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Mechanisms of *Salmonella* pathogenesis in animal models

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Abstract

Animal models play an important role in understanding the mechanisms of bacterial pathogenesis. Here we review recent studies of *Salmonella* infection in various animal models. Although mice are a classic animal model for *Salmonella*, mice do not normally get diarrhea, raising the question of how well the model represents normal human infection. However, pretreatment of mice with oral streptomycin, which apparently reduces the normal microbiota, leads to an inflammatory diarrheal response upon oral infection with *Salmonella*. This has led to a re-evaluation of the role of various *Salmonella* virulence factors in colonization of the intestine and induction of diarrhea. Indeed, it is now clear that *Salmonella* purposefully induces inflammation, which leads to the production of both carbon sources and terminal electron acceptors by the host that allow *Salmonella* to outgrow the normal intestinal microbiota. Overall use of this modified mouse model provides a more nuanced understanding of *Salmonella* intestinal infection in the context of the microbiota with implications for the ability to predict human risk.

Keywords

virulence; microbiota; antibiotic-induced dysbiosis; inflammation; Altered Schaedler Flora

INTRODUCTION

Our understanding of the molecular mechanisms of *Salmonella* infection and pathogenesis has been dependent on good animal models. Here we focus on the mechanisms of *Salmonella* infection gleaned from using the traditional mouse model versus the streptomycin-treated mouse model and other animal systems, and compare these findings with what is known about human infections.

Salmonellosis in humans.

Non-typhoidal *Salmonella* serovars are some of the most common bacterial pathogens in the world, estimated to cause over one million foodborne illnesses per year in the US (Scallan et al. 2011) and 94 million cases per year worldwide (Majowicz et al. 2010). Typically, *Salmonella* causes self-limiting gastroenteritis, but in the very young, elderly or otherwise immunocompromised patients can cause a disseminated systemic infection, septicemia, and death. Certain strains of *Salmonella*, so-called invasive non-typhoidal *Salmonella* (iNTS),

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are adept at causing systemic disease and are of particular concern in HIV-infected adults and in HIV- or Malaria-infected and malnourished children in sub-Saharan Africa (Feasey et al. 2012).

Most cases of salmonellosis are caused by consumption of contaminated food, including chicken, pork, or eggs, but infections are increasing occurring from contaminated fruits, nuts, or vegetables (Painter et al. 2013). Of the confirmed cases, only 6% are associated with outbreaks (CDC 2014). Dose response modeling of *Salmonella* outbreaks suggests an illness ID50 of 36 colony forming units (Blaser and Newman 1982, Boring et al. 1971, D'Aoust 1985, Hennessy et al. 1996, Teunis et al. 2010; Coleman et al., Marks and Coleman, companion papers in this collection). The dose-response relationship depends on multiple factors, including the mode of inoculation, the immune competency of the host, and, as is increasingly appreciated, the intestinal microbiota at the time of infection. Dietert has termed this human/microbiota consortium as the “superorganism” (Dietart, companion paper in this collection).

The first bottleneck that affects *Salmonella* infectivity is the stomach, which has digestive enzymes and a pH as low as 1.5 (Smith 2003). The stomach also contains high concentrations of weak acids which can act as uncouplers of proton motive force. Increasing the pH of the gastric juice using antacids or proton pump inhibitors increases the susceptibility to infection from a variety of oral pathogens, including *Salmonella* (Bavishi and Dupont 2011). Food also protects pathogens in complex ways as they pass through the stomach (Blaser and Newman 1982), either by directly blocking or shielding the organisms from acid, or perhaps by affecting virulence gene expression in the bacteria.

