Trends in Microbiology

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REVIEWS

Review

One for All, but Not All for One: Social Behavior during Bacterial Diseases

Kimberly M. Davis[1] and Ralph R. Isberg[2,*]

It has been known for decades that individual cells within pathogenic bacterial populations have reduced antibiotic susceptibility, which is linked to decreased metabolic rates. A similar phenomenon occurs with virulence-associated proteins, as reduced expression is associated with increased fitness of individual cells. Non-producers within the population can benefit from the virulence proteins produced by others in the population without suffering a fitness cost, thus maintaining a genetically uniform population. Cooperative behavior has been reported for *Salmonella* and *Yersinia*, consistent with selection of social behavior to retain genes associated with pathogenesis; however, cooperation was unclear within *Mycobacterium* populations. This review focuses on these recent descriptions of cooperation, discusses the mechanisms driving heterogeneity, and evaluates the evidence that expression of virulence-associated proteins comes at a fitness cost.

Highlights

Virulence factor expression occurs in subsets of cells within pathogenic bacterial populations.

Stochasticity, spatial location, and cellular interactions all contribute to heterogeneity in gene expression within bacterial populations.

Virulence factor expression may impact the fitness of individual cells, promoting the formation of slow-growing subsets within bacterial populations.

Subpopulations during Bacterial Infection

Perhaps the first description of heterogeneity within bacterial populations was published in 1944 by the Irish physician, Joseph Bigger, who observed that penicillin treatment eliminated a majority of *Staphylococcus aureus* cells within patients, but that there was always a small surviving subset of cells [1]. The survivors were termed **persister cells** (see Glossary) and have been well studied in subsequent years due to their role in relapsing infections and the development of antibiotic resistance [2]. Persister cells represent a subpopulation of bacterial cells with decreased antibiotic susceptibility due to slow growth and decreased metabolic activity [3–5]. The study of persister cells in *S. aureus* and *Mycobacterium tuberculosis* infections has led to the development a variety of tools to identify and study slow-growing cells. The application of these tools to studies of other bacterial species has led to the discovery that growth of many pathogenic bacterial species results in subpopulations of slow-growing cells which have the potential for decreased antibiotic susceptibility [6,7]. The selective advantage of maintaining small subpopulations with slowed growth may be a consequence of cells having reduced fitness, providing selective advantage to the entire population. Several examples of the impact of population heterogeneity on fitness are discussed in this review, in addition to the evidence that either supports or argues against a true impact of gene expression on bacterial fitness.

Bacterial **virulence factors** have been defined as macromolecules that contribute to disease severity within an animal infection model or within a host cell culture model. Virulence factors were traditionally identified by disrupting a gene of interest and determining the ability of this mutant to replicate within a host [8]. Growth attenuation during animal infection models or under conditions that mimic processes taking place during disease indicate that the protein is likely a virulence factor. The mutant strain can then be rescued or complemented with an intact version

[1]W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe St., Baltimore, MD 21205, USA
[2]Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 150 Harrison Ave., Boston, MA 02111, USA

*Correspondence:
Ralph.Isberg@tufts.edu (R.R. Isberg).

of the gene, and if this restores virulence, then this confirms that the gene supports virulence and encodes a component of a virulence factor. This principle, which was clearly an oversimplification of the disease process, was first termed molecular Koch's postulates by Stanley Falkow in 1988 [8,9], and this approach has had an important impact in determining the factors that support bacterial virulence, which in turn identifies candidates for many different drug and vaccine targets. The surprising aspect of this model, however, is that proteins defined as being important for virulence are sometimes expressed only in a subpopulation of bacteria during disease. With recent advances in imaging technologies, researchers have been able to ask which individual bacterial cells within populations produce virulence factors, allowing a surprising level of heterogeneity to be uncovered within **clonal** bacterial populations [10–14]. These studies have primarily focused on individual genes, using fluorescent reporters to determine which cells produce the virulence factor of interest. The spatial organization of expressing cells compared to non-expressing cells was also surprising, as it suggested that individual cells were sensing different microenvironments during growth in tissue sites. These studies also indicated that **cooperation** within bacterial populations replicating within host tissues may occur, as a consequence of some individuals producing proteins necessary for survival in the host, with others reaping the benefits of **shared public goods** (Figure 1, Key Figure). This review focuses on examples of cooperation within pathogenic bacterial populations and the evidence suggesting that cooperation may be costly for producer cells.

