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Opinion

The macrophage–bacterium mismatch in persister formation

Iris Dadole^{1,2}, Didier Blaha^{1,2}, and Nicolas Personnic ^{1,2,*}

Many pathogens are hard to eradicate, even in the absence of genetically detectable antimicrobial resistance mechanisms and despite proven antibiotic susceptibility. The fraction of clonal bacteria that temporarily elude effective antibiotic treatments is commonly known as ‘antibiotic persisters.’ Over the past decade, there has been a growing body of research highlighting the pivotal role played by the cellular host in the development of persisters. In parallel, this research has also sought to elucidate the molecular mechanisms underlying the formation of intracellular antibiotic persisters and has demonstrated a prominent role for the bacterial stress response. However, questions remain regarding the conditions leading to the formation of stress-induced persisters among a clonal population of intracellular bacteria and despite an ostensibly uniform environment. In this opinion, following a brief review of the current state of knowledge regarding intracellular antibiotic persisters, we explore the ways in which macrophage functional heterogeneity and bacterial phenotypic heterogeneity may contribute to the emergence of these persisters. We propose that the degree of mismatch between the macrophage permissiveness and the bacterial preparedness to invade and thrive intracellularly may explain the formation of stress-induced nonreplicating intracellular persisters.

Intracellular persister formation intertwines with bacterial stress responses

Macrophages are major immune cells that play an essential role in sensing and destroying invading microorganisms. Paradoxically, despite serving as the initial line of defense to control bacterial burden, most pathogenic bacteria not only survive within these cells but also undergo replication. Recent research has unveiled an intriguing new aspect of the macrophage–bacteria interactions, demonstrating that these immune cells also act as a niche for the formation of bacterial persisters. A persister is a nonreplicating bacterium, found among a clonal dividing population, that transiently evades the bactericidal activity of antibiotics [1,2]. The formation of intracellular persisters has been experimentally documented for major bacterial pathogens, including *Staphylococcus aureus* [3–5], *Mycobacterium tuberculosis* [6,7], *Escherichia coli* [8,9], *Salmonella enterica* [10,11], *Pseudomonas* spp. [12], *Listeria monocytogenes* [13], *Legionella pneumophila* [14], *Burkholderia pseudomallei* [15], *Yersinia pseudo-tuberculosis* [16], *Streptococcus pneumoniae* [17], and *Brucella abortus* [18]. Although most of the studies have focused on persisters emerging rapidly after phagocytic uptake, recent research has also identified the occurrence of *de novo* persister formation during the pathogen’s intracellular growth [8,19].

Once internalized by macrophages, both **host defenses** (see [Glossary](#)) and bacterial stress responses drive the formation of intracellular persisters. Indeed, they are strongly intertwined with the bacterial resilience to various antibiotics with distinct modes of action, such as β -lactams, fluoroquinolones, aminoglycosides, and macrolides [2]. The bacterial stress responses involved in persister formation include the **stringent response** [3,10] and the acid stress response

Highlights

Intracellular antibiotic persisters represent a fraction of the bacteria that are particularly exposed to host-derived stressors and primarily emerging in macrophages.

The functional heterogeneity of macrophages leads to variability in the stress exposure and intensity experienced by the internalized bacteria.

A pathogen’s maladaptation to intracellular conditions results in increased stress exposure and stress intensity.

Antibiotic persister formation stems from a mismatch between the host defense mechanisms and the individual bacterial ability to cope with or soften them.

¹CIRI - Centre International de Recherche en Infectiologie, CNRS, INSERM, Ecole Normale Supérieure de Lyon, Université Claude Bernard Lyon 1, Lyon, France

²Group Persistence and single-cell dynamics of respiratory pathogens, CIRI, Lyon, France

*Correspondence:
nicolas.personnic@cnsr.fr
(N. Personnic).



[7,10] to **nutrient deprivation** and phagosome acidification, respectively. Additionally, there is the **oxidative stress response** to reactive oxygen or nitrogen species (ROS or RNS, respectively), such as nitric oxide (NO), and peroxyxynitrite, a reactive byproduct of ROS/RNS [4,20–23]. Furthermore, there is the **SOS response**, which is activated in the face of genotoxic attacks [8]. Last, bacterial **quorum sensing**, which orchestrates bacterial responses to a diverse array of stresses, also contributes to the emergence of intracellular persisters [14]. The interplay between host defenses, stress responses, and antibiotic persistence is vividly demonstrated in the context of *S. aureus* intracellular infection. Following bacterial uptake by macrophages, the activation of the stringent response or of the cell-wall stress regulon drive *S. aureus* growth arrest and tolerance to both β -lactams and aminoglycosides and possibly to macrolides [3]. In addition, the macrophage oxidative burst releases a cocktail of ROS and RNS, poisoning the *S. aureus* tricarboxylic acid (TCA) cycle by inactivating aconitase. Collapse of the bacterial TCA cycle reduces the production of ATP, reducing the killing activity of the antibiotic rifampicin [21].