In otherwise healthy individuals, non-typhoid *Salmonella* strains cause what is termed self-limiting gastroenteritis with symptoms of watery diarrhea, abdominal pain, vomiting, nausea, and fever. The extent of diarrhea is variable; a limited number of patients will experience profuse “cholera-like” diarrhea, while in other cases the stools can be more dysentery-like with blood and mucus. Headache, myalgia and fever are also common. Although clearly invasive, the infection is usually limited to the intestine and underlying tissue. Indeed, most people with acute salmonellosis never go to the doctor and are not formally diagnosed or examined and data on the intestinal pathology of otherwise healthy individuals is extremely limited. Rather, pathological examinations have been primarily restricted to biopsies in patients with prolonged diarrhea or excised tissue taken on autopsy from patients who died from *Salmonella* infection (Boyd 1985, Coburn et al. 2007a, Lamps 2007, McGovern and Slavutin 1979). These data suggest that non-typhoid *Salmonella* infection induces primarily colitis, with neutrophil infiltration in the crypt epithelium and the lamina propriae. There is some involvement of the appendix, whereas the distal ileum seems to be involved in only some patients. Experimental infections in rhesus monkeys showed very similar histological appearance (Kent et al. 1966). Thus, acute self-limiting infectious colitis is a better term to describe salmonellosis (Lamps 2007).

Infection in the traditional mouse model.

There are over 2000 serovars of *Salmonella enterica* that differ in their surface antigen structures, their host range, and their disease causing abilities (Ellermeier and Slauch 2006).

The term “Typhimurium” literally means “Typhi of mice” and *Salmonella enterica* serovar Typhimurium (abbreviated *Salmonella* Typhimurium) can be naturally found and isolated from rodents (Hardy 2015). While *Salmonella* Typhi, for example, is a human specific pathogen, *Salmonella* Typhimurium is a generalist, capable of infecting a wide range of animals. Strains of *Salmonella* Typhimurium are one of the leading causes of salmonellosis worldwide (Ao et al. 2015) and *Salmonella* Typhimurium strain LT2 is considered the “type strain” for the species (Ellermeier and Slauch 2006). The use of mice as a natural host and the ability to genetically manipulate both the bacterium and the mouse has provided tremendous insight into the molecular pathogenesis of *Salmonella* Typhimurium. The only limitation is that this is a model for typhoid-like enteric fever and not gastrointestinal disease, as discussed below.

As outlined above, the stomach serves as an important barrier to infection. Enteric bacteria, including *Salmonella*, can activate what is termed the Acid Tolerance Response (ATR); treatment at moderately low pH induces systems that allow survival at yet lower pH. The ATR is composed of acid shock proteins, which are synthesized to prevent and repair damage to proteins (Audia et al. 2001), proton-sodium and potassium-proton anti-porters to export excess protons in the cytoplasm, as well as lysine and arginine decarboxylases, which, upon significant overexpression, can apparently consume protons and regulate intracellular pH (Ryan et al. 2015). The phenomenon has been extensively studied in vitro. Higher levels of acid tolerance have been associated with more highly virulent *S.* Typhimurium DT104 strains (Berk et al. 2005) and acid adaptation helped *S.* Typhimurium survive in pH 1.5 simulated gastric fluid in vitro (Perez et al. 2010). Stress and pathogenicity-related regulatory systems assist in coping with acid stress. The RpoS and Fur regulons protect against organic acid stress induced by weak acids (Baik et al. 1996), while the PhoP and RpoS regulons protect against inorganic acid stress or low pH (Bearson et al. 1998). Despite extensive in vitro analyses of these phenomena, the role of these systems in vivo is not well understood. The identified regulatory systems control multiple virulence genes in addition to those involved in acid tolerance. Thus, it is difficult to determine the specific role of acid tolerance in pathogenesis. Indeed, there is no definitive evidence that this response of *Salmonella* aids passage through the stomach. There are likely multiple systems that have been identified. For example, the PhoP-mediated response is far more likely to be critical in the macrophage phagosome than in the lumen of the stomach (Kato and Groisman 2008). It might also be that passage through the stomach induces some of these systems, which then facilitate survival lower in the intestine (Angelichio et al. 2004). It is important to note that most animal experiments and many human volunteer studies (Kothary and Babu 2001) involve buffering the stomach contents during oral inoculation. Although buffering the stomach acid increases susceptibility to infection, it does not immediately follow that the in vitro identified systems are critical under normal conditions.

