Trends in Microbiology

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REVIEWS

Review

The Role of the Host in Driving Phenotypic Heterogeneity in *Salmonella*

Caressa N. Tsai[1,2] and Brian K. Coombes[1,2,*]

The complex infection environment within hosts exerts unique stresses across tissues and cell types, selecting for phenotypic heterogeneity in bacterial populations. Pathogens maintain variability during infection as a strategy to cope with fluctuating host immune conditions, leading to diversification of virulence phenotypes. Recent improvements in single-cell analyses have revealed that distinct bacterial subpopulations contribute unique colonization and growth strategies across infection sites. We discuss several examples of host-driven phenotypic heterogeneity in *Salmonella* populations throughout the course of infection, highlighting how variation in gene expression, growth rate, immune evasion, and metabolic activity contribute to overall bacterial success at the population level. We additionally focus our discussion on the implications of diversity within bacterial communities for antimicrobial efficacy.

Highlights

The intensity of the innate immune response varies depending on host tissue, cell type, and phase of infection.

Variability in immune stresses selects for diversity in *Salmonella* populations *in vivo* and gives rise to substantial heterogeneity in virulence gene expression.

Salmonella spreads in infected hosts by exploiting the innate immune system, using the natural antimicrobial mechanisms of a host as cues for virulence gene expression.

To maximize pathogenic fitness, *Salmonella* exhibits variation in the extent to which it evades the immune system, grows within and outside host cells, and acquires nutrients for efficient central metabolism.

Microbial phenotypic heterogeneity during infection is a likely cause of antimicrobial failure during therapy, as most antibiotics have single targets that may be dispensable in certain bacterial subpopulations with altered gene expression.

Complexity across the Infection Landscape

During infection, the host environment diversifies into areas with varying degrees of inflammation. The detection of invading pathogens recruits several immune cells to multiple host tissues, which orchestrate the appropriate defense response through antigenic detection, modulation of gene expression, and establishment of immunological memory [1,2]. Traditionally, these processes have been quantified at the population level, contributing to an oversimplified view of the infection process and the host response to it [3]. It is now known that variation in gene expression exists within immune cell populations that otherwise appear homogeneous [4], selecting for equivalent heterogeneity in bacterial subpopulations across microenvironments [5,6].

The development of experimental techniques that offer higher spatiotemporal resolution of the infection landscape has increased appreciation of the phenotypic heterogeneity that manifests in bacterial populations (Box 1). This topic has garnered significant attention in the pathogen *Salmonella enterica* serovar Typhimurium (hereinafter *Salmonella*), which offers a useful case-study into this phenomenon [7–9]. Bacteria are now known to exploit the inherent variability across cell types, using these fluctuating 'environmental signals' to evade host immune pressures and confer resistance to antimicrobial treatment. Thus, bacterial heterogeneity must be carefully considered with the continued exploration of novel treatments such as antivirulence therapies and immunomodulation. In this review we discuss how distinct stages of infection and associated immune pressures select for diverse *Salmonella* phenotypes and host–pathogen interaction outcomes. We emphasize that this heterogeneity represents a critical variable to consider in the context of antimicrobial discovery and development.

Modeling Infection Heterogeneity with *Salmonella*

Depending on host immune status and the infecting strain, exposure to different *Salmonella* serotypes can lead to either self-limiting gastroenteritis or systemic typhoid-like infection, which

[1]Michael DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada
[2]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada

*Correspondence:
coombes@mcmaster.ca
(B.K. Coombes).

Box 1. Experimental Methods for Investigating Bacterial Heterogeneity during Infection

The creation of wild-type isogenic tagged strains (WITS) [95] is one of several methods that facilitated higher resolution studies of bacterial cell individuality. WITS are small libraries of easily distinguishable bacterial strains that can be generated by incorporating signature tag sequences in a neutral location in the genome. These tags act as a barcode for each bacterial strain, as they presumably confer no fitness costs *in vitro* or *in vivo,* and can be PCR-amplified and sequenced to identify strains within populations.

More recently, WITS has been expanded with the development of sequence tag-based analysis of microbial populations (STAMP) [116]. Using STAMP, the number of strains with each individual barcode sequence insertion is multiplied, such that they can be mixed within a founding bacterial population at equal frequency. STAMP can be used to quantify the size of biological bottlenecks during infection, as stochastic sampling of a bacterial population will lead to inequality in the relative frequencies of each barcoded strain following some form of environmental stress *in vivo*.

Understanding the transcriptional response of bacterial populations *in vivo* is critical to capture additional aspects of heterogeneity. RNA-seq has facilitated the assembly of complete transcriptomic profiles for *Salmonella* under infection-relevant host conditions [117–119], and has recently been expanded with the use of dual and single-cell RNA-seq, which allow for simultaneous analysis of pathogens and their hosts, or the profiling of transcriptomic variation between individual cells.

Measurement of bacterial growth kinetics at the single-cell level has been developed with the use of fluorescence dilution [78,120]. Using this technique, bacterial cells are transformed with a plasmid capable of expressing a fluorescent protein under an inducible promoter. Following induction and washout, fluorescence is monitored as a reporter for bacterial replication with dividing cells having their fluorescent signal decreasing by half with each division. Differential intensity in fluorescence between bacterial cells that are nongrowing (persisters) and rapidly dividing can be used to compare the relative abundance across growth phenotypes *in vitro* and *in vivo*.

The use of the TIMER protein [121], which spontaneously alters between green and green/orange fluorescence, has been employed to specifically monitor bacterial growth rate at the single-cell level [90], as slowly growing or nongrowing cells should accumulate green/orange fluorescence over time, but actively growing cells should dilute both forms of fluorescence with continuous cell division.

can both be investigated with several well established animal models [10]. Most of what we know of *Salmonella* pathogenicity has been gleaned from laboratory strains of the nontyphoidal Typhimurium serovar – LT2, SL1344, and ATCC14028s [11] – which produce lethal infections and significant pathology in the spleen and liver following oral, intraperitoneal, or intravenous infections of genetically susceptible, *Nramp*-deficient mice [12]. In the oral infection model, streptomycin pretreatment is often used to transiently suppress the gut microbiota that normally confers substantial colonization resistance [13,14]. Alternatively, genetically resistant mice that harbor the wild-type *Nramp* allele are often used in chronic infection models, which can sustain *Salmonella* infections for months without significant pathology [15]. Additional exploration of the virulence determinants of *Salmonella* has been performed in culture with immortalized fibroblast, epithelial, and macrophage cell lines, as well as in ileal loop and calf infection models of gastroenteritis. For the scope of this review, we focus primarily on results derived from nontyphoidal laboratory strains in cultured epithelial and macrophage cell lines, or from acute murine infection models.

