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Review

The interaction between *Listeria monocytogenes* and the host gastrointestinal tract

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Listeria monocytogenes is a ubiquitous bacterium that causes significant foodborne disease with high mortality rates in immunocompromised adults. In pregnant women foodborne infection can give rise to infection of the fetus resulting in miscarriage. In addition, the bacterium has recently been demonstrated to cause localized gastrointestinal symptoms, predominantly in immunocompetent individuals. The murine model of systemic *L. monocytogenes* infection has provided numerous insights into the mechanisms of pathogenesis of this organism. However, recent application of transcriptomic and proteomic approaches as well as the development of new model systems has allowed a focus upon factors that influence adaptation to gastrointestinal environments and adhesion to and invasion of the gastrointestinal mucosa. In addition, the availability of a large number of complete *L. monocytogenes* genome sequences has permitted inter-strain comparisons and the identification of factors that may influence the emergence of 'epidemic' phenotypes. Here we review some of the exciting recent developments in the analysis of the interaction between *L. monocytogenes* and the host gastrointestinal tract.

Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen that is the causative agent of listeriosis, a disease which manifests primarily as meningitis or meningoencephalitis in non-pregnant individuals, or as infection of the fetus in pregnant women. *L. monocytogenes* normally resides in decaying plant matter in the soil but may be transiently associated with the gastrointestinal (GI) tract in a number of animal species. The pathogen may also cause systemic disease in domestic animals, including sheep and cattle, leading to circling disease in adult animals or spontaneous abortion in pregnant animals (Czuprynski, 2005). Infection by, or indeed carriage of the pathogen by domestic animals poses a risk of zoonotic transmission through contaminated milk or meat. Contaminated raw foodstuffs that are subjected to minimal further processing are likely to pose the greatest risk to human health; such foodstuffs include soft cheeses, pâtés, frankfurters and post-process contaminated milk (Swaminathan & Gerner-Smidt, 2007).

Clearly *L. monocytogenes* encounters the mammalian GI tract at a number of stages during the infectious cycle: potentially during asymptomatic intestinal carriage or prior to causation of animal disease and then during infection of the human host before invasion of the GI epithelium. The genome of the bacterium reflects this ability to adapt to a variety of environments, containing a complex arsenal of genes encoding regulatory proteins as well as genes encoding proteins linked to survival within the GI tract, including bile salt hydrolase (*bsh*) and bile exclusion protein (*bilE*) (Begley *et al.*, 2005; Dussurget *et al.*, 2002; Glaser *et al.*, 2001; Sleator *et al.*, 2005).

We and others have previously described the physiological adaptation of *L. monocytogenes* during passage from external environments to the host GI tract (Gahan & Hill, 2005; Gray *et al.*, 2006). Recently a diverse array of work has either directly or indirectly focused upon the GI phase of *L. monocytogenes* pathogenesis. This includes transcriptomic analysis of the sigma B (σ^B) and PrfA regulons involved in bacterial adaptation and pathogenesis, analysis of the genome sequences of a number of different *L. monocytogenes* isolates and research investigating interactions between the pathogen and commensals in the GI tract. This review analyses recent contributions to our understanding of this most important phase of the infectious process.

Strain variation and the emergence of epidemic strains

The genus *Listeria* includes two pathogenic species: the important human pathogen *L. monocytogenes* and *L. ivanovii,* a species which predominantly infects ruminants.

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The remaining non-pathogenic members of the genus are *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. grayi*. Complete genome sequences are available for a number of strains of *L. monocytogenes*, *L. innocua* and *L. welshimeri* (Glaser *et al.*, 2001; Hain *et al.*, 2006) and are accessible at the TIGR (http://www.tigr.org/tdb/listeria/) and ListiList (http://genolist.pasteur.fr/ListiList/index.html) websites; sequencing of the remaining species as well as a number of further *L. monocytogenes* isolates is nearing completion (Nelson *et al.*, 2004). To date, genome comparisons have shown a very strong conservation of gene organization between members of the genus, which is evidence of a close phylogenetic relationship (Buchrieser, 2007). However, the non-pathogenic species *L. innocua* has clearly lost specific genes associated with infection of the host (Buchrieser, 2007). Such genes include the main virulence gene cluster, the genes encoding InlA and InlB and the bile salt hydrolase (*bsh*) gene (Buchrieser, 2007; Glaser *et al.*, 2001). We have also recently shown that *L. innocua* has lost genes associated with the MEP pathway for isoprenoid biosynthesis, a pathway that is associated with growth in the mammalian host (Begley *et al.*, 2008). The data strongly suggest that *Listeria* species have evolved from a common pathogenic ancestor and that some members of the genus have lost genes associated with pathogenesis as they adapted to non-pathogenic environments (Buchrieser, 2007).

Furthermore there are clear differences between individual *L. monocytogenes* isolates with respect to gene content and ability to cause disease in humans. The species comprises 13 serovars, of which serovars 1/2a, 1/2b, 1/2c and 4b account for the vast majority of cases of human disease (Wiedmann, 2002), with the majority of common-source epidemics caused by serovar 4b strains (Gahan & Hill, 2005). Other studies have defined three distinct phylogenetic lineages of *L. monocytogenes,* with lineage I strains containing isolates that have caused epidemics in humans (including serovars 1/2b and 4b), lineage II strains containing isolates responsible for sporadic human disease (including serovars 1/2a and 1/2c) and lineage III strains comprising mostly animal pathogens (Jeffers *et al.*, 2001; Nightingale *et al.*, 2005). Recent phylogenetic analysis has revealed that within lineage I, serovar 4b evolved from serovar 1/2b (Ragon *et al.*, 2008).