In the traditional mouse model, the primary site of intestinal invasion is the most distal Peyer’s patch in the terminal ileum (Carter and Collins 1974, Jones et al. 1994). Specifically, the bacteria target the M-cells, specially differentiated epithelial cells that overlie the dome of these lymphoid follicles. M cells have several properties that presumably make them particularly good targets for initial intestinal invasion. They have reduced microvilli as well as different glycocalyx and reduced mucus covering, owing to limited goblet cells in the

area. *Salmonella* interact with M cells via long polar fimbriae (*lpf*), one of 14 different fimbriae encoded in the *S. Typhimurium* chromosome (Baumler et al. 1996). This also provides some specificity for attachment. *Salmonella* cells can be seen specifically interacting with M cells within 90 mins of oral infection of mice (unpublished).

Salmonella induces invasion of the M cells using a Type Three Secretion System (T3SS) encoded on the *Salmonella* Pathogenicity Island 1 (SPI-1). This needle-like complex allows the direct injection of bacteria proteins, termed effectors, into the epithelial cell cytoplasm. Greater than 10 different bacterial proteins are injected. These effectors cause actin rearrangement, which leads to engulfment of the bacteria and, independently, induction of the NF- κ B pathway leading to an inflammatory response. The mechanisms of action for specific effectors is reviewed elsewhere (Coburn et al. 2007b, Haraga et al. 2008, LaRock et al. 2015, McGhie et al. 2009).

There are likely several mechanisms by which *Salmonella* cells target the most distal Peyer' patch as the primary site of infection. First, flow through the small intestine is apparently rapid, making it difficult for bacteria to colonize the upper intestine. This flow slows considerably at the distal small intestine prior to ileocecal valve. Second, the M cells themselves are more accessible to the bacteria and the initial attachment is likely somewhat specific for these cells, as mentioned above. Third, the SPI1 T3SS is regulated in response to specific location-dependent signals.

The SPI1 T3SS is tightly regulated in response to a wide array of environmental parameters. Expression of the T3SS causes a growth defect to *Salmonella* cells (Sturm et al. 2011). Therefore, it is thought that the system is expressed only at the appropriate time and place for invasion, presumably the distal small intestine. Numerous environmental cues are integrated to control expression of the system (Golubeva et al. 2012). Examples include both short- and long-chain fatty acids. Long-chain dietary free fatty acids are transported into the bacterial cell where they directly bind to a primary transcriptional regulator of SPI1 to block activation of the system (Golubeva et al. 2016). Importantly, this regulatory effect is independent of the ability of *Salmonella* to use long chain fatty acids as a carbon and energy source. The concentration of dietary fatty acids should decrease along the small intestine as they are absorbed into the body (Carey et al. 1983, Stahl et al. 1999). The short chain fatty acids acetate, formate, propionate, and butyrate, also affect SPI1 expression, but each do so by very different mechanisms. Acetate and formate, which activate expression of the system (Golubeva et al. 2012, Huang et al. 2008, Lawhon et al. 2002, Martinez et al. 2011), are at their highest concentration in the distal ileum (Garner et al. 2009). Propionate and butyrate, which negatively affect expression (Gantois et al. 2006, Golubeva et al. 2016, Hung et al. 2013), are produced as fermentation products by the bacteria in the large intestine. Butyrate, like long chain fatty acids, seems to act solely as a signal; *Salmonella* cannot use butyrate as a carbon source (Gutnick et al. 1969). Thus it is in the distal ileum where the negative acting fatty acids are at their lowest concentration, while the positive acting fatty acids are at their highest concentration. Integration of these signals presumably helps to define the optimal location for expression of the SPI1 system. Although these are just some of the signals being sensed by the bacteria, they have real significance when thinking about infection. The long-chain fatty acids are clearly coming from food (Golubeva et al. 2016), and this would

apparently have implications dependent on the source of *Salmonella* contamination. Moreover, the short-chain fatty acid signals are coming from other intestinal bacteria, implying that one way in which the status of the intestinal microbiota could affect *Salmonella* infection is by altering regulation of virulence gene expression (see below). It follows that variation in the microbiota between individuals could also affect the infectivity of an incoming *Salmonella* cell.

