensively studied for its role in virulence and modulation of host immune responses with many of the Ysc effectors important in dissemination of the *Yersinia* within the host. The recently described, chromosomally-encoded, Ysa TTSS and its effector proteins are important for the colonization of gastrointestinal tissues. The fact that several of the Ysc effectors can be secreted through the Ysa TTSS and the growing evidence that some of the Ysp proteins (YspM) can be directly cytotoxic suggests that the Ysa TTSS may be directly involved in modulation of the host immune response in a manner analogous to the Ysc TTSS. The flagellar TTSS is not as well

described in terms of its direct role in virulence and may play important roles in environmental persistence or other aspects of the *Yersinia* life cycle. All of the TTSS secrete a variety of effector proteins that impact the host in numerous ways, but subversion of host immunity to promote infection is a common theme. Many of the effectors can be secreted by several of the TTSS implying either promiscuous secretion or importance at various times during infection. The Ysc TTSS and its effectors are well described, but there remains much to learn about the Ysa and flagellar TTSS, their effectors, and the role these secretion systems play during human disease.

7 Interaction with the Innate Immune System

A major target of the type three secretion systems of *Yersinia* is the innate immune system. The last 15 years has seen an explosion in our understanding of the innate immune system and a renewed awareness of the importance of innate immunity in the host-pathogen interaction (Karin et al. 2006). Innate immunity provides the first line of defense to infection and is a major target of *Yersinia's* immune evasion mechanisms. The change in our mechanistic understanding of innate immunity occurred with the recognition that microbial pathogens released danger signals that could be recognized by the innate immune system based on the presence of pathogen associated molecular patterns (PAMPs) produced by that pathogen (Akira and Takeda 2004). For example, Gram-negative bacterial pathogens such as *Yersinia* generally produce lipopolysaccharide (LPS) that the innate immune system recognizes as a danger signal and responds by initiating an immune response. The PAMPs are recognized by a variety of pathogen recognition receptors (PRR) such as toll-like receptors and nod-like receptors that generate the initial immune signal (Akira and Takeda 2004). Subsequently, cells activated by PRR produce cytokines and chemokines to recruit and activate more effector cells of the innate immune system including neutrophils, natural killer cells, macrophages, and mast cells. These cells may clear the infection or in most cases they act to control infection until adaptive immune responses can be generated.

As a *Y. enterocolitica* or *Y. pseudotuberculosis* infection progresses, the bacteria are predominantly extracellular and *Yersinia* is challenged by innate aspects of the humoral response and the cellular innate immune response in the form of complement, other opsonins, neutrophils, and macrophages. As discussed previously, *Yersinia* utilizes the Ail and YadA surface proteins to avoid complement and the Ysc TTSS to avoid phagocytosis. A number of the Yops (YopE, YopH, YopT, and YopO) impact signal transduction pathways leading to cytoskeleton rearrangements important for phagocytosis (Cornelis 2002; Viboud and Bliska 2005). After ligation of the β_1-integrin receptor with Inv, signaling is inhibited by YopH; YopH is a protein tyrosine phosphatase that inhibits integrin signaling by targeting FAK, p130-CAS, and paxillin in epithelial cells and SKAP-HOM, Fyb, and the Fak-homolog Pyk in macrophages

(Black and Bliska 1997; Bliska et al. 1991). YopE also targets the cytoskeleton by inhibiting the small G-proteins RhoA, Rac1, and Cdc42 through its GAP activity (Aepfelbacher 2004; Black and Bliska 2000; Schotte et al. 2004). Likewise, YopT and YopO also target small G-proteins with YopT acting as a cysteine protease that cleaves the isoprenyl group from RhoA, thus inactivating it by removing it from the membrane (Shao and Dixon 2003). YopO is a serine/threonine kinase that inactivates RhoA and Rac1 by an unknown mechanism, but recent studies show that kinase activity requires the 1:1 complex of YopO with G actin. Further, the YopO/G actin interaction appears to be required to promote the YopO mediated cytotoxicity (Trasak et al. 2007). Altogether, *Yersinia* injects a minimum of four effector proteins through the TTSS that act to effectively prevent phagocytosis by inhibiting cytoskeletal rearrangements and associated signaling pathways. This then partially protects *Yersinia* from activated macrophages and neutrophils.

Y. enterocolitica and *Y. pseudotuberculosis* interact extensively with many aspects of the innate immune system to ensure their own survival and to promote disease in the host. It is likely that virulence factors are expressed temporally to modulate the host immune response at different stages of infection. Temporal and tissue-specific expression of virulence factors may help to explain some of the discrepancies observed when different aspects of innate immune modulation are observed in vitro compared to in vivo. The majority of the data available on the innate immune responses to *Yersinia* infection have to do with the cytokine response to infection and how *Yersinia* modulates this response. In vitro studies suggest that *Yersinia* is capable of inducing a proinflammatory response at the earliest stages of infection. Ligation of β_1 integrins by *E. coli* expressing either Inv or YadA in vitro leads to the induction of IL-1, MCP-1, and IL-8 suggesting that the initial interaction with the gastric epithelium can be proinflammatory (Kampik et al. 2000). Numerous other studies have suggested that the Ysc TTSS and the Yops are potent inhibitors of proinflammatory signaling with the main effectors responsible for this phenotype being Yops J/P, E, and H (Cornelis 2002; Viboud and Bliska 2005). The enzymatic activities of all three of these effector proteins are known and several of the cellular target proteins involved in inhibiting proinflammatory signaling are known as well.

Yop J/P is a protein acetylase that acetylates serines and threonines present in the activation loops of mitogen-activated kinases (Map kinase) and IκB kinase β (IKKβ) (Mukherjee et al. 2006). There is also some evidence that YopJ is capable of inhibiting signaling through MAPK, NF-κB, and IRF-3 by blocking the ubiquitination of upstream molecules TRAF3 and TRAF6, supporting an earlier claim that YopJ/P is a de-ubiquitinating protease (Orth et al. 1999; Sweet et al. 2007). These data are based on overexpression studies in a reconstituted system and need to be re-examined under infection conditions where physiologically relevant levels of YopJ are present. Currently, the precise mechanisms of YopJ/P mediated inhibition of signal transduction are controversial, but ultimately, YopJ/P activity leads to the inhibition of NF-κB, p38, and ERK-dependent proinflammatory cytokine signaling (Palmer et al. 1999).

YopH, a protein tyrosine phosphatase, is also involved in the inhibition of proinflammatory cytokine signaling by inhibiting the release of MCP-1 through a wortmanin-sensitive PI3 kinase/Akt-dependent mechanism (Sauvonnet et al. 2002). The molecular basis of this observation remains to be determined but it is likely that YopH has a much more involved role in the modulation of inflammatory signaling than previously appreciated (Dube, unpublished data).

YopE, a Rho-GAP, has also been shown to impact the production of proinflammatory cytokines by a poorly understood mechanism (Schotte et al. 2004). YopJ/P appears to be the major effector involved in blocking proinflammatory responses, but unlike YopE and YopH, YopJ/P is dispensable for causing disease in mice (Trulzsch et al. 2004). Inhibition of proinflammatory responses by the Yops may be a critical initial step in the pathogenesis of disease happening in the first few hours post-infection, because in the mouse model, by 48 h post-infection, there is a robust increase in proinflammatory cytokines and noticeable changes in the histopathology of the Peyer's patches (Dube et al. 2003, 2004; Handley et al. 2004, 2006).

During infection of the mouse, a number of cytokines are critical to the disease process, including cytokines that are required to induce pathology and cytokines that are needed to establish an appropriate series of immune responses. A number of IL-1 family members play a central role in the pathogenesis of a *Yersinia* infection, including IL-1α, IL-1β, and IL-18 (Beuscher et al. 1992; Bohn et al. 1998; Dube et al. 2001). IL-1α and IL-1β are potent endogenous pyrogens that are rapidly induced in response to activation of PRR and during a *Y. enterocolitica* infection (Dinarello 1997, 1998; Patarca and Fletcher 1997). More recent data showed that IL-1α expressed in response to a RovA-regulated gene product was critical for inducing inflammatory responses in the gut (Dube et al. 2001, 2003).

IL-1β and IL-18 are both cytokines produced and secreted by macrophages and other cells when there is activation of the inflammasome. The inflammasome is a multiprotein complex that is formed after activation of PRR or other stimuli that ultimately leads to increased levels of IL-1β and IL-18 being secreted from the cell (Gurcel et al. 2006; Martinon et al. 2002). IL-1β and IL-18 are both substrates for the inflammasome associated protease caspase-1. Caspase-1 is critical for the maturation of IL-1β and IL-18 but not IL-1α, which is processed by calpain; however, new evidence suggests that caspase-1 may be important for the secretion of IL-1α from the cell (Dinarello 1998; Keller et al. 2008). IL-18 is known to be important for controlling a *Y. enterocolitica* infection as studies with IL-18-deficient mice demonstrated that these mice were more sensitive to infection than control mice (Bohn et al. 1998; Hein et al. 2001). Although not formally proven, the IL-18 deficient mice are probably more sensitive to infection due to the important role of IL-18 in inducing IFN-γ. Activation of the inflammasome in resting macrophages during *Y. enterocolitica* or *Y. pseudotuberculosis* infection is linked to YopJ/P-dependent macrophage apoptosis and in activated macrophages pyroptosis due to decreased NF-κB-dependent pro-survival signals (BCL-2) (Bergsbaken and Cookson 2007; Zhang et al. 2005). The ability of *Yersinia* to induce cell death in macrophages requires TLR-4, suggesting that cells can recognize the presence

of the bacteria (Zhang and Bliska 2003). This is a significant finding since reports suggest that *Y. pseudotuberculosis* and *Y. pestis* modify their lipid A moieties to be less immuno-stimulatory at 37°C as an immune evasion strategy (Rebeil et al. 2004). In contrast, the lipid A of *Y. enterocolitica* remains immuno-stimulatory at 37°C (Rebeil et al. 2004). Altogether, IL-1 family members play a central role in the pathogenesis of a *Yersinia* infection and may be central mediators of the host response to infection. As such, *Yersinia* has targeted IL-1 responses and the inflammasome as a point of immune evasion. Induced cell death is a potent inflammasome-dependent immune evasion strategy employed by the *Yersinia* that allows the bacteria to escape cell-mediated innate immune responses.

In addition to the IL-1 family members, a number of other cytokines have been shown to be important for the control of the infection. Most notably, TNF-α, IL-12, IL-6, and IFN-γ are critical for the control of a *Yersinia* infection (Autenrieth and Heesemann 1992; Autenrieth et al. 1996; Dube et al. 2004). IL-18 and IL-12 are potent inducers of IFN-γ and probably play an indirect role in macrophage activation and a direct role in Th-1 CD4 T cell polarization during *Yersinia* infection. The ability of activated macrophages to control a *Yersinia* infection is best demonstrated with *Y. pestis* infection; in early studies, it was shown that mice primed with recombinant TNF-α or IFN-γ were resistant to infection (Nakajima and Brubaker 1993). More recently, elegant studies on mice latently infected with herpes virus demonstrated that these mice had higher levels of circulating activated macrophages and were resistant to both *Listeria monocytogenes* and *Y. pestis* infection, suggesting a central role for activated macrophages in control of these infections (Barton et al. 2007). Considerable data suggest that *Y. enterocolitica* infection is ultimately cleared by a IFN-γ-dependent Th-1 T cell response.

TNF-α and IL-6 are both pleiotropic cytokines that can act as endogenous pyrogens, and can activate the endothelium to facilitate the influx of immune cells to the site of infection (Kishimoto 1987; Koj 1985). TNF-α is also an important cytokine in macrophage activation and dendritic cell maturation (Hundsberger et al. 2008). In vitro TNF-α levels are decreased in response to YopJ/P, but in vivo TNF-α is readily detected in the Peyer's patches of mice 24–48 h post-infection, suggesting that if there is any suppression of TNF-α responses in vivo it likely happens early during infection or that the suppressed level is still readily detectable (Dube et al. 2001; Handley et al. 2004; Sauvonnet et al. 2002). IL-6 is a cytokine that is upregulated in response to IL-1 and has a multitude of roles in the host response from inducing fever and acute phase responses to acting as a B cell growth factor (Koj 1985; Van-Snick 1989). We recently reported that mice deficient in IL-6 have a hyperinflammatory response to *Y. enterocolitica* infection, and profound defects in the expression of other cytokines such as IL-10, TGF-β, and IFN-γ during infection (Dube et al. 2004). These defects could be reversed by the administration of recombinant IL-6 to the IL-6-deficient mice linking the defects to the IL-6 deficiency and not to a non-specific response or some other defect in these animals (Dube et al. 2004). The other interesting aspect of this study was that the data suggested that IL-6 might be acting to temper the inflammatory response to infection. This is consistent with a rapidly expanding body of literature indicating that IL-6 can serve as an

anti-inflammatory cytokine as well as a proinflammatory cytokine (Diehl et al. 2000). As we learn more about IL-6 during infection, we are likely to gain greater insights into the control of the inflammatory response by *Y. enterocolitica* during infection. For example, the aberrantly low levels of TGF-β in the IL-6-deficient mice indicate that TGF-β may be important for regulating inflammatory responses to infection. This finding is consistent with older data that demonstrated that mice treated with recombinant TGF-β were protected from infection (Autenrieth et al. 1996). In agreement with these studies, we recently demonstrated that mice immuno-depleted of TGF-β were sensitive to *Y. enterocolitica* infection succumbing to infection more rapidly than control animals (Y. Zhong and Dube, in preparation). TGF-β is a very potent anti-inflammatory cytokine that has many roles in the regulation of immune responses. The fact that both IL-6 and TGF-β appear to function in the negative regulation of proinflammatory responses during *Yersinia* infection illustrates that immune evasion by *Yersinia* can target host responses that activate host immunity and those that temper inflammatory responses.

An important role of cytokines during infection is to appropriately polarize the adaptive immune response to infection. Delaying or inappropriately polarizing an adaptive immune response to an infectious agent is a way of targeting innate immunity for immune evasion that ultimately impacts the adaptive response. As we become more aware of the growing subsets of T cells and the roles cytokines play in their differentiation, it is likely that *Yersinia* is capable of directly modulating T cell differentiation through manipulation of the cytokine environment. For example, it is well established that Th-1-type responses are important for the control of a *Yersinia* infection but IL-6, IL-12p40, TGF-β, and IL-10 are important for the development of two newly described subsets of CD4 T-cells: Th-17 and T-regulatory cells (Awasthi et al. 2008). The role of these T cell subsets during infection remains to be investigated.

8 Interaction with the Adaptive Immune System

Most infections of humans with *Y. enterocolitica* or *Y. pseudotuberculosis* are self-limiting indicating that adaptive immunity adequately controls infection. After infection, *Yersinia* reactive serum antibodies are present and antigen-specific T cells can be cloned from infected mice (Autenrieth et al. 1992, 1993b, 1994; Bottone 1997). In mice, a number of investigations have demonstrated that both CD4+ T cells of the Th-1 phenotype and CD8+ T cells are critical for the control of infection and potential targets for immune evasion (Autenrieth et al. 1992, 1993b, 1994).

YopH targets lymphocytes in vitro for immune modulation by disrupting T cell and B cell receptor activation by dephosphorylating downstream signaling molecules. This ultimately leads to decreased expression of the co-stimulatory molecules B7.2 and CD69 as well as lower levels of IL-2 (Alonso et al. 2004; Yao et al. 1999). Presumably this is a potential mechanism *Yersinia* could use to prevent or

delay clonal expansion during the adaptive response, but this needs to be tested in vivo. The role of the newer subsets of T cells (Th-17 and T-reg) in the pathogenesis of infection remains to be determined.

There is growing evidence that *Yersinia* may modulate adaptive immune response at the level of antigen presentation by dendritic cells. However, much of this work has been done with *Y. pestis* infection or the presentation of *Y. pestis* antigens. It is likely that many aspects of the modulation of dendritic cells by *Y. pestis* will be similar to the mechanisms employed by the enteropathogenic *Yersinia*. However, there are also clear indications that the Yop effectors can have species-dependent effects. This is especially true of YopJ/P and its impact on the apoptosis of dendritic cells. A number of studies have suggested that YopJ-dependent apoptosis of dendritic cells is an important virulence mechanism, but that YopP of *Y. enterocolitica* is significantly more effective at inducing apoptosis in dendritic cells than the *Y. pestis* or *Y. pseudotuberculosis* YopJ (Adkins et al. 2008; Brodsky and Medzhitov 2008; Lindner et al. 2007; Velan et al. 2006). The interaction of *Yersinia* with dendritic cells is currently being investigated with much of the current data suggesting that *Yersinia* can induce apoptosis in dendritic cells thereby reducing the efficiency of the adaptive immune response. It is likely that the interaction of *Yersinia* with dendritic cells is much more complex and may encompass other aspects of pathogenesis such as dissemination.

9 New Insights on Old Paradigms

The application of new technologies to the analysis of the *Yersinia* host–pathogen interaction in the gut has forced a re-evaluation of several paradigms and illustrated numerous unexplored aspects of the pathogenesis of disease. The proliferation of genetically modified mice that are deficient in specific molecules and tissues has allowed for the re-examination of some of the most basic tenets of *Yersinia* pathogenesis. For example, as described in detail above, *Yersinia* utilizes the Inv protein to bind to β_1- integrins expressed on M cells promoting both targeting and invasion of the Peyer's patches. Subsequently, *Yersinia* is capable of disseminating to the mesenteric lymph nodes and in some cases the spleen and liver. However, a number of genetically modified mice are lacking Peyer's patches and other lymph organs allowing for the investigation of the absolute requirement for Peyer's patches in the dissemination of *Yersinia*.

Mice deficient in lymphotoxin-α or the lymphotoxin-β receptor are deficient in organized intestinal lymphoid tissues such as Peyer's patches, isolated lymphoid follicles, and mesenteric lymph nodes (Matsumoto et al. 1996, 1997a,b). These mice do have M cells and villous M cells as well as a normal overall immune cell composition (Jang et al. 2004). When lymphotoxin-α deficient or lymphotoxin-β receptor-deficient mice were infected with wild-type *Y. enterocolitica*, there was normal dissemination of the bacteria from the intestine to the spleen suggesting that

the Peyer's patches are dispensable for dissemination (Handley et al. 2005). Interestingly, dissemination from the intestine to the spleen in lymphotoxin-α-deficient mice is Inv dependent suggesting a role for Inv/M cell interactions in the absence of Peyer's patches (Handley et al. 2005). Isberg and co-workers significantly furthered this concept with a series of elegant genetic experiments that demonstrated colonization of the spleen and liver after *Y. pseudotuberculosis* infection required dissemination from the lumen of the small intestine (Barnes et al. 2006). More significantly, these studies demonstrated that the bacteria colonizing the spleen and liver of wild-type mice were derived from a distinct pool of bacteria separate from those in the mesenteric lymph nodes. Taken together, these studies demonstrate that the ordered progression of *Yersinia* from the lumen of the gut through the Peyer's patches and lymph nodes on route to the spleen and liver is not an accurate reflection of the actual pathogenesis of infection. In reality, neither Peyer's patches nor mesenteric lymph nodes are required for dissemination to the spleen and liver. Actually, colonization of the spleen and liver can result from a distinct pool of bacteria replicating in the intestinal lumen and is dependent on the Inv protein suggesting a more significant role for M cells or villous M cells in this process. These data suggest that dissemination of the bacteria is much more complex than previously appreciated and should be re-examined in the context of this newer data.

9.1 Global Analysis of Host Gene Expression in Response to Infection

The cellular immune response to *Yersinia* infection may also be significantly different from what is currently accepted. The influx of neutrophils, macrophages, and lymphocytes to the site of infection is well established and supported by many studies, but a recent analysis of global gene expression in infected Peyer's patches and mesenteric lymph nodes by Miller and co-workers suggests roles for a number of cells not previously implicated in the response to *Yersinia* infection (Handley et al. 2006). In this study, a micro-array analysis was done on infected Peyer's patches and mesenteric lymph nodes at several times post-infection to monitor changes in host gene expression over time. In many respects this analysis was very informative: (1) it confirmed many previous studies looking at the expression of cytokines and chemokines, (2) it identified multiple unrecognized cytokines and chemokines involved in the host response including IL-17 and IL-11, (3) it identified non-protein immune signaling molecules important in the response to *Yersinia*, and (4) it provided strong evidence for the involvement of histamine receptor signaling in the pathogenesis of disease. Due to limitations in the experimental design, it was impossible to determine if changes in transcript levels were due to increased/decreased gene expression, influx or egress of cells from the tissues, or death of cells in the tissues, but the changes in transcript levels did reveal many targets that would have been missed with a different approach.

One of the genes most highly "upregulated" after infection was histidine decarboxylase, the sole enzyme responsible for making the biogenic amine histamine. Histamine is a potent signaling molecule responsible for many aspects of homeostasis, and in the context of the immune system, histamine is potent effector molecule during allergy and the response to infection. After a *Y. enterocolitica* infection, histidine decarboxylase is rapidly upregulated in the Peyer's patches and histamine is detectable by immunohistochemistry at sites of active infection. Histamine exerts its biological effects through four histamine receptors, H1–H4, present on multiple cell types (Jutel et al. 2005). Using a variety of pharmacological agents, it was determined that histamine signaling through the H2 receptor was critical for the host response to *Yersinia* infection, as treatment with the H2 antagonist cimetidine decreased mouse survival and increased bacterial burdens in the Peyer's patches and mesenteric lymph nodes. Correspondingly, if mice were treated with the H2 agonist dimaprit, survival was increased and bacterial burdens were decreased. One of the effects of H2 antagonism is to decrease gastric acid secretion in the stomach resulting in an increased stomach pH, but when mice were treated with omeprazole, a proton pump inhibitor, and then infected with *Y. enterocolitica*, omeprazole had no impact on survival or bacterial burdens suggesting that the protective effect of histamine-H2 signaling in response to *Y. enterocolitica* infection is independent of effects on stomach pH.

Unfortunately this study was unable to determine the cellular source of the histamine or the responding cells. Because many cells express the histamine receptors, it may be difficult to determine the responding cells, but the cells producing histamine should be readily identified. It is intriguing to speculate that mast cells or basophils may have a role in the host response to infection. There is a significant concentration of both cell types in the gut and they have preformed stores of histamine allowing for a rapid response to infection. Mast cells have been implicated in the host response to other Gram-negative pathogens and it would not be surprising if they were also important for controlling a *Yersinia* infection, but this needs to be formally tested (Abraham and Malaviya 2000; Malaviya et al. 1994, 1996; McLachlan and Abraham 2001).

Altogether, a number of recent studies have cast some doubt on widely accepted aspects of *Yersinia* pathogenesis requiring a re-evaluation of how we think about the infectious process. Under normal situations, the Peyer's patches are significant targets for colonization but other intestinal tissues may be significant contributors to colonization of the spleen and liver. The significant increase in IL-17 in infected tissues could be reflective of a Th-17 T cell response or a neutrophil based IL-17 response; more analysis is required to determine the role of this important cytokine in the pathogenesis of infection. With the identification of histamine and signaling through the H2 receptor, we must now incorporate non-protein immune signaling into our understanding of the pathogenesis of disease and the possibility that mast cells, basophils, or other immune cells not previously implicated in the infectious process may have a significant impact on the pathogenesis of disease.

10 Unresolved Aspects of Pathogenesis

Y. enterocolitica and *Y. pseudotuberculosis* are two of the best-understood human enteric pathogens. We have made significant progress in understanding tissue invasion, regulation of virulence factors, and host responses to infection, yet there are still many unresolved aspects of disease to be investigated. The kinetics of virulence factor expression in vivo and the reconciliation of phenotypes observed in vitro with the actual in vivo manifestations of disease still remain unresolved. The roles of many virulence factors identified in genetic screens remain to be tested in animal models of disease and included in the integration of host responses with the actions of the various virulence factors.

On the host side, there are numerous areas where continued investigation will shed light on the pathogenesis of infectious gastro-intestinal disease. The role of newly described T cell subsets such as Th-17 cells and T-reg cells in a *Yersinia* infection needs to be investigated as well as the recruitment of specific cells to the site of infection. In particular, the role of chemokines and chemokine receptors in the dynamic response to infection is unexplored. The work of Handley et al suggests multiple chemokines and receptors not previously identified are playing a role in disease including some with tissue-specific expression (Handley et al. 2006). The recruitment of immune cells to the site of infection is a well-known target for *Yersinia* immune evasion and these molecules represent possible targets for virulence factors. The same study also illustrated the role of non-protein immune signaling molecules in *Yersinia* infections. Undoubtedly, further analysis of the signaling mediated by histamine, leukotrienes, and other small molecules during a *Yersinia* infection will shed light on the gut's response to infection.

11 Summary

Disease in humans as a result of *Yersinia* infection can range from acute enteritis to chronic manifestations such as reactive arthritis and Grave's disease. *Y. enterocolitica* and *Y. pseudotuberculosis* are two well-studied human enteric pathogens that have been instrumental in our understanding of invasive intestinal pathogens and the resulting immune response to this type of infection. *Yersinia* rapidly senses and responds to the host environment by changing gene expression and elaborating the virulence factors required to survive in the host. These virulence factors include several TTSS and a large variety of chromosomally encoded genes as well as the pYV plasmid-encoded genes. *Salmonella* established a precedence of enteric pathogens utilizing several distinct TTSS at different stages of pathogenesis and it is now clear that *Yersinia* utilize multiple specialized secretion systems to help deliver virulence factors into host cells. Like *Salmonella*, it now appears that each TTSS may be important at a specific stage of infection. There is growing

evidence that tissue-specific virulence factor expression is important for disease, with some mutants being attenuated by natural routes of infection but nearly as virulent as the wild-type bacteria by other routes of infection.

The host detects infection and responds by initially inducing a potent proinflammatory response as part of innate immunity and then a humoral and cellular response to clear the infection. *Yersinia*'s virulence factors effectively neutralize several important aspects of innate immunity, such as the complement attack, giving the bacteria a chance to establish infection. Recent technological advances have allowed us to explore the pathogenesis of infection in greater detail revealing unexpected routes of dissemination and unexplored host responses. These studies have also opened and re-opened many avenues of research ensuring that *Yersinia* will remain an ideal model for exploring the pathogenesis of invasive human bacterial infections.

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Molecular Mechanisms of *Salmonella* Virulence and Host Resistance

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Contents

Abstract *Salmonella* species can cause typhoid fever and gastroenteritis in humans and pose a global threat to human health. In order to establish a successful infection, *Salmonella* utilize a large number of genes encoding a variety of virulence factors. Different animal models of infection have been used to better understand the mechanisms underlying each disease including cattle, rodents, and nematodes. To date, a number of different bacterial virulence factors have been identified using such animal models, most of which are secreted by two type three secretion systems (T3SS) encoded within *Salmonella* pathogenicity islands (SPI) 1 and 2. These proteins alter various host cell pathways, facilitating the invasion of epithelial cells during infection, as well as the survival and replication of *Salmonella* inside phagocytic cells. On the other hand, host genetics and resistance also play a role in

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the susceptibility to *Salmonella* infection. The natural resistance-associated macrophage protein 1 (Nramp1), for example, is critical for host defense, since mice lacking Nramp1 fail to control bacterial replication and succumb to low doses of *S*. Typhimurium. In this chapter, we analyze the different pathogen and host factors that play a role in the dynamic interaction between *Salmonella* and its host and their impact on disease.

Abbreviations

APC	Antigen presenting cell
CD	Cluster of differentiation
DC	Dendritic cell
GALT	Gut-associated lymphoid tissue
IFN-γ	Interferon-γ
IL (e.g. IL-1)	Interleukin
iNOS	Inducible nitric oxide synthase
IκBα	Inhibitory protein κ-B α
Lamp1	Lysosome-associated membrane protein
LM	Lamina propria
LPS	Lipopolysaccharide
M Cell	Microfold cell
M6PR	Mannose-6-phosphate receptor
MAPK	MAP kinase
MHC-II	Major histocompatibility complex II
MLN	Mesenteric lymph node
NF-κB	Nuclear factor κB
Nramp1	Natural resistance-associated macrophage protein 1
PAMP	Pathogen associated molecular pattern
phox	Phagocyte oxidase
PMN	Polymorphonuclear leukocyte
PP	Peyer's patches
PRR	Pattern recognition receptor
RES	Reticuloendothelial system
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S. Enterica	*Salmonella enterica*
S. Paratyphi	*Salmonella enterica* serovar Paratyphi
S. Typhi	*Salmonella enterica* serovar Typhi
S. Dublin	*Salmonella enterica* serovar Dublin
S. Enteritidis	*Salmonella enterica* serovar Enteritidis
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
SCL11A1	Solute carrier family of multimembrane spanning protein 11
SCV	*Salmonella*-containing vacuole

SPI	*Salmonella* pathogenicity island
T3SS	Type three (III) secretion system
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-α
WT	Wild-type

1 Salmonelloses

Salmonelloses are diseases caused by *Salmonella* species. *Salmonella* are Gram-negative, facultative, intracellular bacteria. The genus *Salmonella* is divided into two species: *Salmonella bongori* and *Salmonella enterica*. *S. bongori* resides primarily in reptiles and infrequently causes disease in warm-blooded animals. The species *S.* Enterica contains over 2,000 serovars (Fierer and Guiney 2001), a few of which are etiological agents of two major human diseases causing significant morbidity and mortality worldwide, particularly in the developing world.

Salmonella enterica serovar *Typhi* (*S. typhi*) and *Paratyphi* (*S.* Paratyphi), cause typhoid fever, a systemic disease characterized by fever, intestinal perforation and hemorrhage, enlargement of the mesenteric lymph nodes (MLN), spleen and liver (Parry et al. 2002). *S. typhi* is a highly adapted human pathogen and does not cause disease in other animals. Humans are the major reservoir for *S. typhi*, which is spread through the fecal–oral route, usually by ingestion of food or drinking water contaminated with the bacteria. In the absence of complications, the disease usually is resolved after 4 months, although asymptomatic maintenance and shedding of the bacteria can continue in a minority of individuals (1–6%) for up to a year or longer (Monack et al. 2004b). The disease is endemic in Asia, Africa and South America with an estimated incidence of 17–21 million cases worldwide each year, leading to 600,000 deaths (2003).

Salmonella enterica serovars *Enteritidis* (*S.* Enteritidis) and *Typhimurium* (*S.* Typhimurium) cause gastroenteritis or "food poisoning", a self-limiting disease characterized by diarrhea, abdominal pain, nausea, vomiting, and fever. The acute enteritis is characterized by mucosal edema and inflammation mostly in the large intestine with recruitment of polymorphonuclear leukocytes (PMN) (Santos et al. 2001b). Symptoms occur between 6 and 72 h after consumption of contaminated animal products such as chicken or eggs, last for up to a week, and resolve spontaneously. *S. e*nteritis represents a major economic problem worldwide for both humans and animals and is one of the most frequent causes of bacterial foodborne disease in North America. An estimate of 1.3 billion cases of intestinal disease, including 3 million deaths, has been reported worldwide. (Zhang et al. 2003b). Immunocompromised and young children are particularly vulnerable to severe forms of enteritis, and in isolated cases the infection can spread to systemic sites resulting in death (Wallis and Galyov 2000). Unlike *S. typhi*, which is host

specific. *S. typhimurim* and *S.* Enteritidis have a broad host range, causing acute enteritis in humans and other animals.

2 Animal Models and Overview of the Disease

Animal models are frequently used to develop an improved understanding of the complex mechanisms that lead to salmonellosis in humans (Santos et al. 2001b; Zhang et al. 2003b). These models have been invaluable in defining both the key bacterial virulence factors and the host's responses, thereby clarifying the process and outcome of these diseases. Many models are used to study the two major human diseases, typhoid fever and gastroenteritis, and they encompass human volunteers, monkeys, calf, cow ileal loops, rabbits, rodents, and the nematode *Caenorhabditis elegans* (Alegado and Tan 2008; Paulander et al. 2007; Santos et al. 2001b). The most widely used animal model is the mouse model, since it offers genetic homogeneity, along with well-defined genetic mutants that permit the study of specific genes, cell types, and pathways in host-pathogen interactions (Grassl and Finlay 2008). Below, description of the mouse models for the study of both typhoid fever and gastroenteritis are presented.

2.1 The Typhoid Model

To study the pathogenesis of human typhoid fever, a surrogate host (genetically susceptible inbred mice) and bacterium (*S.* Typhimurium) are used, providing a useful model for this disease (reviewed in Santos et al. 2001b). *S. typhi* does not infect rodents whereas *S. Typhimurium* is a natural mouse pathogen. Importantly, the pathology associated with *S.* Typhimurium infection of mice closely resembles that of *S. typhi* in humans. Hallmarks include enlarged Peyer's patches and thickening of ileal mucosa. Other areas of the small intestine show a diffuse enteritis characterized by mononuclear cell infiltration, with no signs of intestinal epithelium destruction (reviewed in Parry et al. 2002). As in humans infected with *S. typhi*, mice infected with *S.* Typhimurium display disseminated infection and multiplication of bacteria in the liver and spleen, where large granulomatous lesions develop around infected macrophages. The *S.* Typhimurium murine model has become the accepted model for human infections with *S. typhi*. However, it is important to note a key shortcoming of this model. *S.* Typhimurium will infect humans but leads to enteritis rather than the typhoid fever-like symptoms described above. This suggests that, in the context of different hosts (mouse and human), the same pathogen (*S. Typhimurium)* can cause very different diseases (typhoid fever vs infectious colitis). Also, it is known that some of the bacterial pathogenicity genes are not shared between *S. typhi* and *S.* Typhimurium.

For example, *S*. Typhimurium lacks a capsule, yet the Vi antigen is an important virulence factor in *S. typhi*. Therefore, although *S*. Typhimurium can be used to model the pathology associated with *S. typhi* infections in humans, it is not an ideal model to study the role of specific bacterial virulence factors and extrapolate their role to human disease. With this important caveat in mind, the *S*. Typhimurium model is still useful in studying general host responses to typhoid fever-type infections.

With regards to the specific effects of infection in the murine typhoid model, orally-ingested *S*. Typhimurium cross the intestinal barrier by three mechanisms: (1) invasion of specialized epithelial cells, termed M-cells, that are situated in the Peyer's patches (PP) and are known to sample antigens from the intestinal lumen, (2) active invasion of enterocytes, and (3) uptake by intestinal dendritic cells (DCs) that express tight junction proteins and extend dendrites between epithelial cells for direct bacterial uptake (Niess et al. 2005; Rescigno et al. 2001; Sansonetti 2004; Vazquez-Torres et al. 1999).

Once the bacteria cross the mucosal epithelia, they encounter cells of the gut-associated lymphoid tissue (GALT) that include DCs, macrophages, B, and T cells (Rumbo et al. 2004). Contact of *S. Typhimurium* with these cells initiates a series of interactions between the bacteria and the host cells that initiates the disease (Rydstrom and Wick 2007). *Salmonella* gain access to the host circulation within $CD18^+$ cells (Vazquez-Torres et al. 1999). Whether these represent macrophages, DCs, or other myelomonocytic cells remains to be clarified. The bacteria then reach MLN, spleen and liver, and replicate within phagocytic cells in these organs to levels that induce sepsis in susceptible mice.

S. Typhimurium can survive within phagocytic and nonphagocytic cells. During late stages of infection *in vivo*, *Salmonella* can be found within macrophages (Monack et al. 2004a; Salcedo et al. 2001), DCs (Yrlid et al. 2001), neutrophils (Cheminay et al. 2004; Geddes et al. 2007), B cells, T cells, and hepatocytes (Conlan and North 1992; Geddes et al. 2007). In the spleen and liver, *S*. Typhimurium resides in granulomatous foci predominantly within phagocytes.

The dynamics of *S*. Typhimurium spread in the body at the level of individual infected cells is poorly understood. It has been suggested that, in the initial stages of the infection, each focus of infection consists of an individual phagocyte containing only one bacterium and that *Salmonella* growth in the tissues results in the continued passage of the bacteria to uninfected cells. Thus, *S*. Typhimurium growth in the tissues appears to result in an increased number of infected foci with only a small increase in the number of bacteria per cell (Sheppard et al. 2003). The increase in the number of infected foci triggers inflammatory responses, responsible for the recruitment and priming of phagocytes, cytokine release, hepatosplenomegaly, and sepsis, and ultimately leads to the death of susceptible animals (Mastroeni 2002; Mastroeni and Sheppard 2004). Chronic carriers have been reported in *S. typhimurium* infections of resistant mice (nramp+/+, see below) (Monack et al. 2004a, b). Although this model is just beginning to be explored, it has enormous potential since it may more accurately reflect the naturally occurring *S. typhi* carrier state in humans (Monack et al. 2004b).

2.2 The Enterocolitis Model

*S. t*yphimurium infection of calves and of cow ileal loops can induce gastroenteritis with clinical manifestations similar to those found in humans. Although the information obtained from these infections is valuable, the use of these animals presents serious challenges for extensive experimentation. Cattle are outbred, thus creating an inherent variability between subjects. In addition, large animal models are expensive, making the use of these animals costly and less than ideal. This issue was overcome with the development of a mouse model for enterocolitis (Barthel et al. 2003). This model relies on the premise that oral infection of mice with *S. typhimurium* results in modest colonization of the intestine and with little or no inflammation. This was referred to as "colonization resistance." However, this barrier can be overcome by treatment of mice with antibiotics prior to infection with *S.* Typhimurium, resulting in a major increase in bacterial colonization. Although the molecular mechanisms responsible for colonization resistance are still under debate, it is believed that the antibiotics transiently alter the resident gut microbiota, allowing the colonization of the cecum and colon by the incoming pathogen. This colonization is accompanied by significant inflammation that is characterized by the infiltration of PMNs (Barthel et al. 2003) and intestinal pathology and pathophysiology that culminates in watery stools (Woo et al. 2008). These changes closely resemble the features of enterocolitis in humans (Hapfelmeier and Hardt 2005). This model is now being used extensively to examine the course of the disease in a large number of genetically uniform inbred mice. This enables the study of specific cell signaling pathways and the contribution of specific cells of the immune system in the development of colitis. It also provides a means of assessing *S.* Typhimurium mutant strains for their ability to induce colitis or modify the disease course (Coburn et al. 2005; Hapfelmeier et al. 2005, 2008). However, one caveat of this model is the development of "typhoid–like" disease that occurs in parallel to colitis, rendering mice susceptible to systemic infection and death. Thus, this model generates a mixed symptomatology of both typhoid fever and colitis. More recently, the use of genetically-resistant mice has provided valuable information about the dynamics of *S.* Typhimurium and host interactions at later time points, thereby providing information on the resolution of the "colitis-like" component of the disease (Grassl et al. 2008; Stecher et al. 2006; Valdez et al. 2009; Woo et al. 2008).

3 *S.* Typhimurium Virulence Determinants

S. typhimurium has acquired an arsenal of genes that are required to establish a successful infection within the host. Many of the virulence traits of *Salmonella* are directly linked to genes encoded within large regions of the bacterial chromosome called *Salmonella* pathogenicity islands (SPIs). Pathogenicity islands are

discrete chromosomal regions harboring virulence genes. These are common attributes of Gram-negative bacterial pathogens and encode virulence factors, together with the machinery for their regulation and secretion. Such pathogenicity islands are absent from related, nonpathogenic species (Gal-Mor et al. 2006). Pathogenicity islands have DNA G+C contents that are noticeably different from the rest of the bacterial genome, indicating horizontal transmission from other bacteria at some point during evolution. *S*. Typhimurium contains numerous identified SPI, termed SPI-1, SPI-2, etc. SPI-1 and SPI-2 are of particular relevance for pathogenesis, since mutations in these genes impair the bacteria's ability to induce infection in their host by encoding specialized secretion systems (Hansen-Wester and Hensel 2001)

The pathogenicity of *S*. Typhimurium is contingent on specialized machinery called a type III secretion system (T3SS). Effectively, these are molecular syringes used to deliver virulence proteins, called effectors, directly into the host cells where they ultimately manipulate the cellular functions and facilitate the progression of the infection (Galan 2001; Galan and Wolf-Watz 2006). *S*. Typhimurium possesses two distinct virulence-associated T3SS encoded within SPI-1 and SPI-2. Each of the T3SS is used to translocate a unique set of effectors during different phases of the infection in order to manipulate various host pathways (reviewed in Hansen-Wester and Hensel 2001; Hensel 2004). Both T3SS are essential for bacterial virulence, each at different stages of the infection process (Lucas and Lee 2000; Marcus et al. 2000).