Mechanisms Driving Heterogeneity: Differences in Host Cell Encounters

Within clonal, genetically homogeneous populations, there are two broad mechanisms that bacteria utilize to generate phenotypic heterogeneity. One approach is termed 'bet-hedging', in which a bacterial population expresses many different genes within subsets of a population prior to encountering any stresses, allowing a subset of the population to survive and replicate in response to changes in environmental conditions or sudden stress-inducing insults [15]. Bet-hedging occurs due to stochastic differences in gene expression between individual cells, which leads to **bistable expression** of genes [15–18]. Bistable expression occurs when a

Key Figure

Example of Fitness Cost of a Shared Public Good

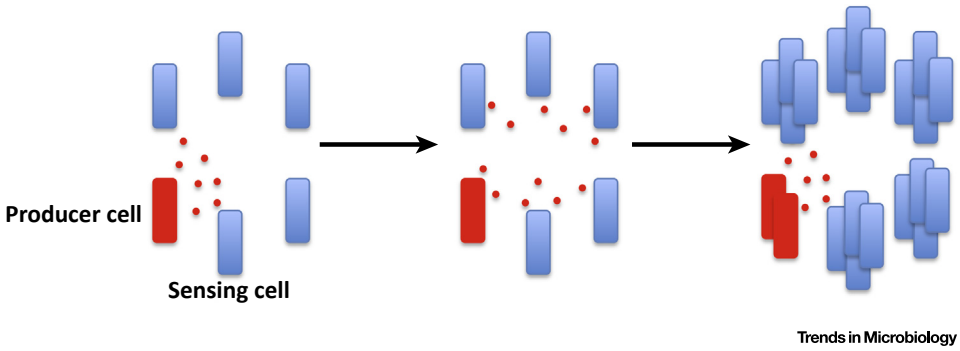

Figure 1. A bacterial producer cell (red rectangle) generates a shared public good (red circles) that promotes the survival and replication of additional bacteria within the population (blue rectangles). Production comes at a fitness cost to the producer cell, so the producer divides only once in the timeframe that sensing cells divide twice.

Glossary

Bistable expression: a population bifurcates expression of a given macromolecule to generate an 'ON'-expressing subpopulation, and an 'OFF' subpopulation that lacks expression. Multiple different mechanisms can lead to this phenotypic output.
Cheater cells: cells that benefit from production of a shared public good (defined below) without the metabolic cost of producing it; typically, cheaters have a mutation that renders the cell unable to produce the gene public good.
Clonal: a population founded by a single, individual cell, resulting in high levels of genetic similarity.
Cooperation: a subpopulation of cells produce a component that is critical for the survival or growth of the population as a whole (shared public good). Production comes at a fitness cost to the producing cells.
Microcolonies: clonal clusters of bacteria surrounded by host inflammation.
Persister cells: bacterial cells that survive following antibiotic treatment, and are typically either slow-growing or nonreplicating, but remain metabolically active.
Shared public good: a product generated by a subset of cells within a population that can benefit all members of the population by promoting survival or replication.
Virulence factor: a macromolecule that is present within a disease-causing strain, and is associated with disease severity within an animal model of infection or in a host cell culture model. Loss of the macromolecule should reduce severity, and restoration of production of the macromolecule should restore disease.

genetic positive feedback loop generates an 'ON', expressing, subpopulation, while other cells within the population remain in the 'OFF', non-expressing state. Phenotypic heterogeneity can also occur when groups of individuals within the population experience microenvironments that are distinct from microenvironments experienced by other individuals [19]. Environmental differences can be caused by the host, or it can be caused by a bacterial subpopulation manipulating the local environment or targeting host cells.