Systemic infection

The bacterial cells traverse the M cells and are taken up by macrophages and/or dendritic cells that are found in the upper dome of the Peyer's patch, underlying the M cells (Hopkins and Kraehenbuhl 1997, Hopkins et al. 2000). Although these cells are naturally phagocytic, engulfment of a *Salmonella* cell that is expressing the SPI1 T3SS leads to a Caspase-1-mediated cell death termed pyroptosis in these phagocytes (Brennan and Cookson 2000, Fink and Cookson 2007, Watson et al. 2000). This is an inflammatory signal and inflammation and destruction of some cells in the Peyer's patch is a hallmark of infection in the mouse (Jones et al. 1994) as well as *S. Typhi* infection in humans (Kohbata et al. 1986, Kraus et al. 1999). This is apparently an important step in *Salmonella* pathogenesis as evidenced by the fact that caspase 1-deficient mice are significantly more resistant to infection (Monack et al. 2000).

The *Salmonella* cells can spread systemically from the Peyer's patch, likely to the draining lymph nodes, and subsequently to all tissues. It is not clear if the bacteria can spread as free cells or are carried by phagocytic cells throughout the body. Evidence certainly suggests that the vast majority of viable *Salmonella* in systemic tissues are found within macrophages and these cells constitute the primary sites of *Salmonella* replication (Mastroeni et al. 2009). However, the data also show that the macrophages contain less than 10 bacterial cells, suggesting that bacteria replicate only a few times in a given cell. How those bacteria get out of one macrophage into new cells is not understood. It is also clear that not all phagocytes are equivalent. Neutrophils likely kill *Salmonella* efficiently via production of large concentrations of hydrogen peroxide and/or hypochlorite (Burton et al. 2014). Highly activated so-called Th1 macrophages also seem capable of killing *Salmonella*. Th2 macrophages, which play a role in tissue repair, are the more likely reservoir. The reservoir could be even narrower, as Detweiler and associates have data suggesting that a subset of macrophages, the hemophagocytic macrophages, which have phagocytized decrepit red blood cells, are the primary replicative niche for *Salmonella*, at least during persistent systemic infection (Nix et al. 2007). Of course replicating in these less lethal phagocytic cells protects the *Salmonella* from other more effective phagocytes, as well as complement and the humoral immune response. In susceptible mouse strains, the bacteria continue to replicate, infecting all tissues in the body, eventually leading to overwhelming septic shock and death.

Replication of *Salmonella* in macrophages has been well-studied. The *Salmonella*-containing vacuole initially acidifies to pH 4–5 (Rathman et al. 1996). This drop in pH and other environmental parameters in the vacuole signal the bacterium to alter gene expression and induce numerous key virulence systems. The PhoPQ two-component regulatory system

in induced, leading to the production of numerous factors important for survival in this harsh environment (Alpuche-Aranda et al. 1994, Alpuche Aranda et al. 1992, Garvis et al. 2001). These include enzymes that modify the outer membrane lipopolysaccharide (LPS) to make it resistant the anti-microbial peptides produced by the macrophage (Groisman and Aspedon 1997, Gunn et al. 1998, Guo et al. 1997), as well as a periplasmic superoxide dismutase that counteracts the phagocytic superoxide produced at high concentrations in the phagosome (Golubeva and Slauch 2006). The SPI1 T3SS is shut down under these conditions and, and partially via PhoPQ, a completely separate second T3SS, encoded on *Salmonella* pathogenicity island 2, is induced. This complex injects proteins across the phagosomal membrane into the macrophage cytoplasm. At least 20 different effector proteins have a number of interesting functions that include alterations in vesicular trafficking to maturing phagosome (Bakowski et al. 2008, Haraga et al. 2008). These alterations in delivery result in the so-called “*Salmonella* containing vacuole”, which is somewhat unique with respect to the various cellular proteins present and, therefore, the vacuolar environment. The *Salmonella* then replicate in this altered vesicle.