3.1 Salmonella *Pathogenicity Island 1 (SPI-1)*

SPI-1 is present in all serovars of both *S*. Enterica and *S*. *bongori* (Ochman and Groisman 1995) and has been shown to be important for the intestinal phase of *Salmonella* infections, whether they progress to a systemic disease or inflammatory diarrhea (Darwin and Miller 1999; Wallis and Galyov 2000). It has been hypothesized that acquisition of SPI-1 allowed *Salmonella* to become an enteric pathogen (Hensel 2004). SPI-1 mutants are attenuated for oral but not intraperitoneal (systemic) infections in the murine typhoid model (Galan and Curtiss 1989), and display attenuated enteropathogenicity in bovine ileal loops (Zhang et al. 2003a). SPI-1 appears to be important mostly for the initial steps of active *Salmonella* invasion of epithelial cells following oral infection, as well as the consequent inflammatory cascade characteristic of intestinal salmonellosis.

The SPI-1 consists of a 40-kb DNA region encoding T3SS structural, effector, and regulatory proteins (Hansen-Wester and Hensel 2001). The T3SS encoded by SPI-1 is regulated in a particularly complex fashion, and responds to several environmental and physiological signals, which are integrated to control the secretion of the effector proteins, both within the SPI-1-encoded region and outside this region (Altier 2005; Ellermeier and Slauch 2007; Jones 2005). The majority of SPI-1 genes are expressed under conditions that resemble the intestinal environment

and are repressed once *Salmonella* colonizes an intracellular compartment. These genes are controlled by five regulators encoded within the SPI-1 region, called HilA, HilC, HilD, InvF, and SprB (Darwin and Miller 1999; Eichelberg and Galan 1999; Rakeman et al. 1999; Schechter et al. 1999). Of these regulators, HilA plays a central role, and a deletion of *hilA* was shown to be phenotypically similar to a deletion of the entire SPI-1 locus (Ellermeier et al. 2005). SPI-1 regulators can also regulate SPI-2 genes. For example, HilA binds and represses the promoter of *ssaH* (Thijs et al. 2007), a SPI-2 encoded gene, and HilD binds and activates the promoter of the *ssrAB* operon (Bustamante et al. 2008), the main regulator of SPI-2 genes. The two-component system PhoP/Q also plays a major role in SPI-1 and also SPI-2 regulation. PhoPQ regulates genes in response to extracellular cation levels (Garcia Vescovi et al. 1996). When the cation concentration is low, PhoQ activates the regulator PhoP by phosphorylation. A low cation environment exists in macrophages, where PhoP activates genes required for survival in macrophages, such as SPI-2-encoded genes (Alpuche Aranda et al. 1992). On the other hand, PhoP has also been shown to repress invasion genes encoded by SPI-1, playing a dual role in *Salmonella* virulence (Bajaj et al. 1996; Behlau and Miller 1993; Pegues et al. 1995).

SPI-1 effectors influence a variety of host cell functions such as cytoskeletal rearrangements (e.g., ruffling) that mediate bacterial uptake by epithelial cells (Hansen-Wester and Hensel 2001), tight-junction disruption macrophage apoptosis (Boyle et al. 2006; Monack et al. 2000; Monack et al. 1996), and neutrophil recruitment (McCormick et al. 1993; Wall et al. 2007) (Table 1). Many SPI-1 effectors appear to promote PMNs migration or fluid accumulation in the bovine gastroenteritis model, possibly by inducing cytokine secretion themselves or by attracting host cells that increase cytokine production (Wallis and Galyov 2000). Their role after bacterial invasion has been a focus of study (Brawn et al. 2007; Giacomodonato et al. 2007). Some SPI-1 secreted effectors like SopB, SopD and SopE2 are present in a wide variety of *Salmonella* serotypes, indicating a major role of these proteins in SPI-1-dependent virulence (Mirold et al. 2001). Recently, SopB, SopD, SopE2, and SipA have also been shown to be synthesized by the bacteria during the final phase of murine infection, arguing that these effectors might have additional roles in the post-invasion stages of the disease (Giacomodonato et al. 2007). The expression of these proteins was detected in the MLNs and spleens of infected mice several days after inoculation; however, their function at late stages of infection remains to be determined.

3.2 Salmonella *Pathogenicity Island 2 (SPI-2)*

SPI-2 is present in *S. enterica* but absent in *S. bongori*, and its acquisition is thought to be a key step in the evolution of *Salmonella* as a systemic and intracellular pathogen (Hensel 2004). SPI-2 mutants are severely attenuated for virulence in the mouse typhoid model and fail to proliferate in internal organs (Hensel 2000). Thus, SPI-2 is essential for intracellular replication, which is necessary for systemic

Table 1 *Salmonella* secreted effectors

Protein	Gene localization	Proposed function	Model of infection	References
AvrA	SPI-1	Inhibition of NF-κB activation; stabilization of cell permeability and tight junctions	Murine colitis model	Collier-Hyams et al. (2002), Jones et al. (2008), Liao et al. (2008), Zhang et al. (2002b)
SipA	SPI-1	Invasion; actin polymeralization; prevention of filament disassembly; induction of membrane ruffles; induction of late endosomes redistribution; SCV localization; neutrophil recruitment across the intestinal epithelium	Bovine and murine colitis models	Brawn et al. (2007), Hapfelmeier et al. (2004), McGhie et al. (2001), Perrett and Jepson (2009), Zhou et al. (1999)
SipB	SPI-1	Translocon component; interaction with caspase-1; macrophages and DC apoptosis	Bovine colitis model	Collazo and Galan (1997), Hayward et al. (2000), Hersh et al. (1999), Santos et al. (2001a), van der Velden et al. (2003), Zhang et al. (2002b)
SipC	SPI-1	Translocon component; actin bundling and nucleation	Murine colitis model	Chang et al. (2007), Collazo and Galan (1997), Hayward and Koronakis (1999)
SlrP	Chromosome (SPI-1/SPI-2 effector)	Peyer's patch colonization; inhibition of antigen presentation	Murine typhoid model and Bovine colitis model	Halici et al. (2008), Tsolis et al. (1999), Zhang et al. (2002b)
SopA	Chromosome (SPI-1 effector)	Invasion; fluid accumulation; leukocytes migration	Bovine colitis model	Raffatellu et al. (2005b), Wood et al. (2000), Zhang et al. (2002a, 2006)
SopB	SPI-5 (SPI-1 effector)	Invasion; increase host inflammatory response; fluid secretion; upregulation of iNOS production; actin regulation; disruption of tight junctions; lysosome trafficking; SCV positioning; anti-apopototic activity	Bovine colitis model	Drecktrah et al. (2005, 2006), Halici et al. (2008), Hayward and Koronakis (2002), Jones et al. (1998), Knodler et al. (2005), Raffatellu et al. (2005b), Zhang et al. (2002b)
SopD	Chromosome (SPI-1 effector)	Invasion; host cell membrane manipulation; virulence in systemic infections	Bovine colitis model and murine typhoid model	Bakowski et al. (2007), Jiang et al. (2004), Raffatellu et al. (2005a), Zhang et al. (2002b)
SopE	Phage (SPI-1 effector)	Invasion; actin regulation, nuclear	Murine colitis model	Boyle et al. (2006), Hapfelmeier et al. (2004), Hardt et al. (1998),

(*continued*)

Table 1 (continued)

Protein	Gene localization	Proposed function	Model of infection	References
		responses; disruption of tight-junctions		Hayward and Koronakis (2002), Patel and Galan (2006), Raffatellu et al. (2005b)
SopE2	Phage remnant (SPI-1 effector)	Invasion; colitis; pathogenesis of diarrhea; regulation of IL-8 production; upregulation of macrophages iNOS; actin regulation; disruption of tight-junctions	Bovine and murine colitis models	Boyle et al. (2006), Cherayil et al. (2000), Hapfelmeier et al. (2004), Hayward and Koronakis (2002), Huang et al. (2004), Raffatellu et al. 2005b, Wallis and Galyov (2000), Zhang et al. (2002a)
SptP	SPI-1	Disruption of actin cytoskeleton; inhibition of MAP kinase trough Raf-1	Macrophage cell line	Fu and Galan (1999), Lin et al. (2003), Murli et al. (2001), Zhang et al. (2002b)
SspH1	Gifsy-3 prophage (SPI-1/ SPI-2 effector)	Downregulation of NF-κB-dependent gene expression	Bovine colitis model	Miao et al. (1999), Zhang et al. (2002b)
SteA	Chromosome (SPI-1 effector)	Virulence	Murine typhoid model	Geddes et al. (2005)
SifA	Chromosome (SPI-2 effector)	Sif formation; SCV integrity and positioning; *Salmonella* replication; mimic host Rab GTPases; host membrane tubulation; LAMP-2 recruitment to SCV and Sifs; inhibition of antigen presentation	Murine typhoid model	Alto et al. (2006), Beuzon et al. (2000), Boucrot et al. (2003, 2005), Brawn et al. (2007), Halici et al. (2008), Ohlson et al. (2008), Roark and Haldar (2008), Stein et al. (1996)
SifB	Chromosome (SPI-2 effector)	Localize to Sifs and SCV	Epithelial and macrophage cell lines	Freeman et al. (2003)
SseF	SPI-2	Sif formation; SCV positioning; protection against macrophage oxidation	Murine typhoid model	Abrahams et al. (2006), Deiwick et al. (2006), Hensel et al. (1998), Kuhle and Hensel (2002), Suvarnapunya and Stein (2005)
SseG	SPI-2	Sif formation; SCV positioning; protection against macrophage oxidation	Murine typhoid model	Hensel et al. (1998), Kuhle and Hensel (2002), Salcedo and Holden (2003), Suvarnapunya and Stein (2005)
SseJ	Phage (SPI-2 effector)	Cholesterol esterification; intracellular bacterial	Murine typhoid model	Lawley et al. (2006), Lossi et al. (2008),

(*continued*)

Table 1 (continued)

Protein	Gene localization	Proposed function	Model of infection	References
		survival; membrane dynamics		Nawabi et al. (2008), Ohlson et al. (2005), Ruiz-Albert et al. (2002)
SseI	Gifsy-2 prophage (SPI-2 effector)	Phagocyte motility	Murine typhoid model	Lawley et al. (2006), Worley et al. (2006)
SseK1	Chromosome (SPI-2 effector)	No virulence effect	Murine typhoid model	Kujat Choy et al. (2004)
SseK2	Chromosome (SPI-2 effector)	Virulence in long-term systemic infections	Murine typhoid model	Kujat Choy et al. (2004), Lawley et al. (2006)
SseL	Chromosome (SPI-2 effector)	Suppression of NF-κB activation; delayed cytotoxic effect on macrophages; virulence	Murine typhoid model	Coombes et al. (2007), Le Negrate et al. (2008), Rytkonen et al. (2007)
SopD2	Chromosome (SPI-2 effector)	Sif formation; virulence; inhibition of antigen presentation	Murine typhoid model	Brumell et al. (2003), Halici et al. (2008), Jiang et al. (2004), Lawley et al. (2006)
SpiC	SPI-2	Translocation; vesicular trafficking; flagellar regulation; activation of MAPK pathway in macrophages; suppression of cytokine signaling 3 in macrophages	Murine typhoid model	Freeman et al. (2002), Knodler and Steele-Mortimer (2005), Uchiya and Nikai (2005, 2008)
PipB	SPI-5	Targeted to Sifs; cecal colonization in chicks; fluid secretion and inflammation	Chicks and bovine colitis model	Knodler and Steele-Mortimer (2005), Knodler et al. (2003), Morgan et al. (2004), Wood et al. (1998)
PipB2	Chromosome (SPI-2 effector)	Sif formation; inhibition of antigen presentation; virulence; kinesin-1 recruitment to SCV	Murine typhoid model	Halici et al. (2008), Henry et al. (2006), Knodler and Steele-Mortimer (2005), Knodler et al. (2003)
SspH2	Phage (SPI-2 effector)	Virulence, actin polymeralization; inhibition of antigen presentation	Bovine colitis model	Halici et al. (2008), Miao et al. (1999, 2003)
GogB	Gifsy-1 prophage (SPI-1 / SPI-2 effector)	Unknown	ND	Coombes et al. (2005b)

ND Not determined

disease (Hensel et al. 1995, 1998; Shea et al. 1996). Specific defects attributed to SPI-2 mutants are a reduced ability to survive in macrophages (Hensel et al. 1998) perhaps due to a failure to form the *Salmonella*-containing vacuole (SCV). This vacuole is thought to be a unique intracellular niche in which *Salmonella* survives and replicates (Holden 2002). SPI-2-mediated secretion impairs trafficking of the oxidase-containing vesicles (Vazquez-Torres and Fang 2001b; Vazquez-Torres et al. 2000b, 2001) and iNOS to the SCV (Chakravortty et al. 2002). These are important mediators of the oxidative and nitrosative burst, thereby enhancing the survival of *Salmonella* within macrophages. SPI-2 also inhibits antigen presentation and T cell activation by DCs (Bueno et al. 2005; Cheminay et al. 2005; Tobar et al. 2006). Thus, SPI-2 allows *Salmonella* to avoid intracellular killing by both the innate and the adaptive immune system.

More recently, it has been shown that SPI-2 also plays an important role in the intestinal phase of *Salmonella* infection in mice and in colitis in the streptomycin-pretreated mouse model (Coburn et al. 2005; Coombes et al. 2005a; Hapfelmeier et al. 2004; Kuhle and Hensel 2004), indicating that it is involved in both typhoid and gastroenteritis.

SPI-2 is a 40-kb locus composed of two distinct regions which are though to have been acquired as independent events (Hensel et al. 1999). The larger region encodes for the major virulent factors including genes for the T3SS apparatus, regulation, chaperones, and effectors. The two-component regulatory system SsrAB is responsible for the regulation of SPI-2 genes and is the only transcriptional regulator encoded within SPI-2 that activates the expression of SPI-2 genes and other genes encoding T3SS effectors located outside SPI-2 (Shea et al. 1996; reviewed in Kuhle and Hensel 2004). Nutritional limitation seems to be sensed by the *ssrAB* system based on the composition of the minimal media that turns on SPI-2 expression *in vitro*, and should be encountered when the bacteria is intracellular. As mentioned before, PhoP/Q and also OmpR/Z (another global regulatory system) play a role in the regulation of SPI-2 genes (Deiwick et al. 1999; Lee et al. 2000).

Proteins that are transported by the SPI-2 T3SS apparatus can be divided into translocon and effector proteins. SseBCD are involved in translocation of SPI-2 effector proteins into the host cells (Kuhle and Hensel 2002; Nikolaus et al. 2001). Evidence suggests that SseA acts as a chaperone for SseB and SseD (Coombes et al. 2003; Zurawski and Stein 2003).

To date, several effector proteins that are secreted through the SPI-2 T3SS have been identified (Table 1); most of them are encoded outside the SPI-2 region. Some of these proteins contain a conserved N-terminal secretion domain that it is unique to SPI-2 effector proteins (Brumell et al. 2003; Miao and Miller 2000; Miao et al. 1999). The function and host cell target of the majority of these effectors remain to be discovered.

One of the best-characterized SPI-2 effectors is SifA, which is required for *Salmonella*-induced filaments (Sifs) formation (Stein et al. 1996). These filaments are specialized LAMP1-rich tubulovesicular structures of unknown function that extend along microtubules from the SCV. SifA is also essential for SCV integrity and *Salmonella* replication (Beuzon 2000). Bacteria lacking SifA have detrimental

motoraccumulation, which triggers abnormal SCV migration toward the cell periphery (Boucrot et al. 2005). SifA might also have the ability to mimic host Rab GTPases, by acquiring a similar eukaryotic membrane-targeting motif (Alto et al. 2006; Boucrot et al. 2003).

Recently, another SPI-2 effector, SseL, was shown to modulate host inflammatory response *in vivo*. Its expression was shown to suppress NF-κB activation of downstream IκBα kinases and to impair IκBα ubiquitination and degradation. Mice infected with an *sseL* mutant showed a stronger inflammatory response, associated with increased production of NF-κB-dependent cytokines (Le Negrate et al. 2008). Another recent study demonstrated that SseJ has serine-dependent phospholipase A and cholesterol acyltransferase activities that seem to be increased in the presence of unknown eukaryotic factors (Lossi et al. 2008). SseJ was recently demonstrated to esterify cholesterol, which is present in high levels in SCV (Nawabi et al. 2008). An *sseJ* mutant is attenuated for virulence, suggesting that SseJ activity is important for intracellular bacteria survival.

Little is known about other SPIs. For example, SPI-3 encodes a high affinity Mg^{2+} uptake system that is thought to be important for *Salmonella* survival in the SCV (Blanc-Potard and Groisman 1997). SPI-4 encodes a nonfimbrial adhesin important for bacterial adherence to the apical surface of polarized cells (Gerlach et al. 2007; Wong et al. 1998). SPI-5 encodes effector proteins that are secreted by SPI-1 and SPI-2 T3SS (Hong and Miller 1998; Knodler et al. 2002; Wood et al. 1998), SPI-6 encodes fimbrial operons (Folkesson et al. 2002) and SPI-9 encodes a type I secretion system (Hensel 2004). Another island called SGI-1 (*Salmonella* genomic island I), found in multidrug-resistant *S*. Typhimurium strains, encodes for antibiotic resistance genes, such as tetracycline, ampicillin, chloramphenicol, streptomycin, and sulfonamides (Boyd et al. 2001), indicating a wide ability to acquire resistance genes.

4 Host Response to *Salmonella* Infection: Resistance/Susceptibility Genes

Susceptibility to *S*. Typhimurium in mice is determined by virulence factors expressed by bacteria as well as by the host genetics (Roy and Malo 2002). Additional experimental factors can influence the severity of the disease. These include route of infection, dose, immunological status, and stress of the host.

The host response to systemic *S*. Typhimurium infection is complex and under the influence of many genes. Several of the genes conferring susceptibility have been identified, and some of the most informative examples of mouse susceptibility are presented in Table 2. These are grouped according to "phases" that correspond to immunological events described by Mastroeni (2002). Compiling studies using sublethal infections, Mastroeni divided the course of the *Salmonella* infection in the mouse typhoid model into four distinct phases (Fig. 1). The first phase involves rapid clearance of the bacteria from the bloodstream (within 2 h after oral

Table 2 Key host genes conferring susceptibility in the murine typhoid model

Phases of *Salmonella* infection	Gene targeted mice	Deficiency function/mechanism of action	References
Phase 2	*gp91phox*$^{-/-}$ mice (NADPH oxidase)	Dramatic exacerbation of *Salmonella* infection in the early phase of infection by inability to produce ROS	Mastroeni et al. (2000)
	Natural mutation in Nramp1 mice: BALB/c, C57BL/6, DBA/1 *Nramp1*$^{-/-}$ mice	Fail in control of bacterial growth in the exponential phase, although controversial, it is believed that Nramp1 alters the intravacuolar environment of the SCV	Govoni et al. (1996), Roy and Malo (2002), Vidal et al. (1995b)
Phase 3	Natural mutation in TLR4 mice: C3H/HeJ *lbp*$^{-/-}$ mice (LPS protein binding) *Cd14*$^{-/-}$ mice *Tlr4*$^{-/-}$ mice	Deficiency in induction of a rapid inflammatory response, decreased expression of TNF-α and IL-6	Bernheiden et al. (2001), Lembo et al. (2003)
	Nos$^{-/-}$ mice (inducible nitric oxide synthase)	Mice can control early *Salmonella* replication, but are unable to suppress bacterial growth later and die earlier than control mice, deficient in generation of RNS	Mastroeni et al. (2000, 2002), Vazquez-Torres et al. (2000a)
	Tnfr55$^{-/-}$ (TNF deficient mice)	Fail to localize NADPH oxidase-containing vesicles to SCV leading to a impaired bacterial killing	Vazquez-Torres et al. (2001)
	Ifngr$^{-/-}$ (IFNγ deficient mice)	Fail in the formation of focal granulomas during *Salmonella* infection. Inability to activate phagocytes and to influence antibody class switching.	Bao et al. (2000)
	Il-12a$^{-/-}$ and *Il12b*$^{-/-}$ (IL-12 deficient mice) Neutralizing antibodies to IL-12	Deficiency in the production of IFNγ and increase production of IL-10 and IL-4	Eckmann and Kagnoff (2001)
	IL-6$^{-/-}$ mice	Lower levels of IgA against *Salmonella* LPS	Dunstan et al. (1996)
Phase 4	*cd28*$^{-/-}$ mice and *tcr-b*$^{-/-}$ mice (lacking mature T a/b T cells)	Impaired T cells activation and reduced T-B cells activation, higher bacterial load and deficient in *Salmonella* clearance	
	xid mice (impaired B cell activation and function) and *Igh-6*$^{-/-}$ (B cell deficient mice)	Defect in antibody production and deficiency in establishment of protective long-lasting Th1 type T cell immunity to *Salmonella*	

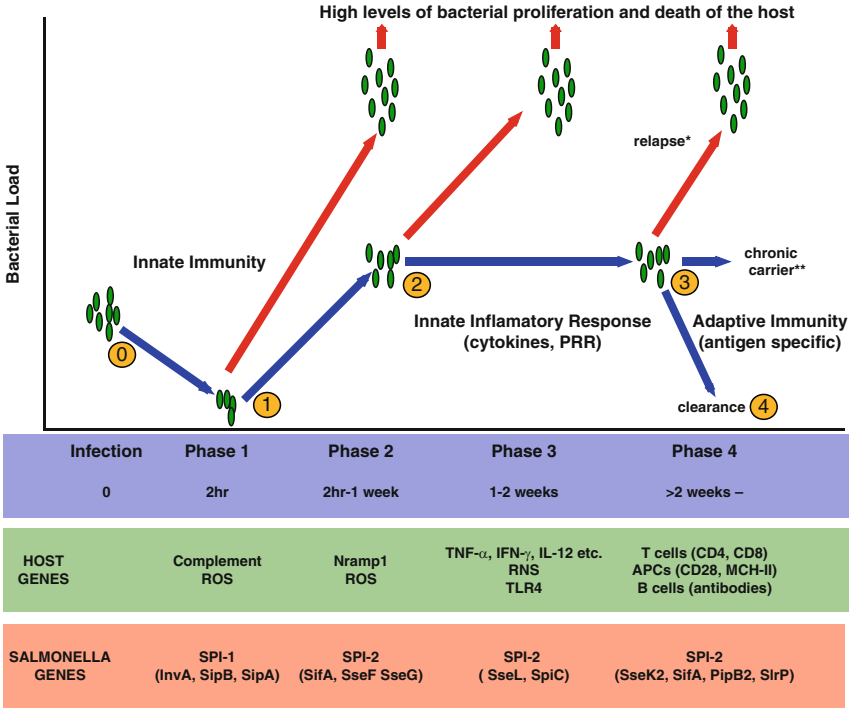

Fig. 1 The four phases of *Salmonella* infection. *Blue lines* show the course of sublethal infection with *S. typhimurium* in WT immunocompetent mice. *Red lines* show the course of the infection when the immunological mechanisms required to control bacterial replication at points 0–4 are absent. The Nramp1 gene and reactive oxygen species (ROS) influence the growth rate from 1 to 2. Their absence causes a shift of the curve and uncontrolled bacterial replication and death of the host occur. Point 2 corresponds to the initiation of cytokine response. Deficiency of innate immune responses in the points 2 and 3 determines failure to suppress bacterial growth. The infection progresses with fatal consequences for the host. Point 4 is the initiation of the adaptive immune responses. This response is required to clear the infection and prevent relapse (*) or the establishment of a chronic carrier state (**). Examples of important host genes known to be involved in the different phases of *Salmonella* infection are shown (green box). Examples of *Salmonella* effectors thought to be involved in the different phases of *Salmonella* infection are shown in red box. Reactive nitrogen species (RNS), toll-like receptors (TLR) and antigen-presenting cells (APC). Modified from Mastroeni (2002)

infection). After clearance from the circulation, *Salmonella* reach intracellular locations within macrophages, polymorphonuclear cells, and DCs in the spleen and liver. Although phagocytes kill some of the bacteria in the next few hours, the surviving bacteria undergo exponential replication, initiating the second phase. The growth rate in this phase is determined by the size of the innoculum and the innate resistance of the host. The natural resistance-associated macrophage protein 1 (Nramp1), plays a critical role in controlling *Salmonella* replication in this phase (Benjamin et al. 1990). Other mediators such as reactive oxygen species (ROS) are also crucial for *Salmonella* killing in this phase. The third phase is initiated by the

activation of the innate immune system, and is characterized by the production of several proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-12 (IL-12). These arise as a consequence of signaling by pattern recognition receptors (PRR) on the immune cells that recognize pathogen-associated molecular patterns (PAMPS) such as LPS. The action of these cytokines is essential for activation of immune cells, which in turn produce antimicrobial mediators such as reactive nitrogen species (RNS), which suppress bacterial growth (plateau phase). This phase is also important for activation of the adaptive immune response. The fourth phase, or resolution of the infection, is a function of the adaptive immune system. The key players in this phase are antigen-presenting cells (APC), B, and T cells and the activation of the antigen-specific humoral immune response (antibody production), as well as T-helper and cytotoxic T-lymphocyte responses. If these responses are not efficient, a relapse or carrier state can develop in the host (Mastroeni 2002).

5 Contribution of Host Phagocytic Cells and Their Role in *S.* typhimurium Infection

Identifying the function of different cell populations involved in *Salmonella* infection is of critical importance to understanding both innate and adaptive immunity induced in response to infection. Unfortunately, deciphering the role of distinct cell populations *in vivo* is complicated. This is particularly true for phagocytes, since they share expression of many of the same surface molecules and play overlapping roles in directing immune responses (Wick 2007). Neutrophils, macrophages, and DCs are recruited early after *Salmonella* infection (Rydstrom and Wick 2007). In addition, it has been demonstrated that these cell populations undergo a rapid expansion/recruitment to the PP and spleens of orally-infected mice (Wick 2004).

5.1 Macrophages and Their Role in S. *Typhimurium Infection*

A key event in *Salmonella* pathogenesis is its ability to survive inside macrophages (Garcia-del Portillo 2001; Monack et al. 2004a). As described earlier, *Salmonella* uses SPI-2 T3SS-secreted virulence proteins to perturb intracellular trafficking of the SCV. SPI-2 mutants are unable to replicate within macrophages and are attenuated in mice. Paradoxically, macrophages are professional phagocytes, recognized as sentinels and first line of defense against bacterial infections (Rosenberger and Finlay 2003). Their importance in resistance to *Salmonella* is evidenced by the fact that depletion of macrophages with silica results in an increase in bacterial load and a substantial decrease in the LD50 (O'Brien et al. 1979). The delicate interplay between *Salmonella* and macrophages has been, and

still is, a fruitful area of research and has broadened our current knowledge of *Salmonella* molecular pathogenesis and served as a model to understand intracellular parasitism (Linehan and Holden 2003).

Most of the studies of the interaction between *Salmonella* and macrophages have relied on immortalized cell lines or primary macrophages cultured ex vivo. Excellent reviews have detailed the macrophage microbicidal activity against *Salmonella* (Vazquez-Torres et al. 2008) and, in particular, the bactericidal activity of phagocyte oxidase (phox) and iNOS (reviewed in (Vazquez-Torres and Fang 2001a)). These enzymes catalyze the synthesis of highly reactive compounds, commonly known as oxidative burst (ROS and RNS), that are crucial to mount an efficient innate immune response to *Salmonella*. Macrophages deficient in either of these enzymes have an impaired ability to control *Salmonella* replication, and phox and iNOS-deficient mice are unable to control bacterial replication and succumb to *Salmonella* infection (Mastroeni et al. 2000; Shiloh et al. 1999; Vazquez-Torres et al. 2000a). Interestingly, *Salmonella* is able to withstand the damaging effects of the above described compounds by inhibiting the localization of ROS and RNS to the SCV; this is contingent to the expression of SPI-2 genes by *Salmonella* (Vazquez-Torres and Fang 2001b).

Salmonella induce macrophage apoptosis, and this process is dependent on expression of the SPI-1 effector, SipB, by *Salmonella* and caspase1/interleukin 1-converting enzyme by macrophages (Monack et al. 1996). The interaction results in rapid macrophage death and release of proinflammatory cytokine IL-1β. Due to its proinflammatory nature, this process has been termed "pyroptosis" (Fink and Cookson 2007). The physiological relevance of this pathway is unclear and controversial. *In vivo* studies using *caspase* $1^{-/-}$ mice suggest that *Salmonella* activation of caspase 1 and IL-1β release is beneficial for the pathogen, since it facilitates *Salmonella* colonization of the PP and translocation across the intestinal barrier to systemic sites. *Caspase* $1^{-/-}$ mice require 1,000-fold higher bacterial doses than wild-type (WT) to induce systemic disease (Monack et al. 2000). In contrast, recent *in vivo* studies contradict the previous results demonstrating that *caspase* $1^{-/-}$ mice are more susceptible to *Salmonella* infection and suggesting that activation of caspase 1 and IL-1β release is protective to the host (Lara-Tejero et al. 2006). In these later studies, the expression of Nramp1 (the main genetic determinant of susceptibility/resistance to *S*. Typhimurium in mice, see below) was carefully assessed. *Caspase* $1^{-/-}$ mice showed higher bacterial numbers in internal organs and died earlier than WT mice, independent of the Nramp1 status (Lara-Tejero et al. 2006). Thus, more studies are needed to clarify and define the specific role of caspase 1 and their contribution during *Salmonella* infection.

5.2 *Neutrophils and Their Role in* S. *Typhimurium Infection*

The protective role of neutrophils during *Salmonella* infection is controversial. While some studies suggest that these cells type are important in defense against

Salmonella (Conlan 1996, 1997), others propose that *Salmonella* uses this cell type for survival and replication (Geddes et al. 2007). To investigate the role of neutrophils in systemic salmonellosis, Conlan et al. (1997) infected WT mice or mice depleted of neutrophils (neutropenic) with *S*. Typhimurium. They found that neutrophils are critical components of the murine host defense against systemic infection. Neutropenic mice have an exacerbated bacterial replication in the spleen and liver, and dissemination of the bacteria to lung and kidney (Conlan 1997). It has been suggested that the actions of neutrophils against *Salmonella* in the liver is primarily by containment of the bacteria within infectious foci, since high numbers of extracellular bacteria were detected in the liver sinusoids of neutropenic mice, whereas no free-growing microcolonies of *Salmonella* were found in livers of control mice (Conlan 1996). Later, another group reported that neutrophils have a supportive, but not essential, function in controlling systemic murine salmonellosis. This group found that neutropenic mice showed increased bacterial burden at day 1 postinfection (Cheminay et al. 2004). However, despite a high infiltration of neutrophils to the sites of infection, a complete clearance of the bacteria was not observed. In contrast to the above reports, a recent study suggests that *Salmonella* specifically targets splenic neutrophils for intracellular survival and replication with viable bacteria recovered from these cells (Geddes et al. 2007). More extensive research is needed in order to clarify the role of neutrophils and its role in salmonellosis.

It has been proposed that neutrophils have distinct roles during the two different diseases caused by *Salmonella:* typhoid fever and gastroenteritis. Rapid neutrophil recruitment to the gut is observed during gastroenteritis. Hence, it has been suggested that the microbicidal action of neutrophils may restrict *Salmonella* to the gut and prevent dissemination of the bacteria to internal organs. In contrast, mild neutrophil recruitment to the gut is observed in the murine typhoid model, and without the protective functions of neutrophils, the bacteria are able to spread rapidly to internal organs causing systemic disease (Cheminay et al. 2004). Now, with the development of a mouse model to study enterocolitis, it will be important to analyze the contribution of neutrophils and their function in gastroenteritis using this model.

5.3 Dendritic Cells and Their Role in S. *Typhimurium Infection*

DCs are professional antigen-presenting cells that play a crucial role in linking innate and adaptive immunity. Immature DCs are situated in peripheral and lymphoid tissues where they are highly phagocytic and able to recognize microbes via PRR on their surface. Following phagocytosis, DCs mature and migrate to the lymph nodes and spleen where they present antigenic peptides to other cells of the immune system (Banchereau and Steinman 1998; Wick 2004). These properties of DCs are important in orchestrating an efficient immune response.

Recent *in vivo* studies have shown that DCs are among the first cells that *Salmonella* encounter when infecting their murine hosts through the oral route (Niess et al. 2005). DCs were shown to sample *Salmonella* in the gut by sending processes between intestinal epithelial cells. These processes maintain the integrity of the epithelial tight junctions and require the expression of the chemokine receptor CX_3CR1. Mice lacking CX_3CR1 display enhanced susceptibility to *S*. typhimurium compared to their WT counterparts, most likely as a consequence of impaired bacterial sampling by DCs. This highlights the importance of DCs in bacterial sampling in the lamina propria and their antibacterial defense during infections (Niess et al. 2005).

In vivo DCs harbor *Salmonella* after oral, intraperitoneal, or intravenous infections (Wick 2003). Furthermore, all three of the major splenic DCs subsets, as well as DCs from Peyer's patches, can take up *Salmonella*. These *in vivo* studies reported an increase in the absolute number of splenic DCs after *Salmonella* infection. In addition, these cells have the capacity to produce TNF-α, IL-12, and IL-6 after *Salmonella* infection (Valdez et al. 2008), and this likely results in the activation of resident immune cells and/or the recruitment of other inflammatory cells to the site of infection, thus facilitating host defense against *S*. Typhimurium (Lalmanach and Lantier 1999).

In addition to their role in innate immunity to microbes and their products, DCs are the most potent antigen-presenting cells (Steinman and Banchereau 2007). This, together with the observation that DCs internalize *Salmonella*, has prompted researchers to study the role of DCs as antigen-presenting cells during *Salmonella* infection. Indeed, it has been demonstrated that DCs can process and present *Salmonella* antigens to specific $CD4^+$ and $CD8^+$ T cells *in vitro* (Svensson et al. 1997; Yrlid and Wick 2002). *In vivo*, DCs expressing $CCR6^+$ in the Peyer's patches are rapidly recruited and activate *Salmonella* specific T cells following oral infection. These data suggest that DCs likely initiate the adaptive immune response by stimulating *Salmonella*-specific T cells during infection. At the same time, these properties of DCs make them attractive targets for intracellular pathogens like *Salmonella*, since successful colonization of their hosts may require inhibition of DC function. In fact, recent reports have suggested that *Salmonella* inhibits antigen presentation and expression of MHCII by DCs (Mitchell et al. 2004; Tobar et al. 2004). This effect was dependent on the induction of inducible NO synthase (iNOS) by DCs and on the function of SPI-2 by *Salmonella* (Cheminay et al. 2005). However, the molecular mechanisms by which intracellular *Salmonella* interfere with DC functions remain to be elucidated.

6 Nramp1 and its Role in Salmonellosis

Many host-resistance factors against *S*. Typhimurium have been identified (see Table 2) (Roy and Malo 2002). Arguably, the most important innate resistance protein in mouse is Nramp1, later renamed Slc11A1 (Vidal et al. 1995a). The

susceptibility of several common inbred mouse strains to *S*. Typhimurium is the result of a single mutation of amino acid 169 of the Nramp1 protein from Gly to Asp (G169D), leading to impaired folding and loss of the mature protein in these mice (Vidal et al. 1993). Susceptible mouse strains succumb to a low dose of *S*. Typhimurium due to uncontrolled bacterial replication, whereas resistant mice control the infection and are thus able to survive. Interestingly, even resistant mouse strains are often unable to completely clear *Salmonella,* and these mice, like some human patients, become chronic carriers of *Salmonella* (Monack et al. 2004b).

Nramp1 is a highly hydrophobic protein and possesses 12 transmembrane domains with divalent cation transporter functions (Jabado et al. 2000). Nramp1 is expressed in the spleen and liver, particularly in the membrane fraction of cells of monocyte/macrophage and granulocyte lineages (Govoni et al. 1997). In addition, we recently characterized the expression of Nramp1 in the lamina propria of the small and large intestine and found that Nramp1 is expressed in a subset of DCs (Valdez et al. 2008). The subcellular localization of Nramp1 was analyzed by double immunofluorescence studies (Gruenheid et al. 1997), which showed that it is recruited to the late endocytic compartment after phagocytosis of latex beads in macrophages. Confocal microscopy in DCs revealed colocalization with $Lamp1^+$ compartments (Stober et al. 2007). Granule fractionation experiments showed that Nramp1 is present in the membrane of gelatinase-positive tertiary granules in neutrophils (Canonne-Hergaux et al. 2002). Together, these experiments suggest that Nramp1 is recruited to endocytic compartments placing it in close proximity to intracellular pathogens.

The mechanisms by which Nramp1 controls the replication of intracellular pathogens at the subcellular level remains controversial (Wyllie et al. 2002). It was proposed that Nramp1 affects the intra-phagosomal microbial replication by modulating the divalent cation content of this organelle, either by depriving the intra-phagosomal bacteria of the availability of Fe^{2+}, Mn^{2+} and Zn^{2+}, which are critical for intra-phagosomal bacterium to grow and mount an effective antioxidant defense, or by increasing the intra-phagosomal Fe^{2+} to generate highly reactive and extremely damaging hydroxyl radicals that kill the bacteria (Forbes and Gros 2001). However, the direction of transport of substrate cations remains controversial. Nramp1 also appears to have an impact on SCV maturation. In Nramp1-deficient macrophages, SCVs fail to acquire M6PR (mannose 6 phosphate receptor), a protein known to regulate the delivery of a subset of lysosomal enzymes for the trans-Golgi network to the prelysosomal compartment, thereby facilitating bacterial killing (Cuellar-Mata et al. 2002). Thus, Nramp1 appears to alter the trafficking patterns of bacteria-containing vacuoles and, as a result, the vacuoles are no longer sequestered from lysosomal trafficking and subject to the full battery of bactericidal agents present in these vacuoles.

In addition to the direct role proposed for Nramp1 in restricting intracellular microbial replication, an indirect function for Nramp1 in priming the immune system has been suggested. Many studies have shown that Nramp1 facilitates innate host defense mechanisms in macrophages such as the production of reactive oxygen

and nitrogen species as well as proinflammatory cytokines (Blackwell et al. 2000). Unfortunately, the influence of Nramp1 on cytokine production in response to *Salmonella* is poorly documented, and many of the studies performed are contradictory. For example, analysis of cytokine secretion in an *in vivo* study suggested that the kinetics and magnitude of the *Salmonella*-induced cytokine response are similar between *nramp1*$^{+/+}$ and *nramp1*$^{-/-}$ mice following infection with *S*. Dublin (Eckmann et al. 1996). In contrast, other studies have suggested that Nramp1 has a potent effect on cytokine responses. For instance, macrophages from *nramp1*$^{-/-}$ mice have a diminished capacity to induce the secretion of IFN-γ by NK cells in response to infection with *S*. Typhimurium. Lower and slower expression of IFN-γ was observed in *nramp1*$^{-/-}$ mice compared to congenic *nramp1*$^{+/+}$ counterparts after *S*. *abortusovis* infection (Lalmanach et al. 2001). In summary, there are conflicting data on the role of Nramp1 in guiding cytokine-dependent immune responses. This may be due, in part, to the failure of the previous studies to be consistent in the use of a model of infection, bacterial doses, and strains of bacteria. Therefore, the exact role of Nramp1 in cytokine-mediated responses to *Salmonella* pathogenesis and disease resistance remains unknown.

Historically, the study of murine typhoid fever has relied on the systemic administration of bacteria either by intraperitoneal or intravenous injection in naturally susceptible mice such like C57BL/6 and BalbC (Nramp1 deficient) (Vidal et al. 2008). This was likely due to the fact that most studies were measuring the outcome of the use of different bacterial mutants or were focused on understanding the pathogenic events in systemic organs where *Salmonella* replicate, such as in the spleen and the liver (Kaufmann et al. 2001; Richter-Dahlfors et al. 1997; Salcedo et al. 2001). Additionally, factors like the synchronicity of the infections were also important for comparing host parameters between mice in these studies, and this is much more easily achieved via systemic injection rather than via the natural oral route of infection. An unintended shortcoming of these studies was the lack of attention to oral infection, which most faithfully recreates the naturally occurring infections in humans. This has limited our knowledge of the initial stages of *Salmonella* infection and their interactions with the host. Consequently, very little attention has been paid to the dynamics of oral *Salmonella* infection in the context of host resistance factors such as Nramp1. A previous publication has shown that Nramp1 is not expressed in the gastrointestinal tract (Vidal et al. 1993). However, in these earlier studies, the expression was analyzed only at the mRNA level using Northern blot assays. Recently, it has been demonstrated that Nramp1 is expressed in the lamina propria of the small and large intestine and this expression modulates the host immune response with important consequences for *Salmonella* replication (Valdez et al. 2008, 2009).