Experiments investigating oral infection of mice with *L. monocytogenes* strains have provided some limited support for a link between serovar and foodborne pathogenesis. Two serovar 4b strains were noted to reach higher levels in internal mouse organs and to cause more severe histopathological damage to organs than did serovar 1/2a and 1/2b strains (Czuprynski *et al.*, 2002). Another study demonstrated that epidemic strains are more invasive than non-epidemic strains following intragastric infection in mice (Kim *et al.*, 2004). Whilst mice are far from an ideal model of foodborne human listeriosis (owing to a single amino acid difference between the murine E-cadherin receptor and that of humans; see below) these studies, combined with evidence from epidemiological analysis of human listeriosis, indicate that genotypic and phenotypic variations between strains can significantly affect virulence potential. In support of this Jacquet *et al.* (2004) reported that clinical strains expressed full-length internalin (InlA) far more frequently than did strains recovered from food products. Furthermore, all strains belonging to serovar 4b that were tested in this study expressed the full-length internalin.

More recent *in silico* analysis of draft and completed sequences from *Listeria* species and various typed strains of *L. monocytogenes* has revealed a number of regions of difference (RDs) (regions greater than 4 kb) between *Listeria* species and strains (Milillo *et al.*, 2009). The study identified 15 RDs present in *L. monocytogenes* but absent from other *Listeria* species, three RDs that are present in lineage I strains and absent from lineage II strains of *L. monocytogenes* and four RDs present in lineage II strains and absent from lineage I strains. The authors deleted three RDs of interest but did not find any differences between mutants and parents using cell-culture-based virulence assays. However, one *L. monocytogenes*-specific RD encoding the arginine deiminase system was demonstrated to play a role in low-pH survival in minimal media, a finding that supports our own recent work on this system (Milillo *et al.*, 2009; Ryan *et al.*, 2009).

Recently Cotter *et al.* (2008) have identified a novel haemolysin, designated listeriolysin S, that is present in approximately half of the lineage I strains tested (including many epidemic isolates), and is consistently absent from lineage II and III strains. This haemolysin is induced by oxidative stress, is essential for full virulence in these strains and is encoded in a gene cluster now designated *Listeria* pathogenicity island 3 (LIPI-3). The work represents the first identification of a virulence factor that is unique to certain epidemic strains of *L. monocytogenes*. In addition to listeriolysin S, subtle differences in gene regulation (including regulation of classical virulence factors) are also likely to influence the capacity of strains to cause disease in humans (Cotter *et al.*, 2008). Overall further *in silico* analysis and functional genetics approaches will undoubtedly uncover the molecular factors affecting strain differences in *L. monocytogenes*.

Transcriptomic analyses and insights into adaptation to the GI tract

The listerial *sigB* gene was independently isolated by Wiedmann *et al.* (1998) and Becker *et al.* (1998) on the basis of sequence homology with the *B. subtilis sigB* gene. While the former study identified a role for the encoded σ^B protein in acid tolerance of *L. monocytogenes* 689426 (Wiedmann *et al.*, 1998), the latter demonstrated that the activity of σ^B in *L. monocytogenes* Scott A is responsive to osmotic up-shift, temperature down-shift, and the presence of EDTA in the growth medium (Becker *et al.*, 1998). The magnitude of the response was greatest after an

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osmotic up-shift, suggesting a role for σ^B in coordinating osmotic responses in *L. monocytogenes*. This observation was later confirmed by a partial gene array study comprising 208 genes, consisting of 166 genes downstream of hidden Markov model (HMM)-predicted σ^B-dependent promoters as well as selected virulence and stress-response genes from *L. monocytogenes* 10403S (Kazmierczak *et al.*, 2003). Fifty-five genes with statistically significant σ^B-dependent expression were identified in the stationary phase or under conditions of osmotic stress, with at least 1.5-fold-higher expression in the wild-type over the *sigB* mutant under either stress condition (Kazmierczak *et al.*, 2003). A more recent whole-genome array analysis of the σ^B regulon in *L. monocytogenes* EGD-e revealed 105 σ^B-positively regulated genes and 111 genes which appeared to be under negative control of σ^B at various stages of the growth cycle (Hain *et al.*, 2008). The σ^B regulon therefore includes 7.6 % of genes in the *L. monocytogenes* genome. Of the genes positively regulated by σ^B, 75 have homologues in *B. subtilis*, but only 33 had been previously described as σ^B-regulated in *B. subtilis*. The data indicate a divergence of the σ^B regulons between these two bacterial genera, possibly reflecting adaptation to differing niches (Hain *et al.*, 2008).

In *Listeria*, the function of σ^B appears to be both species and strain dependent. While σ^B contributes to both stationary- and exponential-phase acid resistance in *L. monocytogenes*, only exponential-phase acid resistance is σ^B-dependent in *L. innocua* (Raengpradub *et al.*, 2008). Furthermore, there is compelling evidence that the *sigB* gene plays a variable role in stress response in the different genetic lineages of *L. monocytogenes* (Moorhead & Dykes, 2003). It was shown that a serotype 1/2a strain is more reliant upon an intact σ^B regulon than a serotype 4c strain across a range of environmental stresses (Moorhead & Dykes, 2003). Such variations in environmental stress resistance among different strains may contribute to disparate survival capabilities in foods and during infection and therefore indirectly to differences in virulence potential (Wiedmann *et al.*, 1998).