A critical factor that affects macrophage survival is the NRAMP1 (*SLC11A1*) metal transport system. NRAMP1 is a phagosomal membrane protein that reportedly transports iron, although there is significantly controversy regarding even the direction of iron transport, let alone the ultimate role in innate immunity (Wessling-Resnick 2015). This factor has been genetically recognized for decades as affecting survival of intracellular pathogens and was previously designated *ity*, *lsh*, or *bcg*, because mice homozygous for this allele show increased sensitivity to *Salmonella*, *Leishmania* and the *Mycobacterium Bcg* vaccine strain, respectively (Vidal et al. 1995). NRAMP1^{-/-} background mice, such as BALB/c or C57BL/6, die within days from an overwhelming systemic *Salmonella* infection, whereas NRAMP1^{+/+} strains, such as 129 or C3H/HeJ, get “persistent” infections in which viable *Salmonella* can be recovered from tissues for up to 1 year, despite lack of any apparent disease (Monack et al. 2004). Genetic polymorphism in the NRAMP locus in humans also affects susceptibility to various infections (Blackwell et al. 2003).

The ability of *Salmonella* to survive in macrophages leads to a lethal infection in sensitive strains of mice. The seriousness of this systemic stage of disease is also reflected in human salmonellosis. The very young, old, or otherwise immunocompromised individuals are particularly sensitive to systemic infection and non-typhoid *Salmonella* is estimated to be a leading cause of death among the food-borne pathogens in the US because of this ability to cause systemic infection in some individuals (Scallan et al. 2011). *Salmonella* Typhi and Paratyphi strains are adapted to and more adept at surviving in human macrophages; the balance is shifted and these organisms cause enteric fever in otherwise healthy individuals.

Events in the intestine in normal and streptomycin-treated mice

There is no evidence that *Salmonella* must replicate prior to invasion of the intestinal epithelium or indeed, in the classic mouse model, that *Salmonella* replicates in the lumen of the intestine at all. First, initial interaction with the M cells of the distal Peyer’s patch is relatively rapid, with transit and binding within 90 minutes (unpublished). Although this does not preclude replication before invasion, it certainly limits it to one or two divisions at

most. Second, the oral LD 50 in mice is $\sim 10^5$ cells. In contrast, if the intestine is bypassed by injected the bacteria intraperitoneally, the LD 50 is <10 organisms (Stocker et al. 1983). This suggests that most of bacteria acquired orally pass through the intestine without ever invading and certainly precludes a model in which a limited number of organisms can replicate to high numbers and then invade. Third, one of the most compelling arguments is the fact that a mutant *Salmonella* that is incapable of replicating in the absence of oxygen is fully virulent in the classic mouse model (Craig et al. 2013). Although this might not preclude one or two rounds of replication in the upper small intestine, the bacteria are certainly not replicating in the anaerobic large intestine. This does not mean that *Salmonella* is not viable in the large intestine, it is just not replicating. The simplest model to explain these data is that *Salmonella* simply traverses the small intestine. A limited number of the bacteria interact with and invade the M cells of the Peyer's patch. Subsequent replication of the bacteria is within the intestinal tissue (which is oxygenated). In addition to spreading systemically, organisms are shed into the lumen of the intestine. They do not replicate in the lumen, but rather are simply excreted in the stool. Bacteria replicating in the liver are also shed into the intestine via the gall bladder. Although some of these organisms can reinvade, most likely pass through and are excreted.

Although much has been learned from the traditional mouse model of *Salmonella* infection, it is a model for typhoid/enteric fever rather than gastroenteritis. Mice do not normally get diarrhea, which has limited our understanding of the most common form of salmonellosis. More in-depth understanding of the gastrointestinal symptoms resulting from *Salmonella* infection has been investigated using cattle (Santos et al. 2001, Tsois et al. 1999), but these experiments are limited by the need for specialized facilities and their expense. More recently, investigators have taken advantage of an old observation (Bohnhoff et al. 1954, 1955, Bohnhoff and Miller 1962, Miller et al. 1956) that treatment of mice with antibiotics, most commonly oral streptomycin, apparently affects the intestinal microbiota. Subsequent oral infection with *Salmonella* leads to an inflammatory diarrhea (Barthel et al. 2003, Que and Hentges 1985).