Bacteria may encounter many different types of cells during growth within host organisms. This may result from bacteria moving into new tissue sites, or the nature of the environment could change over time as the host inflammatory response changes the composition of cells that interface with the focus of infection. This interplay has the potential to trigger gene expression patterns in the bacterium specific to the composition of attacking cells. In some cases, such as systemic infection with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), it has been shown that different subsets of bacteria encounter different immune cell subsets within a single organ [13]. Within the spleen, one *Salmonella* population interacts with macrophages, while other populations interact with neutrophils or inflammatory monocytes, resulting in distinct microbial gene expression profiles [13]. There are also high levels of heterogeneity within a single immune cell subset, which can be defined by differences in activation state of individual cells, or if cells are at different points in the differentiation process [20,21]. For example, high levels of phenotypic heterogeneity have been described in macrophage, monocyte, and neutrophil populations [7,21,22]. This leads to differences in cytokine responses within individual host cells, differences in the production of antimicrobials, and differences in the ability of bacteria to replicate in the presence of host cells [23,24]. All of these factors can contribute to the generation of heterogeneity within a bacterial population, and add to the complex environment sensed by bacteria. Heterogeneity within the innate immune cell populations is predicted to have an important impact on overall disease outcome, as these cells are typically the first host cells that a bacterium encounters within the host environment.

Salmonella System: Clear Cooperation, Unclear If There Is a Fitness Cost

S. Typhimurium is a gastrointestinal bacterial pathogen that initiates human disease after ingestion of contaminated food or water. To establish an infectious niche in the intestine, the bacteria must compete with the microbiota. *S.* Typhimurium invades intestinal epithelial cells to promote transcytosis to the basolateral side of the epithelium, where *S.* Typhimurium can replicate within macrophages. Interactions between *S.* Typhimurium and the intestinal epithelial barrier have recently been reviewed in detail [25]. Inflammation and tissue damage at the intestinal epithelium can lead to bloodstream access, particularly in murine models, which transports *S.* Typhimurium into deep tissues such as the spleen, liver, and kidneys, where infection can be very difficult to treat with antibiotics. *S.* Typhimurium can replicate intracellularly within macrophages in these deep tissue sites.

S. Typhimurium expresses two type-III secretion systems (T3SSs), which are needle-like secretion systems that inject bacterial substrates into host cells to support bacterial replication in tissue sites, and are produced by many Gram-negative bacteria. The two *S.* Typhimurium complexes, T3SS-1 (encoded by *Salmonella* pathogenicity island-1, SPI-1) and T3SS-2 (encoded by SPI-2), play roles at distinct phases of the bacterial life cycle. T3SS-1, and the bacterial substrates injected by this system, promote the initial uptake into intestinal cells and characteristic membrane ruffling associated with the entry process [26–28]. T3SS-1 has also been shown to promote inflammation that is critical for the ability of *S.* Typhimurium to colonize the intestinal tract [29–32]. Many of the T3SS-1$^+$ epithelial cell-invading bacteria appear to be

eliminated following uptake by these cells, but the inflammation they initiate is critical for the pathogen to outcompete the microbiota and establish infection [30,33]. Inflammation leads to the production of tetrathionate and ethanolamine which can be utilized by *S.* Typhimurium as an alternative terminal electron acceptor and as a carbon source respectively, leading to a rapid expansion of the *S.* Typhimurium population [29,32]. Inflammation at the intestinal barrier also causes damage to the barrier integrity, allowing *S.* Typhimurium to access the basolateral site of the epithelium, where the bacteria are internalized by macrophages. Following uptake into phagocytic cells, T3SS-2 promotes the intracellular survival and replication of *S.* Typhimurium by preventing phagolysosomal fusion and establishing an intracellular membrane-bound compartment to house replicating bacteria, called the *Salmonella*-containing vacuole (SCV) [34–37].