The role of Nramp1 following oral infection with *S*. Typhimurium was recently analyzed in the two mouse models previously described. Using the typhoid model, it has been shown that Nramp1 is differentially expressed in distinct subsets of DCs in the lamina propria of the small intestine. Interestingly, Nramp1 expression is higher in a subset of DCs known as "inflammatory" DCs (CD103^{-} DCs), whereas very low expression was detected as "tolerogenic" DCs (CD103^{+} DCs)

(Valdez et al. 2008). Nramp1-positive DCs showed elevated expression of proinflammatory cytokines in response to *S*. Typhimurium infection compared to the Nramp1-negative DCs. These findings suggest that Nramp1 affects the inflammatory status of the host, and it has been proposed that much of the impact of Nramp1 on the severity and outcome of *S*. Typhimurium infection is determined by its influence on the speed and intensity of the host inflammatory response.

The above hypothesis was tested using the *Salmonella* colitis model (Valdez et al. 2009), since pretreatment with streptomycin prior to *Salmonella* infection facilitates bacterial colonization of the lower bowel and induces severe inflammation in these organs (Barthel et al. 2003). Indeed, it has been shown that Nramp1 acts rapidly by inducing an accelerated inflammatory response in the gut mucosa creating an antibacterial environment and limiting spread of *S*. Typhimurium to systemic sites. Nramp1-accelerated inflammatory responses were characterized by: (1) a higher induction of proinflammatory cytokines and chemokines, (2) greater recruitment of PMN cells, and (3) containment of *Salmonella* to the mucosal sites early after the infection. As with the typhoid model described above, most of the previous studies on inflammatory responses to *Salmonella* in the murine colitis model have relied on the use of Nramp1-deficient mouse strains (Coburn et al. 2005; Hapfelmeier et al. 2005, 2008; Stecher et al. 2005). It is important to note that inflammation is an extremely complex and intricate process involving the coordinated interactions of a wide variety of specialized cell types and soluble mediators. Given that (1) Nramp1 plays a key role in generating a more effective immune response in colitis, and (2) the fact that there is no evidence of widespread deficiency of Nramp1 in humans, we would argue that evaluation of colitis in *nramp1*+/+ mouse strains is more likely to give insights into human gastroenteritis and associated diarrhea than the use of Nramp1-deficient mouse models.

Figure 2 represents a schematic model of how Nramp1-dependent cytokine secretion leads to protection against *Salmonella* in the gut. In Nramp1-expressing mice, following *Salmonella* infection, chemokines and cytokines are rapidly released, but accumulate only after a substantial lag in Nramp1-negative mice. The Nramp1-mediated accelerated cytokine response results in a more rapid phagocyte: neutrophil, macrophage, and DCs activation and influx into the lamina propria and lumen of the gut. Nramp1-deficient mice fail to induce this rapid response thus providing the pathogen with sufficient time to replicate and breach the epithelial layer and compromise the ability to contain the infection.

7 Conclusions and Perspectives

It is apparent that the interactions between *Salmonella* and its host are complex, and that the outcome of disease is mediated by these interactions. *Salmonella* encode two T3SS that provide an arsenal of effectors to manipulate various components of host cell biology. Although we have identified many of these effectors, the biological function for most of them remain undefined. Cognate host binding partners

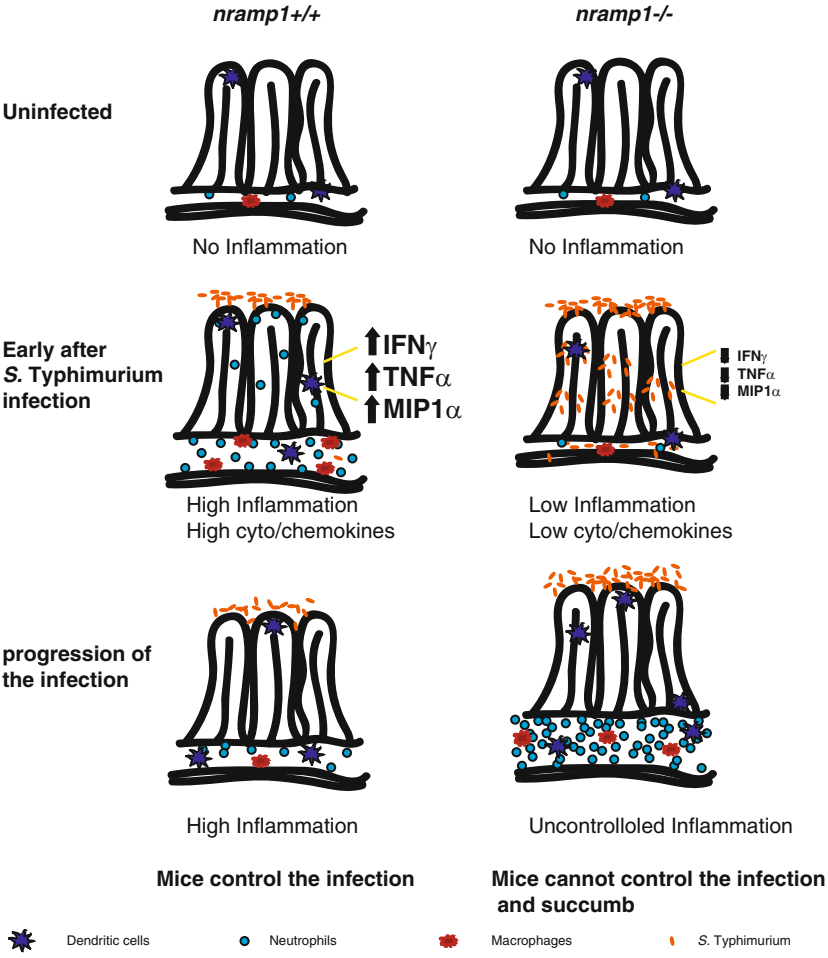

Fig. 2 A model of temporal response by *nramp1* +/+ and *nramp1* −/− mice following *S. typhimurium* infection in the gut. In *nramp1*+/+ mice, within hours following *S. typhimurium* infection, resident cells respond by secreting higher levels of cytokines and chemokines thus activating and attracting new phagocytic cells to the site of infection and creating an antimicrobial environment. In contrast, Nramp1-deficient cells exhibit a delayed response, allowing bacterial replication and penetration to internal layers of the gut. As the infection progresses, both *nramp1* +/+ *and nramp1* −/− mice have responded to the bacteria eliminating them from the deeper layers of the mucosa and sequestered them in the lumen of the gut (although the bacteria have now colonized peripheral tissues). In Nramp1$^{+/+}$ mice, bacterial numbers are substantially lower in both the lumen of the gut and in peripheral sites, likely due to the more rapid, *nramp1*-mediated cytokine response. Nramp1 deficient mice develop an uncontrolled inflammation. This is independent of Nramp1 and likely a compensatory mechanism subsequent to the high proliferation of *S. typhimurium*.

have been found for only a few of these effectors. Moreover, their effects are presumably host cell type-specific, and will probably vary in epithelial cells, macrophages, neutrophils, DCs, etc.

The development of the gastroenteritis murine model has been a major advance for studying infectious colitis. It has also emphasized the key and underappreciated role that microbiota play in determining the outcome of an infection. Previously, most host "resistance" was attributed directly to host genes like the Nramp1 system in mice. However, it now appears that the microbiota also contribute to this resistance, either directly or indirectly. By studying Nramp1, it is now appreciated that host-resistance factors can prevent the infection process by a variety of mechanisms at a variety of stages of infection.

Ultimately, the goal of researchers studying these pathogens is to fully understand the mechanisms they use to cause disease, and to use that information to design effective ways of preventing and/or treating infections. Because of the complexity of disease, studying any particular virulence factor or host defense mechanism in isolation can provide some information, but is probably lacking in establishing the full picture. Instead, a more "systems biology" approach needs to be applied to these studies. This includes studying many effectors at once or in concert in several host cell types in relevant *in vivo* settings. It also includes studying many host cell types in concert as they interface with the disease process. To complicate these studies even further, the role of the resident microbiota also needs to be incorporated into them. Although a difficult task, scientific tools are rapidly being developed for such studies, and their application will provide a much more sophisticated understanding of how *Salmonella* cause disease.

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Virulence Mechanisms and Persistence Strategies of the Human Gastric Pathogen *Helicobacter pylori*

Wolfgang Fischer, Sandra Prassl, and Rainer Haas

Contents

Abstract The human gastric pathogen *Helicobacter pylori* is able to establish an infection in a hostile environment with virtually no competitors. For this purpose, it has elaborated a set of colonization factors which facilitate both survival under acid exposure, motility and orientation in a highly viscous mucus layer, and adherence to epithelial surfaces. A more intimate interaction with gastric epithelia provides the basis to influence gene expression profiles as well as morphological transitions via signaling cascades or via direct activities of virulence factors. *H. pylori* is also one of the most genetically diverse of organisms, and variations are not only found in

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outer membrane adhesins, but also in two major virulence factors, the VacA cytotoxin and the *cag* pathogenicity island. Both factors are able to target different cell types and different interaction partners to induce a wide range of possible cellular effects. Despite the fact that *H. pylori* elicits a strong inflammatory response, the immune system fails to clear the infection, suggesting that immune evasion strategies are used. The mechanisms for immune evasion include the induction of a strongly polarized immune response, a modulation of phagocytosis and neutrophil function, and an inhibition of lymphocyte proliferation. Prolonged inflammation and direct action of bacterial factors may lead to impairment of gland function and eventually to carcinogenesis.

Abbreviations

DC-SIGN	Dendritic cell-specific ICAM-3-grabbing nonintegrin
EGFR	Epidermal growth factor receptor
FAK	Focal adhesion kinase
GSK	Glycogen synthase kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	Jun N-terminal kinase
Le^b	Lewis b
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T cells
PAI	Pathogenicity island
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PMA	Phorbol-myristate-acetate
PMN	Polymorphonuclear cell
ROS	Reactive oxygen species
sLe^x	Sialyl-Lewis x
T4SS	Type IV secretion system
TLR	Toll-like receptor

1 Introduction

Since its discovery 25 years ago (Warren and Marshall 1983), *H. pylori* has gained much attention as one of the most widespread pathogenic bacteria, although its prevalence is decreasing in Western countries and the disappearance of the

organism may be imminent (Genta 2002). A hallmark of *H. pylori* infection of the human stomach is the presence of a chronic active gastritis, generally characterized by both chronic (infiltration of lymphocytes) and active (infiltration of neutrophils) forms of inflammation. It is now well established that *H. pylori* is not only associated with gastritis, but also with peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma. *H. pylori* is not only of major importance as a bacterial inducer of gastric carcinogenesis, but it also represents a paradigmatic system to study the chronicity of bacterial infections and the associated immune modulation capabilities (Monack et al. 2004). Despite the enormous number of studies addressing *H. pylori* infections, the transmission process and the molecular mechanisms of pathogenesis are still poorly understood.

One further aspect of *H. pylori* biology is its unusually high genetic variability (Suerbaum and Josenhans 2007), which, together with its restriction to the human host, has made it possible to trace human migrations and differences in ethnic groups by the sequence diversity found in their *H. pylori* strains (Linz et al. 2007). This review describes the molecular mechanisms that endow *H. pylori* with the ability to colonize its unusual niche in the human body and to build up a delicate balance between the induction of an immune response which is favorable for the bacteria, and an immune suppression or immune modulation required for persistence. Furthermore, the current view on the molecular principles that govern disease development and progression will be summarized. For more comprehensive reviews on *H. pylori* microbiology, immune evasion, and pathogenesis, the reader is referred to several recent reviews (Amieva and El-Omar 2008; Atherton 2006; Kusters et al. 2006; Wilson and Crabtree 2007).

2 Colonization of the Gastric Mucosa

2.1 Acid resistance and Urease as an Essential Colonization Factor

Because of its acidic lumen with considerable concentrations of proteases, the human stomach is a rather hostile environment. Acid resistance is therefore a major colonization requirement. The most important factor of *H. pylori* involved in acid resistance is its highly active and abundant urease (Stingl and De Reuse 2005). Consequently, early studies using gnotobiotic piglets have shown the strict requirement of urease as a colonization factor (Eaton et al. 1991). *H. pylori* urease is a heterooligomer of six UreA and six UreB subunits (Ha et al. 2001), which needs to be activated by incorporation of Ni^{2+} ions (catalyzed by the accessory proteins UreEFGH). Another nickel-containing protein, the hydrogenase HydABC, is required for utilization of hydrogen as an energy source and also seems to play a role in full colonization competence in mice (Olson and

Maier 2002). These two protein complexes are connected, since the hydrogenase accessory proteins HypA and HypB are also necessary for urease activity (Benoit and Maier 2008), and urease and its accessory proteins are part of an interaction network with the Ni/Fe hydrogenase HydABC (Stingl et al. 2008). Accordingly, the uptake of nickel ions as well as their storage and possibly excretion are also important for *H. pylori*. Nickel uptake across the outer membrane is achieved by a TonB/ExbB/ExbD system with FrpB4 as a surface receptor (Schauer et al. 2007), whereas transport across the inner membrane involves the nickel permease NixA and possibly other transporters (reviewed in Belzer et al. 2007). One further accessory protein, UreI, was shown to act as a pH-regulated urea channel, allowing more rapid influx of urea under acidic conditions (Weeks et al. 2000). Interestingly, urease and the UreI channel are also required for persistent colonization, suggesting that urease fulfills additional functions apart from survival in the gastric lumen. One possibility is that urease is required for survival during occasional acid shocks, but it may also mediate different functions at the epithelial surface, such as binding to receptors (Beswick et al. 2006a; Fan et al. 2000) or a disruption of epithelial tight junctions (Lytton et al. 2005; Wroblewski et al. 2008).

2.2 Motility and Chemotaxis

Although urease is the most abundant protein in *H. pylori*, the bacteria survive for only short time periods in the gastric lumen due to a loss of motility (Schreiber et al. 2005). Therefore, successful colonization depends on a rapid orientation and motility of *H. pylori* towards the mucus layer covering the gastric epithelium, where pH values are more neutral (Fig. 1a). In a Mongolian gerbil model of gastric colonization (Schreiber et al. 2004), *H. pylori* cells are mostly found swimming freely in the mucus layer in a narrow range up to about 25 µm away from the epithelial surface, with only a minor percentage adherent to the epithelial surface. Due to the short survival time in the gastric lumen and the highly viscous mucus, flagellar motility is essential for initial colonization (Eaton et al. 1996; Kavermann et al. 2003; Ottemann and Löwenthal 2002). *H. pylori* flagella are composed of two distinct flagellins, FlaA and FlaB, and are enveloped by a membranous sheath containing the lipoprotein HpaA, which is thought to protect the flagella from disassembly (reviewed in Rust et al. 2008). The flagellins are posttranslationally modified by glycosylation involving pseudaminic acid (Schirm et al. 2003).

To reside in the mucus layer, a constant sensing of pH gradients and a corresponding chemotactic behavior is required. Although *H. pylori* has been shown to display positive chemotaxis to urea and bicarbonate in vitro, the major chemotactic signal in the mucus layer seems to be a pH gradient (Schreiber et al. 2004). A defect in the chemotaxis response regulator CheY and in a methyl-accepting chemotaxis receptor that is involved in pH taxis (TlpB) leads to a loss

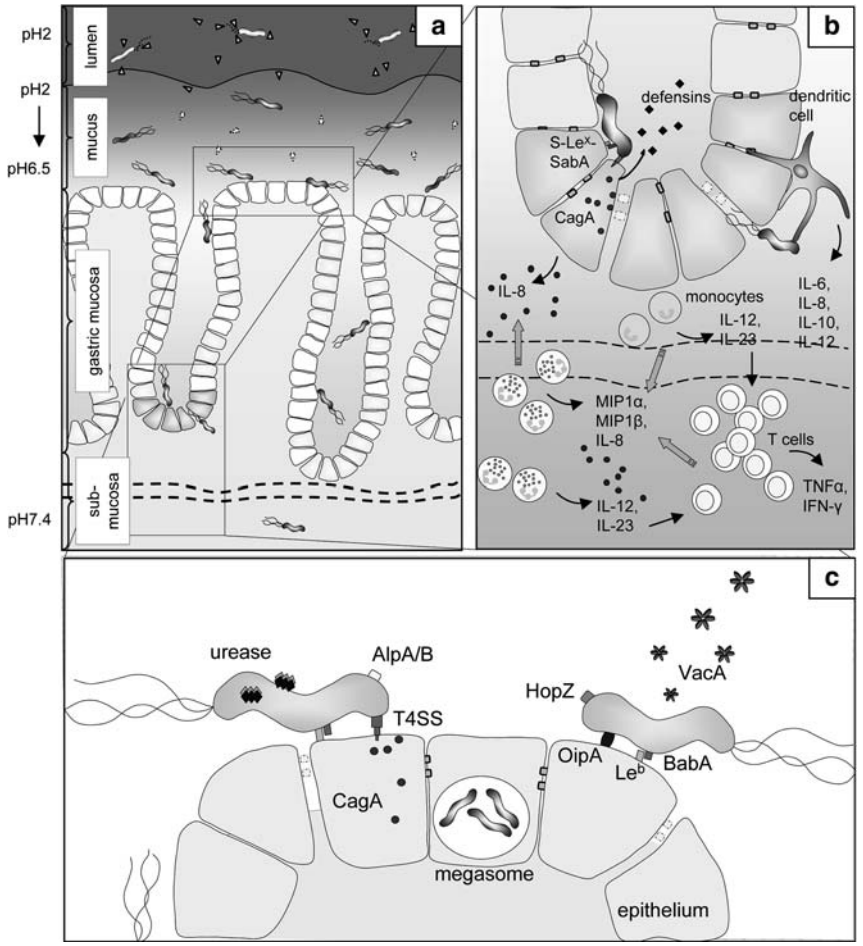

Fig. 1 Colonization of the gastric mucosa by *H. pylori* and induction of inflammation. (**a**) Overview of the colonization processs. In the gastric lumen, where *H. pylori* is attacked by proteases and acid, the bacteria escape into the protective mucus layer, interact with gastric epithelial cells and upon opening of cellular junctions may penetrate into the submucosa. (**b, c**) Contact of *H. pylori* with cellular receptors (Le^b, sLe^x) using bacterial adhesins (BabA, SabA, OipA), or the Cag type IV secretion system (Cag-T4SS) induces IL-8 release. Cellular junctions are opened due to the action of urease, secreted VacA and injected CagA. Granulocytes, monocytes, dendritic cells and T cells are recruited and secrete a number of cytokines and chemokines, resulting in the typical gastritis. *H. pylori* is also able to invade gastric epithelial cells or to move into deeper tissues

of colonization and/or proinflammatory ability in Mongolian gerbils and in mice (Croxen et al. 2006; Foynes et al. 2000; McGee et al. 2005; Williams et al. 2007). Exposure of *H. pylori* to acidic conditions results in upregulation of a number of genes, including those required for acid resistance (reviewed in Pflock et al. 2006).

Further factors that are essential for gastric colonization have been identified using a signature tag mutagenesis approach in the Mongolian gerbil model (Kavermann et al. 2003) and a transposon mutagenesis approach with microarray-based mutant tracking in a mouse model (Baldwin et al. 2007). The results of these two screens showed a considerable overlap and identified, among others, amino acid transporters, a collagenase, and components of type IV secretion systems as essential factors in vivo. Moreover, genes that are transcribed in vivo have been determined by bacterial RNA isolation from human gastric tissue (Graham et al. 2002) or from Mongolian gerbils (Scott et al. 2007), and a recombination-based in vivo expression technology approach has been applied in a mouse model (Castillo et al. 2008). Although a large number of in vivo-induced genes were identified in these screens, there is only little overlap between them. Induced genes included outer membrane proteins, a *vacA* paralogue, *mob* genes, *cag* pathogenicity island genes, and genes that may be involved in horizontal gene transfer. Interestingly, the mobilization gene cluster *mobABD* was found to be essential for mouse colonization (Castillo et al. 2008).

2.3 *Adherence to Epithelial Cells and Associated Signal Transduction Events*

Although most *H. pylori* cells are found in the mucus layer covering the gastric epithelium (Schreiber et al. 2004), a fraction always adheres to the epithelial surface, and some bacteria are even found in deeper tissues or in intracellular locations (Necchi et al. 2007; Dubois and Borén 2007) (Fig. 1a). Adherence is mediated by outer membrane proteins of the Hop family (Yamaoka and Alm 2008). One important adhesin is the blood group antigen-binding adhesin BabA (also called HopS), which mediates binding to the Lewis B (Le^b) receptor, a difucosylated derivative of the H1 antigen defining blood group 0; however, binding to H1 is also possible (Ilver et al. 1998) (Fig. 1c). Le^b antigens are not only present at the epithelial cell surface, but also on mucus glycoproteins such as MUC5AC, which means that BabA may also mediate binding inside the mucus layer (Lindén et al. 2002), with pH-dependent binding patterns (Lindén et al. 2004). Apart from Le^b and H1, BabA proteins from many, but not all, strains are also able to bind to the blood group antigens A and B and their difucosylated derivatives A-Le^b and B-Le^b. These strains have been termed generalists, whereas strains with BabA proteins that bind to H1 and Le^b only, were termed specialists (Aspholm-Hurtig et al. 2004). Specialist strains may have evolved as an adaptation to human populations with a predominance of blood group 0, such as South American Indians. A second outer membrane protein involved in adhesion is the sialyl-Lewis x (sLe^x)-binding adhesin SabA (also called HopP), which may also bind to sLe^a and other sialylated glycans (Aspholm et al. 2006; Mahdavi et al. 2002) (Fig. 1b). Since prolonged gastric inflammation results in replacement of non-sialylated with sialylated Lewis antigens, a phenomenon which depends on upregulation of the β3GnT5

glycosyltransferase by *H. pylori* in a *cag* pathogenicity island-dependent manner (Marcos et al. 2008), adhesion via SabA probably replaces adhesion via BabA in inflamed tissues (Mahdavi et al. 2002). Dependent on the presence or absence of fucosylated blood group antigens on mucosal surfaces (determined by the so-called secretor status), *H. pylori* infection density and sialylation upon inflammation are higher or lower, respectively (Lindén et al. 2008). Although the functions of BabA and SabA are well understood, it should be noted that not all *H. pylori* strains produce functional BabA and SabA proteins. Moreover, BabA may be subject to antigenic variation by recombination between the *babA* and *babB* or *babC* gene loci (Solnick et al. 2004), or to phase variation due to slipped-strand mispairing upstream of the *babA* gene (Bäckström et al. 2004). Likewise, expression of the *sabA* gene can be switched on or off by phase variation at CT dinucleotide repeats in the 5' region of the gene (Mahdavi et al. 2002; Sheu et al. 2006).

Further outer membrane proteins that were shown to be involved in adhesion are AlpA (HopC), AlpB (HopB), and HopZ (Odenbreit 2005; Odenbreit et al. 1999; Peck et al. 1999), although their cellular receptors are unknown. In contrast to BabA and SabA, AlpA and AlpB are not variable and are expressed in all *H. pylori* strains, whereas HopZ is subject to phase variation as well. The outer inflammatory protein OipA may also be involved in adherence (Yamaoka et al. 2002), but is not considered as a major adhesin (Fig. 1c). Instead, binding of *H. pylori* to epithelial cells via OipA results in activation of signaling cascades, such as a STAT1-IRF1 pathway, leading to an interferon-stimulated responsive element-like binding site in the interleukin-8 (IL-8) promoter (Yamaoka et al. 2004), and a p38/AP-1/CRE pathway leading to IL-6 induction (Lu et al. 2005). Although the *oipA* gene is also predicted to be regulated by slipped-strand mispairing, the on/off status of *oipA* expression seems to be more stable than that of *babA* or *sabA* (Yamaoka et al. 2006). Infection with OipA-positive strains is correlated with duodenal ulcer and gastric cancer (Yamaoka et al. 2006). For a more detailed discussion of the association of *H. pylori* outer membrane proteins and development of gastric diseases, the reader is referred to a recent review (Yamaoka and Alm 2008).

Apart from outer membrane proteins, adhesion may also be mediated by LPS. Dependent on the phase-variable expression of different fucosyltransferases, *H. pylori* LPS may contain Le^x or Le^y epitopes (reviewed in Moran 2008), and populations with different Lewis antigen patterns can be found in the same host (Nilsson et al. 2006). The presence of Le^x epitopes was not only reported to modulate the Th1/Th2 balance by interaction with the dendritic cell receptor DC-SIGN (Bergman et al. 2004) (see below), but also to mediate binding at epithelial cell surfaces to galectin-3, a β-galactoside-binding lectin upregulated by *H. pylori* in a CagA-dependent manner (Edwards et al. 2000; Fowler et al. 2006). However, the impact of these interactions for *H. pylori* infections is unclear (Mahdavi et al. 2003). Furthermore, LPS has also been shown to be involved in binding of *H. pylori* cells to the trefoil factor protein TFF1 present in the mucus layer (Clyne et al. 2004; Reeves et al. 2008). In addition to Lewis antigens, other host cell receptors may play a role in pathogenesis. It has been shown that the

complement regulator decay-accelerating factor acts as a receptor for *H. pylori*, and that it is upregulated in epithelial cells by the Cag type IV secretion system in a p38-dependent manner (O'Brien et al. 2006, 2008).

2.4 Genetic Variation and Horizontal Gene Transfer

A striking feature of *H. pylori* is its enormous genetic diversity (Suerbaum and Josenhans 2007). The associated genetic drift during coevolution with its human host has not only allowed to trace human migrations (Falush et al. 2003; Linz et al. 2007), but genetic and phenotypic variation is understood as an important factor driving host adaptation. For example, it has been shown that a family of cysteine-rich, Sel1-like repeat-containing proteins has undergone positive selection to adapt to distinct human populations (Ogura et al. 2007). Genetic variability is caused both by elevated mutation rates and by high recombination frequencies (Falush et al. 2001; Suerbaum et al. 1998). Elevated mutation rates may be due to the lack of some DNA repair systems, such as the mismatch repair and base excision repair systems, but also to phase-variable generation of mutator phenotypes (Kang and Blaser 2006).

Horizontal gene transfer between *H. pylori* cells is mainly mediated by natural transformation competence, but may also involve conjugative transfer events (reviewed in Fischer et al. 2008). In contrast to other naturally competent bacteria, *H. pylori* accomplishes DNA uptake from the extracellular space by a specialized type IV secretion apparatus, the ComB apparatus (Hofreuter et al. 2001). Incorporation of newly acquired DNA further requires an effective recombination system. Interestingly, colonization defects have been found for mutants in competence, recombination, DNA repair, and mobilization genes, even at early stages (Baldwin et al. 2007; Castillo et al. 2008). Although recombination seems to play an important role for *H. pylori* colonization and for maintenance of its genetic diversity, analysis of the published genome sequences indicated that merely parts of the RecBCD and RecFOR complexes, which are typically required for producing single-stranded DNA at sites of damage and for loading RecA onto these sites, are encoded in the genome (Fischer et al. 2001b). However, recent studies have shown that an AddAB-like helicase-nuclease pair fulfills the role of RecBCD (Amundsen et al. 2008), and that *H. pylori* produces a distant orthologue of RecO, which may take part in a RecOR-like complex (Marsin et al. 2008).

It is not clear at present what the role of genetic variability in the infection process might be. It has been shown that intragenomic recombination is used for antigenic variation of outer membrane proteins (Israel et al. 2001; Solnick et al. 2004). Phase variation of genes involved in LPS glycosylation has also been observed during persistent colonization (Nilsson et al. 2006). Such variations have been proposed to allow the bacteria to colonize different niches in the stomach, which may even change in chronic infections (Kang and Blaser 2006; Suerbaum and Josenhans 2007).

3 Pathogenicity Factors Associated with Disease

3.1 *Virulent* H. pylori *Strains and Disruption of Gastric Epithelial Layers*

Although the majority of colonized patients develop only a mild and mostly asymptomatic gastritis, *H. pylori* clearly is not a commensal organism. This is partly due to host factors such as polymorphisms in interleukin-1 gene clusters, but also to bacterial pathogenicity factors (reviewed in Amieva and El-Omar 2008). Major bacterial factors that are associated with the development of diseases, including ulcers or gastric adenocarcinoma, are certain genotypes of the *vacA* gene encoding the vacuolating cytotoxin (*vacA* s1/m1 or *vacA* i1), and the presence of the cytotoxin-associated gene pathogenicity island (*cag*-PAI; see below). However, these genotypes are not independent and they are furthermore associated with the presence of an active *babA* gene, the "on" state of the inflammatory outer membrane protein gene *oipA* and certain alleles of the *hopQ* outer membrane protein gene (Cao and Cover 2002; Gerhard et al. 1999; Yamaoka et al. 2002). Thus, it is difficult to estimate the individual contributions of these virulence factors from epidemiological studies. In the Mongolian gerbil animal model, *cagA* and *oipA* are clearly associated with development of gastric atrophy and therefore gastric cancer (Franco et al. 2008; Ogura et al. 2000; Rieder et al. 2005), and in mouse models, active VacA is associated with development of ulcers (Fujikawa et al. 2003; Telford et al. 1994). Interestingly, both VacA and the *cag*-PAI seem to be restricted to *H. pylori* and are absent even in the very close relative *H. acinonychis* (Eppinger et al. 2006), suggesting that they provide a selective advantage only in humans. Furthermore, the *cag*-PAI is unstable in *H. pylori* mouse infection models, and VacA does not interact with murine T lymphocytes to induce immunomodulation (see below).

Once colonization of the gastric mucus layer including adherence to gastric epithelia has been established, a subset of infections will progress towards disease manifestations. One event that may be considered as a pathogenic process is the disruption of the epithelial barrier (Wessler and Backert 2008), with urease being a possible virulence determinant. It has been shown that production of ammonium by urease mediates processing of occludin, a tight junction complex protein, and disruption of tight junctions in polarized Caco-2 cell monolayers (Lytton et al. 2005). This effect was also observed for gastric epithelial cells and in mice and could be linked to a urease-dependent activation of myosin light-chain kinase (Fedwick et al. 2005; Wroblewski et al. 2008). Furthermore, it has been reported that surface-associated or secreted VacA may cause the loosening of cellular tight junctions (Papini et al. 1998). Finally, translocation of CagA into epithelial cells has also been implicated in disruption of the epithelial barrier and epithelial cell polarity (see below) (Amieva et al. 2003; Saadat et al. 2007). The process of opening tight or adherens junctions might further be supported by recruitment of

inflammatory cells, such as neutrophils or dendritic cells, to the submucosa, as has been described in the *Shigella* infection model (Sansonetti et al. 1999). These changes in barrier function might not only be a means to transport essential nutrients and ions such as Fe^{3+}, or Ni^{2+} to the nutrient-poor habitat in the gastric mucin layer, but they might also enable the transport of bacterial virulence factors, such as VacA, or even whole bacteria into intracellular locations or the gastric submucosa (Dubois and Borén 2007; Necchi et al. 2007).

3.2 The Vacuolating Cytotoxin VacA and its Activities on Epithelial Cells

3.2.1 Structural Organization of VacA

The vacuolating cytotoxin VacA was one of the first *H. pylori* virulence factors to be described (Cover and Blaser 1992; Leunk et al. 1988). It was initially reported to induce formation of characteristic vacuoles in epithelial cell lines, but today, VacA is considered as a multifunctional toxin, displaying numerous activities on different cell types (reviewed in Cover and Blanke 2005). It is produced as a protoxin, secreted by an autotransporter mechanism (Fischer et al. 2001a), and processed to yield a mature 88 kDa protein, which is released to the extracellular environment (Cover and Blaser 1992) or may stay associated with the bacterial surface (Ilver et al. 2004) (Fig. 1c). Secreted VacA can be further processed into N-terminal and C-terminal fragments (termed p33 and p55, respectively). The p33 fragment contains a hydrophobic region close to the N-terminus, which is required for a channel-forming activity in cell membranes, and also for vacuole formation. The p55 domain is involved in oligomerization and binding to target cells (Reyrat et al. 1999). In contrast to typical bacterial AB toxins, the p33 and p55 domains are not independent of each other.

Although the *vacA* gene seems to be present in all *H. pylori* strains, different sequence types have evolved that correspond to VacA proteins with different activity and target cell specificity. These sequence variations include the 5' region encoding the N-terminal signal sequence (s1 and s2 alleles), a mid-region in the p55 domain (m1 and m2 alleles), and an intermediate region (i1 and i2 alleles) (Atherton et al. 1995; Rhead et al. 2007). Similar to the situation with the *cag* pathogenicity island, there is a correlation between *vacA* genotypes and the risk of developing disease, such that s1 and i1 *vacA* alleles are associated with the development of peptic ulcers (Basso et al. 2008). In mouse models, VacA is not strictly required for colonization, but contributes to a selective advantage in co-infection experiments (Salama et al. 2001). Oral administration of VacA results in gastric mucosal injury and inflammation in mice, although high amounts of toxin are required (Fujikawa et al. 2003; Telford et al. 1994). In Mongolian gerbils, *vacA* mutants showed only a weak phenotype with respect to the development of ulcers (Ogura et al. 2000).

3.2.2 Binding to target cells and effects of VacA intoxication

Purified VacA forms flower-shaped oligomeric structures that have to be disassembled by acid or alkaline treatment for full activity. Integration into cell membranes and channel formation involves a renewed hexamerization (Czajkowsky et al. 1999). The recent determination of the crystal structure of the p55 domain and docking into a cryo-electron microscopy map of the oligomer provided a structural model for oligomerization and defined differences between m1- and m2-type VacA molecules at the protein surface (Gangwer et al. 2007). These variants are thought to confer binding specificity to different host cells, but receptors that are responsible for these differences have not been defined. Although VacA is able to insert into artificial membranes, specific interactions with protein receptors are considered as important (Sewald et al. 2008a). It has been demonstrated in vitro that VacA binds to glycosphingolipids (Roche et al. 2007), and particularly sphingomyelin was shown to be involved in binding of VacA to cell membranes (Gupta et al. 2008). As protein receptors, the receptor protein tyrosine phosphatases RPTPα and RPTPβ are used on epithelial cells (Yahiro et al. 1999, 2003), and the β2 integrin subunit CD18 on T lymphocytes (Sewald et al. 2008b; see below). VacA binding induces signal transduction cascades, including the p38/ATF-2 signaling pathway, leading to enhanced expression of cyclooxygenase-2 in epithelial cells, and of IL-8 in monocytes (Hisatsune et al. 2008; Nakayama et al. 2004), and a tyrosine phosphorylation of the G-protein-coupled receptor interactor Git-1 (Fujikawa et al. 2003). Furthermore, PI3K-dependent phosphorylation of Akt and glycogen synthase kinase-3β leads to β-catenin translocation to the nucleus (Nakayama et al. 2008). For these effects, the channel-forming activity and uptake of VacA are not required.

VacA oligomerization at the cell surface leads to receptor clustering in lipid rafts and subsequent internalization. Although the p55 domain is able to bind to the cell surface, it is not internalized due to its inability to oligomerize (McClain et al. 2000). Uptake of VacA is clathrin-independent, but dependent on Cdc42 and the actin cytoskeleton, and is routed to late endosomal compartments (Gauthier et al. 2007; Gauthier et al. 2005). It is not clear whether VacA remains associated with late endosomes, or whether a part of the molecule enters the target cell cytoplasm. Since intracellularly-produced VacA exerts most effects similar to exogenously added VacA, a cytoplasmic activity might be considered. The vacuolation that was originally described as the main VacA activity results presumably from the anion-selective channel activity of VacA in late endocytic compartments (Genisset et al. 2007). At lower doses, VacA also leads to trafficking defects associated with late endocytic compartments, such as inhibition of vesicle trafficking to lysosomes (Satin et al. 1997) or an inhibition of antigen presentation in B cells (Molinari et al. 1998). VacA has also been reported to target mitochondria and to induce apoptosis by cytochrome c release, but these effects require rather high concentrations of VacA (Galmiche et al. 2000; Willhite and Blanke 2004). In addition to the intracellular effects of VacA, transepithelial electric resistance of polarized cell monolayers is reduced by VacA

(Papini et al. 1998). Furthermore, treatment of isolated gastric glands or cultured parietal cells with purified VacA led to an inhibition of acid secretion by channel formation, calcium influx, and calpain-mediated proteolysis of ezrin (Wang et al. 2008). However, a contribution of VacA to a calcium influx into gastric epithelial cells after *H. pylori* infection could not be found in another study (Marlink et al. 2003).

3.3 The Cag Type IV Secretion System as a Mediator of Actin Rearrangements and a Proinflammatory Response

3.3.1 The *cag* Pathogenicity Island and Induction of Disease

The designation *cag* (*cytotoxin-associated gene*) is derived from the *cagA* gene that has been known for many years to be correlated with the development of more severe disease and also with the presence of an active (genotype s1/m1) VacA cytotoxin (Akopyants et al. 1998; Censini et al. 1996). The *cag* pathogenicity island contains roughly 30 genes that encode the CagA protein and a type IV secretion system (T4SS), which is able to translocate CagA from the bacteria to several host cells (Asahi et al. 2000; Backert et al. 2000; Odenbreit et al. 2000, 2001; Stein et al. 2000). The percentage of *cag*-PAI-positive strains varies considerably between different ethnic groups, ranging from complete absence in certain South African strains to ubiquitous presence in East Asian strains (Gressmann et al. 2005).

For the *cagA* gene, variations exist in the 5' (van Doorn et al. 1999) and the 3' parts (Yamaoka et al. 1998, 1999), with the latter regions being different between Western and East Asian strains. The 3' variable region encodes the CagA tyrosine phosphorylation motifs (EPIYA motifs), which can be classified as EPIYA-A, B, C, and D motifs, according to differences in the flanking amino acids (Hatakeyama 2003). Motif ABC-type CagA molecules occur in Western and ABD-type CagA molecules in East Asian strains (Higashi et al. 2002a; Naito et al. 2006) (Fig. 2a). Variations in the 3' repeat region are associated with the severity of disease (Argent et al. 2004; Azuma et al. 2004; Yamaoka et al. 1999). In Western strains, the number of EPIYA-C motifs (mostly ranging from 1 to 3) is correlated with the risk of intestinal metaplasia and of gastric cancer (Basso et al. 2008). East Asian strains with more than one EPIYA-D motif are rare (Argent et al. 2008a), but East Asian-type CagA molecules are more active (see below).

It is currently unknown what triggers expression of *cag*-PAI genes in vivo, but several genes are induced during infection in Rhesus macaques or Mongolian gerbils (Boonjakuakul et al. 2005; Scott et al. 2007), suggesting that the type IV secretion system is used by the bacteria not only under conditions of severe damage in the gastric epithelium.