Experimentally, mice are given a single 20 mg dose of streptomycin orally 24 hours before oral infection with *Salmonella*. This lowers the ID₅₀ at least 5 logs to less than 10 bacteria (Bohnhoff and Miller 1962), although much higher doses are used in the recent studies. *Salmonella* infection in the treated mice induces significant inflammation of the cecum, slightly less inflammation in the colon, and mild inflammation in the distal ileum, as determined by histological analyses, which showed edema of the submucosa and lamina propria, and infiltration of neutrophils into the tissue of intestinal lumen (Barthel et al. 2003). This inflammatory response is dependent on the SPI1 T3SS, but is apparently independent of the organized intestinal lymphoid tissue, such as Peyer's patches, since mice that genetically lack these structures gets colitis upon infection (Barthel et al. 2003). The second *Salmonella* T3SS, encoded on SPI2 is also involved in intestinal inflammation and survival (Coburn et al. 2005, Coombes et al. 2005, Hapfelmeier et al. 2005) and the SPI1 and SPI2 systems are coordinately regulated under certain conditions (Bustamante et al. 2008)

Studies using this infection model have led to a re-evaluation of the inflammatory diarrhea induced upon infection. Simplistically, it was assumed that the immune system somehow knew that *Salmonella* was a pathogen, distinct from the normal intestinal microflora, and that the inflammatory response was induced by the host to fight the infection. It is now clear that *Salmonella* is purposefully inducing inflammation, via injection of proteins by the T3SS. These include specific effectors that lead to induction of the NfκB pathway (Keestra et al. 2011), as well as immune detection of both flagellin and the T3SS per se (Crowley et al. 2016, Sellin et al. 2015, Winter et al. 2009).

Salmonella benefits from the inflammatory response by taking advantage of newly available carbon sources and terminal electron acceptors. Inflammation results in increased production of mucin, which contains high-energy glyco-conjugates and amino acids that can be used as carbon sources for *Salmonella* (Stecher and Hardt 2008). Additionally, there is an influx of neutrophils into the lumen of the intestine that produce reactive oxygen species (ROS), which react with available thiosulfate to generate tetrathionate ($S_4O_6^{2-}$), a terminal electron acceptor that can be utilized by only a limited group of bacteria including *Salmonella*. (Winter et al. 2010). *Salmonella* can also use nitrate, a byproduct of the inflammatory response, for respiration (Winter et al. 2013). These new electron acceptors presumably allow *Salmonella* to metabolize ethanolamine and propanediol, non-fermentable compounds present in the intestine, as carbon and energy sources (Faber et al. 2017, Thiennimitr et al. 2011). Thus, during intestinal inflammation, *Salmonella* can respire using tetrathionate and nitrate, it has access to increased carbon sources including non-fermentable ethanolamine and propanediol and can, therefore, out-compete fermenting bacteria (Clark and Barrett 1987, Lopez et al. 2012).

Chemotaxis is a known virulence factor in a wide range of pathogens, allowing the bacteria to optimize the acquisition of nutrients, avoid toxic substances, and/or move to ideal sites of infection (Ottemann and Miller 1997). For *Salmonella*, chemotaxis is required for full virulence during oral infection. Originally thought to increase the number of encounters between host and bacterial cells (Khoramian-Falsafi et al. 1990), recent work has suggested chemotaxis plays a role in moving towards a more ideal metabolic niche. *Salmonella* lacking a functional *cheY* (chemotaxis) or *fla* (flagellar assembly) are attenuated in the streptomycin pretreated mouse, but only in the presence of inflammation (Stecher et al. 2008) supporting the concept that inflammation provides favorable niche for *Salmonella* replication. The three methyl accepting chemotaxis proteins, Trg, Tsr, and Aer appear to be relevant chemotaxis sensors in the host, sensing galactose and ribose, nitrate, and tetrathionate respectively (Rivera-Chavez et al. 2013). Data suggest that *Salmonella* utilizes chemotaxis in the gut to situate itself near high energy compounds provided by mucin, as well as electron acceptors that allow for anaerobic growth and a growth benefit.