It has been shown that the σ^B regulon includes genes encoding the classical virulence factors InlA and InlB (Hain *et al.*, 2008; Kazmierczak *et al.*, 2003), and σ^B is required for rapid induction of expression of *L. monocytogenes* genes most likely to be important for survival of GI stresses, including reduced pH, elevated osmolarity and bile salts. Indeed, loss of σ^B has been found to result in decreased virulence of *L. monocytogenes* following oral infection in the murine and guinea pig models but not during systemic infection (Garner *et al.*, 2006; Nadon *et al.*, 2002). We have shown that the gene encoding σ^B is transcriptionally upregulated during transit through the mouse GI tract (Begley *et al.*, 2005). Furthermore, the σ^B regulon is induced by many of the stresses encountered during GI passage (Sue *et al.*, 2004) and activates a number of systems that are important for bacterial homeostasis in the gut (Hain *et al.*, 2008). σ^B is therefore critical for optimal pathogenesis of *Listeria* during GI infection. Furthermore, this alternative sigma factor has the potential to promote bacterial survival both outside and inside a host, thus contributing to survival at all stages of the infectious cycle (Gahan & Hill, 2005; Sleator *et al.*, 2003a).

Acid tolerance

It appears that σ^B contributes to acid resistance through at least two mechanisms: a general acid tolerance to which σ^B-regulated systems contribute throughout all growth phases, and a pH-inducible acid-tolerance response mechanism that is at least partially σ^B-dependent in exponential-phase cells (Ferreira *et al.*, 2001; Volker *et al.*, 1999). Becker *et al.* (1998) discerned from primer extension analyses that *sigB* expression in exponential-phase *L. monocytogenes* can be induced from undetectable levels to a level similar to that observed for stationary-phase cells following exposure of cells to mildly acidified media (pH 5.3). The glutamate decarboxylase (GAD) system plays an essential role in pH homeostasis in *L. monocytogenes*, mediated primarily through a σ^B-regulated component GadD2 (formerly GadB) (Cotter *et al.*, 2001; Kazmierczak *et al.*, 2003; Wemekamp-Kamphuis *et al.*, 2004). In addition we have recently shown that the arginine deiminase (ADI) acid tolerance system in *L. monocytogenes* is functional and is regulated by both σ^B and a dedicated transcriptional regulator, ArgR (Ryan *et al.*, 2008, 2009). The influence of these acid-tolerance mechanisms upon *in vivo* gastric transit remains to be established; however, it is likely that the GAD and ADI systems play a significant role in this process.

Bile tolerance

One litre of bile is produced in the liver, stored interdigestively in the gall bladder and secreted into the duodenum each day. The genes encoding the principal listerial bile-resistance mechanisms, BSH and BilE, are preceded by σ^B-dependent promoter sites and are transcriptionally downregulated in a *sigB* mutant (Begley *et al.*, 2005; Sleator *et al.*, 2005). Furthermore, BSH activity is completely abolished in a *L. monocytogenes* Δ*sigB* strain (Begley *et al.*, 2005), thus suggesting a central role for σ^B in coordinating the listerial bile stress response that is necessary for survival of the pathogen in the small intestine (Begley *et al.*, 2005; Dussurget *et al.*, 2002; Sleator *et al.*, 2005).

Osmotolerance

The existence of putative σ^B-dependent promoter sites upstream of the osmolyte transporter genes *betL*, *gbu* and *opuC* suggests that at least a component of osmolyte uptake in *L. monocytogenes* forms part of the σ^B regulon (Sleator *et al.*, 2003b). Indeed, kinetic analysis of transcript accumulation after osmotic up-shift demonstrated that σ^B-dependent transcripts from *gbuA*P2 accumulate for an

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extended period after up-shift, suggesting that σ^B activity may provide a mechanism for sustained high-level expression during osmotic challenge (Cetin *et al.*, 2004; Fraser *et al.*, 2003). Furthermore, Becker *et al.* (1998, 2000) demonstrated that a σ^B mutant of *L. monocytogenes* is significantly impaired in its ability to use betaine and carnitine as osmoprotectants. We have previously shown that the principal carnitine transporter in *L. monocytogenes*, OpuC, is required for full virulence by the oral route (Sleator *et al.*, 2001; Wemekamp-Kamphuis *et al.*, 2002). Recent work has demonstrated that the *in vivo* requirement for this osmolyte uptake system is mediated through a role for OpuC in bile tolerance in the small intestine during oral infection in mice (D. Watson and others, unpublished).

Cross-adaptation during GI transit

Following ingestion, the first physical stress encountered by the bacterium is the low pH of the stomach (~pH 2), followed by elevated osmolarity (equivalent to 0.3 M NaCl) and activity of the biological detergent bile in the upper small intestine. Significantly, pre-exposure to elevated osmolarity (0.3 M NaCl for 1 h) resulted in a dramatic increase in the ability of *L. monocytogenes* to deal with lethal concentrations of bile (adapted cells surviving 1000 times better than naïve cells) (Begley *et al.*, 2002; Sleator *et al.*, 2007). However, a low-pH challenge (as experienced during gastric transit) fails to similarly protect against subsequent osmotic or bile stress. Similarly, pre-exposure to bile fails to protect against acid or salt, but does protect against rechallenge with higher levels of bile salts (Begley *et al.*, 2002). Thus, osmotic stress appears to be at the top of the hierarchy of stress responses during GI transit. Given that *sigB* is transcriptionally upregulated at elevated osmolarity (Becker *et al.*, 1998), it is likely that the increased osmolarity of the GI lumen may be interpreted by *L. monocytogenes* as an environmental cue, signalling gut entry (as is the case for *Salmonella*) (Sleator & Hill, 2002). Furthermore, given that σ^B has recently been shown to modulate expression of PrfA [positive regulatory factor A; the master regulator of the virulence gene cluster which coordinates the intracellular phase of *L. monocytogenes* infection (Vazquez-Boland *et al.*, 2001)] it is possible that osmotically induced stimulation of the σ^B regulon in the upper small intestine may not only facilitate successful GI transit, but also prime the pathogen for the next phase of infection, which, in susceptible individuals, is the systemic invasive disease listeriosis (Ollinger *et al.*, 2009).