One of the first clear examples of cooperation within pathogenic bacterial populations came from studies with *S.* Typhimurium, where it was found that broth-grown *S.* Typhimurium expresses T3SS-1 in only a subset of the population [11,12,14,33]. The expression of T3SS-1 in a subset of broth-grown bacteria was consistent with *S.* Typhimurium expressing this system prior to encountering the host environment, with only a subset of bacteria having assembled the complex in the bacterial envelope. This study showed that T3SS-1 expression varied in a stochastic manner, and that expression was bisTable Subsets of *S.* Typhimurium expressing the T3SS-1 were also more likely to be associated with intestinal tissue, raising the possibility that host cells activate T3SS-1 expression [33]. Conversely, it is also possible that stochastic upregulation of T3SS-1 expression promotes association of the bacterial subpopulation with host cells. After this study was published, spatial regulation of T3SS expression was demonstrated in a *Shigella flexneri* model of intestinal infection, although this was due to lowered oxygen tension levels in the intestinal lumen compared to the more oxygenated surface of intestinal cells [38]. Later, host cells were shown to drive heightened T3SS expression in *Yersinia pseudotuberculosis* infection of the spleen, as is discussed below [10]. These studies support the idea that virulence gene expression may be stochastically regulated, but also may be subject to spatial regulation as well.

Individual *S.* Typhimurium cells encounter multiple different environments within host tissues, as some bacterial cells appear to interact with neutrophils and others are internalized by macrophages during systemic infection. This clearly drives heterogeneous gene expression profiles in the bacterial population, but it is unclear if cell-specific responses protect members of the population that do not experience these stresses. These encounters may simply control the outcome of one individual bacterium and its ability to replicate without affecting the entirety of the population, which would mean that this does not represent cooperation. However, work showing that *Salmonella* stimulates an inflammatory response that results in generation of important components for growth is consistent with the idea that a subpopulation drives a host response that profits the entire population [29,32].

In broth culture, it was shown that expression of the T3SS-1 slowed the growth of individual cells, indicating that expression comes at a fitness cost [14]. Expression of T3SS-1 also appears to result in a fitness cost within host tissues, as avirulent mutants with inactivating mutations in the T3SS-1 emerge during *S.* typhimurium replication within tissues [30]. These are called **cheater cells** because they benefit from the costly production of the T3SS-1 in producer cells. The emergence of cheaters within host tissues suggests that T3SS-1 expression came at a fitness cost to individual bacterial cells [30]. Experiments with a TIMER fluorescent protein to detect slow-growing cells did not specifically connect T3SS-1 expression to slowed growth, although the time frame and tissue sites were different in the two studies [39].

Slow-growing cells express rpoS-regulated genes, which are associated with stationary-phase growth, and it is possible that T3SS-1 expression does not exhibit a metabolic cost during transition to stationary phase. Additional studies will be needed to truly show that *Salmonella* cells expressing T3SS-1 are growing more slowly than non-expressing cells within host tissues.

Yersinia: Clear Cooperation without Clear Fitness Benefit of Non-producers

Three *Yersinia* species are pathogenic to humans: *Yersinia enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis*. *Y. enterocolitica* and *Y. pseudotuberculosis* are foodborne pathogens that infect individuals through contaminated food or water. *Y. pestis* is the causative agent of bubonic and pneumonic plague, and is a relatively recent descendant of *Y. pseudotuberculosis*. *Y. pestis* has experienced severe genome reduction through the accumulation of pseudogenes as well as acquired several additional virulence determinants that have led to an altered infection route in which humans (or small mammals) become infected with *Y. pestis* subcutaneously through a bite from an infected flea. *Y. pseudotuberculosis* is the focus of this section of the review since *Yersinia* research in population heterogeneity has focused primarily on *Y. pseudotuberculosis*, and we expect that many of the findings with *Y. pseudotuberculosis* may also apply to the other *Yersinia* species [40].