A thought-provoking question arises: amid the apparent uniformity within both a clonal bacterial population and their cellular hosts, how do host-derived stressors and bacterial stress responses affect only a fraction of the intracellular bacterial population, leading to the emergence of a subpopulation of stress-induced nonreplicating antibiotic persisters?

In the subsequent sections of this opinion piece, we delve into this inquiry, shedding light on the crucial role played by the intrinsic and regulated heterogeneity of the biological processes at play during the infection of a macrophage.

The functional heterogeneity of macrophages leads to variability in the stress exposure and intensity experienced by bacteria

Coexistence of macrophage subpopulations that exert different pressures on invading bacteria

Macrophages have a central role as the primary cellular niche for persister formation [2]. It is conventionally believed that macrophages exist in a nonprogrammed, neutral state until stimuli dictate their functional phenotype as either type 1 (proinflammatory) or type 2 (anti-inflammatory) activated macrophages (**M1/M2 polarization** paradigm). Recent data support a more nuanced model with high plasticity in cellular states and functional heterogeneity of otherwise homogeneous macrophages [24–28]. Such cellular heterogeneity may diversify the infection outcome for each individual macrophage. A consequence of this would be the emergence of intracellular persisters that experience higher exposure to stressors and/or heightened stress intensity as compared with their kin (Figure 1A,B).

Supporting evidence is provided by *M. tuberculosis* infections [29–31]. Combining single-cell RNA sequencing with a bacterial reporter strain whose fluorescence expression correlates with the amount of environmental stress sensed by *M. tuberculosis*, Pisu *et al.* simultaneously acquired both the host macrophage transcriptome and the associated bacterial fitness phenotype in individual infected macrophages [31]. This approach enabled the identification of coexisting subpopulations of monocyte-derived interstitial macrophages and alveolar macrophages, with distinct expression of the **CD11c** surface marker, each eliciting divergent bacterial stress response intensities. Among these cell subpopulations, the so-called interstitial macrophage subset 3 (IM-3) exhibits a marked M1 inflammatory response with, notably, a high production of NO. The increase in NO results from heightened expression of the inducible NO synthase (iNOS), the absence of the Nfr2-dependent inactivation of the **Toll-like receptor 4 (TLR4)-dependent signaling**, and an upregulation of Slc7a2, a cationic amino acid transporter involved in arginine uptake and necessary for NO production. In addition to NO, IM-3 macrophages produce other ROS or RNS and actively remove iron from the phagolysosome. Therefore, IM-3-associated bacteria trigger various stress

Glossary

- Aging:** a physical system exhibits ‘aging’ if its relaxation after a transient external perturbation depends not only on its macroscopic state at the end of the perturbation but also on the conditions that led to this state.
- Bet-hedging strategy:** where a preexisting diversity of cell states allows rapid population adaptation to an unpredictable environmental change.
- CD11c:** cell surface glycoprotein involved in immune responses and commonly used as a marker in immunophenotyping.
- Collective information coding and transfer:** a diverse ensemble of individuals enables the population to encode and transmit complex information.
- Crowd control:** rare individuals with capacity to respond to perturbation emit local signals that coordinate population behavior.
- CXCL2:** a chemokine contributing to the inflammatory response.
- Defensive virulence program:** term to describe a set of nonaggressive strategies employed to facilitate the pathogen’s ability to invade and replicate. It is mostly based on the production of factors to increase bacterial stress tolerance (e.g., detoxification) and/or the ability to evade host immune surveillance.
- Effectors:** specific proteins produced by pathogens that contribute to the manipulation of host cell processes during infection, often via specific interactions or enzymatic activities.
- Fate plasticity and priming:** uncorrelated, subthreshold fluctuations in regulators of cell fates create subpopulations of cells primed for multiple fate decisions.
- Feedback loops:** regulatory mechanism that can either amplify or dampen the noise in bacterial gene expression. A positive feedback loop amplifies the noise. In this scenario, the expression of a gene product positively regulates its own production. Hence, small stochastic fluctuations in gene expression can be reinforced, leading to sustained expression. A negative feedback loop dampens the noise as the gene product negatively regulates its own production. Complex systems can harbor assembly of positive and negative feedback loops to fine-tune the noise in gene expression.

responses, such as DosR regulon, Fe–S cluster biogenesis system, redox reaction, sulfatases, the NO and ROS detoxification (ergothioneine pathway), and most genes involved in iron acquisition. Such a mosaic of stress responses supports the mycobacterial survival upon exposure to the standard antitubercular drugs isoniazid and rifampicin [7,31]. The coexistence of distinct and pre-existing macrophage identities exerting different levels of stress and contributing to the emergence of persisters was also documented for *S. enterica*. The macrophages harboring persisters displayed an intermediate profile between the M1 and M2 phenotypes. By contrast, macrophages containing replicative bacteria, susceptible to antibiotics, had a clear anti-inflammatory M2 state [32,33].