A hallmark of *Salmonella* pathogenesis is the existence of distinct virulence strategies linked to the activity of two type III secretion systems (T3SSs) encoded on the SPI-1 and SPI-2 pathogenicity islands [16]. Both T3SSs secrete separate effector protein repertoires that functionally constitute the invasive (T3SS-1, SPI-1) and intracellular (T3SS-2, SPI2) pathogenic lifestyles of *Salmonella* [17]. Traditionally, T3SS-1 activity has been associated with enterocyte invasion in the distal ileum and cecum during the early stages of oral infection [18,19], while T3SS-2 activity has been shown to be more important for survival and immune evasion in the intracellular environment (Figure 1). However, recent results from

next-generation sequencing experiments, single-cell analysis, and systematic *in vivo* and culture infections have contributed to a more complex view of the respective roles of these T3SSs to invasion and intracellular survival. The course of systemic *Salmonella* infection in orally infected mice may be viewed in three stages: invasion and inflammation in the intestinal tract, intracellular residence and immune evasion within phagocytic cells, and growth and spread of *Salmonella* in systemic circulation. We aim to highlight several notable examples of heterogeneity that contribute to *Salmonella* virulence in each of these phases of infection.

Invasion and Inflammation of the Intestinal Tract

The initial invasion of the intestinal epithelium is central to *Salmonella* pathogenesis. After oral infection of streptomycin pretreated mice, bacterial colonization is first localized to the terminal ileum, cecum, and colon, inducing pathology and inflammation in these areas [20]. The process of intestinal invasion and colonization has been considered highly T3SS-1-dependent, as SPI-1

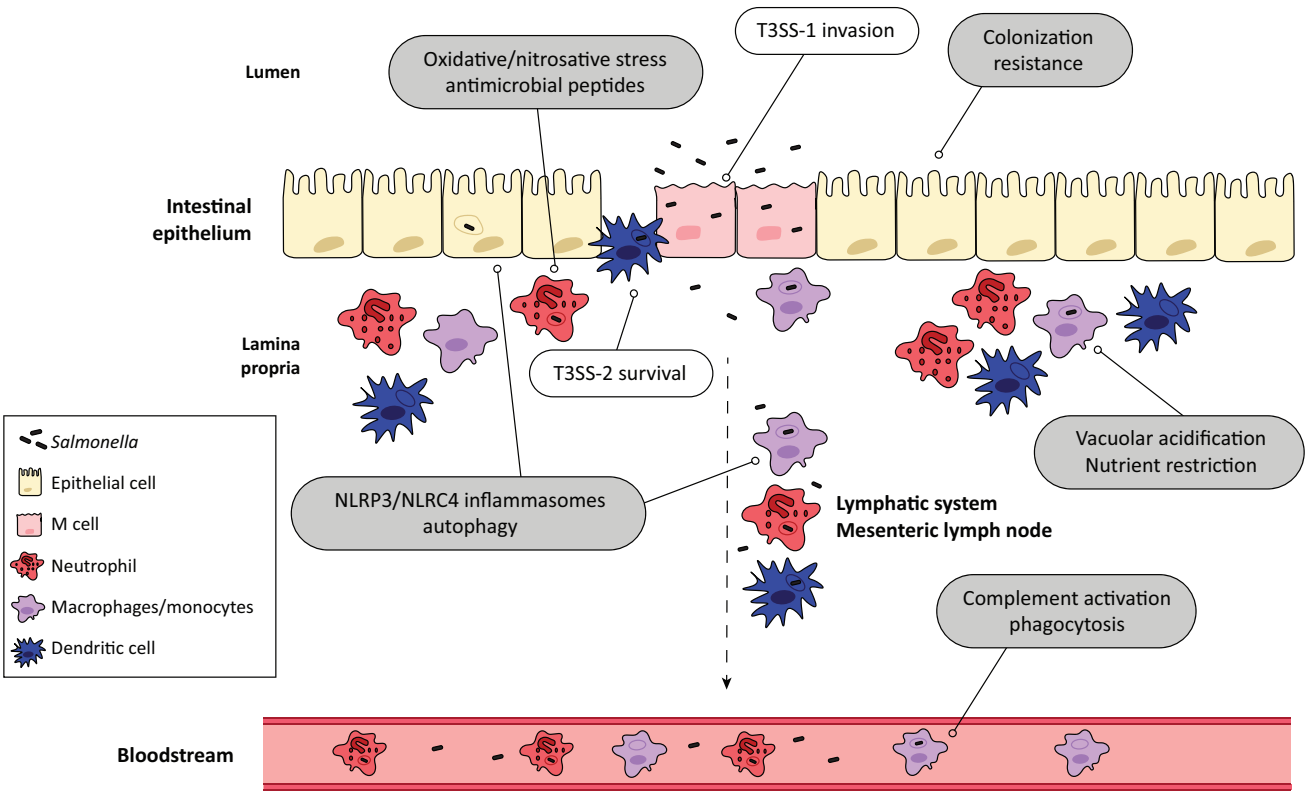

Figure 1. Overview of *Salmonella* Spread during Infection and Localized Immune Stresses. *Salmonella* infections are typically acquired via oral ingestion of contaminated food or water, and after surviving the acidic environment of the stomach, the bacteria will reach the intestinal lumen. The gut microbiome imposes colonization resistance that *Salmonella* must overcome to begin T3SS-1-dependent invasion of the epithelial cell layer. As the monolayer becomes increasingly disrupted, *Salmonella* cells begin to enter into the underlying lamina propria, and rapid recruitment of dendritic cells, neutrophils, and macrophages commences. These phagocytes engulf *Salmonella* and initiate various intrinsic antimicrobial mechanisms in the intracellular environment, including oxidative/nitrosative stress, degranulation and release of antimicrobial peptides, vacuolar acidification, nutrient restriction, inflammasome activation, and autophagy. Survival within these cells is T3SS-2-dependent, requiring the secretion of various effector proteins. Phagocytes facilitate transport of *Salmonella* through the lymphatic system, mesenteric lymph node, and ultimately, the bloodstream. In these areas, phagocytosis continues to occur, with additional potential for complement cascade activation following extracellular escape of *Salmonella* in the bloodstream.

mutants are significantly attenuated for cultured epithelial cell invasion and the induction of intestinal inflammation *in vivo* [21]. However, observations of bistability in T3SS-1 expression and multiple T3SS-1-independent mechanisms of invasion suggest that the dynamics of intestinal colonization are quite complex within this subpopulation, illuminating several interesting examples of microbial heterogeneity.