σ^B interplay with PrfA and other regulators

L. monocytogenes has a profound ability to adapt to a variety of environments, switching from a saprophyte in soil and decaying vegetation, to an intracellular pathogen capable of causing serious infection in humans and in many animal species. This transformation is mediated by a complex interplay between the regulatory networks which modulate the expression of stress- and virulence-associated factors in response to specific environmental cues (Gahan & Hill, 2005; Gray *et al.*, 2006).

In this model σ^B dominates both in the external environment and during GI transit, while it is PrfA which plays the central role during the next phase: intracellular infection. In addition to regulating the expression of stress-response genes, σ^B (in concert with PrfA) also activates the transcription of virulence genes, such as *inlAB*, *bsh* and *bilE* (Begley *et al.*, 2005; Kazmierczak *et al.*, 2003; Sleator *et al.*, 2005). Furthermore, σ^B directly regulates the transcription of *prfA* through a specific promoter, $P2_{prfA}$ (Nadon *et al.*, 2002; Rauch *et al.*, 2005; Schwab *et al.*, 2005). Very recent work reveals a dual role for σ^B as both an initial activator of *prfA* transcription (most likely during the GI phase of infection) and a repressor of the PrfA regulon during intracellular infection. σ^B is proposed as a means of fine-tuning expression of PrfA-regulated genes during cellular infection in order to reduce cytotoxicity in *Listeria*-infected cells (Ollinger *et al.*, 2009).

In addition to modulating the activity of PrfA, σ^B has also been shown to form a regulatory network with the negative regulators HrcA and CtsR (class three stress gene repressor), involved in the regulation of heat-shock genes. Microarray transcriptomic analyses and promoter searches identified at least 40 genes co-regulated by both CtsR and σ^B, including genes encoding proteins with confirmed or likely roles in virulence and stress response. These data demonstrate that interactions between CtsR and σ^B play an important role in *L. monocytogenes* stress resistance and virulence (Hu *et al.*, 2007b). A similar approach revealed 31 genes co-regulated by HrcA and σ^B (Hu *et al.*, 2007a). Thus, σ^B, CtsR and HrcA appear to form a regulatory network that contributes to the transcription of a number of *L. monocytogenes* genes.

The question of motility

Flagellar motility is advantageous to many foodborne bacterial pathogens as it facilitates localization and potentially cellular adhesion, which may be vital for colonization during the infectious cycle (Akerley *et al.*, 1995; Wolfgang *et al.*, 2004). *L. monocytogenes* is capable of motility and produces between four and six peritrichous flagella (Fuhs & Seeliger, 1961). Somewhat unusually for a human pathogen, *L. monocytogenes* is flagellated and motile at temperatures of 30 °C and below but is non-motile at human body temperature (37 °C) and above (Peel *et al.*, 1988). At these physiological temperatures the transcriptional repressor MogR (motility gene repressor) has been identified to inhibit flagellar gene transcription by directly binding to the *flaA* promoter region. At 30 °C and below MogR is inhibited by the antirepressor GmaR, permitting *flaA* transcription and hence motility (Shen & Higgins, 2006). This transcriptional repression is required for virulence of *L. monocytogenes* when the bacterium is administered by the intravenous route of infection, as constitutive expression of *flaA* in ΔMogR strains resulted

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in a 250-fold increase in LD_{50} (Grundling *et al.*, 2004; Shen & Higgins, 2006).

It is unclear whether *L. monocytogenes* expresses flagella and is motile within the GI tract or whether temperature-mediated downregulation of motility is active in this environment. O'Neil & Marquis (2006) showed that listerial flagella do not function as adhesions for invasion of cultured epithelial cells but that listerial motility is important in this context. This is in contrast to other bacteria, such as *Escherichia coli*, for which flagella function as cell-surface adhesions in the absence of motility (Giron *et al.*, 2002). O'Neil & Marquis (2006) also showed that motility plays a major role in the initial colonization of the GI tract by *Listeria* in orally infected mice. They hypothesized that flagella-mediated motility was necessary to maintain contact with host cells until the establishment of high-affinity ligand-receptor binding (such as the interaction between InlA and E-cadherin). As internalin A accumulates at the bacterial poles it may be advantageous for *L. monocytogenes* to interact head on with host cell membranes; hence motility may be important for orientation of bacteria prior to internalization (O'Neil & Marquis, 2006; Rafelski & Theriot, 2006). The apparent requirement for motility for full virulence of *L. monocytogenes* may suggest the existence of a molecular sensing mechanism which de-represses MogR at 37 °C in this environment, allowing for expression of flagella in the GI tract.

Adherence to and invasion of the GI epithelium

L. monocytogenes has recently been demonstrated to bind to a specific human mucin, Muc2, but not to Muc1 (Linden *et al.*, 2008). The pathogen interacts with Muc2 through internalin proteins, with a requirement for these proteins in the order InlB>InlC>InlJ (Linden *et al.*, 2008). This initial interaction may be important for localizing the pathogen in the mucin layer prior to cellular infection. Significantly, InlJ also appears to act directly as a cell adhesin, and heterologous expression of this protein in *L. innocua* promotes significant adherence to HT-29 and JEG-3 cells *in vitro* (Sabet *et al.*, 2008). Expression of a 104 kDa protein called *Listeria* adhesion protein (LAP) is also important for adhesion of the pathogen to the host cell via interaction with host heat-shock protein 60 (Wampler *et al.*, 2004). Expression of LAP in *E. coli* conferred an enhanced ability to bind both Caco-2 cells and purified Hsp60 (Kim *et al.*, 2006). Furthermore the pathogen has the capacity to bind to cell-surface fibronectin through a specific fibronectin-binding protein (FbpA) which is important for murine infection via the oral route (Dramsi *et al.*, 2004).