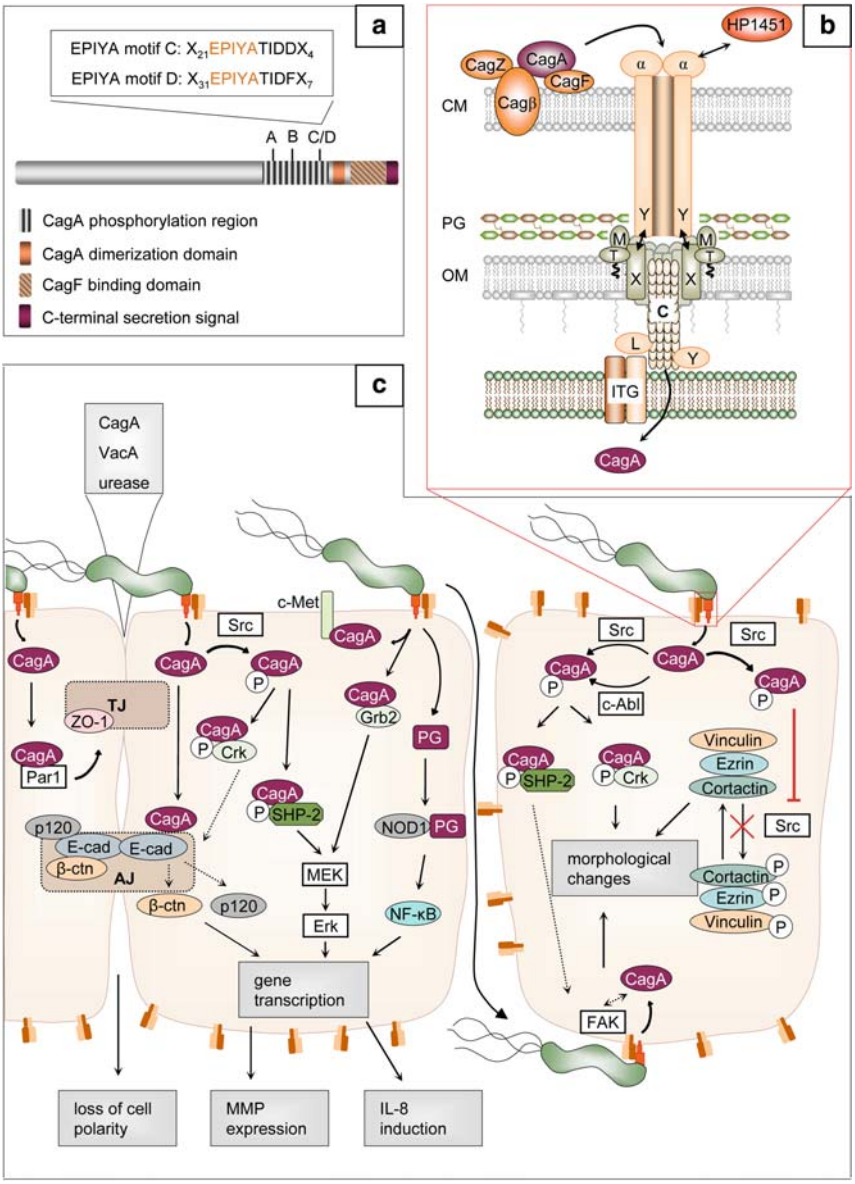

Fig. 2 Molecular events elicited by the Cag-T4SS. (**a**) Schematic representation of the CagA protein and depiction of functional domains. (**b**) Model of the Cag type IV secretion apparatus showing the effector protein CagA, its translocation factors CagF and CagZ, the hexameric ATPases Cagα and Cagβ, and some structural components. HP1451 is encoded outside of the *cag* PAI and interacts with Cagα. A further subassembly of the Cag T4SS is located at the outer membrane consisting of CagX, CagT and CagM. The extracellular pilus consists of CagC and may be covered by the CagY and CagL proteins. CagL is interacting with β1 integrin on the host cell surface. (**c**) *H. pylori* interacts with the β1 integrin receptor to inject the CagA protein and peptidoglycan fragments (PG). Secreted VacA and urease are involved in opening of tight junctions, allowing *H. pylori* to reach the basolateral side of the cells to interact with basolaterally located β1 integrins for more efficient CagA translocation. Injected CagA modulates various signaling cascades and induces actin cytoskeletal rearrangements, disruption of tight junctions and loss of cell polarity. In addition, peptidoglycan, which is translocated in a T4SS-dependent manner, appears to activate the intracellular receptor Nod1, which activates NF-κB. For more details, see text

3.3.2 Components of the Cag Type IV Secretion Apparatus and Translocation Mechanism

The Cag T4SS is mostly compared to well-characterized T4SSs, such as the VirB system of the plant pathogen *Agrobacterium tumefaciens*, and considered as a typical T4SS. Nevertheless, many of its essential components are considerably divergent from their functional homologs in these prototypical systems, or do not have corresponding counterparts at all (Fischer et al. 2008). A comprehensive screen identified 14 *cag*-encoded gene products that are necessary for translocation of the CagA protein into epithelial cells and for induction of IL-8 secretion, and three further gene products that have a supportive role, suggesting that they are components of the secretion apparatus (Fischer et al. 2001c). Four gene products were found to be essential for CagA translocation only, and therefore considered as substrate recognition or translocation factors. Despite the lack of pronounced sequence similarities among most of these gene products, the Cag system seems to contain functional analogs of all typical T4SS components (Fischer et al. 2008; Kutter et al. 2008). Notably, CagL has been considered as a pilus adhesin in analogy to the VirB5 protein of *A. tumefaciens*, and it has been reported to interact with integrins as host cell receptors (Backert et al. 2008; Kwok et al. 2007). Additionally, the Cag T4SS contains essential components without functional counterparts in other T4SSs. Among these, the CagM protein was shown to assemble, together with CagX and CagT, in an outer membrane-associated subcomplex with a putative secretin-like function (Kutter et al. 2008). One further additional factor encoded outside the *cag* pathogenicity island, HP1451, is not necessary for the function of the T4SS, but may modulate the activity of the VirB11-like ATPase Cagα (Hare et al. 2007) (Fig. 2b).

At the bacterial surface, the Cag T4SS system forms unusual sheathed pilus-like appendages that are likely composed of the major pilus component CagC (Andrzejewska et al. 2006). One of the sheath components was identified by immunoelectron microscopy as the secretion apparatus protein CagY (Rohde et al. 2003). Remarkably, the middle region of CagY consists of a regular array of up to 74 sequence repeats composed of six different consensus segments. This region, which is probably the surface-exposed part (Rohde et al. 2003; Tanaka et al. 2003), is rich in cysteine residues and adopts a stable α-helical structure (Delahay et al. 2008) that is probably subject to antigenic variation (Aras et al. 2003). Further surface-associated proteins of the Cag type IV secretion apparatus include parts of the CagT lipoprotein and the CagX protein, which are localized at the base of Cag surface needles (Rohde et al. 2003; Tanaka et al. 2003), and the CagL protein, which has been found at the tip of the needles and was reported to interact with β1 integrins as cellular receptors (Kwok et al. 2007).

Similar to effector proteins of most other T4SSs, CagA has a C-terminal secretion signal (Fig. 2a); however, its N-terminal region is also required for translocation (Hohlfeld et al. 2006). Generally, recognition of type IV secretion substrates is thought to be accomplished by the coupling proteins, cytoplasmic membrane proteins with ATP-binding capacity that are not required for secretion

apparatus assembly (Gomis-Rüth et al. 2004). Consistent with this notion, the coupling protein homologue Cagβ is dispensable for IL-8 induction, but required for CagA translocation (Fischer et al. 2001c). The exact function of Cagβ in the CagA signal sequence recognition process remains unclear. Another *cag*-encoded protein, CagF, has been shown to act as a secretion chaperone-like protein with a recruitment function for CagA to the secretion apparatus (Couturier et al. 2006; Pattis et al. 2007). The structure of CagZ, another CagA translocation factor, has been determined (Cendron et al. 2004), but its function is still unclear (Fig. 2b).

3.3.3 CagA Translocation and Induction of a Proinflammatory Response

The first step of CagA translocation into target cells is interaction of the secretion apparatus with the host cell surface. The Cag apparatus binds to β1 integrins as receptors, and CagL has been postulated as a bacterial ligand (Kwok et al. 2007) (Fig. 2b). Binding of CagL to integrins was reported to result in a fast activation of Src kinase and tyrosine phosphorylation of focal adhesion kinase (FAK Y397) (Kwok et al. 2007); however, FAK Y397 phosphorylation and Src activation seemed to be independent of the *cag* pathogenicity island, but rather dependent on OipA in another study (Tabassam et al. 2008b). Binding of the secretion apparatus to β1 integrins may also induce other signaling cascades, for example leading to JNK activation (Snider et al. 2008). Initial Src and FAK activation results in Erk1/2 signaling and is dependent on epidermal growth factor receptor (EGFR) activation (Tabassam et al. 2008b). EGFR signaling and Erk1/2 activation have also been reported to be induced by transactivation via different mechanisms (Basu et al. 2008; Beswick and Reyes 2008; Keates et al. 2001). It is presently unclear how *H. pylori*, which targets gastric epithelial cells from the apical side, is able to interact with integrin receptors, which are supposed to be exposed at the basolateral side of polarized cells. One possibility is that gastric epithelial cells may contain small amounts of β1 integrins on their apical surfaces (Zuk and Matlin 1996), and that larger amounts of CagA might be translocated only after opening of cell-cell junctions and bacterial access to basolateral membranes. The mechanism of CagA uptake into the host cell is not understood, but it seems to involve detergent-resistant membrane microdomains (Lai et al. 2008).

CagA is the only effector protein that has been found to be translocated by the Cag T4SS. However, bacterial peptidoglycan fragments may also be transferred by the type IV secretion apparatus and subsequently stimulate the intracellular pattern recognition receptor Nod1, resulting in NF-κB activation and induction of chemokines such as IL-8 (Viala et al. 2004) (Figs. 1b and 2c). Since IL-8 induction and the proinflammatory response elicited by *H. pylori* do not only require NF-κB activation but also involve other signaling pathways (reviewed in Naumann 2005), it is likely that Nod1 activation is not the only *cag*-dependent stimulation mechanism. Translocated CagA proteins of some *H. pylori* strains are able to enhance IL-8 expression (Brandt et al. 2005), and transfection of epithelial cells with *cagA*

expression constructs results in IL-8 induction as well (Kim et al. 2006), but the induction of IL-8 secretion is basically independent of CagA translocation (Fischer et al. 2001c). It should also be noted that other bacterial factors, such as the inflammatory outer membrane protein OipA (Lu et al. 2005; Yamaoka et al. 2004) or the adhesins AlpA and AlpB (Lu et al. 2007), may act synergistically with the Cag T4SS to stimulate a proinflammatory response. Furthermore, the gastritis that results from *H. pylori* infection is not only caused by chemokine secretion from epithelial cells, but requires chemokine secretion from other cells, such as T cells (reviewed in Wilson and Crabtree 2007). In mice, the development of gastritis in response to *H. pylori* infection is not possible without prior T cell priming in Peyer's patches (Nagai et al. 2007).

3.3.4 CagA Interference with Host Cell Functions

Multiple cellular consequences of Cag type IV secretion have been reported and numerous interaction partners of translocated CagA have been identified (reviewed in: Backert and Selbach 2008; Bourzac and Guillemin 2005; Hatakeyama 2008). Some of the phenotypes related to Cag type IV secretion have been observed after infection of different cancer cell lines with *H. pylori* strains, and others only after transfection with *cagA*-expressing constructs. CagA-independent effects caused by modulation of different signaling pathways may depend on the cell type used, and CagA-dependent effects may also be indirect due to the massive impact of translocated CagA on gene expression profiles (El Etr et al. 2004). Thus, the relevance of the molecular interactions and cellular consequences under in vivo conditions remains to be determined.

The hallmark of CagA translocation into eucaryotic cells is its tyrosine phosphorylation by kinases of the Src family (Selbach et al. 2002; Stein et al. 2002), and, at later time points, c-Abl kinase (Poppe et al. 2007; Tammer et al. 2007) (Fig. 2c). Tyrosine phosphorylation of CagA results in dephosphorylation of several cellular proteins including cortactin, ezrin, and vinculin (Moese et al. 2007; Selbach et al. 2003, 2004), which is probably due to a feedback inactivation of Src kinase (Selbach et al. 2003; Tsutsumi et al. 2003) (Fig. 2b). Apart from Src and Abl kinases, CagA has been reported to interact in eucaryotic cells with a number of other target molecules, with some interactions being dependent on, and others independent of, CagA tyrosine phosphorylation. As a major interaction partner of phosphorylated CagA, the tyrosine phosphatase SHP-2 has been identified (Higashi et al. 2002b). Binding of CagA to SHP-2 requires tyrosine phosphorylation of EPIYA-D motifs (present in East-Asian strains) or EPIYA-C motifs (with lower affinity) and their interaction with SH2 domains of SHP-2 (Naito et al. 2006), leading to an activation of SHP-2 phosphatase activity (Fig. 2c). Activated SHP-2 in *cagA*-transfected cells induces subsequent dephosphorylation and inactivation of focal adhesion kinase (Tsutsumi et al. 2006), which contributes to a characteristic cell elongation of some epithelial cell lines known as the "hummingbird" phenotype (Segal et al. 1999). The hummingbird phenotype is thought to represent a lack

of focal contact disassembly during Cag-induced cell motility (Bourzac et al. 2007). It requires CagA-dependent activation of Erk1/2 (Higashi et al. 2004) and phosphorylation of the vasodilator-stimulated phosphoprotein VASP (Knauer et al. 2008), as well as other signaling pathway modulations, such as activation of Rac1 via adaptor proteins of the Crk family (Suzuki et al. 2005). Crk family proteins have also been shown to be interaction partners of phosphorylated CagA, and their deregulation leads to adherens junction protein redistribution and thus contributes to epithelial cell motility (Suzuki et al. 2005). However, disruption of adherens junctions upon *H. pylori* infection may also occur in a CagA-independent way and involve shedding of E-cadherin (Weydig et al. 2007). Dephosphorylation of cortactin and vinculin and the concomitant loss of cell-matrix adhesion and redistribution of focal contacts may also contribute to the observed changes in cell morphology (Moese et al. 2007).

Cellular interaction partners of CagA that do not require its tyrosine phosphorylation include the receptor tyrosine kinase c-Met (Churin et al. 2003), the adaptor molecule Grb2 (Mimuro et al. 2002), the adherens junction protein E-cadherin (Murata-Kamiya et al. 2007), and the serine/threonine kinase PAR1b/MARK2 (Saadat et al. 2007; Zeaiter et al. 2007). The interactions with c-Met, Grb2, Crk proteins, and SHP-2 have led to the speculation that CagA may mimic Gab adaptor proteins (Hatakeyama 2003), and in a transgenic *Drosophila* model, CagA was indeed able to partially substitute the Gab homologue Daughter of Sevenless (Botham et al. 2008). Translocated CagA leads to disruption of epithelial tight-junctions, which is accompanied by colocalization of CagA with the junction proteins ZO-1 and JAM, a loss of cell polarity and induction of cell migration (Amieva et al. 2003; Bagnoli et al. 2005). This was explained by interaction of CagA with the polarity-associated kinase PAR1b/MARK2 (Saadat et al. 2007; Zeaiter et al. 2007). This interaction involves a dimerization of CagA via a short C-terminal sequence motif (Lu et al. 2008; Ren et al. 2006) and inhibits the kinase activity of PAR1b/MARK2, resulting in its dissociation from the cell membrane (Saadat et al. 2007). The CagA-PAR1b/MARK2 interaction is also required for efficient interaction with SHP-2 and for induction of the hummingbird phenotype. In the Mongolian gerbil model, *H. pylori* infection was reported to suppress apoptosis of superficial pit cells via CagA-dependent induction of the antiapoptotic factor MCL1, thereby contributing to the pit region hyperplasia that is observed in *H. pylori* infections. This hyperplasia, in turn, may lead to enhanced bacterial colonization (Mimuro et al. 2007).

4 Mechanisms of Immune Evasion

Despite the pronounced immune response, *H. pylori* remains in the stomach mucosa at high densities for years and decades if not treated by antibiotics, indicating that the immune response is ineffective. The chronic infection process can be best explained by the achievement of a delicate balance between stimulation of the

immune system and survival of the bacteria in a milieu of inflammation. To induce a certain degree of inflammation seems to be a more general strategy for pathogenic bacteria in the gut to overcome colonization resistance, as demonstrated for *Salmonella typhimurium* and *Citrobacter rodentium* in the murine model (Stecher and Hardt 2008). For *H. pylori*, inflammation and local damage of the epithelial layer may be a means to get access to nutrients. The orchestrated and coordinated function of *H. pylori* virulence factors might be involved in maintaining the balance between bacterial survival and minimal damage of the host. However, when this balance is disturbed, gastric disease may be the consequence, as found in a subset of infected patients. In the following sections, we will focus on the specific mechanisms of immune interference and control by *H. pylori* virulence factors, allowing *H. pylori* to persist for decades in the human stomach.

4.1 The Innate Immune Response and its Subversion by H. pylori

The probably most important antibacterial property of the stomach is its acidic pH, so that reaching the gastric mucus layer is essential for survival, as described above. There, however, *H. pylori* is confronted with a number of further antibacterial factors, such as the antimicrobial peptides β-defensin 1 and 2 or the cathelicidin LL-37 (Frye et al. 2000; Hase et al. 2003) (Fig. 1b). Interestingly, expression of a set of antimicrobial peptides is actively upregulated in the gastric mucosa, dependent on the presence of the *cag*-PAI, indicating that a so far unrecognized function of the *cag*-PAI might be to induce an antimicrobial host response to increase the competitive advantage of *H. pylori* in the gastric niche (Hornsby et al. 2008). Other, more general, antibacterial factors include the presence of lactoferrin in the stomach mucosa, which restricts the availability of extracellular iron (Luqmani et al. 1991), and surfactant protein D, which is strongly produced on the gastric mucosal surface and binds selectively to microorganisms to induce aggregation and phagocytosis (Murray et al. 2002). Furthermore, certain *O*-glycans present in the mucus overlaying deeper regions of the human gastric mucosa exhibit antimicrobial activity against *H. pylori*, inhibiting biosynthesis of cholesteryl glucosides, which are required for bacterial growth (Kawakubo et al. 2004). Considering the high prevalence of *H. pylori* in the human stomach, the bacteria seem to be well prepared to overcome these innate defense mechanisms of the host.

4.1.1 Interaction of *H. pylori* with Pattern Recognition Receptors

Toll-like receptors (TLRs) represent a group of innate immune system receptors that recognize microbial- or pathogen-associated molecular patterns (MAMPs/PAMPs), and in general play an important role in initiating an innate immune

response against viral or bacterial pathogens. Stimulation of TLRs results in proinflammatory signaling, mostly through NF-κB activation (Takeda et al. 2003). Immunohistochemistry and confocal laser scanning microscopy revealed that epithelial cells in the antrum and the corpus of the human stomach produce TLR4, TLR5, and TLR9 (Schmausser et al. 2004), whereas cultured primary human gastric cells produce TLR2 and TLR5, but not TLR4 mRNA (Bäckhed et al. 2003). *H. pylori* LPS contains a lipid A domain that shows up to 1,000 times lower immunological activity than lipid A of the *Enterobacteriaceae* family (Muotiala et al. 1992). The reduced endotoxicity of *H. pylori* lipid A is thought to arise from its unique chemical structure, lacking the usual 4′-phosphate, as well as the 3′-ester-linked fatty acyl chains. Instead, it is derivatized with a phosphoethanolamine residue at the C-1 position of the proximal glucosamine (Moran et al. 1997). Due to this unique lipid A structure, *H. pylori* is also highly resistant to the antimicrobial peptide polymyxin (Tran et al. 2006). Furthermore, *H. pylori* LPS is unusual in that it contains Lewis blood group antigens, giving rise to a form of molecular mimicry proposed to camouflage the bacterium, which may aid in persistence of the infection (Appelmelk et al. 2000). Thus, *H. pylori* LPS is not well recognized by TLR4 (Fig. 3a), but *H. pylori* LPS induced NF-κB activation in HEK293 cells that expressed TLR2, which indicates that *H. pylori* LPS might be recognized by TLR2 instead of TLR4 (Smith et al. 2003; Yokota et al. 2007). *H. pylori* flagellin is also a poor stimulator of TLR5 (Lee et al. 2003). TLR5 recognizes a region of bacterial flagellin that is involved in subunit-subunit assembly in *Salmonella* and several other pathogenic bacteria, but this short region has diverged in *H. pylori* and *Campylobacter jejuni* flagellin, neither of which are recognized by TLR5 (Galkin et al. 2008) (Fig. 3b). In addition to TLRs, the intracellular pathogen recognition molecules Nod1 and Nod2, which respond to different motifs in bacterial peptidoglycan, have been implicated in recognition of *H. pylori*. Nod1 activation in epithelial cells by *H. pylori* occurred upon intracellular peptidoglycan detection, which was dependent on the function of the *cag*-T4SS. Furthermore, a missense mutation in the leucine-rich region of Nod2 (R702W) is associated with gastric lymphoma in *H. pylori*-infected patients (Rosenstiel et al. 2006).

The C-type lectins represent another class of receptors that recognize specific carbohydrate structures present on the cell wall of pathogens. One of these lectins, which is expressed on dendritic cells, is DC-SIGN (dendritic cell-specific intercellular adhesion molecule grabbing non-integrin). DC-SIGN has been identified as a receptor for viruses, parasites, fungi, and bacteria (Koppel et al. 2005), including *H. pylori* (Bergman et al. 2004), and is involved in cell adhesion as well as antigen presentation to T cells. Binding to the carbohydrate recognition domain of DC-SIGN is dependent on high mannoses or Le sugars. Lewis antigen expression in *H. pylori* is phase variable by translational frame shifts in glycosyltransferase genes that occur during replication. Thus, *H. pylori* Le^+ phase variants can bind to DC-SIGN on DCs in gastric mucosa and induce a shift towards a Th2 polarization (Bergman et al. 2004), whereas Le^- variants escape binding to DCs and induce a strong Th1 cell response (Fig. 3a).

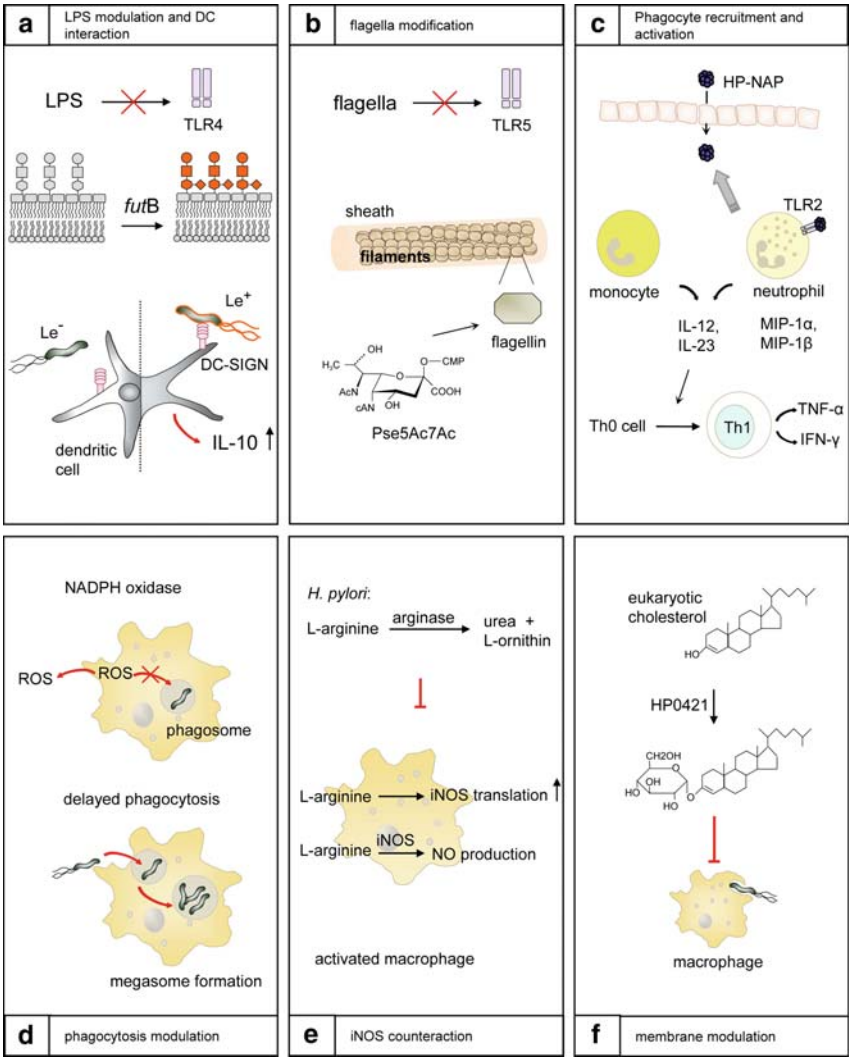

Fig. 3 Innate immune evasion mechanisms. (**a**) *H. pylori* modulates LPS by phase variable expression of fucosyl transferases to generate Lewis antigens, epitopes also found on human epithelial cells. Lewis expression patterns result from the on/off status of three fucosyltransferases (e.g., FutB), which are regulated via slipped-strand mispairing in intragenic poly-A or poly-C tracts. Le^+ *H. pylori* variants are able to bind to the C-type lectin DC-SIGN present on gastric dendritic cells, thereby inducing a Th2 response, whereas Le^- variants escape binding and promote a strong Th1 cell response. *H. pylori* LPS does not interact with TLR4. (**b**) *H. pylori* flagellin is posttranslationally modified by glycosylation involving pseudaminic acid (Pse5Ac7Ac) and is not recognized by TLR5 due to a divergent sequence in the TLR5 recognition region. (**c**) HP-NAP acts as a bacterial chemoattractant, recruits phagocytes to the lamina propria, binds via TLR2 to neutrophils and monocytes and induces secretion of cytokines and chemokines, which finally prime Th0 cells to differentiate into Th1 cells. (**d**) *H. pylori* avoids killing by delayed phagocytosis, dependent on the Cag T4SS and VacA-dependent inhibition of phagosome acidification and formation of megasomes. NADPH oxidase is directed to the plasma membrane to avoid bacterial killing in the phagosome. (E) *H. pylori* induces iNOS expression by phagocytes through urease, but avoids NO damage by producing L-arginase (RocF), which depletes the iNOS substrate L-arginine. L-arginine depletion also blocks translation of iNOS mRNA. (F) *H. pylori* extracts cholesterol from host cell plasma membranes and glucosylates it by the bacterial cholesterol-α-glucosyltransferase (HP0421), a process that abrogates phagocytosis of *H. pylori* and subsequent T cell activation

4.1.2 Resistance to Reactive Oxygen or Nitrogen Species

Phagocytosis is an essential mechanism of the innate immune system involved in destruction of invading microorganisms. Tight binding of Cag T4SS-positive *H. pylori* to the epithelium results in delivery of the effector protein CagA in the cell, but also triggers the synthesis and secretion of the potent neutrophil chemoattractant interleukin-8 (IL-8). Besides IL-8, *H. pylori* urease and HP-NAP are also involved to recruit phagocytes to the lamina propria (Allen 2000). HP-NAP is a 150-kDa dodecameric iron-binding protein that promotes adhesion of PMNs to endothelial cells and stimulates phagocyte chemotaxis, NADPH oxidase assembly, and production of reactive oxygen species (ROS) (Polenghi et al. 2007; Satin et al. 2000). Moreover, HP-NAP acts as a TLR2 agonist and induces the secretion of IL-12 and IL-23 from monocytes and neutrophils, which may result in a Th1-polarized immune response (Amedei et al. 2006) (Fig. 3c). To avoid the antimicrobial activity of ROS, *H. pylori*, like many other bacterial pathogens, produces catalase (Odenbreit et al. 1996) and superoxide dismutase (Spiegelhalder et al. 1993), enzymes involved in detoxification of ROS. In addition to these general mechanisms, *H. pylori* has developed a strategy to disrupt NADPH oxidase targeting and thus directs ROS production to irrelevant compartments. Generally, soluble agonists (e.g., fMLP) direct oxidase assembly to the plasma membrane to release superoxide into the extracellular milieu to kill extracellular bacteria. However, larger phagocytosed particles, such as bacteria or yeast, direct NADPH oxidase to the phagosome membrane, to deliver superoxide inside the phagosome (Dahlgren and Karlsson 1999). Interestingly, during phagocytosis of *H. pylori* by PMNs, strong NADPH oxidase activity is found at the cytoplasmic membrane, but not on *H. pylori* phagosomes. Thus, superoxide is generated at the extracellular environment to promote tissue damage, whereas the ingested bacteria are not harmed by ROS in the phagosome (Allen et al. 2005b) (Fig. 3d).

In addition to NADPH oxidase, *H. pylori* also activates inducible nitric oxide synthase (iNOS) in the human gastric mucosa, which has been associated with an increased risk to develop gastric carcinoma of the intestinal type (Rieder et al. 2003). In the search for bacterial factors involved, the *H. pylori* urease has been implicated in iNOS activation in macrophages (Gobert et al. 2002b). *H. pylori* produces an arginase, RocF, which converts L-arginine to urea and L-ornithine (McGee et al. 1999), and the bacteria make use of urea as a substrate for urease to produce NH_3, which is used to buffer acidity and as an important bacterial nitrogen source (Fig. 3e). Macrophages infected with wild-type *H. pylori* produce significantly less NO than macrophages infected with *rocF*-negative isogenic mutants, and the arginase-defective bacteria are more sensitive to NO-dependent killing by macrophages (Gobert et al. 2001).To further counteract NO production, *H. pylori* also induces eukaryotic arginase II expression in macrophages to induce macrophage apoptosis (Gobert et al. 2002a). L-arginine is limiting at the surface of the gastric epithelium, and L-arginine availability regulates NO production by an effect on iNOS protein translation (Chaturvedi et al. 2007). Thus, *H. pylori* arginase efficiently inhibits iNOS protein expression, and this mechanism might have an

important role for *H. pylori* to evade the macrophage attack. Furthermore, *H. pylori* also encodes AhpC, a member of the bacterial peroxiredoxin family that protects against oxidation of DNA and other molecules by peroxynitrite, a product of NO and O_2^- (McGee et al. 1999).

4.1.3 Modulation of Phagocytosis

A further characteristic of *H. pylori* is its ability to actively retard its own entry into phagocytes (Allen et al. 2000). This delayed phagocytosis is a feature of live, metabolically active, type I *H. pylori* strains and is prevented by opsonisation with specific IgG, or by blockade of bacterial protein synthesis. The pathogenic factor(s) responsible for this effect have not been determined. The process of delayed phagocytosis is different from Fcγ-mediated phagocytosis of opsonized bacteria. It is characterized by a novel signaling cascade, defined by activation of the atypical protein kinase PKCζ, and is PI3K-dependent (Allen and Allgood 2002; Allen et al. 2005a).

Delayed phagocytosis in macrophages results in homotypic phagosome fusion. *H. pylori* persists inside large 'megasomes' due to inhibition of phagosome maturation, mainly induced by *H. pylori* urease and VacA (Schwartz and Allen 2006; Zheng and Jones 2003). Megasomes accumulate coronin, an actin-binding protein, which is recruited to phagosomes in a PI3K-dependent manner, and early endosome antigen 1 (EEA1), a tethering molecule required for endosome clustering and homotypic fusion (Schwartz and Allen 2006; Zheng and Jones 2003). Megasomes are not strongly acidified and acquire only limited amounts of the late endosome membrane protein Lamp-1. *H. pylori* uses a similar strategy to survive inside gastric epithelial cells (Terebiznik et al. 2006) (Fig. 1c).

A further important issue related to bacterial phagocytosis is an unusual lipid composition of *H. pylori* membranes, which are characterized by high concentrations of lysophospholipids and cholesteryl glucosides, a composition probably unique for bacterial membranes (Hirai et al. 1995, 1996). Since *H. pylori* cannot synthesize cholesterol by its own, the bacteria are able to extract the lipid from the plasma membrane of epithelial cells for glycosylation (Wunder et al. 2006) (Fig. 3f). Interestingly, either preloading of bacteria with cholesterol or inactivation of an *H. pylori* glycosyltransferase gene (*hp0421*) markedly enhanced *H. pylori* uptake by serum-starved J774 cells (Wunder et al. 2006). At the moment, it is unclear by which mechanism cholesterol in the bacterial membrane can impact host cell responses or phagocytosis.

Although *H. pylori* is considered primarily a mucosal pathogen, the bacteria can be found in deeper tissues of the lamina propria (Dubois and Borén 2007; Necchi et al. 2007) and even in the associated lymph nodes (Ito et al. 2008). Furthermore, *H. pylori* can invade epithelial cells in vivo and in vitro (Oh et al. 2005; Terebiznik et al. 2006), although the primary niche is the gastric mucous layer. Transient cellular invasion by a subpopulation of *H. pylori* might therefore be a strategy for persistence in the face of unfavorable conditions, such as gastric acid, antibiotics, or the host inflammatory response, similar to uropathogenic *E. coli* invading bladder

epithelial cells in a FimH-dependent manner, followed by organization of coccoid bacteria into a tightly packed cytoplasmic matrix (Anderson et al. 2003). Thus, invasion of host epithelial cells to promote chronic infection may in fact be a common strategy for mucosal pathogens previously thought to have a uniformly extracellular lifestyle.

4.2 Mechanisms of Evasion of the Adaptive Immune Response

H. pylori has not only to deal with innate immune responses but also with a continuous attack of the adaptive immune response. After successful eradication of *H. pylori*, there is little memory or protection against reinfection (Parsonnet 2003). *H. pylori* has evolved complex mechanisms to interfere with many different levels of the adaptive immune response, ranging from antigen presentation to modulation of T cell cytokine signaling. Important bacterial virulence factors involved in modulation of the adaptive immune system, especially the T lymphocytes, are the vacuolating cytotoxin, the γ-glutamyltranspeptidase (γGT), and arginase (RocF).

Several reports, mainly based on in vitro experiments, indicate that live *H. pylori* cells or certain virulence factors are able to interfere with multiple functions in both B and T lymphocytes (Fig. 4). VacA has been initially reported to interfere with antigen processing in B cells and thereby block subsequent presentation of the resulting peptides to T cells (Molinari et al. 1998). VacA inhibited the Ii-dependent pathway of antigen presentation, which is mediated by newly synthesized MHC class II molecules, but did not interfere with the pathway dependent on recycling MHC class II. The inhibition of antigen presentation in B cells is likely the result of a restricted vesicle transport of late endosomal vesicles to the apical surface and thus might be related to the impaired vesicle trafficking effects seen in epithelial cells. VacA was shown to inhibit PMA-induced as well as T cell-induced proliferation of B cells (Torres et al. 2007). Furthermore, ectopic expression of CagA in B cells was reported to inhibit interleukin-3-dependent B-cell proliferation by inhibiting JAK-STAT signaling, which may result in inefficient antibody production and reduced cytokine expression (Umehara et al. 2003). CagA may also play a role in the development of MALT lymphoma by impairing p53-dependent apoptosis in B cells. Although *H. pylori* might come into contact with B cells when invading the gastric submucosa, translocation of CagA into these cells has not been shown under infection conditions.

The effects of *H. pylori* on T cells may also be mediated by different bacterial factors, but the best-characterized modulating factor is VacA. Recently, the β2 subunit of the leukocyte-specific integrin heterodimer LFA-1 (CD18) has been identified as a receptor for VacA on the surface of T cells. Purified VacA interacts with CD18, resulting in VacA internalization into T cells (Sewald et al. 2008b). Uptake of VacA results in a marked inhibition of proliferation of T cell lines upon stimulation (polyclonal or T cell receptor-mediated) as well as a diminished expression of IL-2 and IL-2 receptor (CD25). This effect is due to an interference

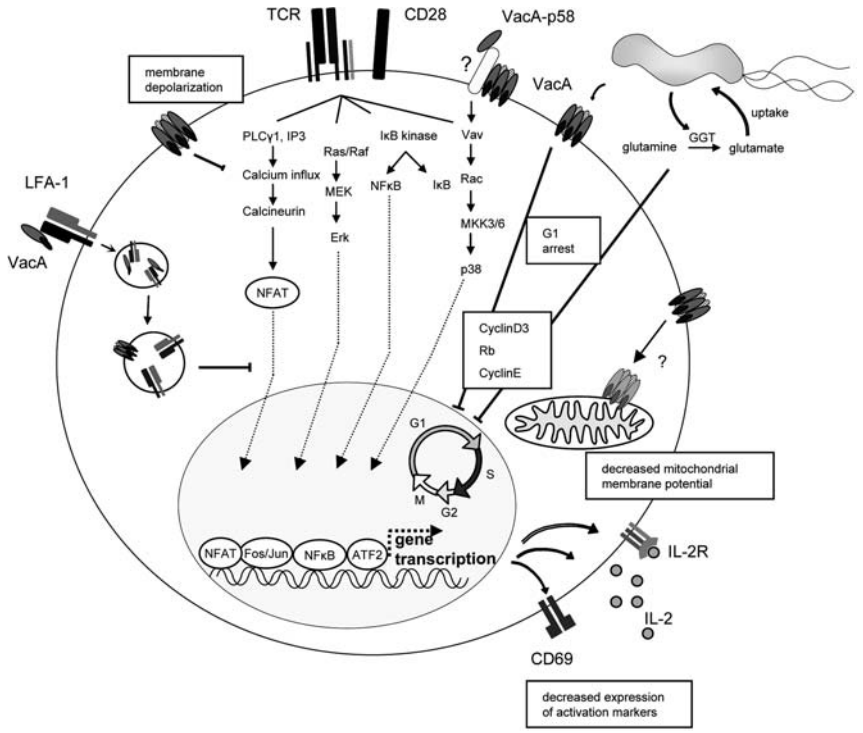

Fig. 4 VacA interactions with T lymphocytes and modulation of adaptive immunity. VacA binds the leukocyte–specific β2 integrin subunit CD18 and exploits β2 integrin recycling of migrating T lymphocytes for uptake and internalization. Due to its channel-forming activity, VacA induces a depolymerization of the plasma membrane. VacA blocks T cell proliferation by interference with the cell cycle (G1 arrest) and induces a decreased mitochondrial membrane potential in T cells. Translocation of the transcription factor NFAT into the nucleus is blocked, resulting in specific abrogation of transcription of a set of cytokine and chemokine genes, including the IL-2 gene. The p58 subunit of VacA is able to bind T cells and to induce a signaling cascade resulting in actin rearrangement

with the activity of nuclear factor of activated T cells (NFAT), a transcription factor that regulates a number of important immune response genes (Boncristiano et al. 2003; Gebert et al. 2003; Sundrud et al. 2004). In contrast, resting primary $CD4^+$ T cells did not show a VacA-mediated IL-2 inhibition (Sundrud et al. 2004); however, activated primary T cells restored this phenotype (Sewald et al. 2008b). The effect of VacA may be mediated by blocking calcium influx, thereby interfering with the activity of the calcium-dependent phosphatase calcineurin, which is required for NFAT activation (Fig. 4). In $CD4^+$ T cells, but not in B cells, a reduction in the mitochondrial membrane potential was suggested as an additional factor involved in blocking T cell proliferation (Torres et al. 2007). Finally, a third effect of VacA involves binding of an unknown receptor on T cells, leading to Rac/p38 activation, actin rearrangement, and inhibition of T cell proliferation (Boncristiano et al. 2003). Since *H. pylori* can disrupt epithelial cell tight junctions, VacA might act

as a distant effector that can reach the lamina propria and block proliferation of T cells in the local gastric environment (Gebert et al. 2004). Inhibition of T cell proliferation and cell cycle arrest has also been attributed to the *H. pylori* secreted factors γ-glutamyltranspeptidase and arginase (Schmees et al. 2007; Zabaleta et al. 2004). The inherent glutamine-hydrolysing activity of γ-glutamyltranspeptidase was also shown to result in deprivation of glutamine, which may be required for activity of lymphocytes and macrophages, and thus could also explain the observed proliferation inhibition (Shibayama et al. 2007).

5 *H. pylori* and the Development of Gastric Cancer

Development of gastric cancer involves several histopathologically characterized steps, including multifocal gastric atrophy, intestinal metaplasia, dysplasia, and, finally, invasive carcinoma (Correa and Houghton 2007). Mouse models indicate that the cancer cells may originate from bone-marrow-derived mesenchymal stem cells, which migrate into stomach epithelia after prolonged inflammation and tissue destruction (Houghton et al. 2004), or from other sources such as gastric progenitor cells (reviewed in Karam 2008). Many conclusions about cancer development were derived from transgenic mouse models, in which *H. pylori* infection enhances the cancer development rates (e.g., Fox et al. 2003; Syder et al. 2004), but it should be noted that it is probably not possible in these models to arrive at conclusions regarding the involvement of pathogenicity determinants, such as the *cag*-PAI, due to their inherent instability. The link between *H. pylori* infection and gastric cancer might simply be induction of gastric atrophy by bacteria-induced long-term inflammation (Atherton 2006), which is associated with oxidative stress, inducing mutations in the gastric mucosa (Touati et al. 2003). This is supported by the fact that defects in DNA repair predispose to development of precancerous lesions in mice (Meira et al. 2008). However, despite the fact that gastric cancer may develop from atrophic gastritis in the absence of *H. pylori*, there is also evidence that bacterial factors may have direct oncogenic effects. For example, *H. pylori* infection of gastric epithelial cells in vitro was shown to induce mutations in the *TP53* tumor suppressor gene, due to increased expression of activation-induced cytidine deaminase (Matsumoto et al. 2007); this effect was dependent on the *cag* pathogenicity island and on NF-κB activation. Since *H. pylori* can be found intracellularly in gastric pit stem cells (Oh et al. 2005), and strain adaptation from atrophic gastritis to gastric cancer has been shown to influence invasion into gastric epithelial progenitor cells (Giannakis et al. 2008), it has also been speculated that the interaction of *H. pylori* with gastric progenitor cells may result in malignant transformation. The Cag T4SS and particularly the CagA protein have been directly associated with development of cancer in animal models (Peek and Blaser 2002). In Mongolian gerbils, a corpus-predominant infection with *H. pylori*, which is considered as a precursor of atrophic gastritis, and also *H. pylori* adaptation to a high carcinogenic potential, are dependent on CagA (Franco et al. 2005; Rieder

et al. 2005). In epithelial cells that were sensitized to carcinogenic agents, transfection of CagA leads to an abnormal activation of MAP kinase pathways, which results in cell transformation (Zhu et al. 2005). Moreover, in a transgenic mouse model, expression of CagA in stomach tissue occasionally resulted in the development of gastric or intestinal cancers, which was not the case upon expression of phosphorylation-deficient CagA (Ohnishi et al. 2008), indicating that CagA alone may function as an oncoprotein. However, during *H. pylori* infections, CagA does not act in isolation, and its activity may be influenced by other virulence factors, as has been found in vitro (Argent et al. 2008b; Yokoyama et al. 2005).