In the streptomycin-treated mice, the primary site of *Salmonella* replication is apparently in intestinal epithelial cells. Sellin et al. (2014) have shown that, after oral infection with $>10^7$ colony forming units, the cecum is colonized by 2–6 hrs with inflammation evident by 8 hrs. The data suggest that bacteria invade the intestinal epithelial cells and replicate primarily within membrane-bound vacuoles. Consistent results are seen in a calf ileal loop model of infection (Laughlin et al. 2014). Infection of the epithelial cells apparently leads to activation

of the NLRC4 inflammasome and caspase 1 via detection of flagella and/or the T3SS needle complex (Sellin et al. 2015). In response, the epithelial cells are extruded into the lumen and undergo pyroptosis, a pro-inflammatory cell death (Knodler et al. 2014a, Knodler et al. 2010). This establishes a situation where *Salmonella* are invading and replicating but the epithelial cells extrude, tempering the bacterial replication (Sellin et al. 2014). In mice lacking components of the inflammasome, mucosal pathogen loads are increased 100–1000 fold (Nordlander et al. 2014, Sellin et al. 2014). Evidence also suggests that in a small fraction of epithelial cells, the *Salmonella* break out of the vacuole and replicate to high numbers within the host cell cytoplasm (Knodler et al. 2014b, Knodler et al. 2010). Although this apparently occurs in only a small fraction of the cells, because of the increased proliferation, these bacteria could represent the majority of total *Salmonella* cells (Knodler et al. 2014b). Moreover, these cytoplasmic bacteria are expressing flagella and the SPI1 T3SS, so they are primed for re-invasion (Knodler 2015).

Oral streptomycin clearly affects the normal microbiota of the intestine (Garner et al. 2009). But how does this actually increase susceptibility to infection by *Salmonella* or other intestinal pathogens? Several non-mutually exclusive mechanisms have been proposed to explain this “colonization resistance”. These include direct effects of the commensal organisms. The intestinal microbiota can compete for nutrients, e.g. carbohydrates and vitamin B12, thereby blocking replication of pathogens. Metabolic byproducts include short-chain fatty acids, which can directly inhibit growth of some organisms as well as, in the case of *Salmonella*, affect expression of the SPI1 T3SS. Some bacteria produce factors like bacteriocins that can directly kill or inhibit other bacteria. It is also likely that some commensal bacteria use type six secretion systems to kill other bacteria with which they come into contact (Schwarz et al. 2010).

Intestinal bacteria can also indirectly affect the ability of pathogens to successfully infect by stimulating both development and function of the mucosal immune system (Buffie and Pamer 2013, Kaiser et al. 2012, Stecher and Hardt 2011). For example, intestinal bacteria promote the production of antimicrobial defensins and REGIIIγ, both of which can directly kill bacteria, or calprotectin, which chelates manganese and zinc. Commensal bacteria also stimulate the differentiation of Th17 T cells, which help to maintain mucosal barrier function. The fact that the effects of antibiotic treatment are relatively rapid suggests that the mechanism is not via changes in immune system development, but rather a direct result of decreased normal microbiota per se or effects on the relative concentration of antimicrobial factors or changes in barrier function, which could respond rapidly. Supporting this concept is the fact that recovery of the normal flora clears the infection (Endt et al. 2010).

It is also clear that normal or experimental variation of the microbiota, independent of antibiotic treatment, can have profound effects on colonization resistance and susceptibility to *Salmonella*. Different mouse strains clearly have different microbiota (Fig. 1; (Krych et al. 2013), but the same mouse strain from different vendors can also differ in microbiota and hence colonization resistance (Stecher et al. 2010, Stecher and Hardt 2011). Some investigators have developed mice with “humanized” microbiota – transplanting a human intestinal mixture into gnotobiotic mice (Turnbaugh et al. 2009). It is not clear if such an

approach will help understand colonization resistance in that a given human microbiota is no better defined than a given mouse microbiota, and this model does not account for evolved and specific genetic interactions between host and respective microbiota, which could be critical to protection against infection.