Bistable T3SS-1 Expression

Despite the well-characterized role of T3SS-1 effector secretion in facilitating epithelial cell invasion, as few as 15% of cells highly express T3SS-1 (T3SS-1^{+}) in both *in vitro* [22,23] and luminal [24] populations of *Salmonella,* with the remainder of the population phenotypically avirulent with respect to T3SS-1 expression (T3SS-1^{-}). Recently, it was shown that T3SS-1^{+} cells pay the cost of virulence with slower growth rates *in vitro* [25] and *in vivo* [26], possibly due to the metabolic costs of T3SS-1 translocon biosynthesis and the complex positive feedback loops and autoactivating transcription factors associated with T3SS-1 regulation [27]. Mathematical modeling, experimental evolution, and *in vivo* studies measuring the frequency of T3SS-1- defector phenotypes have revealed the adaptive value of T3SS-1$^{+/-}$ bistability, suggesting that this phenotype is necessary to stabilize cooperative virulence in the gut [26].

What emerged from these studies is a complex model of heterogeneity in *Salmonella*-specific invasion of the gut, wherein one T3SS-1^{+} subset of cells first induces inflammation and colonizes the epithelial layer at the cost of slow growth and susceptibility to immune-based killing [24], allowing another T3SS-1^{-} wave of bacteria to replicate in the intestinal lumen and thrive in an inflammatory microenvironment induced by T3SS-1^{+} activity [28,29]. The T3SS-1^{+} phenotype was initially viewed as entirely self-destructive, as only T3SS-1^{-} cells appeared to benefit from the expression of this machinery: the ability to infiltrate the mucosal tissue exposes T3SS-1^{+} cells to highly antimicrobial host defense responses, and maintenance of the T3SS-1 translocon poses a metabolic cost and reduces bacterial growth rate [24,25]. However, the slow growth rate of T3SS-1^{+} cells *in vitro* [30] and the ability to form a persistent reservoir in the mucosal tissue *in vivo* [31] have been more recently shown to offer protection from antibiotic exposure. Therefore, these phenotypes exemplify both the division of labour and bet-hedging strategies of microbial heterogeneity (Box 2). T3SS-1^{+} and rapid growth cannot occur simultaneously within single cells but are required for fitness and are both maintained within the intestinal population (division of labor); as well, only slow-growing T3SS-1^{+} cells are able to survive in the event of antibiotic exposure (bet-hedging) (Figure 2).

More work is certainly required to elucidate the genetic basis of bistable T3SS-1 gene expression, as it exhibits heritability across generations [25], and has been linked to *hilD* mutants that can either spontaneously arise [26] or be recapitulated *in vitro* [30]. Unraveling the genetic basis for bistable gene expression in this particular system is complicated by the vast regulatory network controlling SPI-1, that is distinct from other more simplistic forms of prokaryotic bistability [32]. For example, HilD is only one of several transcriptional activators that govern T3SS-1 expression in positive feed-forward loops [33]. One critical implication of this work is that antibiotic treatment of infected hosts likely favors the survival of more virulent (T3SS-1^{+}) bacterial cells [31] due to their slow growth, selecting for a subpopulation of *Salmonella* with an increased capacity to induce host damage. This must be considered in the selection of appropriate infection therapies, necessitating the discovery of new antimicrobials with efficacy against bacterial cells with atypical growth patterns. Bistability in T3SS expression would also be problematic for antivirulence therapies that specifically target secretion systems [34]; however, this may be alleviated with the concurrent administration of traditional antibiotics to target T3SS-1^{-} populations.

Box 2. Mechanisms and Dynamics of Microbial Heterogeneity

The fitness advantages conferred by phenotypic heterogeneity in microbes have been generally described with two distinct community strategies [6]. The first involves a 'bet-hedging' scenario, where two populations are predicted to coexist, with one expressing a phenotype that is optimized for fitness in the current environment, and the other expressing a phenotype that is less fit but is adapted to withstand sudden stress [122]. This scheme is ideal in environments that are expected to fluctuate continuously in an unpredictable manner, in which only the stress-adapted subpopulation will survive environmental change [24]. The second approach involves the 'division of labor', in which multiple subgroups with distinct phenotypes are maintained simultaneously, each performing distinct functions that are required for population-level fitness in the current environment [26,30]. In these situations, the fitness of individual bacterial cells is dependent upon the phenotypes of other cells within the same community. Such a strategy is beneficial when single individuals cannot simultaneously perform multiple functions; thus, the associated phenotypes are partitioned within the population. There are several potential underlying causes of this phenotypic segregation, such as the ease of sharing a freely diffusing metabolite that is produced by only some cells within a community, the cost associated with assembling energetically expensive transporters or protein machines, or the resolution of intrinsic genetic incompatibility between cellular processes [5].

Irrespective of which strategy is adopted within a population, bacterial heterogeneity may be further classified based on heritability. If phenotypic variation is attributed to heritable polymorphic variation at candidate loci, those genotypes with conditional fitness advantages in certain host environments will survive and sweep throughout the population following the sudden onset of immune or antimicrobial-induced stress [123]. However, stochastic processes such as phase variation, differential gene regulation, and bimodal gene expression commonly occur in populations that are otherwise clonal, constituting nonheritable phenotypic diversity [124]. In this case, beneficial phenotypic variation can be maintained using genotypic frequencies that remain stable. The distinction between heritable and nonheritable sources of phenotypic variation is important to consider when interpreting how pathogens respond to *in vivo* selective pressures. Only heritable phenotypic variation is able to offer a direct genetic substrate for evolutionary change; heterogeneity of this nature therefore has the potential to alter genotypic distribution within populations and promote adaptation [125].