The molecular mechanisms which lead to carcinogenesis are not understood. It is well known that the activity of the Cag T4SS, and particularly CagA translocation and phosphorylation, results in massive changes in gene expression profiles of infected cells, which may play a role in carcinogenesis (Cox et al. 2001; El Etr et al. 2004; Guillemin et al. 2002; Mueller et al. 2003). Upregulation of proinflammatory cytokines may lead to stimulation of secondary signaling pathways, as reported for macrophage migration inhibitory factor, which binds to CD74 and induces a proliferative response (Beswick et al. 2006b). Phosphorylation-dependent inhibition of glycogen synthase kinase (GSK)-3β via Akt signaling, which has been reported to be induced by the *cag*-PAI, OipA, or also VacA (Nakayama et al. 2008; Sokolova et al. 2008; Tabassam et al. 2008a), may lead to nuclear translocation of β-catenin and subsequent induction of gene expression. Indeed, infection of gastric epithelial cells with an adapted *H. pylori* strain that displayed a high carcinogenic potential was characterized by activation of gene transcription via β-catenin in a CagA-dependent manner (Franco et al. 2005), and activation of β-catenin was found to involve a tyrosine phosphorylation-independent interaction between CagA and E-cadherin and to result in upregulation of genes involved in intestinal differentiation (Murata-Kamiya et al. 2007).

Many studies have also addressed the upregulation of matrix metalloproteinases, which might be involved in extracellular matrix degradation in the gastric mucosa. Matrix metalloproteinase-7 (MMP-7) is induced in AGS cells, depending on a functional Cag secretion system, but not on CagA (Bebb et al. 2003; Crawford et al. 2003; Wroblewski et al. 2003). MMP-7 upregulation involves Erk activation as well as nuclear translocation of p120-catenin, which in turn is released during disruption of adherens junction complexes (Ogden et al. 2008). Elevated levels of MMP-7 secreted by epithelial cells results in increased epithelial cell proliferation, due to enhanced release of insulin-like growth factor (McCaig et al. 2006). MMP-1 upregulation in AGS cells upon infection is also Erk- and JNK-dependent and relies partly on a functional T4SS and on translocated and phosphorylated CagA (Krueger et al. 2006; Pillinger et al. 2007). Furthermore, increased MMP-2 and MMP-9 activity can be stimulated in AGS cells in a CagA-dependent manner and leads to an invasive phenotype (Oliveira et al. 2006). In mice, however, MMP-2 and MMP-9 are also upregulated by Cag^- strains (Kundu et al. 2006), possibly reflecting the dispensability of the Cag system for mouse infections.

In conclusion, although several molecular events with a possible impact for the development of gastric cancer have been characterized, it is premature to reach

definite conclusions with respect to the oncogenic mechanisms of the Cag T4SS or other *H. pylori* virulence factors.

6 Conclusions

During its long association with humans, lasting back 60,000 years or more (Linz et al. 2007), *H. pylori* has established a balance between generating a comfortable niche and avoiding the immune consequences of its colonization. For this purpose, the bacteria have acquired a set of unique virulence functions, such as a potent urease and a highly efficient motility and chemotaxis system, a set of outer membrane adhesins, the multifunctional toxin VacA, and the Cag T4SS. Effective immune evasion mechanisms concerning the innate as well as the adaptive immunity are of major importance. As a result, the infected human host mounts a vigorous innate and adaptive immune response, which ideally should clear the infection, but in the majority of infected people fails to prevent chronic colonization. The immunosuppressive functions of the bacterial virulence factors counteract the activity of the immune response at several stages. Inducing a mild inflammation seems to be beneficial for *H. pylori* to obtain nutrients and to spread into deeper tissues. However, genetic differences between hosts and external factors (e.g., diet) can tip this balance in either direction. If host responses are no longer able to control the bacterium, virulence factors, such as VacA, HP-NAP, and the Cag T4SS, can result in gastric epithelial damage, which may lead to peptic ulceration or even neoplastic transformation. At least in animal models, vaccination with *H. pylori* antigens and a suitable adjuvant can drive an immune response that confers protection against infection. Understanding the nature of this protective response will be a major challenge. Future studies, directed toward understanding interactions between *H. pylori* and immune cells in vivo, are expected to result in important new insights into the mechanisms of *H. pylori* persistence and also may lead to the development of novel therapeutic approaches.

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Listeria as an Enteroinvasive Gastrointestinal Pathogen

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Contents

Abstract The bacterium *Listeria monocytogenes* is the causative agent of listeriosis, a highly fatal opportunistic foodborne infection. *Listeria* spp. are isolated from a diversity of environmental sources, including soil, water, effluents, a large variety of foods, and the feces of humans and animals. Recent outbreaks demonstrated that *L. monocytogenes* can cause gastroenteritis in otherwise healthy individuals and more severe invasive disease in immunocompromised patients. Common symptoms include fever, watery diarrhea, nausea, headache, and pains in joints and muscles. The intestinal tract is the major portal of entry for *L. monocytogenes,* whereby strains penetrate the mucosal tissue either directly, via invasion of enterocytes, or indirectly, via active penetration of the Peyer's patches. Studies have

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revealed the strategy taken by the bacteria to overcome changes in oxygen tension, osmolarity, acidity, and the sterilizing effects of bile or antimicrobial peptides to adapt to conditions in the gut. In addition, *L. monocytogenes* has evolved species-specific strategies for intestinal entry by exploiting the interaction between the internalin protein and its receptor E-cadherin, or inducing diarrhea and an inflammatory response via the activity of its hemolytic toxin, listeriolysin. The ability of these bacteria to survive in bile-rich environments, and to induce depletion of sentinel cells such as Paneth cells that monitor the luminal burden of commensal bacteria, suggest strategies that have evolved to promote intestinal survival. Pre-existing gastrointestinal disease may be a risk factor for infection of the gastrointestinal tract with *L. monocytogenes*. Currently, there is enough evidence to warrant consideration of *L. monocytogenes* as a possible etiology in outbreaks of febrile gastroenteritis, and for further studies to examine the genetic structure of *Listeria* strains that have a propensity to cause gastrointestinal versus systemic infections.

1 Introduction

The human gut contains a large number of diverse microbiota comprising several hundred different types of bacteria. A balanced relationship between these commensal bacteria and the intestinal immune system and the high species diversity is important in maintaining homeostasis and structural stability. Loss of bacterial diversity as a result of aging and antibiotic treatment impedes the ecosystem's ability to resist the ingress of pathogenic microorganisms. Additionally, accidental introduction of pathogenic bacteria by ingestion of contaminated food or water causes inflammatory changes at the mucosal surface leading to disease. Diseases caused by foodborne pathogens such as the gram-positive bacterium *Listeria monocytogenes* are on the rise and threaten increasing numbers of susceptible individuals, from the unborn to the elderly, worldwide.

The genus *Listeria* consists of a group of Gram-positive bacteria of low G+C content closely related to *Bacillus, Clostridium, Enterococcus, Streptococcus*, and *Staphylococcus*. *Listeria* spp. are facultative anaerobic rods that do not form spores, have no capsule, and are motile at 10–25°C. The bacterium *L. monocytogenes* is the causative agent of listeriosis, a highly fatal opportunistic foodborne infection. *L. monocytogenes* causes serious localized and generalized infections in humans and a variety of other vertebrates, including domesticated and wild birds and mammals. Pregnant women, neonates, the elderly, and debilitated or immunocompromised patients in general are predominantly affected, although the disease can also develop in normal individuals. Clinical manifestations of invasive listeriosis are usually severe and include abortion, sepsis, and meningoencephalitis. Listeriosis can also manifest as a febrile gastroenteritis syndrome. *Listeria ivanovii*, a second pathogenic species of the genus, is specific for ruminants.

Listeria spp. are isolated from a diversity of environmental sources, including soil, water, effluents, a large variety of foods, and the feces of humans and animals.

The natural habitat of these bacteria is thought to be decomposing plant matter, in which they live as saprophytes. Domesticated ruminants probably play a key role in the maintenance of *Listeria* spp. in the rural environment via a continuous fecal–oral enrichment cycle.

Recent outbreaks demonstrated that *L. monocytogenes* can cause gastroenteritis in otherwise healthy individuals and more severe invasive disease in immunocompromised patients. Epidemiological studies of outbreaks of human disease now demonstrate that the pathogen can cause gastroenteritis in the absence of invasive disease and associated mortality. Furthermore, pathogenic *Listeria* have the ability to colonize and persist in the gallbladder suggesting that there may be long-term and chronic infections caused by these bacteria. The ability of the pathogen to survive within the various microenvironments of the gastrointestinal (GI) tract is essential for its ability to cause foodborne infection.

2 *Listeria* as an Enteroinvasive Gastrointestinal Pathogen

Although ingestion of food contaminated with *L. monocytogenes* is the usual mode of transmission, multiple factors likely play a role in the spectrum of presentations, from asymptomatic presentation to mild gastroenteritis to life-threatening invasive listeriosis. These variables include bacterial virulence factors, size of ingested inoculum, and underlying host defenses.

The clinical signs of *L. monocytogenes* infection are very similar in all susceptible hosts. Two basic forms of presentation can be distinguished: perinatal listeriosis and listeriosis in the adult patient. Listeriosis is usually a very severe disease – in fact, one of the most deadly bacterial infections currently known – with a mean mortality rate in humans of 20–30% or higher despite early antibiotic treatment. In each of the outbreaks, listeriosis caused invasive disease with mortality rates that averaged ~30%. These outbreaks illustrate the severe nature of listeriosis, particularly in immunocompromised patients, and highlight the differences between infections caused by *L. monocytogenes* and those caused by other common foodborne pathogens (e.g., *Salmonella* species, *Campylobacter jejuni, Vibrio* species, and *Shigella* species). It has been known for a long time that many patients experience diarrhea antecedent to the development of bacteremia or meningoencephalitis due to *L. monocytogenes*, but it was only recently that convincing evidence was obtained that this organism can cause acute, self-limited, febrile gastroenteritis in healthy persons (Ooi and Lorber 2005). At least seven outbreaks of foodborne gastroenteritis due to *L. monocytogenes* have been reported. Illness typically occurs 24 h after ingestion of a large inoculum of bacteria and usually lasts 2 days. Common symptoms include fever, watery diarrhea, nausea, headache, and pains in joints and muscles. *L. monocytogenes* should be considered to be a possible etiology in outbreaks of febrile gastroenteritis when routine cultures fail to yield a pathogen (Ooi and Lorber 2005).

Table 1 Outbreaks of gastroenteritis due to *Listeria monocytogenes*

Outbreak year	Number of cases	Serotype	Implicated source	Reference
1993	18	1/2b	Rice salad	Salamina et al. (1996)
1994	45	1/2b	Chocolate milk	Dalton et al. (1997)
1997	1,566	4b	Cold corn-and-tuna salad	Aureli et al. (2000)
1998	5	1/2a	Cold smoked trout	Miettinen et al. (1999)
2000	32	1/2	Corned beef and ham	Sim et al. (2002)
2001	16	1/2a	Delicatessen meat	Frye et al. (2002)
2001	48	1/2a	Cheese	Carrique-Mas et al. (2003)

Convincing evidence that *L. monocytogenes* could cause gastrointestinal illness came from an outbreak of febrile gastroenteritis that was associated with the consumption of contaminated chocolate milk (Dalton et al. 1997). Symptoms developed in 75% of persons (45 of 60) who drank chocolate milk that had been served at a picnic. Indistinguishable strains of *L. monocytogenes* were isolated from unopened cartons of chocolate milk, from environmental specimens from the dairy that supplied the milk, and from the stool samples of 14 symptomatic persons. The largest documented outbreak (Aureli et al. 2000) occurred in 1997, when 1,566 students and staff members from two primary schools in northern Italy developed febrile gastrointestinal illness after eating cafeteria food that had been prepared by the same caterer. A total of 292 persons were hospitalized. Cultures of one blood sample and 123 stool samples from hospitalized patients yielded *L. monocytogenes* strains that were identical to strains isolated from food and environmental specimens at the catering plant. In several subsequent outbreaks (Miettinen et al. 1999; Sim et al. 2002; Frye et al. 2002; Carrique-Mas et al. 2003), investigators have shown identity between strains of *L. monocytogenes* isolated from stool samples of individuals with febrile gastroenteritis and strains cultured from the epidemiologically implicated food. The outbreaks of gastrointestinal listeriosis are listed in Table 1.

In general, epidemiological data of foodborne outbreaks of gastroenteritis support the notion of *Listeria* being a diarrheic agent. In animals, an absence of clinical signs except mild diarrhea and staggering gait have been reported in calves and buffaloes after experimental oral infection (Barbuddhe et al. 2000; Chaudhari et al. 2001). Studies on healthy nonhuman primates dosed with various concentrations of *L. monocytogenes* suspended in sterile whole milk revealed symptoms of septicemia, irritability, loss of appetite, and occasional diarrhea (Farber et al. 1991).

3 Entry and Invasion of *Listeria*

Bacteria are generally retained at a distance from the mucus layer, where they generally form biofilm-like structures. Many bacteria produce mucinases to enable contact with the underlying epithelium. *L. monocytogenes* is not known to produce any mucinases; however, intriguingly, a number of surface proteins InlB, InlC, and

InlJ, all of which are members of the internalin family, all bound to MUC2 isoform. Adherence to the mucus layer could provide a beachhead from which secreted virulence factors of the bacterium may engage in modifying and destroying cells of the epithelial lining.

Flagellar structures contribute to the virulence of multiple gastrointestinal pathogens either as the effectors of motility, as adhesins, or as a secretion apparatus for virulence factors. *L. monocytogenes* uses flagella to increase the efficiency of epithelial cell invasion (Bigot et al. 2005). It has also been reported that *L. monocytogenes* flagella are used for motility, not as adhesins, to increase host cell invasion (O'Neil and Marquis 2006).

3.1 Features of Gastrointestinal Interactions

The pathophysiology of *Listeria* infection in humans and animals is still poorly understood. Most of the available information is derived from interpretation of epidemiological, clinical, and histopathological findings and from observations made in experimental infections in animals, particularly in the murine model. The gastrointestinal tract is thought to be the primary site of entry of pathogenic *Listeria* organisms into the host, as contaminated food is the major source of infection in both epidemic and sporadic cases (Farber and Peterkin 1991; Pinner et al. 1992). The clinical course of infection usually begins about 20 h after the ingestion of heavily contaminated food in cases of gastroenteritis (Dalton et al. 1997), whereas the incubation period for the invasive illness is generally much longer, around 20–30 days. Similar incubation periods have been reported in animals for both gastroenteric and invasive disease (Vazquez-Boland et al. 2001a).

Before reaching the intestine, the ingested *Listeria* organisms must withstand the adverse environment of the stomach. The point of entry and the mechanism of intestinal translocation used by *L. monocytogenes* remain controversial. However, in an early study by Racz et al. with guinea pigs infected intragastrically with 10^{10} *L. monocytogenes*, detailed histological analyses revealed that all the animals developed enteritis (Racz et al. 1972). In the initial stages, bacteria were detected mostly in the absorptive epithelial cells of the apical area of the villi, whereas in later phases most were inside macrophages of the stroma of the villi, suggesting that *L. monocytogenes* penetrates the host by invading the intestinal epithelium (Racz et al. 1972). Oral infective doses are lower for cimetidine-treated experimental animals than for untreated animals (Schlech et al. 1983), and the use of antacids and H2-blocking agents has been reported to be a risk factor for listeriosis (Schuchat et al. 1992; Ho et al. 1986). This indicates that gastric acidity may destroy a significant number of the *Listeria* organisms ingested with contaminated food.

Direct evidence that *L. monocytogenes* may indeed penetrate the host via the M cells overlying the Peyer's patches has been provided by a study using a murine ligated-loop model and scanning electron microscopy (Jensen et al. 1998). *Salmonella typhimurium* possesses a highly efficient mechanism for M cell entry that targets and destroys these cells, while *L. monocytogenes* and *Shigella flexneri*

appear to be internalized into M cells in a less disruptive fashion (Jensen et al. 1998). Listeriae have been shown to be capable of infecting the host by translocating from the intestinal lumen through Peyer's Patches (PP); however, results of experiments now indicate that these facultative intracellular parasites may also translocate through PP-independent routes. Listeriae were found to be absent from the PP of mice inoculated intragastrically with *L. monocytogenes*, but were present in the mesenteric lymph nodes of these same mice (Havell et al. 1999). It is known that following the intragastric inoculation of *L. monocytogenes*, listeriae rapidly transit the length of the gastrointestinal tract and reside in the colonic lumen for up to a week. Inoculation of listeriae into the rectum of mice resulted in the infection of the caudal lymph node which indicated that PP was not required for listerial translocation. Shortly after the intragastric inoculation of *L. monocytogenes* into germfree SCID mice, listeriae were found in the mesenteries, livers and spleens indicating that PP are not required for listerial translocation from the intestinal lumen. One possible route of translocation from the intestinal lumen might occur by listeriae entering enterocytes. listeriae have been found to be capable of entering cultured mouse small intestine enterocytes (Havell et al. 1999). Internalized Listeriae were observed to multiply and spread intracellularly between enterocytes.

Intestinal translocation of pathogenic listeriae occurs without the formation of gross macroscopic or histological lesions in the gut of mice (Marco et al. 1992), suggesting that an epithelial phase involving bacterial multiplication in the intestinal mucosa is not required by *L. monocytogenes* for systemic infection. Indeed, a study using a rat ileal loop model of intestinal infection (Pron et al. 1998) has shown that *Listeria* organisms are translocated to deep organs very rapidly, demonstrating that crossing of the intestinal barrier occurs in the absence of prior intraepithelial replication. Translocation was dose dependent, and the presence of Peyer's patches in ligated loops did not affect the rate of translocation, levels of uptake being similarly low for Peyer's patches and villous intestine (50–250 bacteria per cm^2 of tissue after inoculation of the loop with 10^9 bacteria) (Pron et al. 1998). The preferential site for bacterial replication was the Peyer's patches, and the essential listerial virulence factor Hly (hemolysin) was indispensable for this process, showing that *L. monocytogenes* establishes an active local infection in these lymphoid structures of the intestine. The sequence of cellular events leading to the dissemination of *L. monocytogenes* from the gut to draining mesenteric lymph nodes (MLNs) by confocal microscopy of immunostained tissue sections from a rat ligated ileal loop system was studied (Pron et al. 2001). OX-62-positive cells beneath the epithelial lining of Peyer's patches (PPs) were the first *Listeria* targets identified after intestinal inoculation. Listeriae were detected by microscopy in draining MLNs as early as 6 h after inoculation. *Listeria* were transported by DCs from PPs to the deep paracortical regions of draining MLNs and are then transmitted to other cell populations by mechanisms independent of ActA. Another pathway of dissemination to MLNs was identified, probably involving free *Listeria* and leading to the infection of ED3-positive mononuclear phagocytes in the subcapsular sinus and adjacent paracortical areas. The study provided evidence that DCs are

major cellular targets of *L. monocytogenes* in PPs and that DCs may be involved in the early dissemination of this pathogen. DCs were not sites of active bacterial replication, making these cells ideal vectors of infection (Pron et al. 2001).

Experimental observations made with the mouse and rat models of intestinal translocation do not, however, explain how *L. monocytogenes* causes enteritis. The association of gastroenteric symptoms with fever is consistent with invasive intestinal disease, as observed by Racz in the guinea pig model (Racz et al. 1972). Pathogenic *Listeria* organisms pass directly from cell to cell by a mechanism involving host cell actin polymerization. Therefore, regardless of the mechanism of entry used, the bacteria that penetrate the intestinal wall might then invade neighboring enterocytes by basolateral spread, leading to enteritis. This is consistent with in vitro experimental data showing that *L. monocytogenes* enters polarized Caco-2 cells predominantly via the basolateral surface (Gaillard and Finlay 1996). Gross intestinal lesions develop in experimental animals only if large oral doses of *L. monocytogenes* are given (Pron et al. 1998; MacDonald and Carter 1980). Similarly, episodes of listeriae gastroenteritis in humans occur in the form of outbreaks with very short incubation periods and high attack rates among immunocompetent adults (Dalton et al. 1997; Salamina et al. 1996), consistent with the ingestion of a very high dose of bacteria (as high as 2.9×10^{11}, as estimated for one of these outbreaks, caused by the consumption of heavily contaminated chocolate milk; Dalton et al. 1997). Thus, intestinal invasion and the ensuing febrile gastroenteritis syndrome probably result from extensive exposure of the intestine to pathogenic *Listeria* organisms.

3.2 Crossing the Intestinal Barrier

The cellular basis of intracellular parasitism by this bacterium has been to a large extent elucidated. The infection cycle begins with adhesion to the surface of the eukaryote cell and subsequent penetration of the bacterium into the host cell. The invasion of non-phagocytic cells involves a zipper-type mechanism, in which the bacterium gradually sinks into tightly cupped structures of the host cell surface until it is finally engulfed. The target cell membrane closely surrounds the bacterial cell during the process and does not form the spectacular local processes or membrane ruffles characteristic of invasion by *Salmonella* and *Shigella* spp. (Dramsi and Cossart 1998; Karunasagar et al. 1994).

The virulence factors, including the internalins (InlA and InlB), listeriolysin (Hly), phospholipases (PlcA and PlcB), a metalloprotease (Mpl) and a bacterial surface protein that engages the host cell actin machinery, ActA, are encoded by chromosomal genes organized in operons. Six of the virulence factors responsible for key steps of *L. monocytogenes* intracellular parasitism (*prfA, plcA, hly, mpl, actA*, and *plcB*) are physically linked in a 9-kb chromosomal island referred to as LIPI-1 (for *Listeria* pathogenicity island 1) (Vazquez-Boland et al. 2001b). This locus also harbors the PrfA transcriptional regulator, the master regulator of

virulence gene expression in *L. monocytogenes*. The *inl*A and *inl*B genes are located on a chromosomal region distinct from the LIPI-1 locus by are commonly regulated by the transcriptional regulator PrfA. In addition, the expression of both of these genes are independently regulated by the alternative sigma factor σB via a σB-dependent promoter located upstream of *inl*A (Kazmierczak et al 2003; Kim et al. 2005; Hain et al. 2008).

L. monocytogenes uses the two surface proteins, the internalins InlA and inlB, to engage, in a species-specific manner, the adhesion molecule E-cadherin and the hepatocyte growth factor receptor Met, respectively, to induce internalization (Mengaud et al. 1996; Shen et al. 2000). Internalins A and B are members of a large family of 25 proteins that are either located on the bacterial surface or are proteins secreted by the bacteria. These proteins have common structures, comprising of a signal peptide, a N-terminal region comprising of a repeat of 22 amino acid rich in the amino acid leucine, hence leucine-rich-repeats (LRR), as well as a immunoglobulin-like fold juxtaposed to these repeats (Schubert et al. 2001). The 800 amino acid-InlA protein harbors 15 LRR repeats and it is the inter-repeat regions that are now known to engage its receptor E- cadherin (Lecuit et al. 1997; Schubert et al. 2002). The InlA protein is tethered to the bacterial cell wall by a LPXTG amino acid motif present at the C-terminal end of this polypeptide. The receptor E-cadherin engaged by InlA is a member of a large family of transmembrane proteins involved in maintaining the integrity of cell-cell junctions. E-cadherin is a 882 amino acid single pass transmembrane protein of which the N-terminal 555 amino acids are extracellular, possessing a short transmembrane domain followed by a relatively short cytoplasmic tail of 152 amino acids. The extracellular domain which comprises of five 110 amino acid-containing immunoglobulin-like domains (EC1-EC5) is involved in Ca2+-dependent homotypic interactions. It is to the most distal of these repeats that the InlA protein binds.

The InlA-E-cadherin interaction plays a key role in the crossing of the intestinal barrier in humans and is also exploited by *L. monocytogenes* to target and cross the placental barrier. E-cadherin is only expressed by a limited number of cell types, mostly of epithelial origin. It has been reported that InlA (Lecuit et al. 2001), but not InlB (Khelef et al. 2006), plays a critical role in the crossing of the intestinal barrier after infection. However, when animals are infected intravenously with a *inl*A mutant, no defects in the colonization properties of spleens and livers were detected, indicating that InlA plays no role during systemic spread but is clearly a key virulence factor during early oral infections (Lecuit et al. 2001). Following internalization into host cells, the bacteria escape from the phagosomal compartment and enter the cytoplasm. They then spread from cell to cell by a process involving actin polymerisation. In infected hosts, the bacteria cross the intestinal wall at Peyer's patches to invade the mesenteric lymph nodes and the blood. The main target organ is the liver, where the bacteria multiply inside hepatocytes (Vazquez-Boland et al. 2001a).

A number of laboratory strains currently in use, e.g., the LO28 strain, harbor a mutation in the InlA gene leading to the production of a secreted truncated InlA polypeptide. Evidence from epidemiological studies has revealed that about 35% of

strains isolated from food carry an identical mutation in the *inl*A gene. However, from clinical isolates, 96% of all strains encoded a full length InlA polypeptide suggesting that the expression of functional InlA is a prerequisite for translocating from the gut to internal organs during infection.

3.3 Species-Specific Invasion of the Epithelial Barrier

Many pathogenicity tests for studying *L. monocytogenes* have been developed using laboratory animals. A number of small animal species can be experimentally infected with *Listeria*. Mice and guinea pigs can be infected either intragastrically or intravenously. The intravenous route is the most relevant to the human foodborne listeriosis. Not all *L. monocytogenes* strains are equally virulent (Roche et al. 2005). Zachar and Savage (1979) demonstrated that *L. monocytogenes* colonizes the gastrointestinal tract (GIT) of germ-free mice. Generally, *L. monocytogenes* was displaced from the mucosal layer and failed to colonize mice that were not germ free. The rate of peristalsis is slower in germ-free mice so *L. monocytogenes* would be propelled faster through the bowels of normal mice (Abrams and Bishop 1967). Abrams and Bishop (1967) also hypothesized that *L. monocytogenes* may colonize the GIT during an imbalance within the microbial ecosystem of the GIT. Imbalances could be caused by a number of factors including starvation, antibiotics, and other antimicrobials. The role of virulence factors and other mechanisms is outlined in Fig. 1.

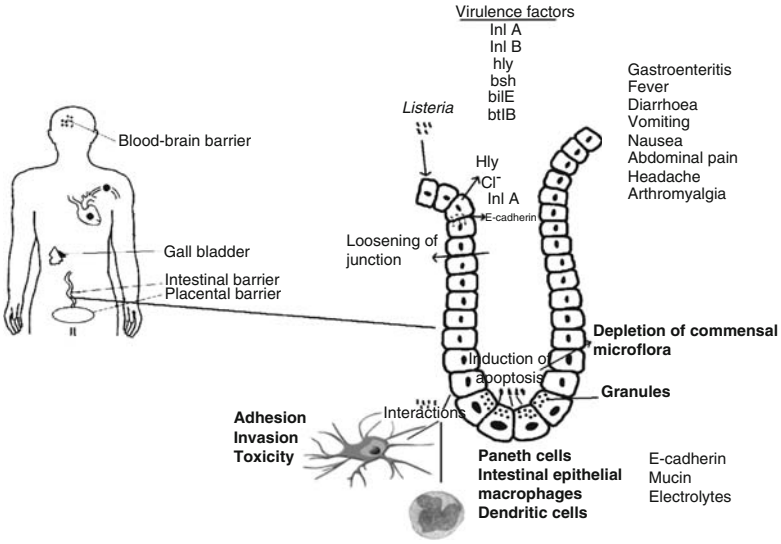

Fig. 1 The host barriers and mechanism of interactions of *Listeria* inside the gut. The various cell types and processes are involved in gastrointestinal listeriosis. InlA plays a central role in crossing the intestinal barrier

Table 2 Host specificity of *Listeria* invasion

Animal	Intestine	Placenta
Mouse	−	−
Rat	−	+
Guinea pig	+	−
Rabbit	+	+
Gerbil	+	+
Human	+	+

The internalin-E-cadherin interaction is species-specific, and relies on the nature of a single amino-acid in the E-cadherin molecule, which is proline in permissive species such as humans, and glutamic acid in non-permissive species such as the mouse (Lecuit 2005). Although there was 85% similarity between mouse E-cadherin and human E-cadherin, no interactions were observed between mouse E-cadherin and InlA. Lecuit et al. (1999) found that substituting a single amino acid in human E-cadherin (proline to glutamic acid) resulted in inefficient interaction between *L. monocytogenes* and E-cadherin. Lecuit et al. (2001) later demonstrated the interaction between internalin and E-cadherin in vivo in a transgenic mouse that expressed human E-cadherin. Recently Wollert et al. (2007) designed a versatile murine model for human listeriosis by substituting two amino acids in InlA (Glu16-Pro and Gln64-Glu) in mice. These mutations adapted the bacterium to infection of the gut epithelium in mouse by modifying InlA to recognize the previously incompatible receptor of mouse E-cadherin. The host specificity of *Listeria* invasion is given in Table 2.

In a more recent study, Lecuit et al. (2001) reported infecting guinea pig cells of epithelial origin with *L. monocytogenes*. In addition, they also sequenced the guinea pig Ecadherin EC1-coding region and found that the guinea pig E-cadherin, like human E-cadherin, harbors a proline at position 16. Even though previous studies had implicated InlA in fetoplacental listeriosis, there was no requirement of InlA in pregnant guinea pigs (Bakardjiev et al. 2005). Additional studies have helped to characterize the role of the bacterial invasion proteins InlA and InlB in these animals. InlB is unable to engage the Met receptor of guinea pigs, thus InlA-dependent fetoplacental crossing is diminished. However, oral infection with two novel and complementary animal models for human listeriosis, the gerbil, a natural host for *L. monocytogenes*, and a knockin mouse line ubiquitously expressing humanized E-cadherin, have been described (Disson et al. 2008). Using these two models, where both receptors for InlA and InlB are functionally expressed at both the gut epithelia and the fetotransplacental barrier, allowed conclusive demonstration of the essential and interdependent roles of InlA and InlB in fetoplacental listeriosis (Disson et al. 2008).

Active translocation of *L. monocytogenes* across the gut epithelial barrier is mediated by interaction of bacterial internalin (InlA) and its species-specific host receptor, E-cadherin, whereas translocation across Peyer's patches through M-cells is InlA-independent. Germ-free transgenic mice expressing the human enterocyte-associated E-cadherin receptor with wild-type (WT) or mutant *L. monocytogenes*

strains, or its nonpathogenic noninvasive relative *Listeria innocua*, or with *Bacteroides thetaiotaomicron*, a prominent gut symbiont, were colonized to define microbial determinants and molecular correlates of the host response to translocation. An analysis of signaling pathways, following infection with wild-type *L. monocytogenes* or isogenic mutant strains lacking listeriolysin (*hly*) or InlA (*inl*A), revealed that the host response was found to be markedly attenuated in a listeriolysin-deficient (Δ*hly*) mutant despite its ability to be translocated to the lamina propria. The mucosal response to the *inl*A mutant was, however, virtually identical to that seen with the wild-type strain. Therefore, *hly*, rather than bacterial invasion of the lamina propria mediated by InlA, is a dominant determinant of the intensity of the host response to *L. monocytogenes* infection via the oral route (Lecuit 2007). Recently, it was demonstrated that listeriolysin can induce diarrheagenic effects by inducing active chloride secretion, and at higher concentrations, affect barrier function. These effects are Ca2+-dependent and can be reproduced by the use of purified toxin, suggesting that this is a remote effect not requiring the presence of the bacterium (J. Richter and J.D. Schulzke, personal communication)

4 Factors Supporting Gastrointestinal Persistence and Survival

Following ingestion into the stomach by a human host, *L. monocytogenes* encounters low pH and low oxygen conditions. Growth, survival, proliferation, and pathogenesis of *L. monocytogenes* under low oxygen or anaerobic conditions have been reviewed by Lungu et al. (2009). Recent studies (Cotter et al. 2001; Ferreira et al. 2003) have shown that *L. monocytogenes* possesses acid resistance systems that enable it to combat low pH conditions occurring in foods as well as in the stomach. Conte et al. (2000) showed that acid-adapted *L. monocytogenes* cells were more capable of infecting and proliferating in Caco-2 cells than their non-acid-stressed counterparts. The alternative sigma factor, σB, has been shown to be necessary for survival of *L. monocytogenes* following exposure to low pH conditions. Ferreira et al. (2001) reported that stationary phase cells of a Δ*sig*B mutant strain were 10,000-fold more susceptible to lethal acid stress (pH 2.5) than the wild-type. *L. monocytogenes* 4b has been linked to most large outbreaks of listeriosis. Czuprynski et al. (2002) suggest that *L. monocytogenes* serotype 4b may possess one or more virulence factors that improve its ability to cause systemic infection following inoculation via the intragastric route. Glutamate enhances the survival of *L. monocytogenes* in gastric fluid and other low pH environments, and this is directly linked to the activity of the glutamate decarboxylase system (GAD). *L. monocytogenes* LO28 possesses two glutamate decarboxylate homologues *gad*A and *gad*B that are differentially expressed. The sensitivity of a Δ*gad*AB mutant to ex vivo porcine and synthetic gastric fluid demonstrated that the GAD system facilitates survival in the stomach following

ingestion as well as in other low pH environments. In addition, *L. monocytogenes* strains that are known to be sensitive to gastric juice exhibit low levels of GAD activity (Cotter et al. 2001).

Survival of the pathogen within gastric acid requires the glutamate decarboxylase (GAD) system. In addition, the carnitine uptake system, OpuC, is essential for adaptation within the murine GI tract and subsequent invasive disease. Survival of bile salts both in vitro and in vivo requires a number of mechanisms including bile salt hydrolase (Bsh) and the recently described bile exclusion system, BilE (previously OpuB). Finally, a number of these systems (including Bsh, OpuC, and BilE) are regulated by both the alternative stress sigma factor, Sigma B, and by the regulator of virulence gene expression, PrfA (Gahan and Hill 2005).

The ability of *L. monocytogenes* to survive the various external environments including low O_2 conditions prior to infection as well as in the various microenvironments of the gastrointestinal tract are essential for this pathogen to cause disease. *L. monocytogenes* encounters various external stresses such as acid stress, anaerobiosis, oxidative stress, and nutrient starvation in the environment; stresses that may also be present during the course of invasion and growth within host cells (Lou and Yousef 1997; Hanawa et al. 1995; Christiansen et al. 2004). For effective colonization or invasion of various environmental niches such as the gastrointestinal tract, soil, silage, and sludges, *L. monocytogenes* has to be able to overcome a number of barriers including anaerobiosis, pH shifts, and high osmolarity. *L. monocytogenes* has also had to develop successful strategies to be able to compete for substrate with other organisms found in these environments. In addition, *L. monocytogenes* must also assimilate nutrient substrates as well as survive under the harsh conditions presented to it in the gastrointestinal tract and the macrophages.

Many of the classical studies of listerial pathogenesis examined the role of bacterial virulence factors on systemic infection in mice following intravenous inoculation. More recent research is beginning to focus upon the GI phase of listeriosis. For instance, recent experiments have established that *L. monocytogenes* colonizes the gall bladder of infected mice (Hardy et al. 2004), that bile salt hydolase activity is essential for pathogenesis (Dussurget et al. 2002), and that carnitine uptake by the pathogen is essential for survival within the small intestine and transient colonization of the murine GI tract (Sleator et al. 2001; Wemekamp-Kamphuis et al. 2002). It is evident that this exciting research is required in order to determine the specific pathogen and host factors that contribute to natural disease following consumption of contaminated foods.

During foodborne infection, *L. monocytogenes* encounters a number of suboptimal microenvironments that constitute elements of the host physico-chemical defense system. In order to transiently colonize the host GI tract prior to invasion the pathogen must survive acid conditions within the stomach as well as elevated osmolarity and the presence of bile salts within the small intestine. Whilst *Listeria* spp. can be isolated from feces, it is not yet clear whether this represents a colonization or carriage state or is due to transient passage through this environment (Hof 2001). The recent finding that *L. monocytogenes* colonizes the gallbladder of infected mice raises the intriguing possibility that the gallbladder may function as a

source of chronic shedding of the organism (Hardy et al. 2004). Examination of the Listerial response to stresses normally encountered in the upper small intestine revealed osmotic stress to be at the top of the hierarchy of stress responses during gastrointestinal residence. Furthermore, the increased osmolarity of the gastrointestinal lumen may be interpreted as an environmental cue signaling gut entry and that the underlying genetic element governing this response is the alternative stress sigma factor, sigma B (Sleator et al. 2007).

There is evidence that treatment of individuals with antacids or cimetidine may reduce resistance to *L. monocytogenes* infection (Donnelly 2001) or increase fecal carriage rates of *L. monocytogenes* (Cobb et al. 1996). This suggests that, under normal conditions, the low pH of the stomach provides a significant and effective barrier to *Listeria* infection.

L. monocytogenes has the capacity to undergo an adaptive response to moderately acidic pH (pH 5–5.5) that enhances survival under conditions of lethal pH (pH 3.5) (Davis et al. 1996; O'Driscoll et al. 1996). The stress hardening response is most likely essential for infection, and it is likely that bacterial gene expression during passage through the stomach will influence subsequent survival in the GI tract. The *L. monocytogenes* GAD system significantly contributes to survival at low pH (Cotter et al. 2001). *L. monocytogenes* encodes two antiporters capable of transporting glutamate into the cell and three GAD enzymes that potentially carry out decarboxylation of glutamate (Glaser et al. 2001). Analysis of the *L. monocytogenes* EGDe genome sequence reveals that the pathogen contains the genes required for operation of the arginine deiminase system (Glaser et al. 2001).

The lumen of the GI tract is a region of relatively high salinity (0.3 M NaCl) (Chowdhury et al. 1996). Passage of bacteria from the stomach into the small intestine therefore represents an osmotic upshift that, in many foodborne pathogens, serves to trigger the expression of genes that are necessary for survival and colonization (Nikaido et al. 1983; Foster and Spector 1995). Functional genetic analysis has determined that the membrane transporters BetL (Sleator et al. 1999) and Gbu (Ko and Smith 1999) are principally responsible for uptake of glycine betaine, an osmolyte predominately associated with plant material. The product of the *L. monocytogenes* gene oppA has been demonstrated to function as a peptide transport system and is required for effective systemic infection of mice (Borezee et al. 2000).

In order to cause systemic illness, *L. monocytogenes* needs to overcome various intestinal barriers including bile stress, volatile fatty acids, osmotic stress, nutrient variability, and intestinal microflora as well as the intestinal wall. To survive in the human intestinal tract during pathogenesis, *L. monocytogenes* must be able to survive bile that is secreted from the gall bladder into the upper small intestine. Begley et al. (2002) demonstrated that, during anaerobic incubation, *L. monocytogenes* LO28 was capable of growth in physiological concentrations of 0.3% human bile, and that further addition of up to 10% human bile did not inhibit growth. This study showed that *L. monocytogenes* LO28 was tolerant to individually conjugated bile acids at 5 mM concentrations.