Localization of surface-associated virulence factors in *L. monocytogenes* is likely to be influenced by Auto, a cell wall hydrolase that is important for remodelling the listerial cell wall and is essential for cell invasion (Bublitz *et al.*, 2009). *L. monocytogenes* expression of the sortase-anchored surface protein Vip is essential for infection of certain mammalian cell lines and for infection of mice by the oral route. The host receptor for Vip has been established to be the endoplasmic reticulum resident chaperone Gp96 (Cabanes *et al.*, 2005). It has also been well established that *L. monocytogenes* requires the surface protein internalin A in order to invade GI enterocytes. Internalin B does not appear to play a role in invasion of enterocytes but is required for invasion of hepatocytes and infection of the fetoplacental unit (Disson *et al.*, 2008; Khelef *et al.*, 2006). It has long been established that the host receptor for InlA is E-cadherin, a protein expressed towards the basolateral surface of polarized enterocytes (Mengaud *et al.*, 1996). Recent work has demonstrated that InlA has the opportunity to interact with E-cadherin during extrusion of apoptotic cells at the villous tips, a process which transiently makes E-cadherin available for binding by *L. monocytogenes* expressing InlA (Pentecost *et al.*, 2006). The molecular mechanisms underpinning InlA-mediated cell entry have been elucidated and extensively reviewed elsewhere (Bonazzi *et al.*, 2009; Hamon *et al.*, 2006; Ireton, 2007; Seveau *et al.*, 2007).

Local defences against *L. monocytogenes*

L. monocytogenes triggers an anti-bacterial immune response in the GI tract through interaction with the host cell cytoplasmic receptor NOD2 (Kobayashi *et al.*, 2005). Mice defective in NOD2 are exquisitely sensitive to intragastric infection by *L. monocytogenes* and fail to induce anti-microbial defensins (cryptins) against the pathogen (Kobayashi *et al.*, 2005). Indeed *L. monocytogenes* is clearly sensitive to the actions of defensins when assayed *in vitro* and it is likely that defensins play an important role in defence against the pathogen *in vivo* (Gottlieb *et al.*, 2008). Intestinal P glycoprotein is also important for host protection against *L. monocytogenes* GI infection, most likely by inhibiting absorption of the pathogen into enterocytes (Neudeck *et al.*, 2004).

Early infiltration of polymorphonuclear neutrophils is essential for limiting local replication of *Listeria* in the gut (Conlan, 1997). Similarly NKT cells (lymphocytes expressing both NK and T-cell markers) play an important role in controlling early enteric infection with *L. monocytogenes* (Ranson *et al.*, 2005). Processing and presentation of listerial antigens occurs via a distinct population of $CD70^+$ dendritic cells, and CD70-mediated co-stimulation is required for the development of a local antigen-specific T-cell response (Laouar *et al.*, 2005). This strong co-stimulation is postulated to be required to activate appropriate anti-listerial T-cells and to overcome tolerance within the generally immunosuppressive intestinal milieu (Laouar *et al.*, 2005). Oral infection with *L. monocytogenes* gives rise to significant local populations of *Listeria*-specific $CD4^+$ and $CD8^+$ T-cells which are likely to play a role in long-term immunity (Huleatt *et al.*, 2001; Kursar *et al.*, 2002). Further work is expected to uncover the intricacies of the enteric immune response to *L. monocytogenes*, and

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newly developed transgenic animal models of listeriosis may prove useful in this endeavour (see below).

Role of the microbiota in resistance to infection

The human GI tract contains a vast array of micro-organisms (500–1000 species) which reach highest levels in the colon (approx. 10^{11}–10^{12} micro-organisms per gram of contents). The small intestine (the site of listerial invasion) also contains a sizeable microbiota, with between 10^4 and 10^8 micro-organisms per gram (Rastall, 2004). The gut microbiota contributes to nutrient acquisition and development of the immune system, and plays a significant role in resistance to foodborne pathogens (Xu & Gordon, 2003). Certainly the presence of a gut microbiota contributes to protection against orally administered *L. monocytogenes* in rodent models of disease when compared to gnotobiotic animals (Czuprynski & Balish, 1981; Vieira *et al.*, 2008). Furthermore, immune responses to *L. monocytogenes* are blunted in mice raised under gnotobiotic conditions, and these mice demonstrate elevated susceptibility to systemic (intraperitoneal) infection (Inagaki *et al.*, 1996).

The concept of using probiotic commensals to protect against oral infection with *L. monocytogenes* has been investigated using a number of model systems. A variety of potentially probiotic strains including *E. coli* Nissle 1917 (Altenhoefer *et al.*, 2004), *Lactobacillus salivarius* UCC118 and *Bifidobacterium breve* UCC2003 (Corr *et al.*, 2007a) have been shown to inhibit *L. monocytogenes* invasion of GI cell culture lines. Oral administration of live *Lactobacillus delbrueckii* UFV-H2b20 has been shown to significantly protect against *L. monocytogenes* infection in a mouse model (Vieira *et al.*, 2008). Similarly, *Lactobacillus sakei* 2a protected gnotobiotic mice against subsequent oral infection with *L. monocytogenes* (Bambirra *et al.*, 2007).