Fluctuations in host-derived pressure at the single-macrophage level

Preexisting subpopulations of macrophages with distinct identities may account for most of the intermacrophage heterogeneity in bacterial exposure to host-derived stressors or stress intensity upon internalization. However, we cannot rule out that variations occurring both at the single-cell and subcellular levels may introduce subtle yet impactful variations in the microbial environment. These changes could influence the formation of pathogen subpopulations and their response to antimicrobial agents. At the cellular level, the expression of genes involved in the antimicrobial defense mechanisms may be essentially stochastic, occurring in bursts separated by silent intervals of variable duration (Figure 1C). For example, at the single-macrophage level, iNOS expression fluctuates over time and is independent of infection or activation by proinflammatory cytokines [34]. These iNOS fluctuations cause increased host pressure in successive waves, the frequency and intensity of which may favor a transient bacterial specialization into antibiotic persisters. Variations in iNOS activity are further increased by the heterogeneous downregulation of its production. This may explain the heterogeneous exit from the persister state observed for intracellular *S. enterica* [23]. At the subcellular level, phagosomal heterogeneity may also exert alternative pressure on bacteria, leading to the coexistence of intracellular antibiotic persisters and susceptible bacteria in the same macrophage (Figure 1D). For instance, varying subcellular membrane trafficking patterns introduce subtle variations in the composition and pH of the *M. tuberculosis* vacuolar compartments [35]. These variations generate many pathogen subpopulations with a gradient of redox potential of the major mycobacterial antioxidant mycothiol (MSH), a functional analog to glutathione. The associated remodeling of sulfur metabolism eventually increases tolerance to the antimycobacterial drug isoniazid [7]. The phagosomes also restrict nutrient supply, such as purine [11], inorganic phosphate [36], or the ions Mg^{2+} and Zn^{2+} , in a heterogeneous manner [37]. Such phagosome-to-phagosome variations may influence the formation, or not, of intracellular *S. enterica* persisters [11]. Finally, variability in the onset of hypochlorous acid production among phagosomes, identified in neutrophils, may apply to macrophages and would explain the coexistence of persister and non-persister bacteria [38,39]. Hence, fluctuations in the host pressure at the cellular and subcellular levels may explain the different fates of individual bacteria within the same macrophage.

Origin of variations in macrophage defense mechanisms

The significance of functional variations among individual mammalian macrophages is being increasingly recognized. Macrophage subpopulations are largely defined by the reversible diversity of their transcriptional profiles. It is not clear how heterogeneous macrophage subpopulations are generated. Cell-to-cell variations are rooted both at the gene transcription level [27] and at the epigenetic level [27,31]. For instance, coexistence of $CXCL2^+$ proinflammatory and $CXCL2^-$ anti-inflammatory alveolar macrophages are determined by an increased expression of the proinflammatory transcription factors Jun and Fos and an increased chromatin accessibility of the anti-inflammatory C1q loci, respectively [27].

At the single-cell level, molecular variation may play a critical role in population-level functionality as it enables the emergence of collective behaviors that would otherwise be inaccessible.

Host defenses: the macrophage arsenal includes (i) the oxidative burst (NADPH oxidase-dependent ROS production; iNOS-dependent RNS production) and (ii) the nonoxidative pathway spanning nutrient deprivation, phagosomal maturation and acidification, autophagy, or antimicrobial peptides or GTPases, such as guanylate binding protein family members.

Macrophage polarization: reversible process whereby macrophages mount a specific functional response to different pathophysiological conditions and surrounding microenvironments (e.g., cytokines). M1 macrophages produce reactive oxygen and nitrogen intermediates, as well as antimicrobial peptides. M2 macrophages have important roles in wound healing and fibrosis and are strongly anti-inflammatory. They also counteract the classical M1 macrophage response.

Noise: stochastic fluctuation in gene product levels due to random processes during cellular activities such as transcription and translation. Essential genes have lower noise, whereas those involved in stress adaptation or regulated by specific factors such as H-NS exhibit higher noise. This correlation indicates that specific functions and regulatory networks are linked to increased gene expression noise.

Nutrient deprivation: active mechanisms to limit the availability of essential nutrients inside the phagosome.