L. monocytogenes isolates are generally relatively bile resistant; however, bile resistance varies significantly between strains (Begley et al. 2002). Using a transposon mutagenesis approach, a total of 12 genes were identified which play a role in bile tolerance in *L. monocytogenes* (Begley et al. 2002). The majority of these genes are predicted to encode proteins that play a role in stress resistance (including *gad*A), maintenance of cell envelope integrity, and transcriptional regulators (Dalet et al. 1999). Interestingly, the majority of these genes can be found within a discrete 50-kb region of the *L. monocytogenes* genome that also includes *opu*C and *bil*E/*opu*B. This region may therefore represent a cluster of genes involved in resistance to conditions encountered within the GI tract (Gahan and Hill 2005).

The gene encoding bile salt hydrolase activity (*bsh*) in *L. monocytogenes* is required for infection of guinea pigs by the oral route and for systemic infection in mice (Dussurget et al. 2002). Furthermore, as Bsh is absent from nonpathogenic *Listeria* species and is regulated by PrfA, the gene product represents a bonafide virulence factor that is indispensable for colonization of the GI tract by pathogenic *Listeria* species prior to invasion (Dussurget et al. 2002). Furthermore, another gene with homology to bile acid dehydratase was demonstrated and hence designated bile tolerance locus B (*btl*B); this is also necessary for colonization of the GI tract in mice (Begley et al. 2005). A two-gene system (*lmo*1421 and *lmo*1422, renamed as *bil*E) plays a role as a potential bile exclusion system, and a mutation in this gene results in significantly reduced resistance to human bile in vitro. BilE is required for full virulence potential in *L. monocytogenes* (Sleator et al. 2005).

A number of reports have documented *L. monocytogenes* cholecystitis in humans (Allerberger et al. 1989; Briones et al. 1992). Whole-animal bioluminescence imaging to track the fate of *L. monocytogenes* cells expressing Lux bioluminescence was used to examine replication of the pathogen in the murine gallbladder (Hardy et al. 2004). The study determined that the gallbladder is a major focus of infection in mice inoculated by the intravenous or oral routes of infection, and that bacteria replicate extracellularly within the lumen of the gallbladder indicating that *L. monocytogenes* must be able to resist high concentrations of bile, and that genes involved in bile resistance may be of paramount importance for survival within the small intestine, and for systemic infection. It was shown that *L. monocytogenes* strains are resistant to high concentrations of bovine bile at neutral pH, the pH of bile within the gallbladder (Begley et al. 2005). However, with a drop in pH, as may be encountered within the intestine, bile becomes much more toxic to the bacterial cell (Begley et al. 2002). Bile may therefore present more of a challenge to Listerial growth in the lumen of the small intestine than in the lumen of the gallbladder.

In addition to competition with resident microflora, *L. monocytogenes* encounters high osmotic conditions (0.3 M) in the small intestine (Chowdhury et al. 1996). Osmotic upshifts trigger the expression of genes necessary for survival under osmotic stress. Three osmolyte transporters have been identified in *L. monocytogenes*, and include glycine betaine porter I (BetL), glycine betaine porter II (Gbu), and a carnitine transporter (OpuC) (Fraser et al. 2000; Ko and Smith 1999; Mendum and Smith 2002). Sleator et al. (2001) linked OpuC to carnitine and glycine betaine uptake and showed that altering OpuC resulted in significant reduction in the ability

of *L. monocytogenes* to colonize the upper small intestine and cause subsequent systemic infection following peroral inoculation.

HtrA is necessary for the survival of *L. monocytogenes* in elevated sodium chloride concentrations, high temperature growth, oxidative stress caused by hydrogen peroxide, and acid sensitivity (Stack et al. 2005; Wonderling et al. 2004). Most of these conditions can be found in the various microenvironments encountered during pathogenesis.

Intracellular gene expression profiling of bacteria from infected macrophages (Chatterjee et al. 2006) revealed upregulation of the *opuBA* gene (lmo1421 and lmo0903, encoding a glycine betaine uptake system) and a gene similar to the *osmC* gene during the intracellular growth. Both genes are also known to be induced under osmotic stress situations (Gutierrez and Devedjian 1991; Wemekamp-Kamphuis et al. 2004). Three genes of the universal stress protein (Usp) family (*lmo*0515, *lmo*2673, and *lmo*2748) and genes for cholate, sodium, and pH homeostasis (*lmo*2378, *lmo*2381, and *lmo*2382) were upregulated intracellularly. Additionally, the upregulation of *lmo*0754 (*btlB*) needed to combat bile stress was detected (Chatterjee et al. 2006).

Apart from the hemolytic toxin listeriolysin a second haemolysin, HlyS, which is present in a subset of strains of lineage I, the evolutionary lineage of *L. monocytogenes* that contributes to the majority of spontaneous and epidemic outbreaks of listeriosis, has been identified (Cotter et al. 2008). This second haemolysin is only induced under oxidative stress conditions and contributes to murine virulence and survival in polymorphonuclear neutrophils. Its role in gastrointestinal infections remain to be explored.

5 Cellular Processes Affecting *Listeria* Survival in the Gastrointestinal Tract

L. monocytogenes was used long before its importance as a risk to public health and food safety was recognized by immunologists, because an infection highly reminiscent of human listeriosis was easily reproducible in laboratory rodents and protection could be transferred in syngeneic mice through spleen cells. The pioneering work of Mackaness in the early 1960s demonstrated that *L. monocytogenes* is able to survive and multiply in macrophages, and this bacterium has been used in immunological research as a prototype intracellular parasite (Machesky 1997). *L. monocytogenes* and *L. ivanovii* are facultative intracellular parasites able to survive in macrophages and to invade a variety of normally nonphagocytic cells, such as epithelial cells, hepatocytes, and endothelial cells.

Bacterial flora and microbial density in the lumen of the intestine are controlled by the Paneth cells, a small intestinal lineage that resides at the base of the crypts of Lieberkühn. These cells contribute to innate immunity by secreting antimicrobial products such as lysozymes, α-defensins, and secretary phospholipase A2.

(Yano and Kurata 2009). Recently, defects in the gene Atg16L1, that is required for autophagosome formation, and the transcription factor XBP1, a key regulator of endoplasmic reticulum (ER) stress response, have led to Paneth cell dysfunction (Saitoh et al. 2008; Cadwell et al. 2008). Mice with an *Xbp*1 deletion in intestinal epithelial cells were unable to control and clear an oral infection with *L. monocytogenes* (Kaser et al. 2008). Escape from autophagy following entry into the host cell cytoplasm is also a strategy used by *L. monocytogenes* even though the molecular mechanisms involved have not been described (Ogawa et al. 2009).

Protective immunity towards intracellular pathogens such as *L. monocytogenes* requires the presence of a cytosolic surveillance pathway via cytosolic nuclear oligodimerization domain (NOD)- like receptors (NLR). Nod2, which recognizes muramyl dipeptide in peptidoglycan, is a critical regulator of bacterial immunity within the intestine and has been found to recognize *L. monocytogenes* following its entry into the host cell cytosol. Recognition by Nod2 induces the expression of a subgroup of intestinal antimicrobial peptides, known as cryptdins. Nod2-deficient mice are susceptible to an infection with *L. monocytogenes* via the oral route but not through intravenous or peritoneal delivery (Kobayashi et al. 2005). Recently, it was discovered that CD147 (also known as BSG and EMMPRIN), a membrane-bound regulator of cellular migration, differentiation, and inflammatory processes, is a protein interaction partner of NOD2. A complex influence of the CD147-NOD2 interaction on NOD2-dependent signaling responses has been observed. The CD147 itself acts as an enhancer of the invasion of *L. monocytogenes*, an intracellular bacterial pathogen, and it is thought that the CD147-NOD2 interaction serves as a molecular guide to regulate NOD2 function at sites of pathogen invasion (Till et al. 2008).

The recognition of lipoproteins on *L. monocytogenes* was shown to be dependent on the presence of the bacterial pro-lipoprotein diacylglyceryl transferase gene *lgt*, which modifies the thiol group of cysteine in the signal peptide during the maturation of lipoproteins (Machata et al. 2008). Mice lacking the TLR2 receptor are also susceptible to infection with *L. monocytogenes* (Janot et al. 2008).

6 Gastrointestinal Listeriosis and Involvement of Other Diseases

Host susceptibility plays a major role in the presentation of clinical disease upon exposure to *L. monocytogenes*. Listeriosis in nonpregnant adults is associated in most cases (> 75%) with at least one of the following conditions: malignancies (leukemia, lymphoma, or sarcoma) and antineoplastic chemotherapy, immunosuppresant therapy (organ transplantation or corticosteroid use), chronic liver disease (cirrhosis or alcoholism), kidney disease, diabetes, and collagen disease (lupus) (Farber and Peterkin 1991; McLauchlin 1990). Many listeriosis patients have a physiological or pathological defect that affects T cell-mediated immunity. Preexisting gastrointestinal disease may be a risk factor for infection of the gastrointestinal tract with *L. monocytogenes* (Schlech et al. 2005).

Inflammatory bowel disease (IBD) has been attributed to aberrant mucosal immunity to the intestinal microbiota. The transcription factor XBP1, a key component of the endoplasmic reticulum (ER) stress response, is required for development and maintenance of secretory cells and linked to JNK activation (Kaser et al. 2008). A stressful environmental milieu in a rapidly proliferating tissue might instigate a proinflammatory response. Xbp1 deletion in intestinal epithelial cells (IECs) results in spontaneous enteritis and increased susceptibility to induced colitis secondary to both Paneth cell dysfunction and an epithelium that is overly reactive to inducers of IBD such as bacterial products (flagellin) and TNFalpha. An association of XBP1 variants with both forms of human IBD (Crohn's disease and ulcerative colitis) was identified and replicated with novel, private hypomorphic variants identified as susceptibility factors. Hence, intestinal inflammation can originate solely from XBP1 abnormalities in IECs, thus linking cell-specific ER stress to the induction of organ-specific inflammation (Kaser et al. 2008). As detailed above, apart from defects in the endocytoplasmic reticulum stress, mice harboring mutations in the NOD2 receptor, in genes involved in autophagy, i.e., ATG16L1. or defective for TLR2 signaling have all been implicated in the susceptibility to inflammatory bowel diseases. The ability of *L. monocytogenes* to target all of these cellular processes makes it a prime suspect among the bacterial pathogens thought to be causative or associated with the onset of disease. The cold chain hypothesis suggests that psychrotrophic bacteria such as *Listeria* spp. contribute to Crohn's disease (Hugot et al. 2003).

7 Persistence in Chronic Listeriosis

Bone marrow has recently been shown to harbor *L. monocytogenes*, which spreads from this location to the central nervous system. Variant strains producing low levels of listeriolysin and which are defective intracellular replication have been found to be capable of prolonged focal infection of the bone marrow for periods of up to several weeks. Bone could, therefore, be an important chronic reservoir (Hardy et al. 2009). Prosthetic hip joints have also been found to harbor *Listeria* (Tabib et al. 2002; Cone et al. 2001), and in addition to previous studies demonstrating persistence of *L. monocytogenes* in the gall bladders of infected mice (see above), suggest the emergence (or realization) of chronic forms of listerial infection.

8 Outlook and Perspective

There is now ample epidemiological, clinical, and biological evidence to implicate *L. monocytogenes* as an important source of diarrhea in infected individuals. Because it is also capable of systemic infection, gastroenteritis due to *L. monocytogenes* should be considered in outbreaks of febrile gastroenteritis when routine

stool cultures fail to yield a pathogen. The role of metabolism and metabolic substrates in the growth and survival of *L. monocytogenes* under anaerobic conditions could yield insights into the causes or triggers of virulence in this pathogen during the gastrointestinal phase. Studies have revealed the strategy taken by the bacteria to overcome changes in oxygen tension, osmolarity, acidity, and the sterilizing effects of bile or antimicrobial peptides to adapt to conditions in the gut. *L. monocytogenes* have evolved species-specific strategies for intestinal entry by exploiting the interaction between the internalin protein and its receptor E-cadherin, or inducing diarrhea and an inflammatory response via the activity of its hemolytic toxin, listeriolysin. The creation of a humanized mouse model and the generation of mouse-adapted InlA are exciting developments that will forge a better understanding of the gastrointestinal phase of listerial infection. The ability of these bacteria to induce ER stress, escape from autophagy, and to induce depletion of sentinel cells, such as Paneth cells that monitor the luminal burden of commensal bacteria, suggest strategies that have evolved to promote intestinal survival. The diarrheagenic effects of listeriolysin have pathophysiological implications, because induction of intestinal secretion allows bacteria to spread to new hosts. Further studies to examine the genetic structure of *Listeria* strains that have a propensity to cause gastrointestinal versus systemic infections is now warranted.

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Molecular Mechanisms of *Campylobacter* Infection

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Abstract *Campylobacter jejuni* is the principal bacterial foodborne pathogen. A major challenge still is to identify the virulence strategies exploited by *C. jejuni*. Recent genomics, proteomics, and metabolomics approaches indicate that *C. jejuni* displays extensive inter- and intrastrain variation. The diverse behavior enables bacterial adaptation to different environmental conditions and directs interactions with the gut mucosa. Here, we report recent progress in understanding the molecular mechanisms and functional consequences of the phenotype diversity. The results suggest that *C. jejuni* actively penetrates the intestinal mucus layer, secretes proteins mainly via its flagellar apparatus, is engulfed by intestinal cells, and can disrupt the integrity of the epithelial lining. *C. jejuni* stimulates the proinflammatory pathway and the production of a large repertoire of cytokines, chemokines, and innate effector molecules. Novel experimental infection models suggest that the activation of the innate immune response is important for the development of intestinal pathology.

1 Introduction

Campylobacter jejuni is estimated to cause approximately 400 million cases of human enterocolitis per year. In developing countries, *Campylobacter* is the most commonly isolated bacterial pathogen from young children with diarrhea (Coker et al. 2002). At older ages, most infections are usually mild or asymptomatic, probably because of immunity that may follow frequent exposure to contaminated food or water (Allos and Blaser 1995; Havelaar et al. 2009). In industrialized nations, *C. jejuni* is the leading bacterial foodborne pathogen and one of the most important causative agents of traveler's diarrhea. Ingestion of as few as 500 bacteria is sufficient to develop symptomatic disease (Black et al. 1988). The bacteria colonize the distal small intestine and the colon, and induce mucosal edema, cellular infiltrates, small abscesses, and focal ulcerations (Colgan et al. 1980). Clinical manifestations are fever, abdominal cramps, and bloody or watery diarrhea (Allos and Blaser 1995). Although the symptoms generally resolve within 5–7 days, the economical burden caused by *C. jejuni* is estimated to be up to 8 billion dollars per year in the US alone (Buzby and Roberts 1997). This is partly attributed to severe complications that can follow *C. jejuni* infection, such as reactive arthritis and the paralyzing autoimmune neuropathies, Guillain-Barré syndrome and Miller Fisher syndrome (Yuki et al. 2004). The association between *C. jejuni* infection and the occurrence of irritable bowel syndrome (Spiller 2007) and immunoproliferative intestinal lymphomas (IPSID) (Lecuit et al. 2004) is still under investigation.

In comparison to other intestinal pathogens of global importance, *C. jejuni* pathogenesis is still poorly understood. Recent genomics, proteomics, and advanced infection biology approaches, however, have led to the discovery of important bacterial traits including the presence of a polysaccharide capsule and sophisticated protein glycosylation machineries. Furthermore, metabolic adaptation in response to changing environments, flagella-driven motility, chemotaxis, protein secretion,

colonization of mucus, bacterial infection of mucosal cells, and toxin production appear key steps in the establishment of infection. Here, we will discuss the state-of-the-art of the molecular pathogenesis of *C. jejuni* infection of the gut.

2 *C. jejuni* Genetics and Diversity

2.1 Genome Variation

C. jejuni is a Gram-negative spiral-shaped bacterium that needs a microaerophilic growth environment of 30–44°C under laboratory conditions. The bacterium is highly motile due to the presence of a single flagellum at each pole. The *C. jejuni* genome is relatively small (1.6–1.7 Mb) (Parkhill et al. 2000), but shows considerable genetic diversity among individual *C. jejuni* isolates. Horizontal gene exchange and natural competence for DNA uptake likely contribute to the largely nonclonal nature of the species. Genome-wide analysis of multiple *C. jejuni* isolates suggests that about 20% of the *C. jejuni* genome varies between strains with the presence of unique sets of genes in different isolates (Pearson et al. 2003; Poly et al. 2005; Hofreuter et al. 2006). Approximately 50% of the variable gene pool is located in hypervariable loci involved in the biosynthesis and posttranslational modification of flagellin, in capsule and lipo-oligosaccharide (LOS) production, and in DNA restriction/modification. A relatively large number of *C. jejuni* genes within the variable DNA regions contain tracts of repetitive nucleotide repeats (Wassenaar et al. 2002; Parkhill et al. 2000). These homopolymeric tracts are prone to undergo high rate slipped-strand mispairing resulting in high frequency on-off switching of gene function.

The *C. jejuni* genome does not contain typical pathogenicity islands. However, individual strains may contain one or more *C. jejuni*-integrated elements (CJIEs) with phage characteristics (Fouts et al. 2005; Parker et al. 2006; Clark and Ng 2008) and/or different types of cryptic plasmids (Miller et al. 2007). Conjugative plasmids are frequently found, but generally poorly characterized. A small subset of isolates carries the pVir plasmid (Bacon et al. 2002; Tracz et al. 2005). This plasmid contains elements of a putative type IV secretion system that in other bacterial species is involved in DNA export, conjugation, and protein secretion. In *C. jejuni*, the pVir plasmid is not required to establish an infection and does not appear to be associated with the development of bloody diarrhea (Louwen et al. 2006) in contrast to earlier suggestions (Tracz et al. 2005).

2.2 Phenotype Diversity in C. jejuni

The variable gene content in *C. jejuni* isolates generates differences in bacterial phenotype and adaptation potential. At the metabolic level, this is nicely

exemplified by the variable ability of strains to utilize glutamine and asparagine as nutrients due to variable presence or allelic variation in the genes encoding gamma-glutamyltranspeptidase (GGT) and a periplasmic asparaginase, respectively (Hofreuter et al. 2008). This may influence bacterial colonization (Barnes et al. 2007). Similarly, strains may secrete isoforms of the FspA protein that differ in their ability to induce host cell apoptosis (Poly et al. 2007), while variable presence of CJIEs contributes to the difference in natural transformability between *C. jejuni* strains due to encoded DNase activity (Gaasbeek et al 2009). Strain variations in the composition of the flagellar locus can lead to both differences in the flagellin protein backbone and in variable post-translational modifications of flagellin. This variation influences antigenicity and flagella function, i.e., autoagglutination behavior. Even more marked strain diversity originates from the variable composition of the capsular and LOS biosynthesis loci. This results in the presence of many capsule types (Karlyshev et al. 2005) and a huge repertoire of produced surface lipo-oligosaccharides (Karlyshev et al. 2005; Parker et al. 2008). The capsule surface variation is often accompanied by a change in different antigenic properties (Karlyshev et al. 2005) and may contribute to the variable susceptibility of *C. jejuni* to bacteriophages (Coward et al. 2006). The clinical importance of the LOS diversity is illustrated through the association between the distinct LOS glycoforms that mimic host cell gangliosides and the development of Guillain–Barré syndrome (Yuki et al. 2004). The LOS structures may also differentially interact with host lectin receptors and thus influence the pathogen–host interaction. Thus far, phenotype diversity of *C. jejuni* is rarely taken into account in molecular pathogenesis studies, most of which are performed with a limited set of strains (e.g., strains 11168, 81–176, and 81116).

2.3 *Intrastrain Phenotype Variation*

In addition to differences in gene content that may explain diversity in behavior between *C. jejuni* isolates, individual strains display extensive phenotype variation. Two major mechanisms contribute to the intrastrain phenotype diversity, genetic variation and gene regulation. The genetic variation is largely based on the large number of homopolymeric DNA repeats in the genome. This often leads to uncontrolled variation in promoter activity or a shift in open reading frames. The seemingly random on-and-off switching of genes in the population yields a bacterial progeny that is heterogeneous in the production and/or structure of major surface components including the capsular polysaccharide, LOS and flagellin (Bacon et al. 2001; Linton et al. 2000; Guerry et al. 2002; van Alphen et al. 2008b). This diversity can be highly beneficial to the *C. jejuni* isolate as a heterogeneous set of bacterial phenotypes can prepare the bacterial population to survive changing environmental conditions.

Apart from via (random) genetic variation, the *C. jejuni* can switch phenotype by controlled regulation of gene expression. This type of regulation usually acts at the

level of the entire population rather than of individual bacteria and typically occurs in response to distinct environmental cues. Illustrative examples are the availability of iron and phosphate, which regulate the biosynthesis of iron and phosphate acquisition systems (Palyada et al. 2004; Wösten et al. 2006). Other traits of *C. jejuni* that appear to be subject to gene regulation are capsule production, flagella synthesis, flagella-mediated protein secretion, and biofilm formation. The molecular mechanisms that drive these events and their importance for *C. jejuni* colonization and virulence largely remain to be determined.

2.4 Metabolic Adaptation

C. jejuni encounters a variety of environmental niches ranging from surface water to the gut of animals and humans. Survival under these conditions requires intricate adaptation machineries that enable *C. jejuni* to switch between, e.g., different nutrient sources, and to respond to alterations in oxygen availability and temperature such as exist in the intestine of different hosts. Microarray analysis of *C. jejuni* cultured under different environmental conditions demonstrates major differences in gene expression after growth at 37 and 42°C, simulating the body temperatures of human and chicken, respectively (Stintzi 2003). Similarly, in a limited oxygen environment, *C. jejuni* can switch to alternative electron acceptors including fumarate and nitrite (Sellars et al. 2002), and utilize alternative amino acids as preferred carbon source (Guccione et al. 2008; Wright et al. 2009). The change in metabolic state in different environmental niches may alter *C. jejuni* virulence properties. In chickens, which are a major reservoir of *C. jejuni*, colonization of the cecum occurs without apparent intestinal pathology. The bacteria preferentially reside in the mucus in close proximity to the epithelial cells but apparently do not adhere to or invade the intestinal tissue (Beery et al. 1988; Meinersmann et al. 1991). The altered body temperature and the much more abundant presence in the chicken cecum of the amino acids serine, proline, aspartate, and glutamate, that are preferentially metabolized by *C. jejuni*, may influence *C. jejuni* behavior such as bacterial growth and chemotaxis and thereby alter bacterial virulence.

The environmental changes in *C. jejuni* behavior appear mainly driven via sophisticated two-component signal transduction systems that control the expression of distinct metabolic regulons (reviewed in Wösten et al. 2008) and posttranscriptional regulatory mechanisms (Yun et al. 2008; Fields and Thompson 2008). Infection experiments in chickens with *C. jejuni* with genetically defined defects in different two-component signal transduction systems demonstrate that they are essential for bacterial colonization and/or persistence in the intestine (Brás and Ketley 1999; Svensson et al. 2009; MacKichan et al. 2004; Wösten et al. 2004). Future expression profiling of human *C. jejuni* intestinal isolates may reveal which adaptation machineries are activated in the human intestine and whether these systems affect *C. jejuni* virulence traits.

3 *C. jejuni* Virulence Repertoire

3.1 Campylobacter *Infection in Humans*

Human volunteer studies using clinical isolates confirm that *C. jejuni* causes dysenteric symptoms with high numbers of leukocytes in the feces (Black et al. 1988). Pathology on intestinal biopsies and experimental animal models show damage to columnar epithelial cells, increased exfoliation, necrosis, diffuse neutrophil infiltration of the lamina propria with superficial crypt abscesses, and histopathological features similar to *Salmonella*- and *Shigella*-induced colitis (Black et al. 1988; Russell et al. 1989). *C. jejuni* is also found inside colonic mucosal cells (van Spreeuwel et al. 1985), indicating that *Campylobacter* is able to invade human epithelial cells in vivo. The molecular basis of *C. jejuni* intestinal pathology is not completely understood. The major bacterial traits that are thought to contribute to *C. jejuni* colonization and pathogenesis are outlined below.

3.2 Flagella and Flagella-Mediated Motility

Flagella-mediated motility is highly important for the successful *C. jejuni* colonization of the gastrointestinal tract of experimental animals and human volunteers (Morooka et al. 1985; Walker et al. 1986). Bacterial motility is conferred through a single unsheathed flagellum that is present at each pole. Over 40 genes are involved in *C. jejuni* flagella biogenesis and assembly (Wösten et al. 2004, 2008). *C. jejuni* produces two different (approximately 59-kDa) flagellin subunits, FlaA and FlaB, that are both incorporated into the flagellum although not in equal amounts. The expression of FlaA and FlaB is controlled by different transcription factors, namely the alternative sigma^{28} (FlaA) and sigma^{54} (FlaB) transcription factors (Wösten et al. 2004; Hendrixson and DiRita 2003). The biological advantage of the differential regulation of the expression of the two flagellins is unknown. Both types of flagellin can be assembled into a filament structure and differ in only 9–10 amino acids in their otherwise conserved domains that form the backbone of the flagella fiber. It cannot be excluded that the amino acid differences influence the properties and/or structure of the hollow fiber or perhaps the protein secretion through the filament, but this awaits further study.

Flagella assembly in *C. jejuni* is unusual as the filament consists of 7 rather than 11 protofilaments (Galkin et al. 2008) and because flagella formation requires glycosylation of the flagellin monomers (Linton et al. 2000; Karlyshev et al. 2002; Goon et al. 2003) (Fig. 1). The molecular basis for the altered architecture of the flagellum and the need for glycosylation are unknown. The altered packaging of the flagellum may have co-evolved with the inability of *C. jejuni* flagellins to activate the innate Toll-like receptor TLR5 (Galkin et al. 2008). The glycosylation of the flagellins may enforce structural requirements important in the export and/or

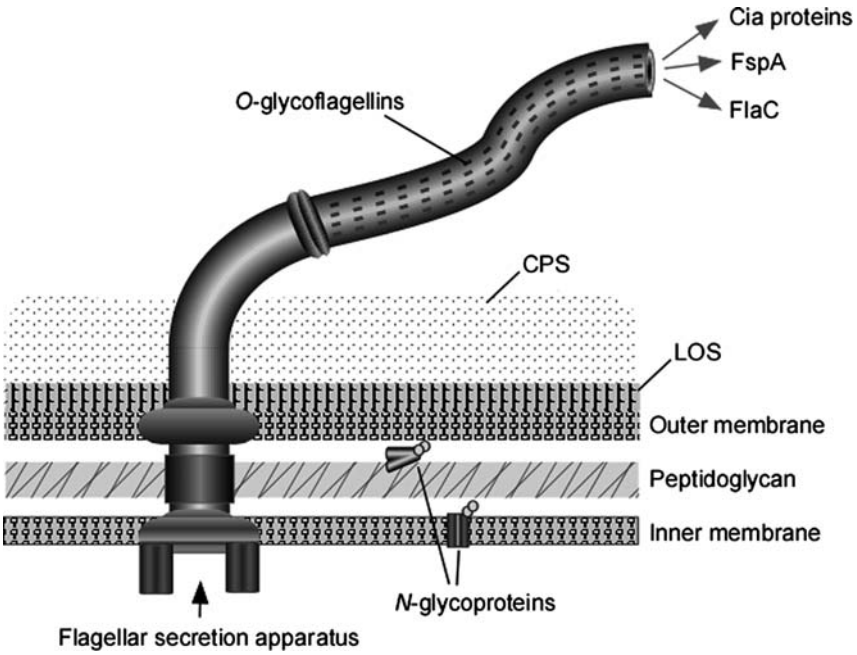

Fig. 1 Schematic structure of the flagellar secretion apparatus and the different types of carbohydrates structures of *C. jejuni*. The flagellum is composed of a basal body embedded in the membrane and an extracellular fiber consisting of thousands of flagellin polymers. The *C. jejuni* flagellins are decorated with variable *O*-linked carbohydrates. Other variable carbohydrate surface structures are the outer membrane lipooligosaccharide (LOS) and the polysaccharide capsule (CPS). The largely conserved *N*-linked glycans are attached mainly to proteins in the periplasm. In the absence of a type III secretion system, the flagellar secretion apparatus appears to secrete several putative virulence proteins including FspA, FlaC and up to eight Cia proteins

polymerization of flagellins, although this would not explain the existing variation in glycans used to decorate the flagellins. Alternatively, the sugar coat on the flagellum may provide strength, rigidity, and charge that facilitate the *C. jejuni* to access more viscous environment.

The composition in the surface-exposed regions of both FlaA and FlaB is highly variable among isolates due to horizontal exchange and recombinatorial events (Wassenaar et al. 1995) and heterogeneity of the attached glycan moieties. The glyco-modifications are encoded by the *O*-glycosylation locus (Guerry et al. 2006). This locus varies in gene composition between strains and contains several contingency genes that contribute to intrastrain carbohydrate heterogeneity (Guerry et al. 2006). State-of-the-art chemical analysis indicate that the attached carbohydrates predominantly consist of pseudaminic acid or legionaminic acid derivatives that are attached to distinct serine and threonine residues in the variable domain (Thibault et al. 2001; Logan et al. 2002, 2009; McNally et al. 2006; Guerry and Szymanski 2008). Variable substitution of the acetamido group of pseudaminic acid with acetaminido or hydroxyproprionyl groups causes further

microheterogeneity in the carbohydrates moieties (Thibault et al. 2001). While the variation in amino acid sequence of the variable domain does not seem to influence flagella-mediated motility, the attached glycans do influence flagella-mediated auto-agglutination and microcolony formation (Guerry 2007; Guerry et al. 2006; van Alphen et al. 2008b) and thus are functionally relevant. The diversity in the surface-exposed region of *C. jejuni* flagellins may also serve to escape attack from the immune system (Andersen-Nissen et al. 2005) and bacteriophages (Coward et al. 2006).

3.3 Chemotaxis

With their flagella, *C. jejuni* can specifically swim towards a variety of substances or away from harmful environments. The directed motility appears to be driven by at least two types of taxis systems, namely chemotaxis that responds to environmental stimuli, and energy-taxis that drives motility towards environments promoting optimal electron transport. Major chemoattractants include mucin and the amino acids serine, proline, aspartate, glutamate (Hugdahl et al. 1988). Identified activators of the *C. jejuni* energy taxis system are fumarate and pyruvate, which yield high levels of electron transport and ATP (Hendrixson et al. 2001). In silico analysis of the *C. jejuni* genome sequence predicts the presence of at least nine putative chemotaxis sensing receptors (methyl accepting receptors, MCPs), the energy-taxis proteins CetA and CetB, one chemotaxis protein with potential histidine kinase activity (CheA), and multiple proteins with a CheY-like response regulator domains (CheY, CheA and CheV), as well as CheZ, CheV, CheB and CheR orthologs that may be involved in signal amplification and/or adaptation to chemotactic stimuli (Marchant et al 2002).

At the functional level, *C. jejuni* CheA and CheY are required for directional motility in soft agar (Golden and Acheson 2002; Hendrixson et al. 2001; Yao et al. 1997) as noted for other bacterial species. A *C. jejuni* CheY mutant is hyperinvasive in cultured cells (Golden and Acheson 2002; Hickey et al. 1999) possibly because this mutant is still capable of flagella-based movement but is no longer directional (Yao et al. 1997). Both CheY and CheA mutants are deficient in colonization of the mouse or chicken gastrointestinal tract (Takata et al. 1992; Yao et al. 1997; Hendrixson and DiRita 2004), indicating that chemotaxis is essential for colonization.

The energy-taxis system of *C. jejuni* involves the sensory protein complex CetB and CetA (Hendrixson et al. 2001). The complex shares features with the energy-taxis receptor Aer of *E. coli* including the presence of domains of Aer (divided between CetA and CetB), a sensory PAS domain in CetB, and a predicted transmembrane region, HAMP domain and HCD domain in CetA. Interestingly, CetA and CetB are co-transcribed independently of the flagellar regulon (Elliott and DiRita 2008). While CetA and CetB mutants display normal colonization of the chicken intestine (Hendrixson and DiRita 2004), inactivation of CetA but not CetB

causes a moderate (five-fold) reduction in invasion of cultured human epithelial cells (Elliott et al. 2009). Thus, the energy-taxis system may be important for virulence rather than colonization and/or may display host-specific activation. The signals that activate the *C. jejuni* CetA/CetB energy-taxis complex in vivo and the molecular basis of the altered invasive properties are still unresolved.

3.4 C. jejuni *Capsule and LOS*

Like most Gram-negative bacterial species, *C. jejuni* produces surface glycolipids including capsular polysaccharide (CPS) and lipo-oligosaccharide (LOS) (Fig. 1). The polysaccharide capsule was first discovered after analysis of the *C. jejuni* genome sequence (Parkhill et al. 2000) and confirmed by electron microscopy (Karlyshev et al. 2001). The capsule consists of repeating oligosaccharide units attached to a dipalmitoyl-glycerophosphate lipid anchor (St Michael et al. 2002; Corcoran et al. 2006). The CPS is extensively substituted with variable *O*-methyl phosphoramidate, methyl, ethanolamine, and *N*-glycerol groups (McNally et al. 2007). The structural variation in CPS is consistent with the noted genetic diversity in the *cps* gene cluster and is the basis of the Penner serotyping used to distinguish *C. jejuni* isolates (Karlyshev et al. 2005). Environmental regulation of capsule biosynthesis as present in many other bacterial pathogens has not been investigated in great detail. However, capsular biosynthesis undergoes frequent phase variation, suggesting that the *C. jejuni* capsular phenotype is variable (Bacon et al. 2001). Capsule-deficient *C. jejuni* show increased surface hydrophobicity and serum sensitivity, and reduced invasion of INT-407 cells and virulence, in ferrets (Bacon et al. 2001).

The majority of the *C. jejuni* cell wall consists of oligosaccharides attached to a lipid A anchor (LOS). The structure of *C. jejuni* lipid A follows the same architectural principle as in most other Gram-negative species, although the lipid A backbone of *C. jejuni* is composed of a phosphorylated disaccharide containing diaminoglucose and glucosamine as the major molecular species with slightly longer N- and O-linked acyl chains (Moran 1995). The disaccharide is variably substituted with phosphate or phosphoethanolamine (Moran 1997). The biological significance of the different core structure is unknown. *C. jejuni* lipid A seems to have a lower fluidity at 37°C than *Salmonella* LPS, possibly because of the different acyl chain characteristics. *C. jejuni* LOS activates the proinflammatory TLR4/MD2 pathway (de Zoete and van Putten, unpublished results), but is less biologically active than *Salmonella* LPS with regard to toxicity, pyrogenicity, and the induction of TNFα (Moran 1995).

The LOS inner core region consists of two heptose residues attached to one KDO molecule. The distal heptose can contain O-linked glycine (Dzieciatkowska et al. 2007). The outer core of *C. jejuni* LOS is highly variable in structure. Frequent horizontal gene exchange and rearrangements of LOS genes has resulted in mosaic-like

organization of the LOS gene cluster with different strains each have their own LOS gene repertoire (Parker et al. 2008; Gilbert et al. 2002). Additional intrastrain LOS heterogeneity stems from the variable expression of genes with homopolymeric nucleotide tracts that are prone to slipped-strand mispairing (Parkhill et al. 2000). Other mechanisms contributing to the LOS diversity are mutations leading to gene inactivation or different acceptor specificities of glycosyltransferases (Gilbert et al. 2002). Of particular biological importance is the ability of most *C. jejuni* strains to variably incorporate sialic acids into its LOS (Godschalk et al. 2007; Guerry et al. 2002; St Michael et al. 2002). This can result in the formation of ganglioside-like structures as reported for *Helicobacter pylori*, *Haemophilus influenzae*, *Haemophilus ducreyi*, and the pathogenic *Neisseria* species. The ganglioside mimics may elicit pathogenic antibodies that cross-react with host cell glycolipids and contribute to the development of the autoimmunity-based Guillain-Barré syndrome (Yuki et al. 2004). *C. jejuni* LOS may promote adhesion to and invasion of host cells (Fry et al. 2000; Guerry et al. 2002) and may target host cell glycan receptors with immunomodulatory functions (Louwen et al. 2008; Avril et al. 2006). The variable oligosaccharide structure may thus aid *C. jejuni* to colonize different hosts or intestinal niches.

In addition to capsule and LOS, *C. jejuni* may produce another type of polysaccharide that may be involved in biofilm formation (Kalmokoff et al. 2006; Joshua et al. 2006). The nature of this surface polysaccharide is unknown but its biosynthesis may require carbamoylphosphate synthase (McLennan et al. 2008). Biofilm formation is upregulated under anaerobic conditions and in a *C. jejuni* SpoT mutant. This mutant is defective for the stringent response that is important for survival of environmental stress (McLennan et al. 2008). The formation of *C. jejuni* biofilms may contribute to survival in aquatic environments. Its role in *C. jejuni* virulence remains to be determined.

3.5 Surface Proteins and Protein Glycosylation

The number of identified surface-exposed membrane proteins in *C. jejuni* is limited. One principal protein is the major outer membrane porin, MOMP (Moser et al. 1997; Dé et al. 2000). *C. jejuni* MOMP is a β-barrel protein with surface exposed loops that are hypervariable in amino acid composition between isolates (Clark et al. 2007), suggestive of selective pressure by the immune system. A second porin protein, Omp50, is upregulated during in vivo growth of *C. jejuni* (Stintzi et al. 2005). Other *C. jejuni* surface proteins include the fibronectin binding protein CadF (Konkel et al. 1997) and the lipoproteins Omp18 (Burnens et al. 1995; Konkel et al. 1996) and JlpA (Jin et al. 2001). A separate class of proteins are the PEB proteins implicated in amino acid and phosphate transport as well as bacterial adherence to eukaryotic cells (Pei et al. 1998; Leon-Kempis Mdel et al. 2006). How these proteins exert this dual function remains to be established.

C. jejuni produces two putative autotransporters, CapA and CapB (Ashgar et al. 2007). In other pathogens, autotransporters represent an extensive and rapidly growing family of secreted virulence-associated proteins. The identified *C. jejuni* autotransporters each contain a homopolymeric tract and are therefore predicted to undergo phase variation. Insertional inactivation of CapA results in reduced adhesion and invasion of Caco-2 cells and loss of the ability to colonize chickens, suggesting that the protein may play a role in *C. jejuni* colonization and virulence (Ashgar et al. 2007).

In addition to the *O*-glycosylated flagellins, *C. jejuni* contains at least 35 different *N*-linked glycoproteins (Young et al. 2002). Genes involved in the *N*-linked protein glycosylation pathway are encoded by the protein glycosylation (*pgl)* locus (Szymanski et al. 1999). In contrast to the *O*-glycosylation locus, this locus is conserved among *C. jejuni* strains which explains why all *N*-glycoproteins appear to carry the same heptasaccharide moiety, GalNAc-α1,4-GalNAc-α 1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac, where Bac is bacillosamine (Young et al. 2002). The oligosaccharide is attached to asparagine residues that are part of the specific glycosylation consensus sequence, Asp/Glu-Y-Asn-X-Ser/Thr, where Y and X is any amino acid except proline (Kowarik et al. 2006). Important here is that virtually all *N*-glycosylated proteins appear to be located in the periplasm (Fig. 1). Their limited (if at all) surface exposure in the intact bacterium may explain the conserved nature of the oligosaccharide. The function of the *N*-linked protein glycosylation is still an enigma. Disruption of the glycosylation pathway reduces *C. jejuni* adherence and invasion in INT-407 cells and the colonization of the intestinal tracts of animals (Szymanksi et al. 2002; Karlyshev et al. 2004). The molecular basis of the attenuated *C. jejuni* behavior remains to be defined, but may reflect a general dysfunction of the *C. jejuni* membrane.

3.6 Secreted Factors

C. jejuni secretes several putative virulence factors into its environment. Most identified factors are secreted through the flagellar secretion apparatus (Fig. 1). Apart from flagellar components, this machinery secretes the FlaC, FspA, and at least eight *C. jejuni* invasion antigens (Cia). The 26-kDa FlaC protein is predicted to resemble FlaA and FlaB except that it lacks the variable central domain of the flagellins (Song et al. 2004). Structurally, the protein FlaC is not required for flagellum formation or motility, but was shown to bind to HEp-2 cells, both when secreted by the bacteria during in vitro infection of HEp-2 cells and as purified recombinant protein (Song et al. 2004).

The approximately 18-kDa FspA protein is present in *C. jejuni* in either of two variant forms, FspA1 or FspA2. The FspA proteins display considerable heterogeneity between strains. FspA2, but not FspA1, binds eukaryotic cells and induces apoptosis in epithelial cells (Poly et al. 2007).