Y. pseudotuberculosis associates with the small intestinal lumen and passes through specialized cells of the intestinal epithelium, called M cells, which transcytose pathogens and other antigenic particles to the basolateral side of the intestinal epithelium. Specialized lymphoid organs, called Peyer's patches, are located directly under M cells to sense and respond to antigen or pathogenic insults, and *Y. pseudotuberculosis* replicates within these tissues after transit. Movement to the basolateral side of the intestinal epithelium also provides *Y. pseudotuberculosis* access to the lymphatic system, which allows bacteria to access and colonize mesenteric lymph nodes. Bacteria within the intestinal lumen are passed along the intestinal tract into the cecum, where *Y. pseudotuberculosis* can colonize for periods of several weeks [41,42]. In humans, neutrophil recruitment is typically sufficient to limit *Y. pseudotuberculosis* growth within intestinal tissues. However, neutrophils are not sufficient to contain bacterial replication in immunocompromised patients or individuals with haemochromatosis (iron-overload disorder) [43–45]. In the absence of restriction, bacteria from the intestinal lumen can also access the bloodstream and spread systemically to colonize deep tissues, such as the kidneys, liver, and spleen [46–48].

Y. pseudotuberculosis replicates extracellularly within host tissues, and forms replicating **microcolonies** [49]. These small clusters of bacteria are founded by a single bacterium, resulting in a clone in which replicating bacteria are surrounded by recruited neutrophils [10,50–52]. In the mouse model of infection, the neutrophils fail to restrict bacterial growth in the spleen, and the microcolonies continue to grow despite neutrophil and subsequent macrophage recruitment to these sites. In addition, high bacterial loads cannot be contained within the spleen, and eventually the animal will succumb to the infection [53].

Virulence factor expression in *Y. pseudotuberculosis* is tightly regulated by temperature and involves multiple pathways and proteins, which collectively trigger expression of many different virulence genes during a shift to mammalian body temperature. Several recent studies have explored virulence factor expression during *Y. pseudotuberculosis* infection, and have found that expression in a subset of bacterial cells is critical to support the replication of the population as a whole, which defines cooperative behavior.

RovA is considered a master virulence regulator because of its direct impact on virulence-gene regulation by promoting expression of invasin, which is associated with bacterial adhesion and uptake, and its role in promoting expression of YmoA, which regulates temperature-dependent T3SS expression through the LcrF positive regulator. Temperature increases alter RovA conformation, releasing the repressor from DNA, and rendering it susceptible to Lon-mediated proteolysis [54,55]. It was recently shown that RovA acts as a bistable switch, which results in RovA ON and RovA OFF subpopulations of bacteria, and represents a bet-hedging approach for the population prior to encountering the host environment [56]. RovA ON and OFF populations were also observed as bacteria replicate within Peyer's patches and the cecum. Interestingly, altering turnover of RovA attenuated *Y. pseudotuberculosis*, indicating that fine tuning of this switch, and the ability to turn virulence gene expression on and off during infection, is critical for optimal virulence [56]. *Y. pseudotuberculosis* RovA has 100% sequence identity with *Y. pestis* RovA, so it is very likely that RovA regulation results in distinct subpopulations during *Y. pestis* infection as well. RovA is a homolog of the *Salmonella* master regulator, SlyA, which is known to control expression of SPI-2 [55], so it is possible that similar bistability could lead to heterogeneous expression of SPI-2 during animal infections, perhaps being regulated in a manner similar to SPI-1 mentioned above. However, SlyA protein remains stable at mammalian body temperature, suggesting that its regulation would not be temperature dependent, but expression could also be bistable and driven by a different cue.