Offensive virulence program: term to describe a set of aggressive strategies employed to facilitate the ability of the pathogen to invade and replicate. It is mostly based on the translocation of effector proteins that exquisitely hijack many host cell functions.

Quorum sensing: interbacterial communication system based on cell density.

Regulatory motifs: nucleotide sequence controlling the degree of noise of a gene. In the promoter, it affects the affinity of transcription factor/RNA polymerase binding. At the ribosome binding site, it modulates translation efficiency. A heritable trait that may evolve.

Response distribution: cell-to-cell variation in binary decisions that allows a fractional population response.

Secretion systems: bacterial nanomachines delivering proteins across the envelope and the host cell membranes. They play crucial roles in bacterial pathogenesis, notably by facilitating the secretion of effectors.

These behaviors include **bet-hedging strategy**, **response distribution**, **fate plasticity and priming**, **collective information coding and transfer**, and **crowd control** [40]. Experimental validation of such phenomena and the associated functional consequences remain scarce and largely concern cultivated epithelial cells [41,42]. Using high-resolution multiplexing technology based on iterative staining and elution of antibodies, Kramer *et al.* [42] recently explored the heterogeneity of epidermal growth factor (EGF) signaling responses among individual human epithelial cells. Remarkably, they unveiled that, at a single-cell level, the cellular state (cell cycle, local cell density, cell spreading, abundance/localization of transcriptional regulators, etc.) had a stronger effect on these responses than changes in growth factor concentration. In other words, cells can sense and interpret a range of signal strengths and combine that information with their internal state to make cellular state dependent decisions. It is tempting to speculate that similar mechanisms are at work in immune cells such as macrophages. By integrating information about the cellular state into the decision-making process, macrophages may exhibit extensive cell-to-cell variations in their functionalities and exert distinct pressures on invading bacteria (Figure 1B). In that regard, recent exploratory work has probed single-cell macrophage activation in response to TLR4 stimulation by lipopolysaccharide (LPS). Interestingly, single-cell heterogeneity in interleukin (IL)-10 and tumor necrosis factor (TNF)- α production, controlled at least partly by cell density, was observed [43].

Hence, it is likely that each individual macrophage possesses a preexisting program. This program is constrained by epigenetic imprinting and **noise**, which are further fine-tuned by intrinsic factors such as cellular state and extrinsic cues such as evolving cytokines and the cellular environment. Together, these factors determine the spectrum of activities for each macrophage. For phagocytosed bacteria, it means different host pressures and the coexistence of stress-induced antibiotic persisters and antibiotic susceptible counterparts.

The bacterial phenotypic heterogeneity generates individual bacteria with distinct performance to cope with or soften host pressure

Stress-induced antibiotic persisters and the underachiever hypothesis

In our prior exploration, we delved into how subtle distinctions among seemingly identical macrophages could result in varied exposure of internalized isogenic bacteria to the host-derived stressors or stress intensity. Now, we pose the question whether the bacteria, in turn, influence their own destiny as intracellular persisters. We scrutinize this query through the lens of an underachiever hypothesis.

Bacteria are members of large microbial communities, the functionality of which lies in the ability of individual bacteria to specialize in performing different tasks [44]. During the infection process, a robust clonal pathogen expansion leads to a genetically identical population of bacteria. Bacterial specialization then occurs as the result of phenotypic heterogeneity (i.e., the transient expression of alternative phenotypes to improve the performance of the entire isogenic population) [45]. Recent research has explored the variability of individual isogenic bacteria in terms of their molecular composition, investigating how it shapes their individual performance in performing specific tasks. This research ultimately investigates the distribution of phenotypes as a potential component of the bet-hedging strategy. In this strategy, different phenotypic variants adapt to various environmental conditions, collectively enhancing the overall functional performance of the population within complex systems [46]. In relation to the topic of interest here and considering the central role of the host-derived stress and the bacterial stress responses in intracellular antibiotic persistence, we propose that these intracellular persisters embody individuals maladapted to macrophage invasion and therefore overwhelmed by the host cell-derived stress. Underachievers thrive better in other tasks (e.g., antibiotic survival) or niches (e.g., extracellular) crucial to the life

