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Current Opinion in Microbiology

Heterogeneity of *Salmonella*-host interactions in infected host tissues

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Infected host tissues have complex anatomy, diverse cell types, and dynamic inflammation. Traditional infection biology approaches largely ignore this complex host environment and its impact on pathogens, but recent single-cell technologies unravel extensively heterogeneous host-pathogen interactions *in vivo*. *Salmonella* are major model pathogens in this field due to the availability of excellent mouse disease models and facile molecular biology. The results show how *Salmonella* stochastically vary their virulence, exploit differential nutrient availability, experience and respond to widely varying stresses, and have disparate fates ranging from vigorous proliferation to eradication within the same host tissue. Specific *Salmonella* subsets drive disease progression, while others persist during antimicrobial chemotherapy. Further elucidation of the underlying mechanisms could provide a basis for improved infection control.

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Current Opinion in Microbiology 2017, **39**:57–63

This review comes from a themed issue on **Bacterial systems biology**

Edited by **Dirk Bumann** and **Christoph Dehio**

http://dx.doi.org/10.1016/j.mib.2017.09.008

Introduction

During infection, pathogens often colonize host tissues with complex anatomy, diverse cell types and microenvironments with different physico-chemical parameters and divergent molecular composition. Pathogens can adopt a large variety of physiological states and stress defense programs in these highly heterogeneous environments. This externally triggered pathogen heterogeneity will add to the inevitable internal variation due to stochastic molecular fluctuations in the pathogen. The resulting rich diversity of pathogen behavior has been largely ignored until recently, in part because available methodology provided only bulk average readouts that could not resolve variation between pathogen subpopulations. However, in the past few years, single-cell approaches have been starting to reveal fascinating diversity of host-pathogen interactions in infected tissues [1–3].

These data provide the basis for a paradigm shift to single-cell pathogen infection biology for better understanding fundamental mechanisms that determine course of disease and treatment outcome. Individual pathogen-host encounters involve divergent cellular and molecular mechanisms that lead to disparate outcomes within the same tissue that range from local pathogen eradication to vigorous proliferation in adjacent infection foci [1–3]. Disease progression hence does not reflect a general inability of host immunity to control the pathogen. Instead, the host seems often have powerful effector mechanisms that efficiently kill pathogen, but fails to employ these mechanisms against all dispersed pathogens, resulting in local lack of control. Likewise, antimicrobial chemotherapy might rapidly kill a large fraction of pathogens, but some pathogen subsets might hide in microenvironments that are poorly reachable for drugs [4], or adopt physiological states that make them tolerant against antibiotics [5–7]. Such surviving pathogens will require extended treatments to minimize the risk of relapses. We need to understand better the pathogen subsets that escape efficient immune control and antimicrobial chemotherapy to enable more efficient infection control strategies.

Salmonella infections in mice provide unique opportunities for developing concepts and approaches that might be broadly applicable to other infection models. In particular, well-characterized mouse infection models, facile *Salmonella* genetics and suitability for numerous experimental approaches, as well as extensive literature make *Salmonella* one of the best-studied pathogens. The mouse is a natural host of various *Salmonella enterica* serovars. Low doses of *Salmonella enterica* serovar Typhimurium can cause systemic infections in genetically susceptible mice that reproduce some aspects of human typhoid fever (which is caused by human-adapted serovars Typhi and Paratyphi) [8], and the recent re-establishment of experimental human models of typhoid/paratyphoid fever [9,10•] offers exciting possibilities to compare at murine and human infections under well-controlled infection conditions. Infection of genetically resistant mice can lead to chronic infections with low but stable *Salmonella* tissue loads in spite of a strong immune response [11]. Mice do not normally develop diarrhea, but disruption of the normal gut microbiota by a single dose of

streptomycin overcomes *Salmonella* colonization resistance, and provides a versatile and widely used enteritis model [12].