Additional virulence factors in *Y. pseudotuberculosis* are regulated based on the response of individual bacteria to environmental signals, and the interactions of individual bacterial cells with host cells. A critical virulence factor for *Yersinia*, the plasmid-encoded T3SS, is regulated by both temperature and host cell contact-dependent mechanisms, which is linked to calcium sensing in culture [57,58]. The mechanism that leads to contact-dependent expression still remains unclear, but the interaction of individual *Y. pseudotuberculosis* cells with host cells has been shown to increase T3SS expression in tissue culture models and during bacterial growth within the spleen [10,58]. In the spleen environment, bacterial cells in direct contact with neutrophils express heightened levels of the T3SS [10], allowing this bacterial subpopulation to translocate effector proteins into neutrophils and prevent both phagocytosis and the release of reactive oxygen species [59–62]. Bacteria within microcolonies also sense the presence of nitric oxide (NO), which diffuses towards microcolonies from a layer of macrophages located in a ring outside the neutrophil layer. Sensing of NO upregulates expression of the NO detoxifying gene, *hmp*, which occurs specifically within peripheral cells of the microcolony. Expression of *hmp* at the periphery leads to NO detoxification, which prevents further NO diffusion into the microcolony, and protects the bacterial subsets at the interior of these clusters. Thus, peripheral bacteria produce multiple different virulence factors that protect the interior population, potentially at a fitness cost to the peripheral subpopulation. T3SS expression has been shown to slow the growth of *Y. pseudotuberculosis*, but since all cells express some level of this virulence factor, it is unclear if the heightened levels at the periphery are sufficient to impact growth rates [10,63]. Additionally, constitutive expression of the NsrR regulon, which is expressed in response to NO, has been shown to reduce bacterial fitness, indicating that the response to NO could slow the growth of peripheral bacteria relative to the internal population [10,64]. Both NO and cell contact appear to be sensed by peripheral cells simultaneously, so it is likely that cells on the periphery are a slow-growing subpopulation. Additional work is necessary to determine if this population grows slower relative to interior cells, possibly by using tools generated in other pathogenic bacterial systems, such those used to interrogate *Mycobacterium tuberculosis* growth [65–67].

Mycobacterium tuberculosis: Differences in Growth Rates between Subpopulations without Clear Cooperation

Mycobacterium tuberculosis is a respiratory pathogen that replicates intracellularly within macrophages. Upon inhalation, human alveolar macrophages are the first cells that encounter *M. tuberculosis* within the lung. Bacteria form an intracellular, vacuolar niche within alveolar macrophages, which supports bacterial replication. Replication releases proinflammatory bacterial fragments that are sensed by the host, resulting in the recruitment of additional cells to the site of infection. Lesions develop over time to form granulomas, which contain many different host immune cell types, including both innate and adaptive immune cells. Each lesion in the lung develops independently, and there is a different outcome within each lesion in terms of control of bacterial replication, containment, or release of bacteria into the airways [68,69]. Some lesions become caseous and will contain slowly replicating bacteria for decades. Other lesions will liquefy, releasing bacteria that can be spread via the aerosol route to new human hosts.