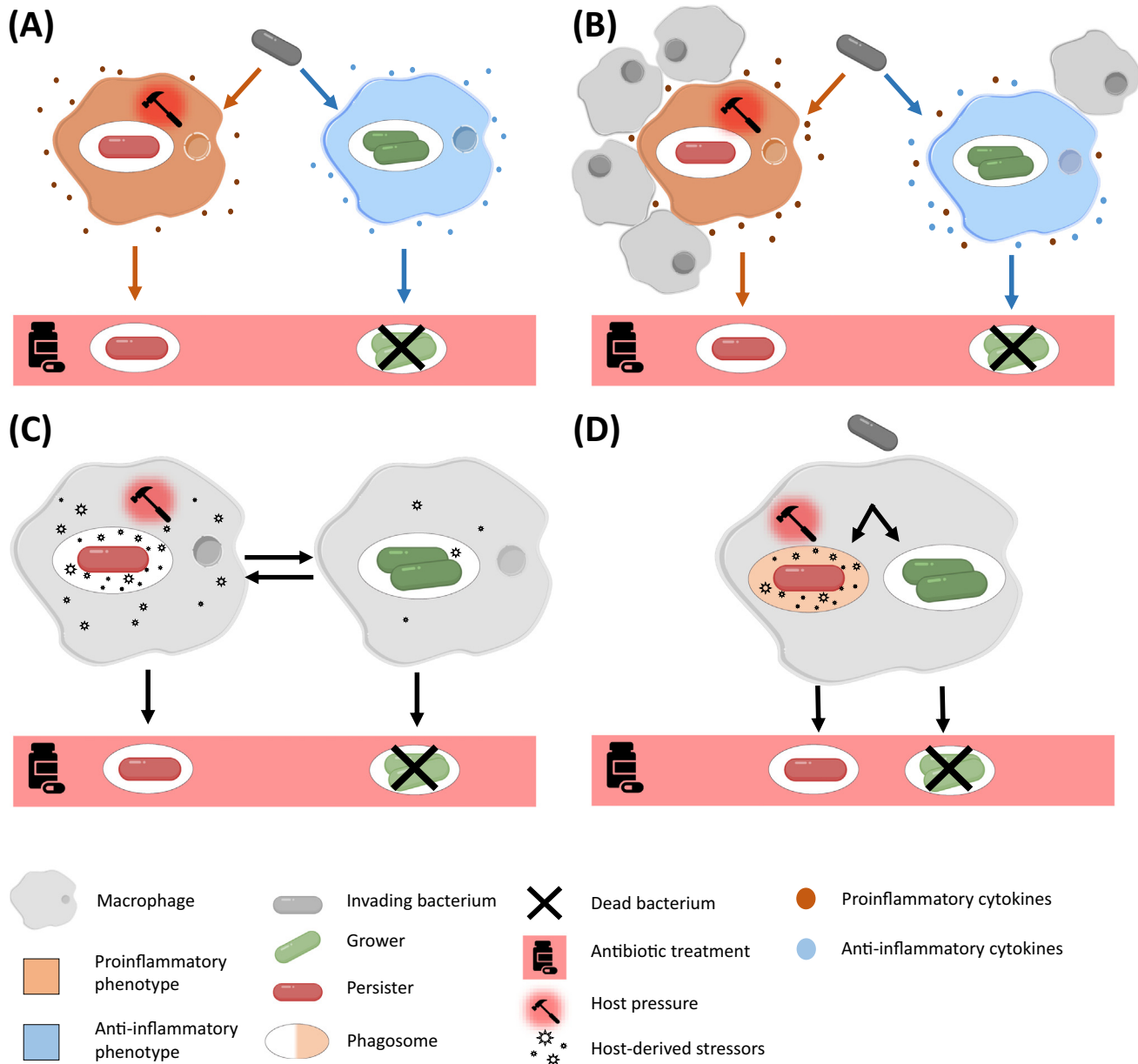
SOS response: global response to DNA damage associated with growth arrest, DNA repair, and mutagenesis.

Stringent response: response to nutrient limitation and various other stresses. Mediated by the alarmone guanosine pentaphosphate ([pppGpp]) to reduce bacterial growth and metabolism.

Toll-like receptor 4 (TLR4)-dependent PRR signaling: PRR found on the surface of macrophages. TLR4 recognizes bacterial LPS and mediates the macrophage proinflammatory response to infection.

Two-component system: bacterial phosphorelay that allows bacteria to sense and respond to changes in their environment.

Virulence program: coordinated set of molecular mechanisms employed by pathogenic bacteria to enhance their ability to invade a host. In this opinion, we discriminate between the defensive and offensive virulence programs.



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Figure 1. Macrophage functional heterogeneity generates distinct stress exposure and stress intensity. Macrophages exert degrees of stress, giving rise to persisters or susceptible kin. (A and B) Coexistence of macrophage subpopulations that exert different pressures on invading bacteria. (A) **Macrophage polarization** states and (B) cellular state dependent decisions (e.g., density) may explain alternative antimicrobial pressure between host cells. (C and D) Fluctuations in host-derived pressure at the single-macrophage level. (C) Stress exposure/intensity varying over time (e.g., iNOS expression) at the single-cell level and (D) at the subcellular level (i.e., phagosome heterogeneity). Abbreviation: iNOS, inducible nitric oxide synthase.

cycle of the pathogen. We find compelling support for this concept with the human pathogen *L. pneumophila*. The heterogeneous activation of the *Legionella* α -hydroxyketone LAI-1-dependent signaling pathways differentially primes individual bacteria to grow either within infected phagocytes (LAI-1 'OFF' individuals) or in extracellular biofilms (LAI-1 'ON' individuals). However, when there is a mismatch between the initial priming and the actual environment, such as the invasion of a host cell

by an LAI-1 'ON' bacterium, the formation of a 'maladapted' intracellular bacterium occurs. This bacterium experiences excessive stress, giving rise to an antibiotic persister [14,47].