Hyper-replication and Extrusion of the Epithelium

The subset of T3SS-1$^+$ *Salmonella* that is capable of epithelial cell entry continues to diversify, as some of these bacteria are transcriptionally reprogrammed and begin hyper-replicating in the epithelial cell cytosol [35]. Although *Salmonella* initially occupies *Salmonella*-containing vacuoles (SCVs) after host cell entry, as many as 20–30% of bacteria in cultured epithelial cells have been observed to lyse the nascent SCV and infiltrate the cytosolic subcompartment [36,37]. Once cytosolic, *Salmonella* is able to hyper-replicate in polarized epithelial monolayers [38,39], accounting for up to half of the intracellular population by 7 h after infection [36]. This phenotype has been genetically attributed to increased T3SS-1 and flagellar expression [38], which adds a nuanced view to the previously held dogma that T3SS-1 is repressed and T3SS-2 is activated in the intracellular environment [40,41].

This intraepithelial phenotype extends beyond rapid bacterial growth rate and may also be a cue for host surveillance. Cytosolic *Salmonella* induces proinflammatory cell death (pyroptosis) in cultured epithelial cells, leading to the expulsion and 'luminal' release of enterocytes from polarized monolayers [38]. Initially, this process was proposed as beneficial for the spread of *Salmonella* across the intestinal epithelium, as the release of 'invasion-primed' bacteria into the lumen may allow for reinfection of other epithelial cells. However, it is now known that pyroptosis and subsequent expulsion is caused by Naip/NLRC4 inflammasome activation within epithelial cells, which is ultimately restrictive for bacterial replication [42–44]. Inflammasomes are multiprotein complexes that detect and respond to the presence of intracellular bacterial ligands with Caspase-mediated processing of cytokines and the pore-forming Gasdermin-D protein, cumulating in pyroptotic lysis of the infected cell [45]. The restrictive role of the epithelial inflammasomes in *Salmonella* pathogenesis has been explored with mice lacking various sensor/caspase components (Caspase-11 [42], Naip/NLRC4 [43], Caspase-1/8/11, Gasdermin-D, Nlrc4 [44]), which exhibit hypersensitivity to intestinal pathology and bacterial

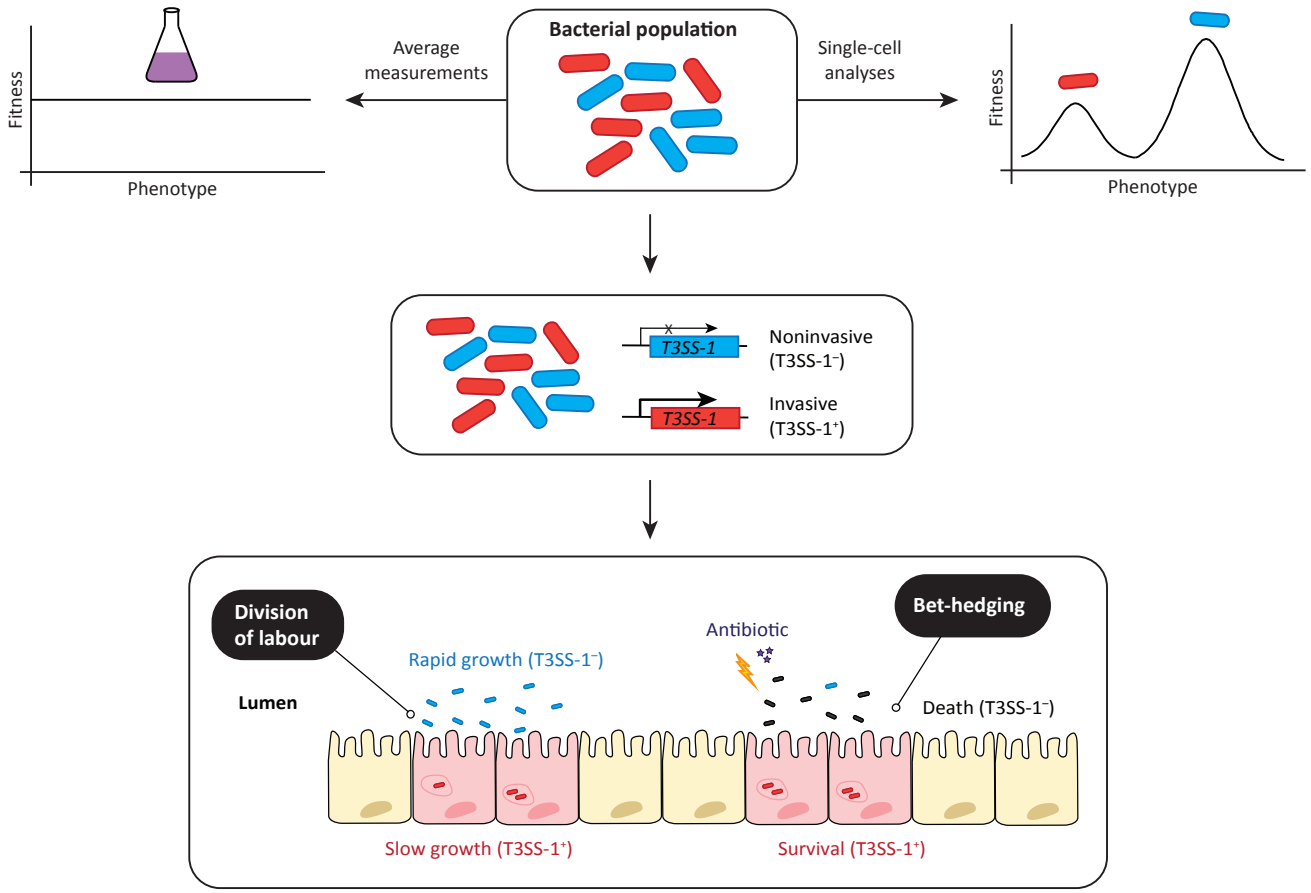

Figure 2. Cooperative Virulence Strategies Facilitated by Phenotypic Heterogeneity of *Salmonella*. Given a bacterial population in which multiple phenotypes (red, blue) coexist and are stably maintained, different conclusions may be drawn depending on experimental approach. In the case of conventional bulk average measurements, the population in aggregate may appear homogeneous, displaying a phenotype that is an intermediate (purple) of the two that truly exist. Conversely, single-cell measurements and analysis of population dynamics may reveal the existence of both phenotypes with different fitness. The coexistence of multiple phenotypes may be explained by a 'division of labor' strategy in which each phenotype performs a different function that cannot coexist within a single individual cell. This is exemplified by the coexistence of virulent (red) and rapid growth (blue) phenotypes in *Salmonella,* regulated by bistable T3SS-1 gene expression: T3SS-1 expressing (red) bacteria are virulent but grow slowly, and T3SS-1 repressing (blue) bacteria are avirulent but grow rapidly. Alternatively, a 'bet-hedging' strategy may also explain bacterial heterogeneity, wherein multiple phenotypes are maintained because only one will survive the induction of environmental stress.

burdens after infection. A second layer of intestinal protection is also likely derived from the concomitant release of proinflammatory cytokines with inflammasome activation, promoting neutrophil and natural killer cell recruitment with significant antimicrobial potential [43,46]. Together, these observations suggest a protective role for epithelial cell pyroptosis in *Salmonella* infection, despite initial bacterial hyper-replication.