In *M. tuberculosis*, the cell division process generates heterogeneity within the bacterial population. Cells divide asymmetrically, which generates intrinsic differences between individuals in bacterial populations prior to a host encounter [65,70]. Interactions within the host, and antimicrobial therapy, can amplify this heterogeneity by selecting for the survival of a subset of the daughter cells. For instance, antibiotics penetrate some lesions better than others, with the consequence that in some lesions there are subsets of bacteria that experience lower antibiotic concentrations, allowing for the emergence of resistant subpopulations among those seeing the lowest doses of drug [71,72]. Host immune pressures also play a critical role in altering the composition of the *M. tuberculosis* population, as vaccinated hosts contain bacteria with slower replication rates than non-vaccinated animals [67]. Production of cytokines, such as interferon γ, and immune cell activation can also result in the production of heterogeneous *M. tuberculosis* populations, as bacterial populations from *Ifnγ*$^{-/-}$ mice have decreased heterogeneity relative to populations from wild-type (WT) mice, based on the relative growth rate of individual cells [65]. Individuals within *M. tuberculosis* populations have heterogeneous growth rates under normal bacteriological growth conditions, based on a ribosomal RNA reporter, but this heterogeneity is amplified in response to stresses, including nutrient limitation, intracellular growth within macrophages, and growth within the mouse lung [65]. A subpopulation of nongrowing, metabolically active cells develops in the presence of IFNγ in the WT mouse lung, and this subpopulation preferentially survives following antibiotic treatment, indicating a potential link between this cell population and the formation of a persistent or chronic infection [65]. Nondividing cells can also go on to replicate when they encounter permissive conditions, consistent with the hypothesis that persisters can re-emerge in the absence of antibiotic pressure [73]. Many mechanisms can lead to the formation of slow-growing, or nongrowing persister cell populations, such as expression of toxin/antitoxin systems or passage through stationary phase into dormancy [3,5,73–76]. It remains unclear which of these pathways leads to the formation of persisters within *M. tuberculosis* granulomas. Based on the described heterogeneity within these lesions, it is likely that the formation of persister subpopulations is a consequence of the complex interplay between a number of factors that control growth and microbial survival.

There are high levels of heterogeneity within *M. tuberculosis* populations during infection, but it remains unclear if the development of distinct subpopulations is required to maintain the infectious state. It is also unclear if there is heterogeneity in virulence factor expression in *M. tuberculosis* populations as seen with *S.* typhimurium and *Y. pseudotuberculosis*. For this reason, it would be particularly important to investigate the regulatory properties of the bacterial

type VII secretion systems that are known to be virulence associated [77–79]. Within the human lung, each individual lesion can develop differently, and it will be interesting to determine the extent of bacterial heterogeneity within distinct lesions in the same tissue. The differences in the developmental outcome of each lesion is likely the result of slightly different host cell–bacterial cell interactions at the earliest phase of infection, again emphasizing the heterogeneity within both the host cell and bacterial populations [66]. Critical to understanding the disease process will be investigating if heterogeneity within a single lesion leads to cooperation between subsets of bacteria located in close spatial proximity to each other. There may be parallels here to *Y. pseudotuberculosis* lesions, although *Y. pseudotuberculosis* remains extracellular and *M. tuberculosis* replicates within macrophages in the center of lesions.

Mechanisms Preventing Heterogeneity

Although bacteria maintain mechanisms to promote the diversity of gene expression profiles of their population as a bet-hedging approach, there are also mechanisms in place to limit diversity. These mechanisms prevent fast-growing mutant cells, such as cheater cells, from taking over the population. During *S.* Typhimurium infection, fast-growing cheater cells emerge with mutations in T3SS-1, but these bacteria are avirulent and cannot infect another host [30]. When avirulent mutants predominate within the population this leads to premature clearance of the infection, thus eliminating the population as a whole. Population stability occurs when the phenotypically avirulent, fast-growing T3SS-1 non-expressing cells predominate within the population [30]. Cheaters also emerge in *Pseudomonas aeruginosa* chronic lung infections during cystic fibrosis (CF) and in bacteriological culture, in which fast-growing mutant cells that lack quorum sensing (*lasR* mutations) utilize the shared goods produced by other members of the population that produce quorum sensing signals [80–83]. *P. aeruginosa* can also police itself by eliminating or reducing the levels of cheater subpopulations [82]. This prevents the emergence of genetic variants within the population, but it is unclear if mechanisms also exist to prevent phenotypic diversity. If a phenotype is energetically costly, then it would only emerge in a slow growing subset, and would not reach a high population density due to its slow replication