In the next sections, we explore how the maladaptation of the pathogen, primarily in the **offensive** and **defensive virulence programs**, may result in increased exposure to host-derived stressors or heightened stress intensity for some intracellular individuals.

Bacterial underachievers potentiate host pressure

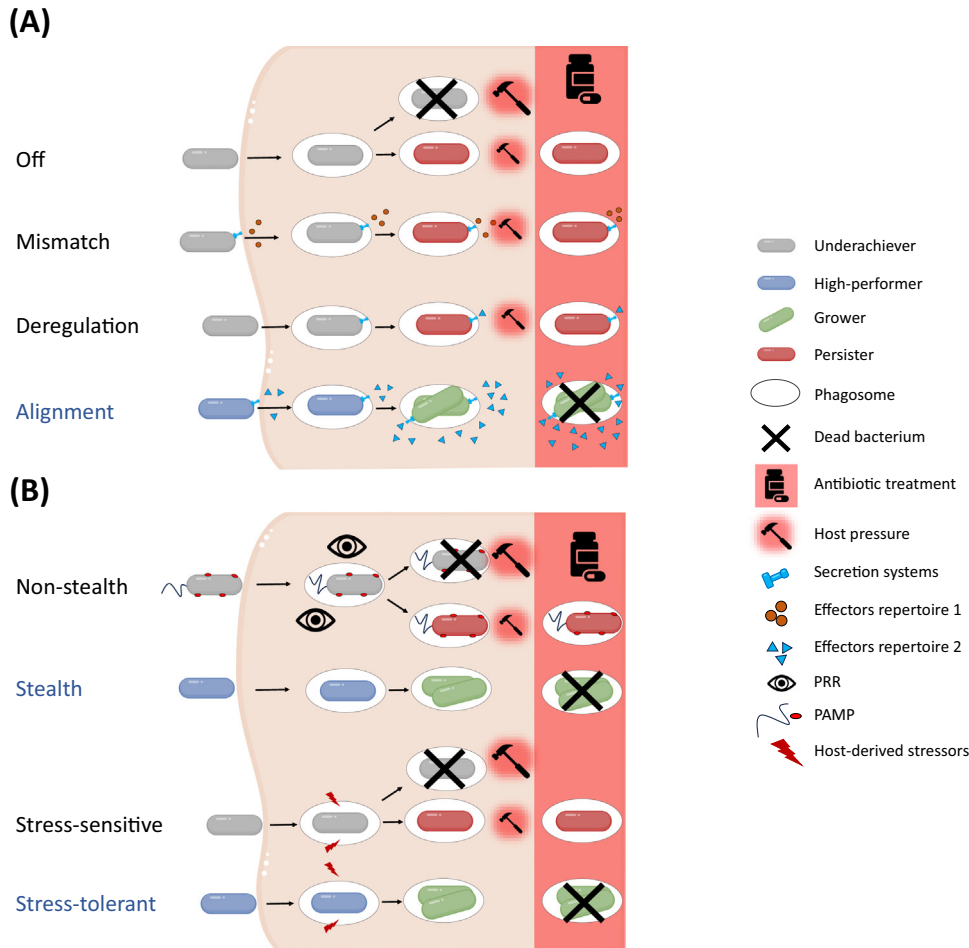
Pathogenic bacteria have evolved multiple strategies to actively dampen host-derived bactericidal attacks. These strategies can take the form of an elaborated offensive virulence program that involves a portfolio of **effectors** that are translocated in a timely manner into the host cell to hijack cellular functions. Across species, both the production of effectors [13,14,33,48,49] and **their secretion systems** [12,50–54] exhibit cell-to-cell variability. It is reasonable to assume that, if the **virulence program** is not executed adequately and/or not timely, the level of stress experienced by an internalized individual will rise, increasing the chance of it becoming an intracellular persister (Figure 2A). An example is provided by *S. enterica*. Within its macrophage host, the bacterium exploits a type 3 secretion system encoded by the *Salmonella* pathogenicity islands 2 (SPI-2) to translocate the effector SteE. SteE sustains the activation of STAT3, a master transcriptional regulator that redirects the macrophage toward a more permissive M2 anti-inflammatory phenotype [55]. However, the macrophages hosting an *S. enterica* antibiotic persister exhibit an altered immune program with a profile between the M1 proinflammatory and M2 anti-inflammatory polarization states [32]. This apparent partial loss of control of host polarization may reflect the lower ability of the persisters to produce and translocate effectors in sufficient amounts (as documented in Figure 1C and in Figure S4a in [33]). This maladaptation could be caused by the fact that, at a given moment, not all *S. enterica* assemble the SPI-2 needle on their surface [52]. This maladaptation is compounded by the remarkably low intracellular bacterial load induced by the persisters, thereby diminishing the injection of effectors into host cells as compared with macrophages hosting actively replicating individuals.