The mechanisms by which potentially probiotic strains may impede colonization of pathogens have also been investigated. Competitive exclusion of pathogens by probiotic commensals has been suggested as a mechanism for displacing pathogens from the host cell surface (Collado *et al.*, 2005). In addition, *Lactobacillus*-mediated stimulation of mucin production (Muc3) by gut HT29 cells has been shown to protect against enteropathogenic *E. coli* (Mack *et al.*, 2003). This work is interesting in the light of recent studies demonstrating that *L. monocytogenes* has the potential to interact with human Muc2 (Linden *et al.*, 2008); however, the role of the microbiota in inducing *muc2* expression has not yet been established. We have recently demonstrated that *Lb. salivarius* UCC118 mediates protection against *L. monocytogenes* through bacteriocin production. Mice receiving orally administered wild-type UCC118 bacteria demonstrated significant protection against subsequent oral infection with *L. monocytogenes* and this protection was abolished in mice receiving a UCC118 mutant which lacks the ability to produce the bacteriocin. Furthermore, we created a bacteriocin-resistant variant of *L. monocytogenes* which was immune to protection by wild-type UCC118. This work demonstrates that bacteriocin production represents a mechanism for probiotic/commensal-mediated protection against *L. monocytogenes* infection *in situ* within the GI tract (Corr *et al.*, 2007b).

A recent study defined the intestinal host responses to *L. monocytogenes* infection in a gnotobiotic humanized (hEcad) mouse model (Lecuit *et al.*, 2007). Transcriptional responses in ileal cells were compared across mice monocolonized by *L. monocytogenes*, *L. innocua* or the commensal *Bacteroides thetaiotaomicron*. In this setting *L. monocytogenes* initiated a profound immunoinflammatory response that was not induced by either *L. innocua* or *Bact. thetaiotaomicron*. This response included the stimulation of chemokines (Cxcl9 and Cxcl10) necessary for recruitment of T lymphocytes and identified TNFα as a central regulator of the response. Furthermore, lack of host cell invasion (in Δ*inlA* and Δ*inlAB* mutants) or lack of cytoplasmic replication (in a Δ*hly* mutant) significantly reduced the ileal immunoinflammatory response even though luminal numbers of bacteria were comparable. This comprehensive study therefore revealed details of the cellular response evoked by *L. monocytogenes* infection of the small intestine and verified that the host response is specific to the pathogen and distinct from that induced by commensals or non-pathogens.

New research tools for the analysis of the GI phase of *L. monocytogenes* infection

It is well established that mice offer a poor model for the analysis of oral infection by *L. monocytogenes*. Commonly used inbred strains of mice (e.g. BALB/c or C57Bl/6) require administration of exceptionally high oral doses of the pathogen in order to achieve a significant invasive infection. It is clear that this initial resistance to infection is mediated by an amino acid difference between the murine E-cadherin protein and the listerial InlA invasion (Lecuit *et al.*, 2007). Murine E-cadherin has a glutamate at position 16 whereas humans and other permissive species have a proline at this position (Lecuit, 2007). This single amino acid change in E-cadherin has rendered mice and rats relatively resistant to oral infection and this resistance can be overcome through the generation of transgenic mice expressing 'humanized' (E16P) E-cadherin within enterocytes (*iFABP-human E cadherin* mice) (Lecuit *et al.*, 2001). This model (Fig. 1) was used to definitively demonstrate a role for InlA (but not InlB) in the translocation of *L. monocytogenes* across the GI barrier and has proved invaluable in the analysis of other factors (Vip, FbpA and InlJ) involved in GI translocation (Lecuit, 2007). However, in *iFABP-human E cadherin* mice expression of humanized E-cadherin is localized to the GI tract and this model therefore does not permit analysis of subsequent infectious processes involving E-cadherin–

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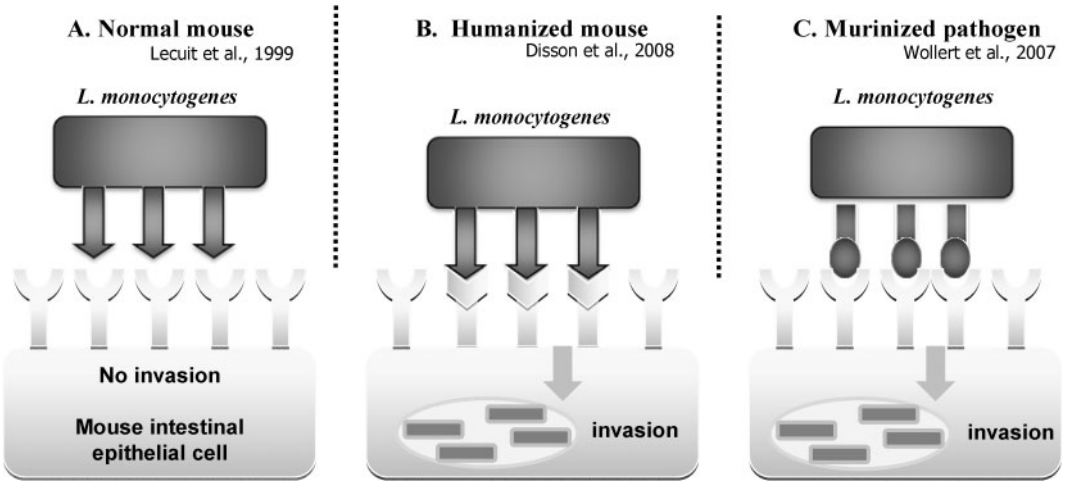

Fig. 1. Design of new model systems for analysis of *L. monocytogenes* infection in animal models (adapted from Bhaskaran & Stebbins, 2007). A. The nature of species specificity of *L. monocytogenes*. The internalin molecule InlA is unable to bind to the murine E-cadherin receptor. B. Human E-cadherin can be expressed ubiquitously (Disson *et al.*, 2008) or in specific tissues in transgenic mice (Lecuit *et al.*, 2001). C. InlA can be modified through rational protein engineering to bind murine E-cadherin.