Stecher and colleagues have taken several approaches to define further the microbiota components that confer colonization protection. In one study (Stecher et al. 2010), they started with mice colonized with the Altered Schaedler Flora, a mixture of eight mouse intestinal bacterial strains. This mixture is routinely used by breeders to provide microbiota to caesarian born mice (Norin and Midtvedt 2010). Mice with this reduced so-called low complexity microbiota are susceptible to inflammatory diarrhea upon oral infection with *Salmonella*. They then housed some of these mice with conventional mice, allowing them to be colonized by additional intestinal organisms and then correlated the acquired microflora with the level of colonization resistance. In general, they found that the mice that had relatively high levels of *E. coli* were susceptible to *Salmonella* induced inflammatory diarrhea.

In a separate study (Brugiroux et al. 2016), Stecher and colleagues colonized mice with a microbiota consisting of 12 cultured and defined bacterial strains isolated from mice. This microbiota stably colonized mice and conferred partial colonization resistance. By performing metagenomic analysis, they compared the metabolic capabilities of this defined microbiota with that of a conventional microbiota and inferred that the defined microbiota lacked certain respiratory pathways. They attempted to compensate by supplementing this defined microbiota with three facultative anaerobes, including an *E. coli* strain. This apparently more metabolically complete microbiota conferred more complete colonization resistance. Interestingly, the two studies could be interpreted as coming to opposite conclusions. In the first case, the levels of other Enterobacteriaceae correlated with susceptibility to *Salmonella* and this was interpreted as indicating that the conditions were ripe for colonization by this group of organisms. In the second case, it was the intentional addition of facultative anaerobes that conferred protection against *Salmonella*, presumably because these organisms effectively compete for nutrients. Obviously more work is required, but it is likely that the overall consortium contributes to colonization protection, rather than the presence or absence of a given class of organism, and different consortia might be equally effective. These reductionist approaches provide important experimental tractability to address these fundamental questions.

Remaining questions

The streptomycin mouse model seems to mimic many aspects of human salmonellosis and provides a critical tool to further understand *Salmonella* infection. But important questions remain. First, how does it work? What does reducing the microbiota do at the molecular level? What is the similarity between the streptomycin-treated mouse intestine and the normal intestine of humans, which are naturally susceptible to *Salmonella* induced inflammatory diarrhea? Second, where is the initial interaction/invasion of *Salmonella* within the intestine? In the normal mouse model and Typhi in humans, it is clearly the Peyer's patch in the distal small intestine. In the streptomycin-treated mouse, the cecum and

colon are apparently the primary infection sites, with *Salmonella* invading and replicating in intestinal epithelial cells. Is this correct? Is this what happens in the normal human intestine? Third, how do the differences in mouse and human anatomy affect the interpretation of these results. The differences in cecum/appendix architecture and function are striking examples (Nguyen et al. 2015). Fourth, how do differences in microbiota affect the outcome? There are differences noted between mice from different sources because of their unmatched microflora and mouse microbiota and human microbiota are obviously different (Krych et al. 2013, Stecher and Hardt 2011); Fig. 1. Of course humans also have “unmatched” microflora. Fourth, is there any bacterial replication prior to intestinal invasion? This has a significant impact on understanding risk of infection at low doses. Fifth, after inflammation is initiated, what is the relative replication rate of intracellular bacteria versus *Salmonella* in the lumen? Answers to these questions and further refinement of the mouse model with full appreciation of the role of microbiota in the “superorganism” will certainly provide important insight into human disease.

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Figure 1.

Principal Coordinate Analysis (PCoA) plot based on (A) unweighted and (B) weighted distance matrices, each calculated from 10 rarefied (4500 reads per sample) operational taxonomic unit (OTU) tables. A) Qualitative information used to generate principal coordinates enables for clear clustering according to host that samples were collected from. B) Quantitative information used to generate principal coordinates enables for clear separation of human and both BALB/c samples and less distinct but significant separation of the NOD and two B6 mice gut microbiota profiles. Labels “BALB/c (f)” and “BALB/c (c)” stand for the gut microbiota of BALB/c mice determined using fecal and caecal samples respectively. The degree of variation between 10 jackknifed replicates of PCoA is displayed with confidence ellipsoids around each sample. Reprinted from Krych et al. 2013, doi: 10.1371/journal.pone.0062578.g002.