It is important to note that the cytosolic subpopulation of intestinal *Salmonella in vivo* has not been thoroughly interrogated, despite considerable evidence from infections of cultured epithelial cells. Observations of large cytosolic burdens of *Salmonella* in the epithelial cells of infected animals are therefore quite limited, although extruded epithelial cells containing small bacterial populations have been identified in the murine gallbladder [38] and bovine ileal loop [47] infection models. Additionally, while pyroptotic cell death is likely only restrictive for bacterial replication, delayed T3SS-1 effector secretion in cytosolic *Salmonella* may be able to delay the

onset of epithelial cell death, perhaps conferring an advantage to intraepithelial bacteria [48]. Several questions remain in the consideration of this unique phenotype, necessitating future investigation into the frequency of hyper-replication *in vivo,* the extent to which epithelial cells in different areas of the intestinal tract activate the inflammasome, and the potential environmental cues within infected epithelial cells that induce hyper-replication.

Invasion Preferences and Exit from the Intestinal Tract

Another form of heterogeneity in intestinal *Salmonella* exists in the targeting of specific epithelial cell sites for invasion. Membrane ruffling of cells within the epithelial monolayer has been shown to induce preferential invasion [49,50], while others have suggested that targeting is linked to the stage of cellular division, and therefore surface cholesterol content, in enterocytes [51]. Recently, the vulnerability of epithelial cells in culture has been attributed more to infection status, as cells already infected with *Salmonella* are more susceptible to reinfection, even in the absence of membrane ruffling [52]. Together, these observations emphasize the importance of exploring *Salmonella* invasion directly in oral infection models that extend beyond monocultured epithelial cells, in which intraintestinal cell heterogeneity may be more appropriately accounted for.

After intestinal colonization and epithelial cell invasion, *Salmonella* infections are thought to proceed through infiltration of the gut mucosa, and ultimately exit from the intestinal tract. Although this specific stage of infection is incompletely characterized, several lines of evidence suggest that this process may be entirely independent of T3SS-1 expression and epithelial cell invasion. Monocytic phagocytosis in the terminal ileum [53], M cell-mediated uptake and translocation [54,55], and antigen-sampling dendritic cells [56–58] have all been shown to offer T3SS-1-independent routes of infiltration into the mucosal tissue across the epithelial layer. T3SS-2-dependent basolateral trafficking across the monolayer has also been shown to be sufficient for entry into the gut mucosa in the absence of intraepithelial replication, even after T3SS-1-dependent invasion [59]. This is consistent with more recent reports of the highly redundant roles of individual T3SS-1 effectors, which are individually only minimally important for bacterial invasion [60].

Overall, these observations suggest that intraepithelial survival and replication are not strict prerequisites for crossing the epithelium, infiltrating the mucosal tissue, or inducing pathology *in vivo.* Considered with the examples of heterogeneity we discuss above, it is tempting to speculate that only the T3SS-1^{-} subset of cells traverses the epithelium through these T3SS-1-independent pathways, while the T3SS-1^{+} subset of cells never enters systemic circulation, remaining confined within the inflamed intestinal tract. Determining the frequency and subpopulation specificity of T3SS-1-dependent or -independent mechanisms of mucosal infiltration *in vivo* remains an important question for future characterization.

Survival and Replication in the Intracellular Environment

Irrespective of the mechanisms underlying epithelium traversal and extraintestinal exit, the next stage of *Salmonella* pathogenesis is dependent upon intracellular adaptation, with *Salmonella* populations mostly occupying dendritic cells, macrophages, neutrophils, and fibroblasts [61]. Once infected, these host cells attempt to restrict the growth of internalized *Salmonella* with several antimicrobial activities. Here we highlight only a few of the diverse outcomes of *Salmonella*–macrophage interactions, as this subset of host cells represents a primary niche for bacterial replication *in vivo.* We note that additionally fascinating examples of heterogeneity exist within fibroblasts, dendritic cells, neutrophils, and nonphagocytic lymphocytes, which we do not include here due to space constraints.

Inflammasome Activation

Above, we described the activation of the canonical and noncanonical inflammasomes in response to cytosolic, intraepithelial *Salmonella*. Contrary to this, the vast majority of intramacrophage *Salmonella* exists as a vacuolar subpopulation confined within the SCV [37]. In the rare event of cytosolic *Salmonella* escape within macrophages, the canonical caspase-1 Naip/NLRC4 inflammasome rapidly responds to flagellin (with Naip5/6) [62,63] or T3SS-1 needle components (with Naip1/2) [64] by initiating pyroptosis. *In vivo,* this process is thought to release cytosolic *Salmonella* into the extracellular milieu, trapping bacteria within the products of cellular lysis and recruiting additional secondary phagocytes to engulf and kill these cells [65]. The noncanonical caspase-11 NLRP3 inflammasome is also active within macrophages and responds in a similar way, although its activation has been partially attributed to oxidative stress and may not require cytosolic bacterial ligands [66,67].

Although inflammasomes are one of the most important host defense responses that limit *Salmonella* replication in cultured macrophages, it is important to note that there is considerable heterogeneity in the activation of these antimicrobial systems. Indeed, long-standing observations of *Salmonella* in cultured infections have revealed that only some macrophages undergo cell death in response to bacterial internalization, while others survive and allow bacteria to replicate. On one hand, this may be due to bacterial variability in gene expression: *Salmonella* can be internalized within macrophages through either T3SS-1-dependent invasion or phagocytic uptake, with the former more likely to predispose macrophages towards inflammasome activation. This is due to the spurious pore-forming activity of the T3SS-1 machinery [68], which destabilizes the SCV membrane in ~10% of productive infections, increasing the likelihood of detecting flagellin and/or whole bacterial cells in the host cell cytosol [37]. To overcome this, *Salmonella* has evolved several mechanisms to repress T3SS-1 and flagellar gene expression in the intracellular environment [64,69–71].