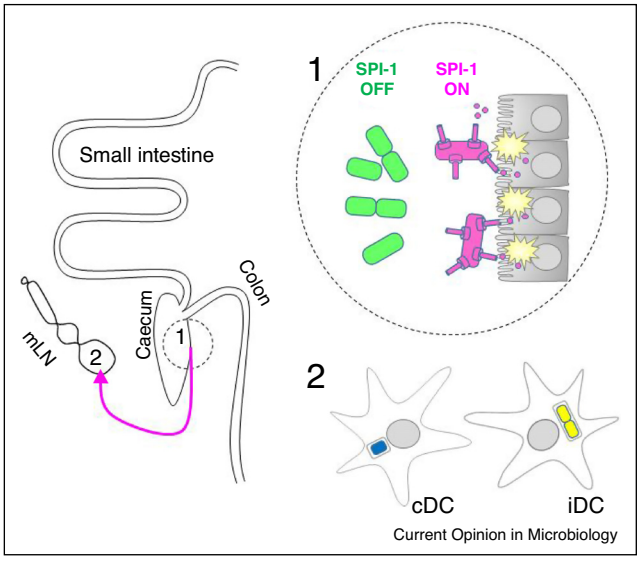
Suitable animal and cell-culture infection models, together with facile molecular biology have made *Salmonella* a prime pathogen for developing numerous innovative approaches. This includes *in vivo* expression technology (IVET) [13], signature-tagged mutagenesis (STM) [14], differential fluorescence induction (DFI) [15], *ex vivo* proteomics [16], population dynamics with wild-type isogenic tagged strains (WITS) [17], fluorescence dilution (FD) [18], TIMER growth rate reporter [19••], *ex vivo* isolation of pathogen subpopulations [20••], dual RNA-seq [21], single-cell RNA-seq of infected cells [22•,23••], etc. As part of this general history of *Salmonella* as a suitable model pathogen for developing novel methodology, single-cell techniques such as confocal microscopy, flow cytometry coupled with informative fluorescent reporter constructs, and single cell RNAseq are starting to yield unique insights into *Salmonella in vivo* heterogeneity in expression, growth rate, stress exposure, antimicrobial tolerance, and single-cell fates. Some of these methods have been recently covered in other reviews [2,24–26]. Here, we will focus on the results that have been obtained and open questions in this new field.

Salmonella growth rate

Early studies revealed extensive differences in *Salmonella* growth rate, gene expression, and proteome in gut lumen vs. mucosal tissues and spleen of infected mice [12,16,27–29]. A major switch occurs in *Salmonella* that invade gut epithelial cells and turn on expression of a type three secretion system encoded on *Salmonella* pathogenicity island 2 (SPI-2), which is associated with intracellular growth. A recent study showed that many *Salmonella* originating from the gut lumen and arriving at mesenteric lymph nodes are in an extended lag phase [30••], perhaps while they re-program their gene expression as required for the new tissue microenvironment.

Maybe more surprisingly, *Salmonella* shows also extensive heterogeneity even within a single host organ. In the enteritis model, *Salmonella* splits into two intestinal subpopulations with one rapidly proliferating subset with low virulence gene expression and another more slowly growing subset with high levels of the invasion-associated type three secretion system encoded on *Salmonella* pathogenicity island 1 (SPI-1) [31] (Figure 1). This heterogeneity seems to reflect stochastic variations in *hilD* gene expression [32], but the underlying molecular mechanisms remain unclear. The SPI-1 ON subset invades the mucosa, causes inflammation, and is partially cleared by the host immune system. However, the gut inflammation that is triggered by this SPI-1 ON subset suppresses competing gut microbiota thus enabling the SPI-1 OFF subset to thrive. This is a striking example of "division of labor" or cooperative virulence among pathogen subpopulations. Another advantage of bistable expression of SPI-1 is the maintenance of a well-growing subset. This subset competes effectively against cheater mutants that completely switch off SPI-1, but exploit benefits generated by wild-type subsets with high SPI-1 activity [33].

Figure 1



Salmonella heterogeneity in gut-associated tissues in a widely used mouse enteritis model. *Salmonella* colonizes the caecum lumen (which is partially equivalent to the human colon) and splits into two subsets (1). One subset actively proliferates with low virulence gene expression, whereas the other subset has low growth rate and high expression of virulence genes associated with the SPI-1 type III secretion system ('SPI-1 ON'). The SPI-1 ON subset invades the caecum mucosa and triggers inflammation that diminishes the density of competing normal gut microbiota, thereby enabling the SPI-1 OFF *Salmonella* subset to thrive. Although many invading *Salmonella* are killed, some SPI-1 ON *Salmonella* manage to travel inside dendritic cells from the gut to mesenteric lymph nodes (mLN). Many *Salmonella* residing in classical dendritic cells (cDC) do not divide enabling them to tolerate high doses of antimicrobials, whereas *Salmonella* in interstitial dendritic cells (iDC) might proliferate at higher rates and remain sensitive to antibiotics (2).

Salmonella also shows highly heterogeneous growth rates when they reside mostly intracellularly in systemic mouse tissues such mesenteric lymph nodes, spleen, and the gall bladder epithelium [18,19••,34••,35]. Early after oral infection and tissue invasion, a small *Salmonella* subset remains in an extended lag phase with no detectable cell division [18]. This extensive lag phase is triggered by induction of toxin proteins of toxin/anti-toxin modules such as an aminoacyl-tRNA acetylase TacT [36], possibly in response to low pH and poor nutrient availability in intracellular *Salmonella*-containing vacuoles [34••]. However, as disease progresses such growth-arrested subsets become very rare [19••,34••] due to overgrowth