Ensuring the timely deployment of appropriate effectors is crucial for a well-adapted virulence program. *L. pneumophila* stands out as an interesting model in this regard. The bacterium uses a vast arsenal of over 400 effectors to disrupt various host cell functions. The effector translocation process is intricately regulated and coordinated by c-di-GMP, ensuring precise effector activity at specific times [56]. Therefore, any glitches in the production or translocation of effectors may lead to ineffective management of host pressure. It is thus interesting to note that *L. pneumophila* intracellular persisters exhibit both a distinct set of effectors and an altered c-di-GMP metabolism compared with their actively multiplying counterparts [14].

Macrophages possess pattern recognition receptors (PRRs), including TLRs and NOD-like receptors, which identify pathogen-associated molecular patterns (PAMPs) and initiate macrophage defense mechanisms. Consequently, variations in the ability of the bacterium to evade detection may intensify or dampen the host defense program (Figure 2B). In agreement, seemingly identical invading *S. enterica* drive radically different host immune responses by modifying the LPS in a subset of bacteria [57]. Additionally, enteropathogenic *E. coli* heterogeneously expresses bundle-forming pili, rendering the producer more exposed to detection by the immune system [54]. Finally, the flagellum is another PAMP that frequently displays a bistable production during an infection, as observed in *S. enterica* [58], *L. pneumophila* [19,47], and *P. aeruginosa* [59].

Bacterial underachievers struggle with host-derived defenses

In recent years, quantitative measurements at the single-cell/molecular level have unveiled extensive bacterium-to-bacterium variations in the activation of one or multiple costly stress responses



Trends in Microbiology

Figure 2. Bacterial phenotypic heterogeneity generates individual bacteria with distinct performance to cope with or soften host pressure. Bacterial phenotypic heterogeneity allows the reversible specialization of some individuals among an isogenic population of bacteria. Mismatch between the task to perform (i.e., successfully invading and replicating within a macrophage) and the task being performed produces a bacterial underachiever overwhelmed by host pressure. In the right conditions, the underachiever can specialize as an antibiotic persister; however, when the mismatch is too great, bacterial death is inevitable. Various scenarios are proposed. (A) The ability to execute an offensive virulence program in an adequate and/or timely manner varies from bacterium to bacterium. The translocation of effector proteins into the host cell is exemplified. Off: lack of effectors and/or translocation machinery. Mismatch: the biological effect of the translocated effector repertoire is not appropriate to the host cell context. Deregulation: inefficient or untimely translocation of necessary effector repertoire. Alignment: efficient and timely translocation of required effector repertoire. (B) Bacterium-to-bacterium variations in the defensive virulence program. Heterogeneity in the ability of a bacterium to avoid immunological detection (stealth versus non-stealth bacteria) and bacterial stress tolerance is depicted. Abbreviations: PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor.

[60]. Such heterogeneity in the defensive virulence program manages the energy expenditure at the population level without compromising performance. Yet, upon phagocytosis, these variations may result in an increased vulnerability of a few maladapted individuals confronted with an acute, although not necessarily lethal, defensive host program (Figure 2B).

The phagocytic vacuole is immediately acidified by the macrophage to neutralize the engulfed bacteria. Naturally, all bacterial species possess one or multiple acid resistance systems. The

Gad system in particular is highly conserved and is heterogeneously activated, thereby preemptively generating a range of acid-resistant and acid-susceptible individuals in *E. coli* [61]. The latter may constitute a subpopulation of intravacuolar persisters induced by acid stress, where the decreased intracytosolic pH may be offset by an upregulated expression of multidrug efflux pumps [62,63]. On the flip side, for *S. enterica*, performing too well when it comes to protecting the bacterium from an acidic environment may be counterproductive. Indeed, increased cytosolic pH inactivates the SsrA/B **two-component system** that plays a crucial role in regulating SPI-2 gene expression [64]. Consequently, high performers in terms of acid stress resistance may display low performance regarding effector injection into the host. This diminished capacity to actively alleviate the host pressure and to tailor an intracellular replicative niche could promote the adoption of a persister phenotype.