Conversely, the macrophage-specific determinants of inflammasome activation may be linked to phenotypic diversity in localization (bone-marrow-derived, tissue-resident) or polarization (classically activated/proinflammatory M1, alternatively activated/anti-inflammatory M2). Preliminary investigation into this area has suggested that peritoneal macrophages produce more eicosanoids after Naip/NLRC4 inflammasome activation relative to bone-marrow-derived macrophages [72], and that the expression of the NLRP3 [73] and NLRC4 [74] inflammasomes is augmented in M1 polarized macrophages. Moreover, the presence of cytosolic *Salmonella* in macrophages has been shown to not always result in cellular lysis, as activation of the caspase-1/11 inflammasomes can occur to restrict intracellular bacterial replication without pyroptosis [75]. A fruitful line of investigation would appear to be in taking inventory of the spatiotemporal distribution of host cells with different antimicrobial potencies, as it is likely that this host-driven heterogeneity produces much of the bacterial phenotypic diversity during *Salmonella* infection (Figure 3). Indeed, though not specifically related to inflammasome activation, others have recently reported that variability in bacterial lipopolysaccharide modification induces drastically different type I interferon immune responses in subsets of macrophages [76], in support of the substantial heterogeneity possible within these host cells.

Persister Formation

The relationship between macrophage polarization and bacterial heterogeneity is also exemplified with the formation of 'persister' bacterial cells that stop replicating in response to stress [77]. Intramacrophage adaptation has been shown to induce persister formation in culture [78] and *in vivo* [79], in which growth-arrested bacteria exhibit antibiotic tolerance while retaining metabolic activity. Interestingly, nongrowing populations of intracellular *Salmonella*

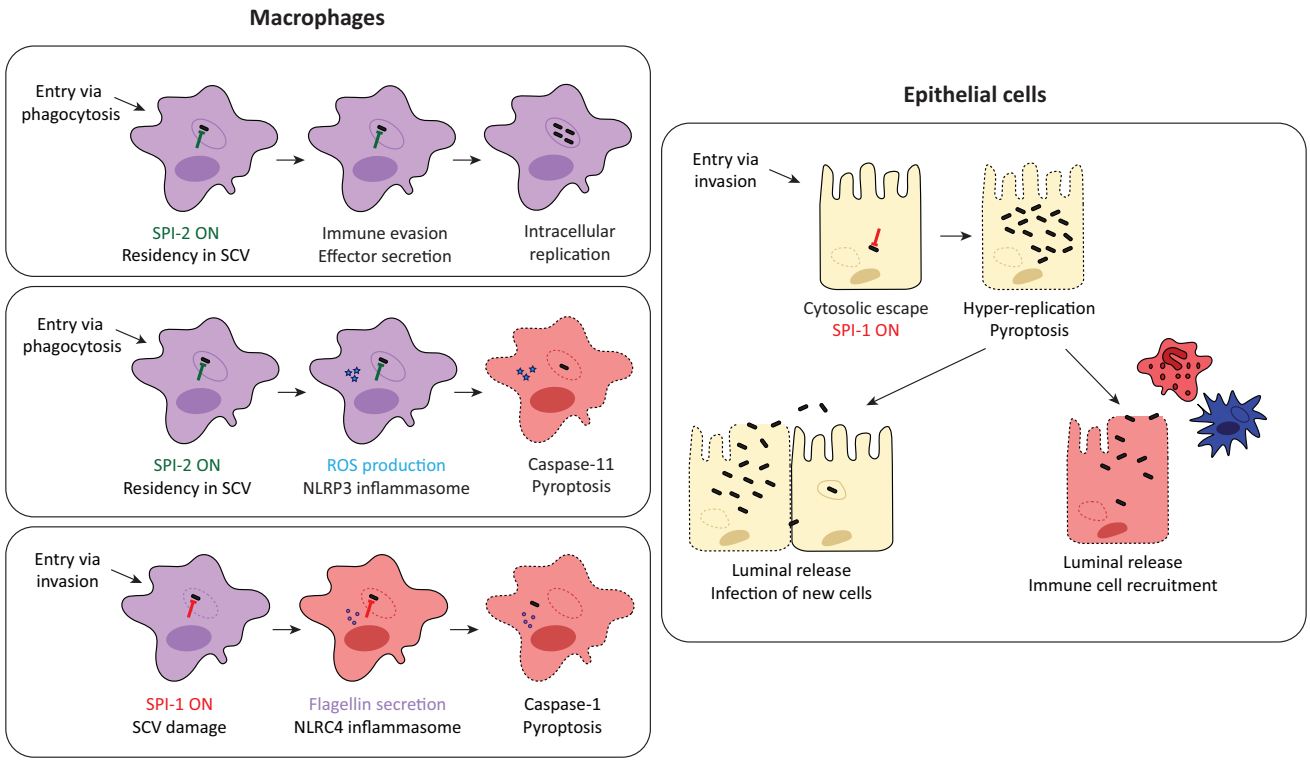

Figure 3. Intracellular Behavior of *Salmonella* in Epithelial Cells and Macrophages. In macrophages (left panel), *Salmonella* entry can occur through either phagocytic uptake or T3SS-1-dependent invasion. In the event of entry via phagocytosis, bacterial cells repress T3SS-1, upregulate T3SS-2, and maintain integrity of the *Salmonella*-containing vacuoles (SCV). Under these conditions, the production of reactive oxygen species (ROS) can initiate the intracellular NLRP3 inflammasome, leading to caspase-11 activation and pyroptosis of the infected macrophage. Alternatively, if macrophage entry proceeds via invasion, the T3SS-1 needle can damage the SCV, allowing for the accidental secretion of flagellin into the host cell cytosol. This, in addition to detection of T3SS-1 components or cytosolic escape of bacteria, can initiate the NLRC4 inflammasome, caspase-1 activation, and pyroptosis. In epithelial cells (right panel), SCV damage can occur following uptake of invasive *Salmonella,* permitting cytosolic escape and subsequent hyper-replication. This process can lead to pyroptosis of the infected epithelial cell, releasing bacteria into the intestinal lumen, where they will either reinfect neighboring enterocytes or be ingested by recruited phagocytic cells.

in murine bone-marrow-derived macrophages have been shown to preferentially occupy proinflammatory M1 macrophages, while actively dividing bacterial cells are more often found within anti-inflammatory M2 macrophages [80], consistent with similar observations in human monocytes [81]. Most recently, intramacrophage persisters in culture and *in vivo* have been shown to retain the ability to secrete T3SS-2 effectors, which interact with inflammatory signaling pathways to polarize macrophages towards an anti-inflammatory M2 state and counteract M1 activation [82].