In contrast to FlaC and FspA, secretion of the Cia proteins requires contact with host cells or the presence of mucin or serum (Rivera-Amill et al. 2001). Deoxycholate induces the transcription of the *ciaB* gene, and *C. jejuni* harvested from Muller-Hinton agar plates supplemented with deoxycholate also secrete Cia proteins (Malik-Kale et al. 2008). The apparent diversity in signals that drive Cia secretion suggests that the secretion event may be related to environmental changes rather than a distinct environmental cue. In some *C. jejuni* strains (but not strain 129108), inactivation of *ciaB* prevents secretion of other Cia proteins and blocks bacterial invasion of cultured epithelial cells (Konkel et al. 1999).

Like several other enteropathogens, *C. jejuni* secretes a cytolethal-distending toxin (CDT) (Smith and Bayles 2006). This toxin is comprised of three subunits, CdtA, CdtB, and CdtC, all of which are essential for toxin activity (Lara-Tejero and Galán 2001). CdtA and CdtC are essential for binding to host cells (Lee et al. 2003), while CdtB internalization by the eukaryotic cells is essential for toxicity (Lara-Tejero and Galán 2001). CdtB, which has DNase I-like activity, is translocated to the nucleus and induces eukaryotic cell cycle arrest in the G2 phase (Whitehouse et al. 1998). Cytotoxicity or cell cycle arrest can be achieved by adding a combination of the three purified toxin proteins to cultured epithelial cells (Lee et al. 2003; Lara-Tejero and Galán 2001). In addition to its cytotoxic effect, CDT also appears to stimulate the proinflammatory NF-kappaB pathway and elicits IL-8 secretion (Hickey et al. 2000; Zheng et al. 2008).

C. jejuni also secretes several nonproteinaceous molecules. One important factor may be the auto-inducer AI-2. This compound influences *C. jejuni* swarming motility, autoagglutination, biofilm formation, sensitivity to hydrogen peroxide, and the transcription of the cytolethal-distending toxin genes (He et al. 2008). *C. jejuni* AI-2 production, which is dependent upon *luxS*, is maximal during mid- to late-exponential growth, but rapidly decreases at high cell concentrations during entry into the stationary growth phase (Quinones et al. 2009).

4 Mucosal Infection by *C. jejuni*

4.1 Penetration of the Mucus Layer

The first barrier *C. jejuni* encounters in the gut is the mucus layer (Fig. 2). *C. jejuni* can effectively penetrate this barrier. Pathology studies frequently identify large numbers of bacteria in the mucus layer and in intestinal crypts without apparent attachment to the microvillus (Beery et al. 1988; Lee et al. 1986). Mucin is a strong chemoattractant for *C. jejuni* (Hugdahl et al. 1988) and, instead of being trapped and transported out of the intestinal tract, the bacterium freely moves in parallel streams along the mucus strands (Lee et al. 1986; McSweegan et al. 1987). This efficient mobility in mucus may be due to its spiral-shaped morphology and the presence of putative mucin-degrading enzymes. In addition, *C. jejuni* swims at higher speeds in environments of high rather than low viscosity and under

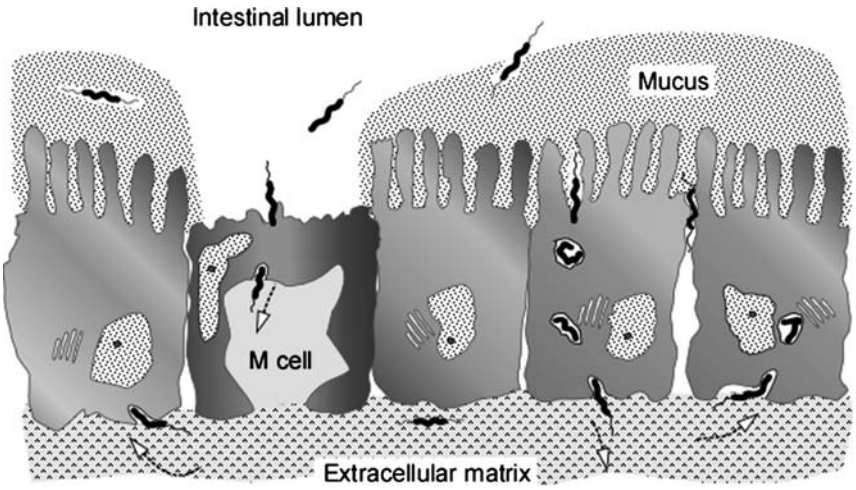

Fig. 2 Colonization and invasion strategies of *C. jejuni*. The pathogen can actively swim into the mucus layer and survive in intestinal crypts. Uptake and transport across M cells may enable *C. jejuni* to migrate into the subcellular environment and to invade epithelial cells at the cell basis. Transient disruption of tight junctions between intestinal cells may enable penetration of the epithelial lining via the paracellular route. Bacteria-directed uptake into epithelial cells may lead to transcellular transport and exocytosis at the cell basis, as well as to trafficking to a unique intracellular compartment in proximity of the Golgi complex. The contribution of the various invasion strategies of *C. jejuni* to the establishment of a natural infection is unknown

conditions that immobilize conventional rod-shaped bacteria (Ferrero and Lee 1988). In media with high viscosity, *C. jejuni* shows longer periods of straight swimming with increased velocity followed by pauses, resembling the swimming pattern of spirochetes rather than of other monotrichous bacteria (Shigematsu et al. 1998). Whether the change in swimming mode is related to the observed down-regulation of the flagellin (*flaA*) promoter in viscous conditions (Allen and Griffiths 2001) awaits further study.

The use of mucus or high-viscosity medium during in vitro infection tends to increase *C. jejuni* adherence and invasion of epithelial cells. On other hand, mucus of rabbits previously colonized with *C. jejuni* impede the bacterial adherence to INT-407 cells due to the presence of *Campylobacter*-specific IgA, which causes aggregation of *C. jejuni* within the mucus layer (McSweegan et al. 1987). This observation may at least partially explain why frequent *C. jejuni* exposure protects against intestinal pathology.

4.2 Cellular Infection

After mucus penetration, *C. jejuni* can come in close contact with the intestinal epithelial cells. Despite excellent work, the molecular interaction of *C. jejuni* with

eukaryotic cells is still poorly understood. Diverse behavior of the various *C. jejuni* phenotypes and/or the different strategies that *C. jejuni* exploits to adhere, invade, and survive in different cell types may explain, but also complicate, comparison and interpretation of reported results. Methodological issues may further blur scientific progress. A typical example is the gentamicin survival assay often used to estimate bacterial invasion into eukaryotic cells. Results obtained with this assay do not accurately measure the number of intracellular bacteria at a given time. Instead, they in fact reflect the outcome of a series of events including bacterial internalization, the resistance against the hostile intracellular milieu, and the adaptive capabilities of *C. jejuni* that are needed to survive the transition from the intracellular environment to growth on nutrient-rich agar plates in a different gas atmosphere. Recently, discovery of novel *C. jejuni* invasion and intracellular trafficking pathways was successful because these issues were taken into account (Watson and Galán 2008; van Alphen et al. 2008a). Despite these technicalities, several key steps in the *C. jejuni* infection of eukaryotic cells have been identified and are outlined below.

4.3 Adherence of C. jejuni *to Mucosal Cells*

Once close to the epithelial cells, *C. jejuni* can adhere to the cell surface through a number of adhesins (de Melo and Pechere 1990; Konkel et al. 1997; McSweegan and Walker 1986; Kervella et al. 1993). One identified adhesion is the 42-kDa protein JlpA. which mediates adherence to HEp-2 cells. This event can be inhibited with JlpA-specific antibodies or preincubation of the eukaryotic cells with purified JlpA protein (Jin et al. 2001). JlpA interacts with surface-exposed heat shock protein Hsp90α and is blocked by the Hsp90 inhibitor geldamycin (Jin et al. 2003). A JlpA-GST fusion protein triggers nuclear translocation of the transcription factor NF-kappaB and phosphorylation of p38 MAP kinase. This suggests that JlpA not only confers *C. jejuni* adherence but also elicits a proinflammatory response in the infected host cell (Jin et al. 2003).

A second protein with adhesive properties is CadF (Konkel et al. 1997). This protein, which may belong to the OmpA-like protein family, confers bacterial adhesion via binding of host cell fibronectin (Fn). The Fn binding domain of *C. jejuni* CadF has been identified as a single exposed amino acid domain of four residues (Konkel et al. 2005). Apart for CadF, Fn binding has also been proposed for *C. jejuni* flagellin, the major membrane protein MOMP, and LOS (Moser and Schroder 1997). The significance of this Fn binding for *C. jejuni* adherence is unknown.

Other putative *C. jejuni* adhesins are PEB1 (Pei et al. 1998) and certain LOS glycoforms (McSweegan and Walker 1986; Avril et al. 2006). A PEB1 mutant shows a 50- to 100-fold reduction in bacterial adherence to epithelial cells and a reduced colonization of mice (Pei et al. 1998). The protein binds to HeLa cell membranes (Kervella et al. 1993). More recently, PEB1 has been shown to be a conserved asparate/glutamate-binding protein that belongs to the family of cluster

three binding proteins of bacterial ATP transporters (Leon-Kempis Mdel et al. 2006). Indeed, purified recombinant PEB1 binds L-aspartate and L-glutamate which may indicate that the protein is important in the utilization of in vivo carbon sources. Biochemical studies demonstrate that the majority of PEB1 protein resides in the periplasmic space and only a small portion is transported across the outer membrane (Leon-Kempis Mdel et al. 2006). The crystal structure of the protein further strengthened its role as periplasmic amino acid binding protein by demonstrating a ligand binding cleft, which could explain the high binding affinity for L-aspartate and L-glutamate (Muller et al. 2007). Whether the adherence-promoting properties of PEB1 relate to its importance in the uptake of amino acids necessary for bacterial growth is unknown. The function of *C. jejuni* LOS (McSweegan and Walker 1986) and capsule polysaccharide (Bachtiar et al. 2007) in bacterial adherence has not been systematically investigated, partly because their extensive intra- and interstrain structural heterogeneity.

At this time, the relative contribution of the various *C. jejuni* adhesins to the infection of mucosal cells is difficult to discern. In many studies, inactivation of each of the putative adhesins strongly reduces the association of *C. jejuni* with eukaryotic cells, indicating that they all are dominant adhesins. This suggests that either they act in a complex or display cell-type specificity. To our knowledge, tissue- or host-specific *C. jejuni* adhesins have thus far not been identified, but receptor identification for each of the adhesins may resolve this issue.

4.4 Mechanisms of C. jejuni *Entry into Eukaryotic Cells*

Analysis of intestinal biopsies of infected patients and primates (van Spreeuwel et al. 1985; Russell et al. 1993; Babakhani et al. 1993) as well as results from numerous in vitro studies (Newell and Pearson 1984; de Melo et al. 1989; Konkel et al. 1992a,b; Wassenaar et al. 1991; Babakhani et al. 1993; Oelschlaeger et al. 1993) indicate that *C. jejuni* is internalized by eukaryotic cells. Reported *C. jejuni* invasion levels, as mostly determined by the gentamycin assay, display huge variation between laboratories and are strongly dependent on multiplicity of infection used as an inoculum. The often-used *C. jejuni* strain 81–176 typically enters young semiconfluent INT-407 and Caco-2 cells in 2 h with an invasion efficiency of 1–2%, but efficiencies range from 0.001 to 4% (Hu and Kopecko 1999). In most studies, even with the most invasive strains, only one to three bacteria are internalized per cell (Biswas et al. 2000), much less than reported for other enteropathogens. However, when selected for the appropriate phenotype and dependent on the environmental conditions, uptake levels of 70–80% of the inoculum can be obtained within 2–4 h of infection (van Alphen et al. 2008a), suggesting that *C. jejuni* has the intrinsic ability to efficiently enter eukaryotic cells.

A key factor in virtually all *C. jejuni* uptake studies is the presence of functional flagella (Grant et al. 1993; Wassenaar et al. 1991; Yao et al. 1994; Szymanski et al. 1995; van Alphen et al. 2008a). *C. jejuni* that lack flagella, or carry a short flagellum consisting of only FlaB, show reduced invasion of epithelial cells (Wassenaar et al.

1991; Szymanski et al. 1995; Yao et al. 1994). Furthermore, *C. jejuni* with paralyzed flagella adhere but do not enter eukaryotic cells, suggesting that motility is a prerequisite for bacterial internalization (Yao et al. 1994). The hyperinvasiveness of CheY mutants, that display enhanced directional motility (Yao et al. 1997), also points in this direction.

The signals that drive the actual internalization process are still poorly defined. Upon contact with the cell surface, *C. jejuni* triggers membrane ruffling and invaginations, and is taken up with its polar tip first (Krause-Gruszczynska et al. 2007; Hu et al. 2008). The uptake process may require de novo protein synthesis (Oelschlaeger et al. 1993) and the flagellar secretion of the aforementioned FlaC, FspA, and Cia as the proteins. Inactivation of FlaC causes reduced invasion of *C. jejuni* into cultured epithelial cells but does not affect bacterial adherence (Song et al. 2004). The production and secretion of the Cia proteins is triggered by contact with host cells. However, the role of these proteins in the internalization event remains to be elucidated, as CiaB is obligatory for *C. jejuni* invasion in certain strains only (van Alphen et al. 2008a; Goon et al. 2006) and delivery of the secreted molecules to the host cells has not been demonstrated. Thus far, no function has been assigned to any of these proteins.

Recently, a novel highly efficient *C. jejuni* invasion mechanism has been identified that acts independently of CiaB or FlaC (van Alphen et al. 2008a) (Fig. 2). The highly invasive *C. jejuni* phenotype displays a remarkable route of invasion that yields on average 10–15 intracellular bacteria per epithelial cell. The *C. jejuni* first swims towards the subcellular space of cultured epithelial cells (a process termed "subvasion") and then accesses the cell at the basal cell side. The subvasion process requires functional flagella. Molecular analysis of the selected highly subvasive bacteria indicated a change in the bacterial taxis system. This led to the discovery that *C. jejuni* subvasion can be directly controlled by the availability of nutrients (van Alphen and van Putten, unpublished results). The precise mechanism via which subcellular *C. jejuni* enters the eukaryotic cells, is still under investigation. One possible regulatory factor is the post-transcriptional regulator carbon starvation regulator CsrA. Inactivation of the protein, which is required for resistance of *C. jejuni* to oxidative stress, causes a strong increase in *C. jejuni* invasion despite reduced motility (Fields et al. 2008).

Other molecules uniquely involved in *C. jejuni* invasion are gamma-glutamyltranspeptidase (GGT) (Barnes et al. 2007), polysaccharide capsule (Karlyshev et al. 2000; Kanipes et al. 2004; Bacon et al. 2001), and sialylated LOS (Louwen et al. 2008). The underlying mechanisms, however, remain to be explored. The presence of the virulence plasmid pVir can enhance but is not required to trigger *C. jejuni* invasion (Bacon et al. 2002).

4.5 Cellular events Accompanying C. jejuni *Internalization*

Most studies are consistent with the scenario that *C. jejuni*-directed entry into epithelial cells proceeds via the local depolymerization of cortical actin

filaments and the formation of microtubuli-based membrane projections (Krause-Gruszczynska et al. 2007; Watson and Galán 2008; Konkel et al. 1992a, b; Monteville et al. 2003; Oelschlaeger et al. 1993; Hu and Kopecko 1999). Microtubule depolymerizing agents, such as nocodazole, block *C. jejuni* invasion (Hu and Kopecko 1999; Oelschlaeger et al. 1993). The involvement of the actin cytoskeleton in *C. jejuni* uptake is not always found. Whether this relates to the presence of cortical cytochalasin D-insensitive actin filaments in certain cell types (Godman et al. 1980; Horvath and Kellie 1990) is unknown. Efficient *C. jejuni* internalization also requires caveolin-1 (Krause-Gruszczynska et al. 2007; Wooldridge et al. 1996; Hu et al. 2006a, b). However, as the entry process was shown to be dynamin-independent (Watson and Galán 2008), *C. jejuni* internalization is unlikely to occur via caveolea-mediated endocytosis. Instead, the caveolin-1 containing lipid membrane domains may be important for proper *C. jejuni* activation of tyrosine kinases (Watson and Galán 2008; Hu et al. 2006b) and of the small Rho GTPases Rac 1 and Cdc42 (but not RhoA) that could drive the cytoskeletal rearrangements (Krause-Gruszczynska et al. 2007, Wooldridge et al. 1996; Hu et al. 2006a, b). Both the activation of the GTPases and *C. jejuni* uptake is blocked by the kinase inhibitors genistein, tyrphostin-46, wortmannin, and staurosporin. At least nine proteins become phosphorylated in *C. jejuni*-infected cells, including phosphoinositol 3-kinase and heterotrimeric G proteins (Wooldridge et al. 1996; Biswas et al. 2004). Their exact role in the uptake process, as well as the requirement of the release of Ca^{2+} from intracellular stores for *C. jejuni* entry (Hu et al. 2005), remain to be defined. Cell signaling studies with different mutants indicate that CadF and PEB1 are not essential for activation of the Rho GTPases (Krause-Gruszczynska et al. 2007). CadF does increase tyrosine phosphorylation of paxillin in focal adhesions and thus may contribute to cytoskeletal rearrangements during the Fn-mediated uptake at the basolateral cell surface (Monteville and Konkel 2002).

4.6 Intracellular Trafficking of C. jejuni

Once ingested by eukaryotic cells, *C. jejuni* resides in a membrane-bound cellular compartment and co-localizes with microtubules and the microtubule motor protein dynein during the entire invasion process (Hu and Kopecko 1999; Konkel et al. 1992b; Oelschlaeger et al. 1993; Russell and Blake 1994). In INT-407 cells, *C. jejuni* is able to replicate after an initial decline in the number of intracellular bacteria. Replication ultimately results in the deterioration of the epithelial monolayer. Bacterial iron acquisition is essential for intracellular survival in this cell line (Naikare et al. 2006). In HEp-2 epithelial cells, *C. jejuni* ultimately localize in vacuoles that show signs of phago-lysosome fusion and change from spiral to coccoid forms with a concomitant decrease in viability (de Melo et al. 1989).

A novel trafficking pathway was recently identified in semiconfluent T84 intestinal epithelial cells. In these cells, *C. jejuni* appears to traffick into a unique intracellular compartment and avoid delivery to lysosomes (Watson and

Galán 2008) (Fig. 2). Once formed, the *C. jejuni*-containing vesicle is transported along microtubules to close proximity of the Golgi apparatus in the perinuclear region. Recovery of intracellular bacteria from this compartment requires oxygen-limiting conditions, a finding that could change conclusions from previously described invasion studies. The trafficking of *C. jejuni* to the non-lysosomal compartment is related to its entry mechanism as infection of Fc receptor-transfected cells with antibody-opsonized *C. jejuni* results in delivery of the bacteria to endosomes rather than the unique intracellular compartment (Watson and Galán 2008).

In polarized epithelial cells, *C. jejuni* is able to translocate to the basolateral surface to be released in the subcellular space (Fig. 2). During transit, *C. jejuni* remains with a membrane-bound compartment and there is only very limited replication intracellularly (Hu et al. 2008). The signals that drive this transcellular route are unknown.

4.7 Translocation of the Intestinal Mucosa

As well as through cellular invasion and the transcellular pathway, *C. jejuni* is also able to (transiently) disrupt tight junctions between polarized cells and thereby gain access to the subepithelial tissue via the paracellular route (Brás and Ketley 1999; Grant et al. 1993; Konkel et al. 1992b; MacCallum et al. 2005a) (Fig. 2). Infection of polarized T84 cells with *C. jejuni* decreases transepithelial electric resistance and causes a redistribution of the tight junction transmembrane protein occludin from an intercellular to an intracellular location, indicating alterations in the tight junctions (Chen et al. 2006). This event is enhanced in the presence of IFNγ (Rees et al. 2008). Once the tight junctions have been passed, *C. jejuni* can enter the eukaryotic cells at the basolateral surface as shown in EGTA-treated polarized cells (Monteville and Konkel 2002). The molecules that drive the transcellular migration and subsequent internalization process are largely undefined, although it can be imagined that the binding of Fn by CadF promotes basolateral uptake of *C. jejuni* through interaction with integrin receptors.

Several observations suggest that in vivo *C. jejuni* may exploit M cells to penetrate the intestinal barrier (Fig. 2). M cells are an important port of entry for a variety of pathogens including *Salmonella* (Siebers and Finlay 1996). In rabbit intestinal loop models, *C. jejuni* selectively associates with M cells (Everest et al. 1993; Walker et al. 1988). In differentiated polarized Caco-2 cells, the bacteria efficiently invade only approximately 5% of the cells that may represent M-like cells (Hu and Kopecko 1999; van Alphen and van Putten, unpublished results). The observed active penetration of the subcellular space by *C. jejuni* (van Alphen et al. 2008a) may reflect the spread of the pathogen from infected M cells into the subepithelial layer. These bacteria can subsequently invade the epithelial cells at cell basis. It has been speculated that, at low inocula (500–800 bacteria), M cells are primarily exploited by *C. jejuni* to traverse the epithelium, while at high inocula, the

pathogen may invade villus epithelial cells, perhaps as a secondary event after loss of tight junction integrity (Everest 2005; Chen et al. 2006).

4.8 Interaction of C. jejuni *with Professional Phagocytes*

Whether *C. jejuni* can survive within professional phagocytes is still under debate. Both survival and replication inside monocytes and macrophages, but also induced CDT-dependent apoptosis, has been reported (Hickey et al. 2005; Kiehlbauch et al. 1985; Siegesmund et al. 2004). Clinical isolates of *C. jejuni* survive for several days in murine macrophages, whereby catalase plays an important role in providing resistance to hydrogen peroxide (Day et al. 2000). Others report that *C. jejuni* survival in human monocyte-derived macrophages is donor-dependent: in cells from most donors, *C. jejuni* is killed within 24–48 h inside the cells, while 10% of the donors carry monocytes that are unable to kill *C. jejuni* (Wassenaar et al. 1997). Intriguingly, *C. jejuni* DNA fragments can be detected in circulating human blood cells of distinct individuals for a period of up to 12 months, although no viable bacteria could be detected (van Rhijn et al. 2002). This may indicate the presence of either viable, non-culturable *C. jejuni* within these cells, or that the bacterium resides in a thus far unidentified niche within the body that serves as a reservoir for continuous *C. jejuni* infection of monocytes. In murine bone marrow-derived macrophages, *C. jejuni* is delivered to a lysosomal compartment and killed within 24 h of infection (Watson and Galán 2008). *C. jejuni* is also readily internalized by human dendritic cells and induces maturation and cytokine production in these cells (Hu et al. 2006a, b). In mice, this results in a Th1-effector response against *C. jejuni* (Rathinam et al. 2008).

5 Cellular Response to Infection

Despite its global relevance, the molecular basis of the *C. jejuni* intestinal pathology is still an enigma. One candidate bacterial factor contributing to the inflammatory pathology is CDT. The toxin not only induces cell cycle arrest but also activates the NF-kappaB inflammatory pathway (Whitehouse et al. 1998; Lara-Tejero and Galán 2001; Zheng et al. 2008). It can be imagined that the CDT-induced growth arrest affects the constant renewal of the epithelial cell lining and thereby disrupts the integrity of the intestinal barrier. This may allow bacterial tissue penetration and the induction of an inflammatory response (Whitehouse et al. 1998). Transcription profiling on Caco-2 cells infected with *C. jejuni* for 6 h indicated upregulation of genes involved in cell growth, gene transcription, steroid biosynthesis, and inflammation, but also in cell polarity, water movement, and solute transport (Rinella et al. 2006). The *C. jejuni* -specific altered gene expression

was not observed in murine intestinal CT-62 cells, suggesting that the response may be species-specific. On the other hand, *C. jejuni* stimulates Na^+ and Cl^- secretion in infected rat ileum in a calcium-dependent and possibly protein kinase C-dependent fashion (Kanwar et al. 1995). Whether this effect is related to observed transcriptional alterations and/or the development of diarrhea awaits more knowledge of the bacterial factors that contribute to these responses

Analysis of the cellular signaling pathways indicates that *C. jejuni* induces a potent innate immune response that may contribute to the inflammatory pathology. *C. jejuni* infection of intestinal cells activates the transcription factors NF-kappaB and AP-1, causes phosphorylation of ERK and p38 mitogen-activated protein kinases and of JUN N-terminal protein kinase, and induces the basolateral secretion of proinflammatory mediators (Mellits et al. 2002; Jones et al. 2003; MacCallum et al. 2005b; Chen et al. 2006). Activation of the ERK and p38 mitogen-activated protein kinases requires de novo protein synthesis of *C. jejuni* factors that are produced upon contact with the host cells (Watson and Galán 2005). However, CDT (Zheng et al. 2008) and boiled extracts of *C. jejuni* also induce a proinflammatory transcriptional response in intestinal cells (Mellits et al. 2009). Other factors implicated in induction of the inflammatory response are *C. jejuni* lipoproteins and LOS, which activate the innate TLR2 and TLR4/MD2 signaling pathways, respectively (Zheng et al. 2008; Hu et al. 2006a, b). These effects appear particularly profound with damaged bacteria or isolated compounds (Hu et al. 2006a, b; de Zoete and van Putten, unpublished results). Maximal induction of the innate response with live *C. jejuni* appears to involve the intracellular Nod1, but not Nod2 innate immune receptor (Zilbauer et al. 2007). *C. jejuni* flagellin is a poor activator of TLR5 (Watson and Galán 2005; Andersen-Nissen et al. 2005; Johanesen and Dwinell 2006). Reconstitution of a recombinant *C. jejuni* flagellin that is able to activate TLR5, identified three small conserved regions in the flagellin backbone as the basis of the *C. jejuni* evasion of the TLR5 response (de Zoete and van Putten, unpublished results).

The activation of the innate immune response by *C. jejuni* results in the production of an array of proinflammatory cytokines and chemokines including IL-1α, IL-1β, IL-6, IL-6, and TNFα, but also of the neutrophil chemoattractant IL-8 (Hickey et al. 1999, 2000; Jones et al. 2003; Hu and Hickey 2005; Bakhiet et al. 2004; Johanesen and Dwinell 2006). These factors likely promote tissue damage and the recruitment of neutrophils and monocytes. The influx of these cell types at the site of infection is confirmed by immunohistochemistry on biopsies of infected patients. Produced innate effector molecules such as beta-defensins have potent antibacterial activity against *C. jejuni* (Zilbauer et al. 2005).

As chickens do not develop intestinal inflammation during *C. jejuni* colonization, a comparison of the avian and human innate immune system might shed light on molecular basis of inflammation. Analysis of the functional TLR repertoire of chickens indicates that this species has functional TLR4 and TLR5 receptors but lacks a TLR9 orthologue (Keestra and van Putten 2008; Keestra et al. 2008). The TLR2 complex differs from the mammalian species as it consists of a heterodimer

of TLR2 and TLR16. TLR16 combines the specificity for di- and triacylpeptides of mammalian TLR1 and TLR6 in a single molecule (Keestra et al. 2007). Remarkably, the avian species appear to lack a functional MyD88-independent pathway needed for β-interferon production in response to LOS. This feature may explain the resistance of chickens to endotoxin shock (Keestra and van Putten 2008). Preliminary analysis of the response of individual chicken TLR receptors to *C. jejuni* provides, except for the different LOS response, no obvious basis for the lack of inflammation in *C. jejuni* infected chickens (de Zoete and van Putten, unpublished results).

6 Experimental *Campylobacter* Infection Models

Despite the identification of a number of putative virulence factors, their biological significance for *C. jejuni* pathology remains largely unknown. Understanding the pathogenesis of *C. jejuni* infections in vivo has long been hampered by the lack of suitable infection models. In its natural habitat of the avian species and other warm-blooded animals, *C. jejuni* colonizes the intestine but rarely causes disease (Beery et al. 1988). Thus, these species are suitable to investigate *C. jejuni* colonization rather than virulence. Similarly, murine infection models generally yield variable *C. jejuni* colonization but virtually no intestinal pathology unless the gut flora is limited or the immune system is compromised (Jesudason et al. 1989; Hodgson et al. 1998). In C3H SCID mice with limited gut flora, *C. jejuni* causes severe inflammation of the colon and cecum, but not diarrhea (Chang and Miller 2006). Intestinal colonization and pathology are observed only for motile, chemotaxis-proficient *C. jejuni*. NF-kappaB-deficient C57BL/29 mice display moderate intestinal inflammation, but only after infection with CDT-positive *C. jejuni* strains (Fox et al. 2004). Experimental infection of congenic C57BL/6 IL-10-deficient mice produces pathological lesions similar to those seen in humans, with *C. jejuni* present at paracellular junctions and at the basolateral surface of the epithelium (Mansfield et al. 2007). Mice deficient in the Toll-like receptor adaptor protein MyD88 can be persistently colonized by *C. jejuni* but do not develop pathology (Watson et al. 2007). In these mice, colonization requires *N*-glycosylation of proteins and capsule production (Watson et al. 2007), indicating these traits are required for colonization even when the innate immune response is attenuated.

The *C. jejuni* animal model closest resembling human infection is the oral infection of nonhuman primates. In experimentally infected macaques, *C. jejuni* causes an acute enterocolitis with bacteria invading the mucosa before the development of inflammation (Russell et al. 1989, 1993). Microscopy showed bacteria located in intestinal crypts, within surface epithelial cells and in the subepithelial tissue, indicative of mucosal penetration (Russell et al. 1989, 1993). These animals also display several clinical symptoms as observed during human infection, including bloody diarrhea. However, the nonhuman primate animal model is rarely used,

mainly on ethical grounds. A promising alternative model involves the use of gnotobiotic piglets. Infection of colostrum-deprived newborn piglets with *C. jejuni* results in clinical symptoms and histopathology similar to those observed in humans infected with *C. jejuni*. The bacteria cause gross lesions in the large intestine with edema, neutrophil infiltration, and sloughing of epithelial cells, and hemorrhage or increased mucus production. At the cellular level, bacteria are found inside intestinal epithelial cells as well as in the underlying tissue (Babakhani et al. 1993; Boosinger and Powe 1988).

Overall, the in vivo infection models suggest that in most animals the indigenous gut flora and/or a well-developed host immune system may limit *C. jejuni* intestinal pathology. The use of animals with limited gut flora and/or immune deficiencies as *C. jejuni* infection models may shed light on the molecular basis and species-specificity of the *C. jejuni*-induced pathology, and provide information on the bacterial phenotype and metabolic status of the bacteria compared with those isolated from the human intestine.

7 Conclusions and Outlook

A primary challenge in *C. jejuni* research remains the discovery of the molecular basis of the pathogenic behavior of *C. jejuni* in humans compared to the commensal behavior in most other species, in particular poultry. Dissection of the virulence strategies of *C. jejuni* is seriously hampered by the huge phenotype diversity. Extensive surface variation and the broad adaptation potential appear to provide the pathogen with an array of seemingly redundant tools to exploit host cell biology. Strain differences further complicate functional analysis of *C. jejuni* pathogenicity. Yet, current knowledge indicates flagella-mediated motility, chemotaxis, and penetration of mucus as key determinants of bacterial colonization in all species. The induction of pathology appears to require damage of the integrity of the epithelium lining the mucosa, either via toxin production, invasion of epithelial cells, weakening of cellular tight junctions, and/or traversal of intestinal M cells. Bacterial products as well as cellular damage may elicit an inflammatory response that contributes to the development of pathology. One key determinant of the course of an infection may be the local microenvironment at the site of infection. *C. jejuni* appears to be able to quickly adapt its phenotype to changing microenvironments like the availability of oxygen and nutrient sources. Recent progress in the understanding of the *C. jejuni* genome diversity, gene regulation, and its dynamic behavior in different environments has paved the way to finally understand and attack the virulence strategies of this important pathogen.

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Shigella Infection of Intestinal Epithelium and Circumvention of the Host Innate Defense System

Hiroshi Ashida, Michinaga Ogawa, Hitomi Mimuro, and Chihiro Sasakawa

Contents

Abstract *Shigella*, Gram-negative bacteria closely related to *Escherichia coli*, are highly adapted human pathogens that cause bacillary dysentery. Although *Shigella* have neither adherence factors nor flagella required for attaching or accessing the

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intestinal epithelium, *Shigella* are capable of colonizing the intestinal epithelium by exploiting epithelial-cell functions and circumventing the host innate immune response. During *Shigella* infection, they deliver many numbers of effectors through the type III secretion system into the surrounding space and directly into the host-cell cytoplasm. The effectors play pivotal roles from the onset of bacterial infection through to the establishment of the colonization of the intestinal epithelium, such as bacterial invasion, intracellular survival, subversion of the host immune defense response, and maintenance of the infectious foothold. These examples suggest that *Shigella* have evolved highly sophisticated infectious and intracellular strategies to establish replicative niches in the intestinal epithelium.

1 Introduction

Shigella is a genus of highly adapted human pathogens that cause bacillary dysentery (shigellosis), a disease that provokes severe bloody and mucous diarrhea. In tropical areas of developing countries, shigellosis is endemic and a major killer of children under 5 years of age. Shigellosis occurs following the ingestion of a very small number (100–1,000) of bacteria, thus permitting the easy spread of the disease under conditions of poverty, crowding and poor sanitation through person-to-person contact as well as by drinking contaminated water (Kotloff et al. 1999; Jennison and Verma 2004).

Shigella, a Gram-negative bacillus, comprises four species: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. *Shigella* is closely related to *Escherichia coli* based on DNA-DNA homology; however, the group of bacteria causing shigellosis is idiomatically called *Shigella*. Shigellosis is also caused by enteroinvasive *E. coli* (EIEC), a pathogenic *E. coli,* albeit the resulting symptoms are not as severe as those caused by *Shigella*. Indeed, *Shigella* and EIEC both possess a large 220-kb plasmid on which the major virulence-associated proteins (effectors and some other virulence factors) and the type III secretion system (TTSS) required for the delivery of the effectors are encoded. In addition, they also possess a 3-kb ColE1-type cryptic plasmid (known as pHS-2 in *S. flexneri*); the presence of this plasmid is associated with Reiter's syndrome, which causes reactive arthritis and occasionally occurs in shigellosis patients possessing a special human leukocyte antigen (HLA) background, such as HLA-B27 (Stieglitz et al. 1988; Stieglitz and Lipsky 1993).

Shigella have neither adhesins for attaching to the upper intestinal epithelium nor flagella required for directly accessing the intestinal epithelial surface. Therefore, upon infection by means of the fecal–oral route, *Shigella* move down to the colon and rectum directly, where they translocate through the epithelial barrier via M cells, which overlie solitary lymphoid nodules and sample foreign antigens in the intestinal lumen, presenting the antigens to the immune system (Wassef et al. 1989; Cossart and Sansonetti 2004). When they reach the underlying M cells, *Shigella* infect the resident macrophages and dendritic cells that

reside within the M cell pocket. Within the phagosomal membrane, *Shigella* secrete effectors, such as IpaB and IpaC, that are translocated via the TTSS; the secreted proteins then break the phagosomal membrane, thus allowing the bacteria to escape into the cytoplasm. In the macrophage-cell cytoplasm, the bacteria multiply and induce rapid cell death by activating caspase-1-dependent and caspase-1-independent cell death pathways (Zychlinsky et al. 1992, 1994; Hilbi et al. 1998; Suzuki et al. 2005).

Meanwhile, *Shigella* released from the dead macrophages enter into the surrounding enterocytes (polarized epithelial cells) from the basolateral surface by inducing membrane ruffling, which finally leads to macropinocytosis. Once a bacterium is surrounded by a membrane vacuole within an epithelial cell, it immediately disrupts the membrane vacuole and escapes into the cytoplasm. Within the cytoplasm, *Shigella* can multiply and move both intra- and intercellularly by inducing actin polymerization at one pole of the bacterium (Suzuki and Sasakawa 2001). Multiplying *Shigella* release large amounts of lipopolysaccharide (LPS) and peptideglycan (PGN); PGN in turn is sensitized by the Nod1/CARD-family, eventually leading to the activation of NF-κB (Girardin et al. 2001, 2003). In response to the activation of NF-κB, the colonic epithelium expresses a large array of proinflammatory cytokines and chemokines, especially IL-8, thus further promoting local inflammation and attracting more PMNs (Perdomo et al. 1994; Pédron et al. 2003). Consequently, bacterial infection leads to the inflammatory destruction of the intestinal epithelium, which causes mucopurulent bloody diarrhea. Figure 1 shows the whole infectious process of *Shigella* in the intestinal epithelium.

2 Invasion of Intestinal Epithelium

2.1 Shigella *Entry into Polarized Enterocytes via M Cells*

Shigella have neither adherence factors nor flagella; nevertheless, they are capable of efficiently entering the intestinal epithelium. *Shigella* invasion of epithelial cells occurs not from the apical surface of polarized enterocytes but rather from the basolateral surface. Indeed, when polarized MDCK (Madin Darby Canine Kidney) or Caco-2 cells were infected with *Shigella*, the bacteria were unable to enter from the apical surface of the cells. If, however, the cell-to-cell junctions were opened by treating the cells with EGTA, which disrupts the Ca^{2+}-chelating E-cadherin-E-cadherin homophilic interaction, *Shigella* was able to enter the cells efficiently (Mounier et al. 1992; Watarai et al. 1995). In agreement with this manner of bacterial entry, when *Shigella* was used to infect rabbit ligated ileal loops, the bacteria moved to the basolateral surface via entry through the M cells (Wassef et al. 1989; Perdomo et al. 1994). Thus, *Shigella* possess a highly evolved system to promote bacterial entry into epithelial cells by inducing membrane ruffles

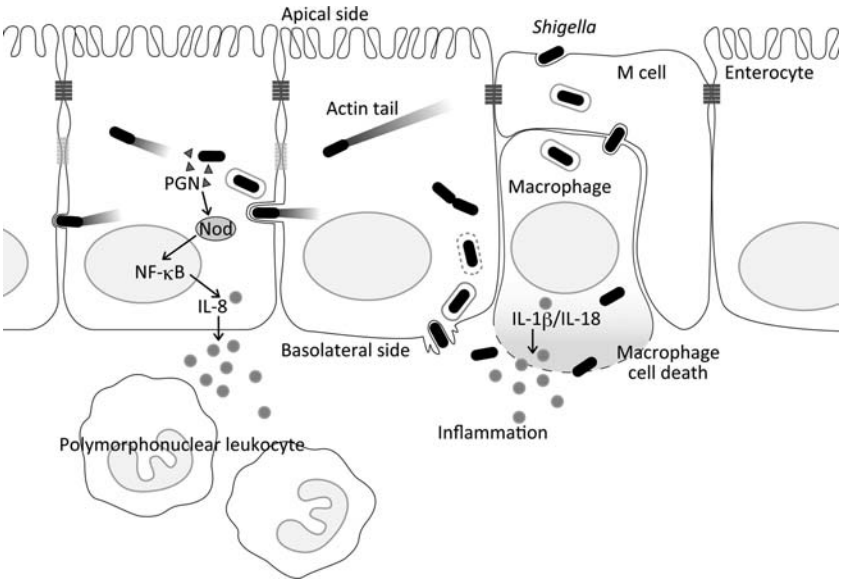

Fig. 1 A schematic model showing the infection of colonic epithelial cells by *Shigella*. Please refer to the text for details

around the bacterial entry site. Consequently, the ability of *Shigella* to recognize the M cell surface and the basolateral surface of enterocytes must be important for determining the route of bacterial invasion in intestinal epithelium.

2.2 *Regulation of* Shigella *Invasion of Epithelial Cells*

The invasiveness of *Shigella* requires a subset of effectors secreted via the TTSS; these effectors are encoded by the *ipaA*, *ipaB*, *ipaC*, *ipaD*, *ipgB1*, *ipgB2*, *ipgD*, and *virA* genes located on a large 220-kb plasmid (Buchrieser et al. 2000; Venkatesan et al. 2001). The large plasmid is highly conserved among *Shigella* species and is thought to contain 50–60 virulence-associated genes, including genes that encode effectors, chaperons, components of the TTSS, and regulatory proteins (Buchrieser et al. 2000; Venkatesan et al. 2001). The activity of the TTSS and the production/secretion of the effectors are tightly regulated by two major modulator genes, *virF* and *mxiE*. At 37°C, the local DNA supercoil structure around the *virF* promoter allows an increase in *virF* transcription, which in turn activates the *virB* promoter (Sakai et al. 1988; Adler et al. 1989; Tobe et al. 1993; Dorman and Porter 1998). Since VirB directly controls the transcription of a subset of genes that includes *ipaA*, *ipaB*, *ipaC*, *ipaD*, *ipgB1*, *ipgD*, *icsB*, *ospB*, *ospC1*, *ospC2*, *ospC3*, *ospC4*, *ospD1*, *ospD2*, *ospF*, and *virA*, the activation of *virB* transcription via VirF results in an increase in the production/secretion of the effectors (Le Gall et al. 2005).