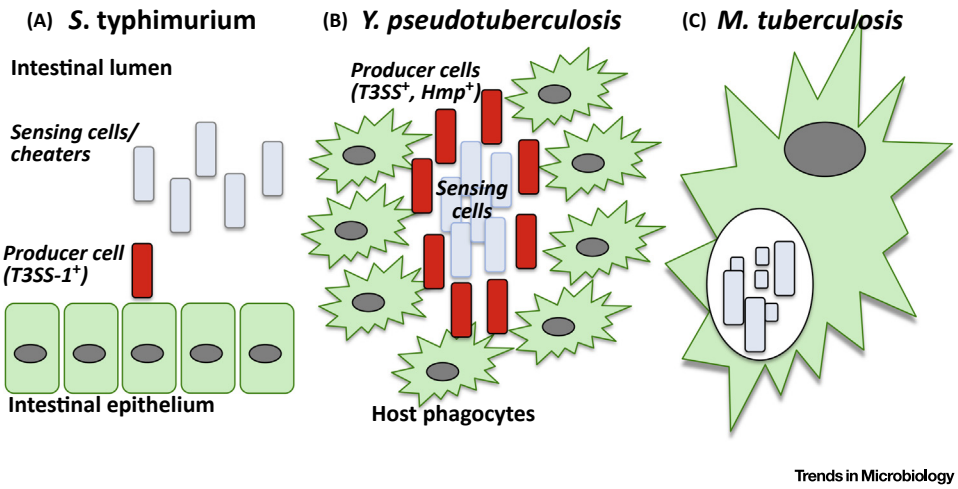

Figure 2. Social Behavior within Host Tissues. (A) *Salmonella enterica* serovar Typhimurium producer cells (red, T3SS-1$^+$) interact with the intestinal epithelium (green) and support the growth of sensing cells and cheaters (blue) within the intestinal lumen. (B) *Yersinia pseudotuberculosis* producer cells (red, T3SS$^+$ Hmp$^+$) interact with host phagocytes (green) to support the growth of sensing cells (blue). (C) *Mycobacterium tuberculosis* generates heterogeneous populations (blue) as it replicates within the phagosomal membrane (black line) of host macrophages (green). T3SS, type III secretion system.

rate. It is possible that slowed growth, and the ability to switch between different phenotypes, may allow bacteria to phenotypically diversify at very little cost to the overall population.

Concluding Remarks and Future Perspectives

The presence of a heterogeneous bacterial population on its own does not necessarily mean that bacteria are cooperating within this population. To truly show that cooperation exists, one subset of the population must rely on another subset to support its growth (Figure 2). The examples we have highlighted above demonstrate that this occurs with *S.* typhimurium and *Y. pseudotuberculosis* populations, and possibly with *P. aeruginosa* in the CF lung, but there will likely be many more examples of this as we continue to explore interactions between individual cells within bacterial populations.

One additional component of this interaction is determining whether or not the production of a given factor is costly for the individual producer cell. There are clearly subsets of slow-growing cells in *S.* typhimurium and *M. tuberculosis* populations, but it has been difficult to link slowed growth with virulence factor production during growth in host tissues. It is possible that virulence factor production elicits a more subtle fitness cost, and the current methods for detecting slow growth are not sensitive enough to detect this small change (see Outstanding Questions). The methods for detecting differences in growth rates have recently been reviewed elsewhere [19,84].

This review has focused on examples of cooperation within a single, related population, but there are also many examples of cooperation between bacterial populations of different species. These cooperative events occur frequently in the environmental communities, in which interactions are critical to form biofilm structures, or to stabilize multispecies communities in general. Looking to future work regarding understanding how interactions between individual family members contribute to disease, recent advances in imaging technologies and strategies for marking individuals in the population will be critical for future advances. These include using fluorescent reporters and CRISPR-based systems that can allow bacterial cell division over time to be followed as well as growth rates to be determined [85].

Outstanding Questions

Does virulence factor expression truly impact fitness of individual, expressing, cells?

Can we detect differences in replication or cell division rates within virulence-factor-expressing bacteria?

Are the mechanisms driving cooperative virulence also controlling the efficiency of persister cell formation within host tissues? Are multiple pathways simultaneously contributing?

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