Overall, the mechanisms governing phenotypic transitions between *Salmonella* replication or persister formation appear to be a function of macrophage polarization, suggesting that bacterial cells evade the host immune system by reducing growth rate when residing within more proinflammatory host cell subtypes. This aligns with previous observations of the tropism of *Salmonella* for anti-inflammatory macrophages in the spleen and mesenteric lymph nodes of infected mice [83,84]. The molecular mechanisms that *Salmonella* uses to reprogram the intracellular environment is a rich avenue of future investigation. It also seems warranted to investigate the potential for persister formation in nonmacrophage host cell types, as the ability

of *Salmonella* to enter a nonproliferating state in fibroblasts has been recently linked to dampened NF-kB signaling [85], adding new mechanistic understanding into this well-known yet enigmatic phenomenon.

Metabolic Strategies across the Infection Landscape

Within and outside immune cells, infected hosts restrict access to amino acids, essential transition metals, and other nutrients in an attempt to prevent pathogen growth. Collectively, these processes make up the 'nutritional immunity' defense strategy [86]. Because nutritional limitation negatively impacts bacterial replication, adaptation of pathogens to the *in vivo* environment often includes the acquisition of novel metabolic capabilities to circumvent host-mediated sequestration of free nutrients. To this end, intracellular *Salmonella* exhibits heterogeneity in the metabolic pathways deployed to acquire diverse nutrients from host cells, allowing for subversion of the nutritional limitation imposed by the host immune system.

Salmonella is a prototroph that can synthesize essential macromolecules in the presence of a simple carbon source. The replication of intracellular *Salmonella* suggests that SCV-confined bacteria have access to all nutrients required for growth, or at least a reliable carbon substrate. This is thought to be facilitated mostly by *Salmonella*-induced filaments [87] that extend from the SCV and establish an endosomal network for metabolite exchange between the host cytosol and the SCV [88]. Indeed, this is supported by proteomic and metabolomic characterization suggesting that *Salmonella* has access to at least 31 different nutrients during infection [89]. However, single-cell analysis of *Salmonella* populations inside host cells has indicated that nutrient supply exhibits heterogeneity even in single tissues, such that different subsets of nutrient limitation and stress response proteins are elevated in *Salmonella* internalized within neighboring host cells [90]. Cultured macrophage infections with *Salmonella* have also revealed variable expression of genes that participate in different catabolic pathways for carbon utilization, suggesting the existence of different bacterial subpopulations using glucose, gluconate, or fatty acid substrates in different abundances to support central metabolism [91].

Heterogeneity in metabolic gene expression suggests that *Salmonella* has access to several metabolites *in vivo,* but that different host cell subsets restrict different nutrients from *Salmonella*. It seems likely that the ability to simultaneously exploit multiple metabolites is required to fully support growth and virulence, selecting for subpopulations of bacteria that can deploy different metabolic pathways depending on the type of nutrient limitation. While the exact host cell determinants that produce heterogeneity in nutrient supply to intracellular *Salmonella* are unknown, it is tempting to speculate that macrophage polarization is one contributing factor. Amino acid metabolism, glycolysis, oxidative phosphorylation, fatty acid synthesis, and fatty acid oxidation have all been shown to differ between M1 and M2 polarized macrophages [92]. It is therefore possible that the metabolic activity of differentially polarized macrophages supplies intracellular bacteria with diverse sets of nutrients, which may not only select for heterogeneity in bacterial metabolism, but also explain the differences in permissiveness of M1/M2 macrophages for bacterial growth [93]. Further investigation is required to explore the spatial distribution of M1 and M2 macrophage subtypes *in vivo* in an effort to better link metabolic availability to bacterial heterogeneity.

Growth and Spread of *Salmonella* at Systemic Sites

During the first day after infection, the total *Salmonella* burden within infected hosts is thought to initially decrease, due to a net balance of host-mediated killing over bacterial replication [94,95]. However, as infection proceeds, bacterial survival is favored by the transcriptional

reprogramming that occurs in intracellular *Salmonella* to express genes that confer resistance to the natural antimicrobial mechanisms of the host innate immune system. This includes the expression of enzymes that detoxify reactive oxygen species, and others that fortify the outer membrane with modifications to decrease susceptibility to host defense peptides [96]. These adaptations help to create and sustain a partitioned bacterial population, with a fraction of cells remaining in the intestinal tract, invading enterocytes and intensifying inflammation, while others are shuttled to systemic sites through the reticuloendothelial system [97]. Again, the extra-intestinal pathogenesis of *Salmonella* exemplifies microbial heterogeneity during infection, as subpopulations in the lymph nodes, spleen, and liver adopt unique colonization dynamics to facilitate the infection and reinfection of new host cells.

Colonization of the Lymph Nodes and Antibiotic Tolerance

In infected mice, the migration of *Salmonella* from the gut mucosal tissue to the mesenteric lymph node imposes a significant bottleneck in the transition to systemic infection. More recently, several studies using next-generation sequencing of wild-type isogenic tagged strains (WITS) (Box 1) have added to our understanding of the individual contributions of virulence determinants and host cell subsets to this critical stage of infection. It is now known that neutrophil infiltration to the gut mucosal tissue is primarily responsible for the replication of intestinal *Salmonella* [98], while the movement of dendritic cells, and not macrophages, is most important in facilitating the eventual migration of bacterial cells to the mesenteric lymph node [99–101].

Interestingly, several independent lines of evidence have suggested that bacterial growth rate is reduced after cells have reached the mesenteric lymph node, at least in the earlier stages of infection. While activity of the T3SS-1 appeared to be unimportant for lymph node migration, the maintained expression of this system was associated with decreased replication rates of bacteria in this location [99], consistent with observations of reduced growth of T3SS-1^{+} cells in the gut. Additionally, several nondividing or slowly dividing subpopulations of *Salmonella* have been shown to arise in the first 24 h after infection of mice, the majority contained within the mesenteric lymph node [79,102], but some in the spleen and liver [90]. These findings are in line with results derived from *Salmonella* infections of cultured macrophages, in which intracellular adaptation is associated with persister formation [78,79,82]. However, it is important to note that fast-growing subsets of *Salmonella* coexist with those cells that have slow-growth phenotypes [90], suggesting that heterogeneity in growth rate must be maintained in pathological lesions during infection.