Although the roles of these proteins as effectors have not yet been completely elucidated, some of them, such as IpaA, IpaB, IpaC, IpaD, IpgB1, IpgD, and VirA, have been characterized as effectors involved in the bacterial invasion of epithelial cells. The genes encoding the TTSS are also, if not fully, expressed at 37°C and become further activated upon the contact of *Shigella* with the host cells. Upon activation of the TTSS in *Shigella* or in TTSS-deregulated mutants, the transcription of nearly a dozen genes encoding effectors, such as *ospB*, *ospC1*, *ospD3*, *ospE1*, *ospE2*, *ospF*, *ospG*, *ipaH4.5*, *ipaH7.8*, *ipaH9.8*, and *virA*, are induced (Demers et al. 1998; Mavris et al. 2002a; Kane et al. 2002). The transcription of effector-encoding genes requires MixE, a *cis*-acting transcriptional activator of the AraC family; MixE is encoded by the *mixE* gene, which exists among the TTSS genes on the large plasmid. The *cis*-acting sites are known as "MixE-boxes"; these 17-bp sequences are located between −49 and −33 bp upstream of each transcription start site (Mavris et al. 2002b). Although the roles of each effector regulated by MixE remain partly unknown, the production/secretion of the effectors is believed to be required for intracellular bacterial survival, intracellular multiplication, evasion from the host innate defenses, and the prolonged survival of the infected host cells (Le Gall et al. 2005).

2.3 Role of Effectors in Bacterial Invasion of Host Cells

The effectors secreted from extracellular *Shigella* via the TTSS during the initial stage of infection interact with various host target proteins and stimulate host cell signal pathways to direct local actin polymerization, which is required to change the cell surface architecture and to entrap the bacteria. IpaB and IpaC, which are secreted in the surrounding bacterial space via the TTSS of extracellular *Shigella*, interact with host surface molecules such as β1 integrin and CD44, which act as receptors (Watarai et al. 1996; Skoudy et al. 2000; Lafont et al. 2002). IpaB interacts with CD44 and cholesterol, which occurs within lipid-rich rafts, to stimulate cell signaling involved in the promotion of local actin polymerization (Skoudy et al. 2000; Lafont et al. 2002). IpaC, which is integrated into the host plasma membrane, can stimulate actin polymerization. Actin foci at the site of bacterial entry into the epithelial cells have been shown to accumulate c-Src (Duménil et al. 1998, 2000; Bougnères et al. 2004; Tran Van Nhieu et al. 2005). A recent study has revealed that the IpaC carboxy-terminal domain induces the recruitment of Src and actin polymerization, resulting in ruffling formation during *Shigella* invasion (Mounier et al. 2009). The invasion of *Shigella* into the epithelial cells also elicits the phosphorylation of cortactin (Dehio et al. 1995), which activates the Arp2/3 complex and induces actin polymerization, in a Src-dependent manner (Bougnères et al. 2004). Crk is also phosphorylated by Abl kinase to activate Rac1 upon the invasion of *Shigella* into epithelial cells (Burton et al. 2003). Thus, IpaC is assumed to play a pivotal role in the induction of actin polymerization. IpgD exhibits phosphatidylinositol (4,5) biphosphate phosphatase activity, which catalyzes the

hydrolysis of PI(4,5)P2 to PI(5)P (phosphatidyl-inositol 5-monophosphate), thereby contributing to local actin polymerization (Niebuhr et al. 2002; Pendaries et al. 2006). IpaA binds to the vinculin head, which stimulates actin depolymerization, and also interacts with β1-integrin to stimulate RhoA activity, thus facilitating the recycling of the free actin pool through the destruction of stress fibers and contributing to the production of membrane ruffles (Bourdet-Sicard et al. 1999; Hamiaux et al. 2006; Izard et al. 2006; Demali et al. 2006). IpgB1 plays a major role in the invasion of *Shigella* into the epithelial cells, since IpgB1 activity mimics RhoG in the host cell, which is required for the activation of the ELMO and Dock180 complex, thus leading to the activation of Rac1 and ruffle formation (Ohya et al. 2005; Handa et al. 2007). Indeed, upon the ectopic expression of IpgB1 in HeLa cells, for example, large membrane ruffles are produced. Under *in vitro* conditions, recombinant IpgB1 protein competitively binds to the N-terminal portion of ELMO with its binding by RhoG protein, suggesting that IpgB1 mimics the function of RhoG in the host. Although its biological role in bacterial entry remains unknown, IpgB2, an IpgB1 homolog, binds to mDia1 (facilitating actin nucleation) and ROCK (Rho-kinase) via a GBD (GTPase binding domain) region, thereby mimicking RhoA activity in the induction of stress fiber formation (Alto et al. 2006). VirA, which belongs to the EspG/VirA family and is found in enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, and *Shigella*, is delivered to the vicinity of the bacterial entry site (VirA can also be secreted from intracellular *Shigella*) (Elliott et al. 2001; Yoshida et al. 2002). VirA (and EspG) can induce the degradation of local microtubule networks (MTs) under both *in vitro* and *in vivo* conditions. Since the degradation of MTs by EspG results in the release of various MT-associated proteins, including GEF (GTP exchange factor)-H1 (which activates RhoA; Yoshida et al. 2002; Matuzawa et al. 2004), VirA activity is thought to contribute to ruffle formation during *Shigella* invasion via cross-talk between RhoA and Rac1. Together, these studies strongly indicate that synergistic activities arising from the interplay between bacterial effectors and target host proteins orchestrated by Rho-GTPases and tyrosine kinases are the key factors responsible for promoting bacterial invasion in epithelial cells.

3 Dissemination of *Shigella* Among epithelial cells

3.1 Actin-Based Intracellular Motility

Some cytoplasmic invading bacterial pathogens, including *Shigella*, *Listeria monocytogenes*, *Rickettsia*, *Mycobacterium marinum*, and *Burkholderia pseudomallei*, are capable of inducing local actin polymerization at one pole of the bacterium, enabling them to gain a propulsive force that they can use to move within the cytoplasm and into adjacent host cells; this mechanism is an important bacterial system for renewing replicative niches and serves as a portal of entry into

deeper tissues (Gouin et al. 2005; Stevens et al. 2006). In the case of *Shigella*, the activity is crucial for the expansion of the replicative foothold in the colonic epithelium. The means by which the pathogens mediate actin polymerization are distinct for each pathogen in terms of bacterial factors; however, they share the ability to recruit and activate the Arp2/3 complex (actin-related protein 2 and 3) in the vicinity of the bacterial surface, inducing local actin polymerization (Gouin et al. 2005; Stevens et al. 2006). The actin-based movement of *Shigella* depends on a special interplay between VirG (IcsA) and N-WASP (neural Wiskott-Aldrich syndrome protein) (Suzuki and Sasakawa 2001). VirG is a 1102-amino acid outer membrane protein encoded by the *virG* gene on the large plasmid (Makino et al. 1986; Bernardini et al. 1989; Lett et al. 1989). Inside the host cells, the multiplication of *Shigella* ultimately results in the accumulation of VirG at one pole of the bacterium. The N-terminal domain of VirG, which is composed of 706 amino acids (α-domain), is exposed on the bacterial surface, while the 344 amino acid C-terminal domain (β-core) is embedded in the outer membrane through the formation of a membrane pore, which acts as an auto-transporter to expose the α-domain (Lett et al. 1989; Goldberg et al. 1993; Suzuki et al. 1995).

The N-terminal VirG region specifically binds to N-WASP, one of the WASP family proteins (Suzuki et al. 1998). VirG interacts simultaneously with vinculin and, as described later, also interacts with its own bacterial secreted IcsB effector via the TTSS as well as with Atg5, an autophagic protein required for autophagosome formation (Suzuki et al. 1996; Ogawa et al. 2005). N-WASP acts as an adapter for interactions with the Arp2/3 complex. The binding of VirG and Cdc42 to N-WASP activates N-WASP, which in turn leads to the recruitment of the Arp2/3 complex (Suzuki et al. 1998, 2000; Egile et al. 1999). To initiate and sustain the active conformation of N-WASP, Toca-1, which mediates Cdc42-dependent N-WASP activation, must be recruited in the vicinity of the motile bacterial surface through an interaction with N-WASP (Leung et al. 2008). Once N-WASP that has been recruited in the vicinity of the bacterial surface becomes activated, it can then interact with and activate Arp2/3, together with monomeric actin, and profilin, thus enabling the bacterium to gain a propulsive force in the host cytoplasm (Mimuro et al. 2000) (Fig. 2). During bacterial movement, some motile bacteria impinge on the host plasma membrane and cause membrane protrusion. The tips of these bacteria-containing protrusions are then engulfed by neighboring uninfected cells, leaving the bacteria transiently contained within double host plasma membrane-bound vacuoles. The bacteria then disrupt the protrusion vacuoles, thereby releasing *Shigella* into the cytoplasm of the neighboring epithelial cells. *Shigella* is disseminated from one cell to another in this manner.

The motile behavior of bacteria in the cytoplasm is highly variable and depends on the cellular location; some bacteria can rapidly move along the surface of the nuclear membrane or beneath the plasma membrane, while others suddenly change direction, spin around, or stop moving. The motile behavior of the bacteria also depends on the subcellular location, since bacterial movement within the cytoplasm is severely hindered by MTs. However, motile bacteria can destroy the surrounding MTs by secreting VirA via the TTSS (Yoshida et al. 2006). Smooth bacterial

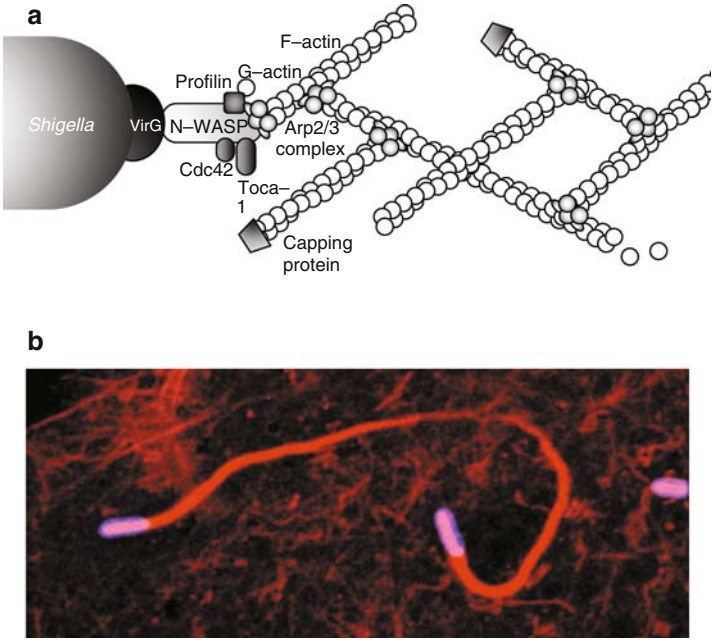

Fig. 2 *Shigella* movement within the host cell cytoplasm requires actin polymerization. (**a**) The asymmetric distribution of VirG (also known as IcsA) on the bacterial surface is essential for the polar movement of *Shigella* in epithelial cells. VirG at one pole of the bacterium recruits N-WASP (neural Wiskott-Aldrich syndrome protein), which is activated by the attachment of Cdc42 and Toca-1, resulting in the activation of the Arp2/3 complex. (**b**) A confocal image of the actin comet tail from one pole of *Shigella* in the cytoplasm

movements thus depend on the ability of VirA to degrade the MTs (Yoshida et al. 2006). Consistent with this mechanism, *virA*-deleted mutants cannot move as smoothly within the host cytoplasm as wild-type bacteria. Consequently, *virA* mutants are incapable of intercellular spreading and become attenuated when inoculated into mice via the nasal route. Thus, as described above, VirA has dual roles in both bacterial invasion and intracellular spreading via MT degradation, in which VirA's ability to collapse local MT structures is a key factor in promoting bacterial infection (Yoshida et al. 2002, 2006).

3.2 Bacterial Cell-Cell Spreading

The cell-cell spreading of *Shigella* is a sequential process that resembles the intercellular transport of a large double-membrane vesicle. This process is thought to require many bacterial and host factors, though the molecular basis of the process is still poorly understood. The whole process of bacterial movement from one cell to another consists of at least three distinctive stages; first, the bacterium attaches to

the plasma membrane and impinges upon the membrane so that it protrudes as a filopodium; second, the protruding filopodium penetrates a neighboring cell; and third, the double plasma membranes are lysed (Fig. 1). Several bacterial effectors secreted via the TTSS, such as IpaB and IpaC, as well as VacJ, a chromosome-encoded lipoprotein, are reportedly involved in bacterial dissemination (Suzuki et al. 1994; Page et al. 1999). Intriguingly, a motile bacterium wrapped at the tip of a filopodium that is not entrapped by a neighboring cell membrane cannot be released into the extracellular medium. Thus, motile bacteria may have a sensitizing system that recognizes a wrapped environment. IpaB possesses an RTX motif that is required for membrane disruption, while IpaC that has been integrated into the host cell plasma membranes can trigger cell signals in the initial cell that induce the actin polymerization required for the invasion of *Shigella* into epithelial cells (High et al. 1992; Page et al. 1999; Tran Van Nhieu et al. 1999, 2005). These activities are thus thought to participate in the lysis of the double-membranes containing the motile bacteria and the plasma membrane engulfed by the adjacent, uninfected cell. Although the mechanism responsible for this is still unclear, a lipoprotein-encoding chromosomal Tn*5*-insertion mutant of *S. flexneri*, called *vacJ*, reportedly results in the inability of the mutant to move to adjacent epithelial cells (Suzuki et al. 1994). *Shigella* lacking the *vacJ* gene can extend filopodium; however, the mutant bacteria within the protrusion cannot escape into the new host. Instead, the bacteria continue to multiply within the trapped vacuole until the filopodium begins to resemble a large balloon filled with bacteria. This mutant phenotype suggests that the VacJ protein participates either directly or indirectly in the lysis of the protrusion membrane after the filopodium has been entrapped by the adjacent cell membrane.

In bacterial cell-cell spreading, cell-cell junctions such as tight junctions and adherence junctions play structurally and functionally important roles. An early study indicated that E-cadherin, the major connector at adherence junctions, is involved in the mediation of *Shigella* intercellular spreading. However, as epithelial cells lacking E-cadherin still allow *Shigella* cell-cell movement, the exact role of E-cadherin in bacterial spreading remains to be elucidated (Vasselon et al. 1992; Sansonetti et al. 1994). Recently, connexin (Cx) proteins, such as Cx26, have been shown to play an important role in promoting the invasion and subsequent dissemination of *Shigella* into epithelial cells (Tran Van Nhieu et al. 2003). *S. flexneri* infection can induce transient peaks in the intracellular calcium concentration, triggering the opening of Cx26 hemichannels and allowing the release of ATP into the medium (Tran Van Nhieu et al. 2003). The ATP promotes calcium signaling, which in turn somehow promotes bacterial invasion and dissemination. The hemichannel activity of Cx26 and the ATP released during *Shigella* infection are functionally important for promoting bacterial dissemination. For example, when an anti-Cx26 antibody was used to block the extracellular loop of Cx26, which is involved in the transmission of hemichannel signaling, *Shigella* dissemination was hindered, while the addition of ATP to the medium significantly increased bacterial cell-cell spreading (Tran Van Nhieu et al. 2003). Cx26 is predominantly expressed from the basolateral side of polarized epithelial cells

and interacts with ZO-1 at tight junctions. *S. flexneri* dissemination can also be inhibited by treating the host cells with suramin, an antagonist of purinergic receptors (Tran Van Nhieu et al. 2003). Thus, these results together suggest that both hemichannel and extracellular ATP mediate some unknown signaling mechanism involved in the promotion of bacterial spreading.

4 Intestinal Inflammation Caused by *Shigella* Infection

The ability of *Shigella* to invade the resident macrophages and intestinal epithelium and the resulting inflammation are the major pathogenic features of *Shigella*. Bacterial activity resulting in the induction of an inflammatory response has been demonstrated using various *in vivo* and *in vitro* infection models; for example, the inoculation of *Shigella* into rabbit ileal loops caused acute inflammation and subsequent destruction of the intestinal villi (Perdomo et al. 1994). The inoculation of *Shigella* onto guinea pigs eyes, known as the "Sereney test," caused ulcerative keratoconjunctivitis (Serény 1957). Similarly, the inoculation of *Shigella* into the rectums of guinea pigs caused diarrhea (Shim et al. 2007). In mice pulmonary model (since mice intestine is naturally resistant to *Shigella* infection), intranasal infection with *Shigella* caused an acute broncho-pneumonia accompanied by a massive neutrophil infiltration, resulting in the production and secretion of MIP-2 (a murine IL-8), IL-1β, and IL-6 (Voino-Yasenetsky and Voino-Yasenetskaya 1961; Phalipon et al. 1995). The ability of *Shigella* to induce inflammatory responses in these animal models depends on the invasiveness of the bacteria and intracellular multiplication, suggesting that bacterial components released and secreted from intracellular *Shigella* stimulate the inflammatory signal pathways (Phalipon and Sansonetti 2007).

4.1 Macrophage Killing and Inflammatory Response

Shigella invade the resident macrophages (and the dendritic cells) residing in the M cell pocket, where they burst out of phagocytic vacuoles and multiply within the cytoplasm. The bacterial multiplication induces a strong inflammatory response via the recognition of bacterial components by the host innate immune system, which activates caspase-1 and induces macrophage cell death (Franchi et al. 2008). An early study reported that macrophages infected by *Shigella* undergo apoptosis via the interaction of IpaB, secreted via the TTSS, with caspase-1 (Zychlinsky et al. 1992; Chen et al. 1996; Hilbi et al. 1998). However, the cell death of macrophages induced by *Shigella* has recently been characterized and identified as a new type of programmed cell death associated with an inflammatory response, which is accompanied by plasma membrane permeability and nuclear condensation; this type of programmed cell death is termed pyroptosis (Fink and Cookson

2005; Suzuki et al. 2007; Bergsbaken et al. 2009). Interestingly, Ipaf and the adaptor protein ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain), which are nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) that can sense the presence of microbial components in the cell cytoplasm, appear to be required for caspase-1 activation and macrophage cell death, and Ipaf and ASC-mediated caspase-1 activation seem to occur as a result of sensing through some unknown bacterial factor(s) independently on flagellin (Suzuki et al. 2007). Recently, Ipaf has also been reported to mediate caspase-1 activation, IL-1β processing, and caspase-1-dependent cell death through the detection of bacterial flagellin in macrophages infected with *Salmonella typhimurium* or *Legionella pneumophilla* (Mariathasan et al. 2004; Franchi et al. 2006, 2009; Miao et al. 2006; Amer et al. 2006). Finally, at later time points, NLRP3 (also known as Cryopyrin) seems to mediate an additional cell death in *Shigella*-infected macrophages. Interestingly, this NLRP3-mediated cell death, termed pyronecrosis, is caspase-1-independent, thus meaning it is inflammasome-independent (Willingham et al. 2007). Consequently, proinflammatory chemokines and cytokines are produced in macrophages after infection with *Shigella* and become a major cause of strong inflammation.

4.2 Epithelial Invasion and Inflammatory Response

During the multiplication of *Shigella* within epithelial cells, the bacteria release LPS and PGN as well as nucleic acids (Sansonetti 2004; Phalipon and Sansonetti 2007; Ogawa et al. 2008). These bacterial components are recognized by the host innate immune system through a process mediated by Toll-like receptors (TLRs) and Nod-like receptors (NLRs). As a result, these components stimulate the inflammatory signal cascade, activating the host innate defense systems and leading to cellular and humoral immune responses and the production of antimicrobial peptides, such as LL-37 and β-defensins (Lehrer and Ganz 2002; Selsted and Ouellette 2005). Therefore, bacterial multiplication within the intestinal epithelium (as well as within macrophages) becomes a major cause of strong inflammation in the intestine (Fig. 3). The intestinal epithelium expresses a wide range of pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) released from intracellular bacteria (Fritz et al. 2006). When *Shigella* multiply within the epithelial cells, the Nod1-RICK-dependent NF-κB pathway is stimulated through the recognition of PGN by Nod1, a NLR family protein, that activates inflammatory signal cascades, such as mitogen-activated protein kinases (MAPKs) and NF-κB (Girardin et al. 2001, 2003; Inohara and Núñez 2003). Intriguingly, Nod1 is recruited to the bacterial entry site by moving from the cytoplasm to the plasma membrane, raising the possibility that efficient PGN sensing may occur around the site of bacterial entry (Kufer et al. 2007). Furthermore, a recent study indicated that NLRX1 (a newly identified cytoplasmic NLR protein) is localized in the mitochondria via its N-terminal mitochondrial

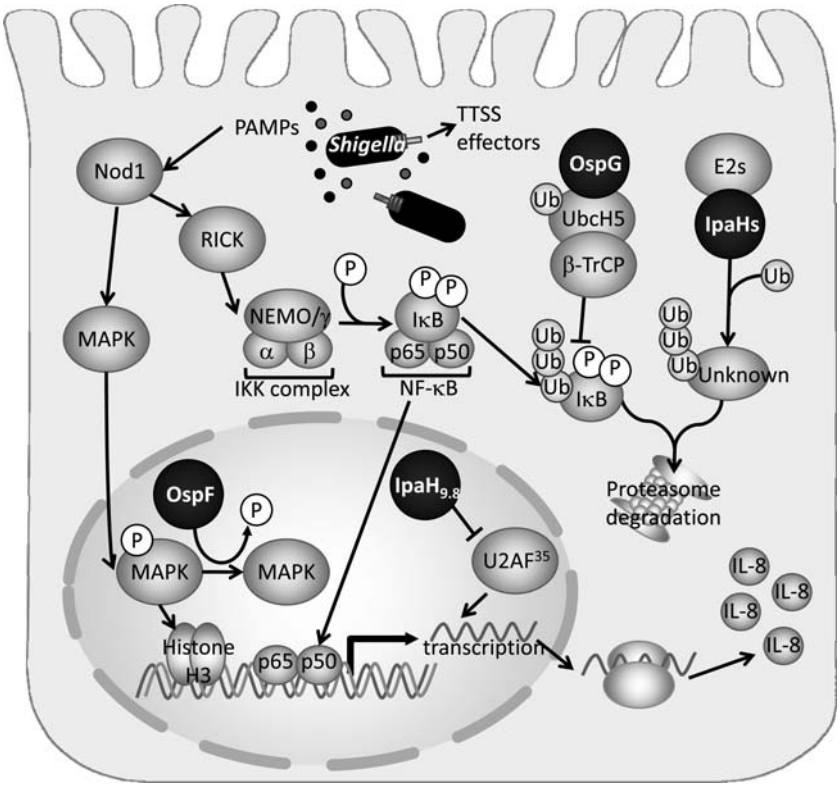

Fig. 3 *Shigella* downregulates the host inflammatory response induced by PAMPs (pathogen-associated molecular patterns). During the multiplication of *Shigella* in epithelial cells, the bacteria shed PAMPs, such as peptidoglycan (PGN), into the cytoplasm. The recognition of PGN by Nod1 activates the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK)-dependent inflammatory signals. *Shigella* delivers a set of effectors, including IpaHs, OspF and OspG, into the host cytosol through the type III secretion system (TTSS), enabling these effectors to circumvent the host inflammatory response and inactivate the innate immune system. See the main text for details

targeting sequence and promotes reactive oxygen species (ROS) production when epithelial cells are infected with *Shigella*, although the PAMPs sensitized by NLRX1 and the mechanism of ROS production induced by *Shigella* infection remain unclear (Tattoli et al. 2008). In contrast to macrophages as described above, the cell death mechanisms in *Shigella*-infected epithelial cells remain unknown. However, a recent study has revealed that *Shigella* induce mitochondrial dysfunction, resulting in caspase-independent necrotic cell death through a pathway dependent on Bnip3 and cyclophilin D, two key regulators of mitochondrial permeability transition and cell death during oxidative cell stress, in epithelial cells (Carneiro et al. 2009). This mitochondrial dysfunction-mediated cell death mechanism is tightly counterbalanced by a Nod1-dependent NF-κB activity by maintaining the Bnip3/Bcl-2 expression ratio, demonstrating the existence of

specific checkpoints that convert at mitochondria to determine prodeath and pro-survival signaling. Nevertheless, as will be described later, *Shigella* (and many other bacterial pathogens) that have infected the intestinal mucosa are capable of circumventing the host innate immune response, allowing them to survive and multiply within the epithelium; this ability implies that *Shigella* possess some activities that dampen the host inflammatory signals.

5 Modulation of Host Inflammatory Response

5.1 Bacterial Strategy for Modulating the Host Inflammatory Response

The modulation of the host inflammatory responses and the circumvention of the host immune system are key factors in the survival of bacterial pathogens during the infection of the intestinal epithelium (Sansonetti and Di Santo 2007; Mattoo et al. 2007). To modulate the host inflammatory response, *Shigella* release more than a dozen different effector proteins while multiplying within epithelial cells. These effectors include the IpaH family proteins, OspG, and OspF (Fig. 3) (Ashida et al. 2007; Ogawa et al. 2008).

One member of the IpaH family, IpaH9.8, is secreted from intracellular *Shigella* via the TTSS and is translocated into the nucleus (Venkatesan et al. 1991; Toyotome et al. 2001). The nuclear translocated IpaH9.8 protein interacts with $U2AF^{35}$, a mRNA splicing factor, and interferes with the $U2AF^{35}$-dependent splicing reaction. Upon the expression of the *ipaH9.8* gene in epithelial cells or during the knockdown of $U2AF^{35}$ production using siRNA, the levels of proinflammatory cytokines decrease considerably (Okukda et al. 2005). In mice lung infection model, infection with the *ipaH9.8* mutant caused a more severe inflammatory response and a greater production of proinflammatory cytokine than infection with wild-type *Shigella*. Of importance, the colonization rate of the *ipaH9.8* mutant in the lung tissue was greatly reduced to less than one-thirtieth of the wild-type level (Okukda et al. 2005). IpaH9.8 and IpaH homologs produced by *Salmonella* and *Pseudomonas* species can act as E3 ubiquitin ligases, an activity that is encoded by the highly conserved C-terminal region of these molecules (Rohde et al. 2007). A recent crystal structural analysis of IpaH proteins revealed that IpaHs represent a new class of E3 ligase that differs from the typical RING- and HECT-types of E3 ligases. Furthermore, the IpaH C-terminal region also confers a catalytic E3 ligase activity, while the N-terminal leucine-rich repeat domain is responsible for substrate recognition (Singer et al. 2008; Zhu et al. 2008). E3 ligase activity of IpaH9.8, as examined in a yeast system, is required for interference with the pheromone response signal cascade through the ubiquitination of MAPKK Ste7, which undergoes proteasome degradation (Rohde et al. 2007).

OspG interacts with ubiquitinated E2s (Ub conjugating enzymes), such as UbcH5b, that are required for phospho-IκBα ubiquitination and subsequent proteasome degradation (Kim et al. 2005). OspG activity can thus inhibit IκBα degradation, which is required for NF-κB activation, leading to the suppression of NF-κB activation. Consistent with the activity of OspG, *in vivo* and *in vitro* infection models with an *ospG* mutant have indicated that OspG plays a role in downregulating the inflammatory response to bacterial infection (Kim et al. 2005).

OspF secreted from intracellular *Shigella* is translocated into the epithelial cell nucleus. OspF possesses a phosphatase activity that allows it to dephosphorylate and inactivate MAPKs, such as ERK1/2, JNK, and p38; this, in turn, leads to the inhibition of phosphorylation at the Ser10 residue of histone H3, which is involved in activating the transcription of a group of genes regulated by NF-κB (Arbibe et al. 2007). Furthermore, *Shigella* OspF, *Salmonella* SpvC and *Pseudomonas syringae* HopAI1 are all capable of dephosphorylating MAPKs through their phosphothreonine activities, enabling them to interfere with MAPK activity (Li et al. 2007). *In vivo* and *in vitro* infection models with an *ospF* mutant revealed that OspF also downregulates the inflammatory response to bacterial infection required for the promotion of bacterial colonization (Arbibe et al. 2007; Kramer et al. 2007). Antimicrobial peptides are also important bactericidal components of the host innate defense system (Zasloff 2002; Selsted and Ouellette 2005). Recent studies have indicated that *Shigella* can suppress the transcription of several genes encoding antimicrobial peptides and chemoattractants, such as human β-defensin, LL-37, and CCL20, in a *Shigella* TTSS-dependent manner (Sperandio et al. 2008). Although the exact number of effectors involved in the modulation of inflammatory responses to *Shigella* infection in the intestine remains uncertain, the numerous host molecules and signal pathways that are engaged in the induction of an inflammatory response likely mean that *Shigella* require many effectors to modulate various inflammatory signal pathways at different time points and cascade levels during bacterial infection. These findings also strongly suggest that the inability to control cytokine production during bacterial infection would be detrimental to bacterial colonization.

6 Intracellular Survival Strategies

6.1 Escape from Autophagic Recognition

Autophagy is a ubiquitous degradation system in eukaryotic cells that is a crucial cellular response to starvation and stress as well as the removal of damaged or surplus organelles (Mizushima 2007; Levine and Deretic 2007; Deretic and Levine 2009). Autophagy is a highly conserved pathway during which a double-layered isolation membrane wraps around undesirable cytoplasmic contents. The enclosed material is delivered to an autophagosome and degraded after the autophagosome

fuses with a lysosome. Autophagy is also a pivotal component of the host innate defense system for eliminating invading cytoplasmic microbes. For example, Group A *Streptococcus* (GAS) can invade epithelial cells but is usually targeted and eventually destroyed by autophagy (Nakagawa et al. 2004). *Mycobacterium tuberculosis*, an intracellular parasitic pathogen that survives in vacuolar compartments within the macrophages, can also be targeted by autophagy at an early stage of infection as long as the host innate immune response is intact (Gutierrez et al. 2004). *Rickettsia* is also sequestered in autophagosome-like double-membranes, in which bacterial replication is limited and the bacteria eventually undergo degradation (Rikihisa 1984). Although a number of controversial reports exist, some intracellular pathogens, such as *Legionella pneumophila*, *Coxiella burnetti*, and *Porphyromonas gingivalis*, are enclosed by vacuoles that they then modify to resist fusion with lysosomes, allowing the pathogens to survive and multiply unless autophagy is stimulated (Kirkegaard et al. 2004; Amer and Swanson 2005; Gutierrez et al 2005; Bélanger et al. 2006).

Listeria monocytogenes and *Shigella* are able to multiply and move within the host cell cytoplasm via the activities of their respective surface-expressed proteins ActA and VirG/IcsA; this process is called actin-based bacterial motility. They are also capable of escaping autophagic recognition though distinct means. In the case of *Shigella*, IcsB, one of the effectors secreted via the TTSS by intracellular *Shigella*, plays a pivotal role in disguising the bacterium against autophagic recognition (Ogawa et al. 2005; Ogawa and Sasakawa, 2005). An *icsB* mutant is still capable of invading epithelial cells, but it cannot, multiply within the host cells; although the mutant multiplies and moves normally for the first 3 h after the infection of BHK cells, intracellular multiplication eventually plateaus at 4 h after infection. At this stage, approximately 40% of the intracellular *icsB* mutant (and approximately 8% of the intracellular wild-type) are colocalized with acidic lysosomes (Lysotracker) and autophagosomes (monodancyl-cadaverin and LC3). When MDCK cells are infected with the *icsB* mutant (or wild-type *Shigella*) under an amino acid-starved condition (which stimulates autophagy), the number of LC3-positive bacteria significantly increases in the MDCK cells in response to amino acid deprivation. Conversely, when MDCK cells are treated with a known autophagy inhibitor, such as 3-methyladenine, the LC3-positive *icsB* population is greatly reduced. Electron microscopic examination reveals that 3–4 h after infection, the *icsB* mutant is frequently enclosed by lamellar membranous structures, though the wild-type *Shigella* have long actin tails; these observations suggest that, unless *Shigella* produce IcsB, they readily succumb to autophagy. Intriguingly, the VirG protein required for bacterial intracellular motility is targeted for autophagic recognition by binding to Atg5. In *in vitro* binding assays, both IcsB and Atg5 exhibited some ability to interact with VirG, and IcsB and Atg5 share the same interacting region on VirG. Importantly, the affinity of IcsB for VirG is relatively strong, compared with that of Atg5, suggesting that IcsB plays a pivotal role as an anti-Atg5 binding protein, thereby camouflaging the target VirG protein from autophagic recognition (Ogawa et al. 2005).

6.2 Control of Replicative Foothold

The intestinal epithelium self-renews every several days, and the exfoliation of infected cells from the basal membrane provides an important intrinsic defense system that limits bacterial colonization (Stevens and Leblond 1953; Sansonetti 2004; Oswald et al. 2005). The rapid turnover of intestinal epithelial cells forms a crucial physical as well as a functional barrier, and renewal is sustained by the vigorous proliferation of epithelial progenitors that migrate upwards from the bottom of the intestinal crypts. Nevertheless, many pathogenic bacteria, including *Shigella*, are capable of colonizing the intestinal epithelium. Recent studies have indicated that a growing family of bacterial toxins, effectors, and small compounds known as "cyclomodulins" are capable of modulating the host cell cycle (Nougayrède et al. 2005). For example, Cif is secreted via the TTSS of EPEC and inhibits host cell mitosis. Cells transformed by Cif accumulate 4n DNA and re-initiate DNA synthesis without dividing, resulting in cells that contain 8-16n DNA (Marchès et al. 2003; Taieb et al. 2006; Samba-Louaka et al. 2008). CDTs are cytolethal distending toxins produced by *Shigella dysenteriae*, *Campylobacter jejuni*, *E. coli* and *Salmonella typhi*, and one of the CDTs produced by *C. jejuni* possesses a deoxyribonuclease I-like activity that causes limited DNA damage when delivered into a host cell nucleus, leading to the activation of ATM (PI3 kinase protein) and eventually resulting in cell-cycle arrest (Lara-Tejero and Galán 2000; Nougayrède et al. 2005; Ge et al. 2008). Although the biological significance of each cyclomodulin and its target host cells in bacterial infection remain to be elucidated, some of the cyclomodulins are assumed to prolong the pathogen's presence by interfering with the rapid turnover of epithelial cells. A recent study indicated that IpaB secreted from intracellular *Shigella* via the TTSS into the epithelial progenitor cytoplasm causes cell cycle arrest by targeting Mad2L2, an anaphase-promoting complex/cyclosome (APC) inhibitor (Iwai et al. 2007). Indeed, a rabbit ileal loop infection model showed that, during an intermediate stage of *Shigella* infection, the bacteria can directly access the intestinal crypts and infect the cryptic epithelial progenitor cells. At this stage, while few progenitor cells are detected in the crypts after wild-type *Shigella* infection, abundant progenitor cells are detected after infection with the *ipaB* mutant. Cell cycle progression is stringently controlled by cell cycle-specific proteolysis; this process involves the ubiquitination of target proteins by two major types of E3 ligase complexes, namely, the APC complex and the Skp1-culin-F-box protein (SCF) complex. The APC complex is a multi-subunit complex that targets substrates for degradation only during mitosis and the G1 phase; it also targets mitotic Cyclin A and Cyclin B1, allowing mitotic progression. Cyclin B1 ubiquitination assays have shown that APC undergoes unscheduled activation in response to IpaB interaction with Mad2L2. Synchronized HeLa cells infected with *Shigella* fail to accumulate APC substrates, such as Cyclin B1, Cdc20, and Plk1, causing cell cycle arrest at the G2/M phase in an IpaB/Mad2L2-dependent manner. IpaB/Mad2L2-dependent cell cycle arrest by *Shigella* infection can be visualized in the intestinal crypt

progenitors of rabbit ileal loops, and the IpaB-mediated arrest contributes to the efficient colonization of the host cells. Thus, bacterial activity resulting in the retardation of intestinal epithelial renewal is assumed to be pivotal for prolonging the infectious foothold and promoting the bacterial colonization of the intestinal epithelium (Iwai et al. 2007).

The rapid exfoliation of infected intestinal epithelial cells and the rapid sealing of neighboring cells are important for maintaining the epithelial integrity, which also serves as an innate defense system against bacterial colonization. Nevertheless, many pathogenic bacteria, including *Shigella*, are capable of efficiently colonizing the epithelium. Recent studies have shown that OspE, delivered into epithelial cells via the TTSS of *Shigella*, accumulates at the focal adhesion (FA), reinforcing the host cell's adherence to the basement membrane by interacting with integrin-linked kinase (ILK) (Miura et al. 2006; Kim et al., 2009). Interestingly, OspE is highly conserved among enteropathogenic *Escherichia coli*, enterohemorrhagic *E. coli*, *Citrobacter rodentium* and *Salmonella* strains (Tobe et al. 2006). The formation of focal adhesion (FA) and the level of cell surface β1-integrin are augmented by OspE-ILK interaction-dependent manner. The interaction between OspE and ILK suppresses the phosphorylation of FAK and paxillin, which are required for the rapid turnover of FA in cell motility, then OspE promotes targeting of the membrane-associated ILK. Thus, the disassembly of FA from the matrix such as fibronectin during *Shigella* infection can be repressed through the interaction of OspE with ILK (Kim et al. 2009). The infection of polarized epithelial cell monolayers by an *ospE* mutant caused more rapid cell exfoliation than infection by wild-type *Shigella*, indicating the importance of the OspE-mediated maintenance of *Shigella*-infected cell architectures. Infection of guinea pig colon with wild-type *Shigella* corroborates the pivotal role of the interaction of OspE with ILK in repressing epithelial detachment, resulting in increased bacterial cell-to-cell spreading and the promotion of bacterial colonization (Kim et al. 2009).

7 Conclusion

Shigella possess highly evolved invasive as well as intracellular survival systems. Furthermore, they are equipped with various offensive systems against the host innate defense and immune systems. These bacterial infectious activities are mostly exerted through effectors, of which more than 50 may exist, delivered via the TTSS into host cells. These effectors play pivotal roles from the onset of bacterial infection through to the establishment of the colonization of the intestinal epithelium. According to characterization studies of these effectors, their roles in infection can be categorized into at least four classes: (1) bacterial entry into host cells, such as macrophages and epithelial cells; (2) intracellular survive and multiplication; (3) modulation of the inflammatory response; and (4) maintenance of the infectious foothold. Since many effectors have yet to be characterized, some of these uncharacterized effectors may play roles in other infectious aspects that are not yet known. Nevertheless, the biological roles of individual effectors are exerted

in at least two ways: some possess distinctive enzymatic activities that are capable of directly modulating cellular function in the host, while others exert their activity via targeting (binding to) host proteins. Some effectors utilize both of these (Galán and Wolf-Watz 2006; Galán 2007). More complicatedly, some effectors possess two or three intracellular activities. For example, IpaB secreted at the tip of a TTSS needle not only acts as the translocater required for the delivery of other effectors (Blocker et al. 1999), but also acts as a *Shigella* invasive protein for promoting the bacterial invasion of epithelial cells by interacting with CD44 (Skoudy et al. 2000; Lafont et al. 2002). Furthermore, IpaB is secreted from intracellular *Shigella* after the pathogen has invaded intestinal epithelial progenitors, where it then interacts with Mad2L2 and downregulates cell cycle progression (Iwai et al. 2007). IpaH9.8, which is secreted from intracellular *Shigella*, interacts with $U2AF^{35}$ and dampens the expression of numerous genes, including genes that encode inflammatory chemokines and cytokines, while also downregulating NF-κB activity in a $U2AF^{35}$-independent pathway (Haraga and Miller 2003; Okuda et al. 2005). Thus, these examples suggest that bacterial effectors are highly adaptive proteins capable of interacting with the host cellular system to promote bacterial infection and modulate the host immune response in a manner that benefits the pathogen.

Clearly, further studies of the biological activities of bacterial effectors will provide us with not only the opportunity to uncover novel bacterial infectious strategies, but also the tools needed to further understand host cellular and immune systems. Such knowledge would serve as a strong basis for the development of an attenuated *Shigella* vaccine, drugs to control infection, and new animal models for studying shigellosis.

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