InlA interactions. This limitation was recently overcome by Disson *et al.* (2008), who developed knock-in mice ubiquitously expressing humanized E-cadherin. This humanized mouse strain was used to demonstrate a role for both InlA and InlB in fetoplacental listeriosis. Furthermore their work demonstrated that gerbils, which are a naturally InlA and InlB permissive species, may also provide a useful model for analysis of *L. monocytogenes* infections (Disson *et al.*, 2008). Overall this work has led to the development of infectious models which more closely resemble *L. monocytogenes* infection in humans and which will be invaluable for the future analysis of the GI phase of infection (reviewed by Lecuit, 2007).

Another approach to the development of improved interactions between InlA and E-cadherin in the mouse is to 'murinize' the InlA molecule in *L. monocytogenes* to provide engineered strains of the pathogen that can be used in any existing mouse model. Wollert *et al.* (2007) recently analysed the crystal structure of the complex between InlA and E-cadherin and made rational amino acid modifications that improve the interactions between these proteins. This work has resulted in a murinized strain (Lmo-InlA^{m}) with significantly enhanced invasive potential in orally infected mice (C57Bl/6 J mice) (Wollert *et al.*, 2007) (Fig 1). We have recently used a random mutagenesis approach to enhance the interaction between InlA and murine E-cadheren. Whilst we isolated some interesting mutants with enhanced affinity for murine E-cadherin we did not improve upon the murinized strain created through the rational protein design approach of Wollert *et al.* (2007) (I. Monk, unpublished). As a control we recreated the Lmo-InlA^{m} strain described above and confirmed that this strain demonstrates significantly enhanced invasion following oral inoculation in BALB/c mice. Overall the murinization of *L. monocytogenes* is a significant step towards mimicking oral infection in humans and has the benefit that the strain can be easily constructed and used in existing mouse models (including existing knockout and transgenic mouse strains).

A suite of new molecular genetic research tools is now available that will undoubtedly enhance research into the GI phase of *L. monocytogenes* infection. Andersen *et al.* (2006) have developed a set of fluorescence plasmids for differentially tagging strains of *L. monocytogenes* prior to competitive index experiments in murine virulence studies (Andersen *et al.*, 2006). We have created a range of integrative vectors expressing IPTG-inducible antibiotic and phenotypic markers that allow comparison of virulence potential across four differentially labelled strains in a single mouse (Monk *et al.*, 2008a). We have also created a suite of new vectors which allow IPTG-dependent gene expression and significant gene overexpression of selected genes in *L. monocytogenes* (Monk *et al.*, 2008b).

lux gene expression coupled with bioluminescence imaging approaches have the potential to provide significant insights into *L. monocytogenes* infection via the GI tract. A variety of approaches have been used to create stable *L. monocytogenes* strains constitutively expressing bacterial *lux*, including random *lux*-transposon insertion (Hardy *et al.*, 2004) and expression of *lux* from a constitutive promoter on an integrative plasmid (Riedel *et al.*, 2007). These approaches have been used for *in vivo* localization of luminescent *L. monocytogenes* in complex environments including the gall bladder (Hardy *et al.*, 2004), murine tumours (Riedel *et al.*, 2007) and fetoplacental tissues (Disson *et al.*, 2008). Furthermore, *lux* expression from a specific cloned promoter may be used to measure

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expression levels of individual genes (promoters) in living animals. Indeed we have described an integrating plasmid system (pPL2*lux*) which facilitates cloning of individual gene promoters upstream of the *luxABCDE* operon (Bron *et al.*, 2006). We have recently used this system to measure gene expression levels for a number of genes expressed within the murine GI tract (see Fig. 2). Such bioluminescence imaging approaches therefore have the potential to determine both the location of the bacterium and levels of gene expression of infecting strains.

L. monocytogenes as a model for the patho-biotechnology concept

The patho-biotechnology concept describes the use of pathogenic stress survival and virulence factors in biotechnology, medicine and food (Sleator & Hill, 2006, 2007, 2008a, b). The availability of the complete genome sequence of *L. monocytogenes* and its genetic tractability, coupled with an ability to cope with stress, traverse the GI tract and induce a strong cellular immune response, makes *L. monocytogenes* an ideal model organism for demonstrating the patho-biotechnology concept.

In particular, *L. monocytogenes* is one of the most promising vaccine delivery platforms currently in development (Roland *et al.*, 2005). Brockstedt *et al.* (2004) showed that it is possible to segregate vaccine immunogenicity, due to uptake by antigen-presenting cells, from toxicity due to infection of non-phagocytic cells by deleting ActA and internalin B, thereby limiting the pathogen's tropism for non-professional phagocytes while abrogating its ability to undergo cell-to-cell spread (Brockstedt *et al.*, 2004). Attenuated vaccine strains of *L. monocytogenes* have been exploited as delivery vehicles for anti-cancer vaccines in human trials (Shahabi *et al.*, 2008; Wallecha *et al.*, 2009). The pathogen has also been used as the platform for the development of killed but metabolically active (KBMA) microbes: a new vaccine paradigm that exploits mutants in genes required for nucleotide excision repair (*uvrAB*) to maintain bacteria in a viable but nonreplicative state for eliciting effector T-cell responses and protective immunity (Brockstedt *et al.*, 2005).