Cellular Tropism and Intracellular Spread of *Salmonella*

In systemic circulation, *Salmonella* appears to maintain the ability to discriminate between different subsets of host cells, consistent with similar observations in cultured epithelial cells. For example, single-cell analysis has revealed that only a subset of macrophages cultured with *Salmonella* display susceptibility to bacterial internalization, such that multiple points of contact between bacteria and phagocytes are required before infection is achieved [103]. This model was more recently expanded further to emphasize the importance of bacterial shape [104] and length of contact time [105] in increasing the likelihood of successful bacterial infection of macrophages. It is unclear whether this discrimination is driven more by heterogeneity within host or bacterial cells, although the ability for *Salmonella* to manipulate macrophage polarization with T3SS-secreted effectors [82] and preferentially replicate within anti-inflammatory host cell types [80,83,84] would suggest that bacterial variability is at least partially responsible. Others have demonstrated that *Salmonella* discriminates between specific cell types *in vivo* with the secretion of different T3SS-1/2 effectors, as nonphagocytic lymphocytes are subject to

increased bacterial secretion, while mature splenic macrophages do not harbor intracellular populations of *Salmonella* in the later stages of infection [61].

The selective targeting of certain host cell types in systemic circulation is indicative of paralleled heterogeneity between *Salmonella* and its intracellular environments, such that the former preferentially occupies host cells most permissive for bacterial growth, while the latter exhibits diversification in antimicrobial phenotypes to exert multiple forms of immune stress. Although the ability for *Salmonella* to sense different immune pressures to initiate virulence gene expression has been demonstrated [106,107], the molecular basis of this detection across host cell types remains unknown. However, it seems likely that distinct nutritional or immune microenvironments between cytosolic and vacuolar subcompartments of epithelial cells, macrophages, and other host cell types provide specific cues for differential gene expression in *Salmonella,* driving unique immune evasion profiles. For example, differences in nutritional availability, oxidative stress, pH, and other signals between subcellular compartments may be detected by one or more of the two-component regulatory systems in *Salmonella* that are already known to sense and respond to multiple host environmental cues (e.g., SsrA–SsrB [108], EnvZ–OmpR [109], PhoQ–PhoP [110], PmrA–PmrB [111], amongst others). Signaling through the sensor systems of *Salmonella* may facilitate the identification of host cell type and subsequent repression or activation of the appropriate genetic pathways required for intracellular survival.

The importance of intracellular survival of *Salmonella* for systemic spread suggests that, during infection, internalized bacteria must be able to escape and reinfect new host cells. Indeed, several lines of evidence have suggested that the eventual increase in overall bacterial burden within infected hosts is more dependent upon transfer between, rather than replication within, individual phagocytes [95,97,112,113]. How does *Salmonella* spread between phagocytes, and how often? The highly proinflammatory nature of inflammasome-mediated pyroptosis is unlikely to be beneficial for bacterial spread, although it offers a route of exit from macrophages. However, some have suggested that intracellular *Salmonella* is able to escape from macrophages in a flagella-dependent manner by inducing a form of cell death, called 'oncosis', that is distinct from inflammasome-induced pyroptosis, although the frequency of this *in vivo* is unknown [114]. Earlier studies have also suggested that the SCV is capable of centrifugal movement within epithelial cells and localizes to the host cell periphery after 24 h of infection [115], offering a potential mechanism of cell–cell transfer that remains uninvestigated within macrophages. Lastly, caspase-3-mediated apoptosis of infected macrophages may offer a route of intraphagocytic spread without extracellular exposure, as apoptotic host cells remain intact after death and are then engulfed by other phagocytes [94,95]. Continuing to investigate this process *in vivo* will contribute significantly to our understanding of the population dynamics of *Salmonella* infections.

Concluding Remarks

The asynchronous nature of bacterial infection within hosts creates a pool of phenotypic diversity that permits the exploitation of a wide range of niches, as well as the evasion of environmental and immunological stresses. Consistent with this, distinct subpopulations of both immune and bacterial cells emerge in the complex landscape of infection, creating microenvironments with different host–pathogen interaction outcomes. Despite being one of the most well-studied pathogenic organisms, the vast majority of *Salmonella* infection biology has been gleaned from population-wide averages that have overlooked information on phenotypic heterogeneity across infection foci. Our understanding of within-host bacterial diversity has matured in recent years with advances in systems-level analyses of bacterial

Outstanding Questions

In the systemic phase of infection, how do individual bacterial cells spread between host phagocytes?

How frequently are epithelial cells or macrophages lysed via pyroptosis (or other mechanisms) versus allowing bacterial cells to remain intracellular and replicate?

How do intracellular populations of *Salmonella* distinguish between the vacuolar and cytosolic environments of epithelial cells versus macrophages?

What regulatory cues used by *Salmonella* control fate decisions such as intracellular replication, vacuolar rupture, cytosolic escape?

Is bacterial heterogeneity that develops during infection caused by the host immune response, or does it pre-exist within subpopulations to thwart environmental stress?

Is it possible to eradicate infections by developing antimicrobials selective for bacterial persisters?

Can immunomodulatory therapies be used to direct the more potent subset of host immune cells to appropriate locations during infection?

Can antimicrobials be used to dampen or enrich the inflammatory response at different times during infection?

communities, single-cell labeling techniques, next-generation transcriptomics, and predictive *in silico* and mathematical models. Here we have summarized some new aspects of host-driven phenotypic heterogeneity within *Salmonella* populations during infection.

We currently lack a complete understanding of how *Salmonella* detects host microenvironments that present different immune pressures. Bacterial detection of the host environment is an important step in the infection cycle, as it sets up unique gene expression programs within bacterial cells that produce phenotypic diversification *in vivo*. With the continued application of higher resolution experimental techniques to characterize pathogenic heterogeneity, we may be able to leverage knowledge of the regulatory requirements for growth and virulence across cellular compartments and tissues to identify novel antimicrobial targets (see Outstanding Questions). In this context, antibiotic adjuvants and combinations may prove useful, as multiple antimicrobials with unique bacterial targets could be coadministered to inhibit a range of processes across multiple host sites. Continuing to investigate host-mediated pathogenic heterogeneity is essential to fully understand *in vivo* bacterial population dynamics and encourage the development of antimicrobial therapies most optimized for rapid clearance of infection.

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