An alternative to using attenuated *L. monocytogenes* for disease prevention or therapy involves equipping non-pathogenic bacteria with the genetic elements necessary to survive the many stresses encountered outside the host (Maa & Prestrelski, 2000; Shahidi & Han, 1993) as well as the myriad of antimicrobial hurdles faced during host transit and/or colonization. To illustrate the potential for exploiting pathogens such as *L. monocytogenes* to improve probiotics, we recently demonstrated that controlled heterologous expression of the listerial betaine uptake system, BetL, in *Lb. salivarius* UCC118 increases the resistance of the probiotic to several biotechnologically relevant stresses, including elevated osmo-, cryo-, baro- and chill-tolerance, as well as increasing its resistance to spray- and freeze-drying (Sheehan *et al.*, 2006). Significantly, heterologous expression of *betL* in *Bif. breve* also improved the gastric transit, GI persistence and therapeutic efficacy of the probiotic strain (Sheehan *et al.*,

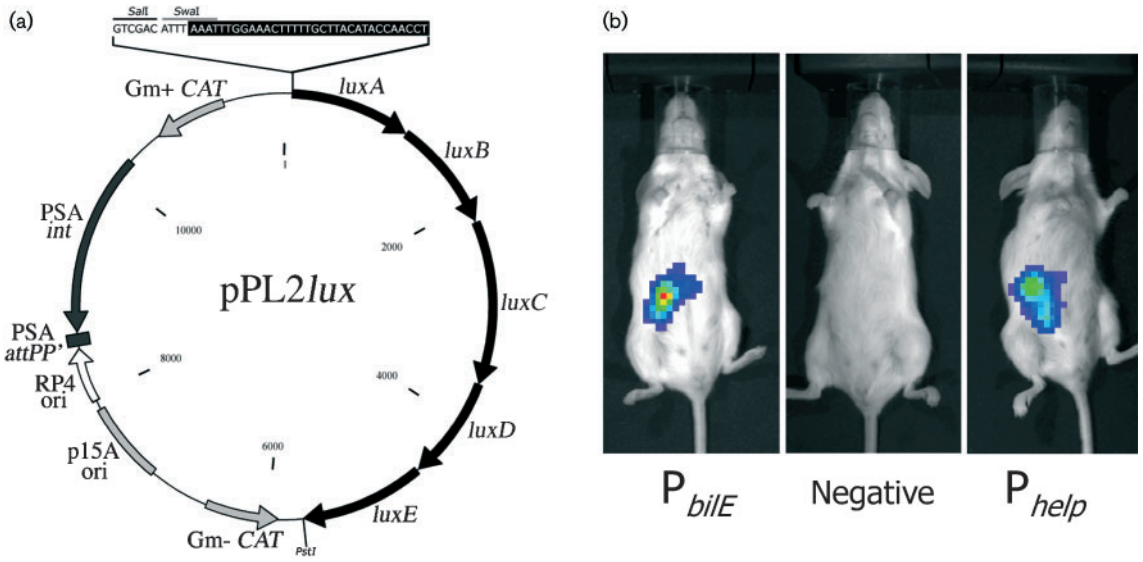

Fig. 2. Example of the use of bioluminescence imaging for monitoring bacterial gene expression during GI transit by *L. monocytogenes*. (a) The pPL2*lux* plasmid for creating translational promoter fusions to *luxABCDE* (Bron *et al.*, 2006). (b) Lux expression 30 min following oral inoculation of mice with pPL2*lux* fusion strains expressing *lux* from the *bilE* promoter, empty plasmid (no promoter) or the constitutive highly expressed listerial promoter (P_{help}) respectively.

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2007). We have also recently demonstrated that heterologous expression of the bile tolerance locus *bilE* in *Bif. breve* or *Lactococcus lactis* can significantly enhance bile tolerance and can increase survival of the engineered strains in the murine GI tract (Watson *et al.*, 2008).

In addition to improving cell viability, certain non-pathogenic cultures can be engineered to function as vaccine or drug delivery vehicles which, unlike attenuated pathogenic platforms, lack the possibility of reverting to a more virulent phenotype (Seegers, 2002). Cloning the listerial *inlA* gene (encoding internalin A) into the avirulent food-grade lactic acid bacterium *Lc. lactis* renders the otherwise non-invasive strain capable of entering intestinal cells and mediating gene delivery (of a GFP marker gene) (Guimaraes *et al.*, 2005). In addition, *Lc. lactis* strains expressing the *L. monocytogenes* LLO molecule are capable of entering the cytoplasmic class I MHC antigen processing pathway and driving a $CD8^+$ T-cell response (Bahey-El-Din *et al.*, 2008). Further exploitation of the patho-biotechnology concept (combined with improved knowledge of listerial GI survival mechanisms) is expected to deliver robust and safe vaccine and therapeutic delivery vehicles that can be administered via the oral route.

Conclusions

L. monocytogenes has long been utilized as a model intracellular pathogen with which to examine microbe–host interactions and the stimulation of specific anti-microbial immune responses (Cossart, 2007). However, recent work outlined in the current review demonstrates that the pathogen may also provide a tractable bacterial model with which to examine factors required for microbial survival in the GI environment. It is clear, for example, that bacterial factors such as BSH may be shared between pathogens (including *L. monocytogenes*) and the host microbiota (Jones *et al.*, 2008). In addition, bacterial systems involved in adaptation to local microenvironments in the gut have been shown to play a role in transient colonization by the pathogen (Sleator *et al.*, 2001, 2005) and many of these are regulated by the central regulator of stress adaptation, σ^B (Chaturongakul *et al.*, 2008; Hain *et al.*, 2008). Many of the systems involved in stress adaptation also contribute to survival in the external environment and in the food matrix and therefore contribute to the entire infectious cycle (Gahan & Hill, 2005). Indeed, there remains the intriguing possibility that foods with high levels of glutamate (the substrate of the GAD system) may enhance acid survival by *L. monocytogenes* in the stomach, or that foods containing high levels of carnitine (the target of the OpuC uptake system) may enhance survival of the pathogen in the intestine. The investigation of this phenomenon, the analysis of strain differences and the functional dissection of components of the σ^B and other regulons will undoubtedly be enhanced by the exploitation of new improved models and tools for the examination of the GI phase of *L. monocytogenes* infection.

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