Heterogeneity in the bacterial preparedness to deal with the host-derived stressors also applies to other stresses and bacterial responses. Carbon source limitation is a common stress in the phagosome [65]. Professional pathogens can hijack the host's cellular functions to bypass host nutrient deprivation mechanisms. Nonetheless, extensive interbacterial variations in the ability to recycle macromolecules [66] and the coexistence of diverse metabolic strategies (e.g., respiration versus fermentation) may affect cell growth following temporary carbon starvation, leading, in turn, to the formation of non-growing bacteria [67], a hallmark for antibiotic persistence. Metabolic maladaptation may thus contribute to the pool of intramacrophagic persisters.

In the phagosome, exposure to ROS and RNS damages bacterial DNA. In *E. coli*, prior to any genotoxic stress, the SOS response master regulator, LexA [68], displays substantial cell-to-cell variation in abundance. This triggers spontaneous SOS pulses throughout the population that vary in intensity and frequency and creates a broad spectrum of isogenic individuals more or less prone to anticipate and resolve DNA damage [69]. Such a mixed preexisting population is likely to perform heterogeneously once engulfed in a phagosome. On the one hand, non-SOS-primed bacteria (LexA^{High}, low performers) may accumulate DNA damage for extended periods of time, giving rise to acute oxidative stress-induced persisters. On the other hand, SOS-primed bacteria (LexA^{Low}, high performers) may accumulate less DNA damage over shorter durations, facilitating swift recovery from stress and fast resumption of growth [22,70]. Interestingly, in rare cases, LexA abundance falls below a critical threshold, instigating an abrupt SOS response linked to bacterial growth arrest [69]. It is thus conceivable that an apparent improved capability for DNA damage repair may well result in a diminished capacity to colonize a host, thereby fostering the development of persisters regardless of the stress encountered in the phagosome.

Molecular constraints and cellular context determine bacterial performance

Performance to achieve a specific task varies extensively across an isogenic bacterial population. Regarding cellular infection, the occurrence of high and low performers as defined earlier likely is rooted in the molecular constraint or the bacterial cell context that renders bacterial functions noisier.

Bacterium-to-bacterium variations are primarily fueled by the stochastic nature of the biochemical reactions underlying gene expression, from mRNA transcription to translation and protein degradation. This is particularly true for genes involved in the stress responses. For instance, stochastic self-cleavage of the SOS repressor LexA occurs among a clonal population of *E. coli*. The consequent cell-to-cell variation in LexA abundance and thus, in the SOS response regulon impacts bacterial preparedness to genotoxic stress [69]. Furthermore, **regulatory motifs** (DNA/RNA binding sequences) as well as the architecture of regulatory circuits (positive/negative **feedback loops**) have evolved to control (i) the extent of noise in gene expression and (ii) the generation of

sustained oscillations resulting in cell fate decision [71]. These mechanisms explain the coexistence of virulent and avirulent subpopulations among isogenic *S. enterica*. For instance, occasional spurious transcription initiation eludes Rho termination and dislodges the gene silencing protein H-NS from its path. This action opens a short window during which the master regulator for virulence, *hilD*, is expressed. Strong positive autoregulation of *hilD* locks the system in a positive feedback loop, turning transient gene expression into the stable expression of a virulent phenotype [72]. Similar mechanisms may account for the bistable activity observed in Gad-mediated acid stress resistance (*E. coli*) [73], as well as the *phoPQ* (*S. enterica*) and *perABC* (enteropathogenic *E. coli*) regulons, which govern different bacterial envelope immunogenic potencies [54,57].

Regulated molecular stochasticity generates bacterial diversity and offers a convenient explanation for the coexistence of high and low performers regarding the infection process. Yet, asymmetry in the pathogen developmental program may be another contributor in both the early and late stages of macrophage infection. Bacterial asymmetry corresponds to the partitioning of cellular components during cell division to produce two daughter cells that exhibit distinct regulatory networks and may therefore perform differently when facing the host defenses. For instance, *M. tuberculosis* sequesters irreversibly oxidized proteins (IOPs) into aggregates in association with the chaperone ClpB, followed by the asymmetric distribution of these aggregates between daughter bacteria. Progeny born with minimal IOPs cope better with stress and chemotherapy than their IOP-burdened siblings [74]. In *E. coli*, asymmetrical segregation of proteins can impact precise functions such as the efflux capacity. The progeny with the old pole inherits a greater number of AcrAB-TolC efflux pumps and consequently is better able to expel toxins, including host-derived antimicrobial agents [75]. Asymmetry may also trigger distinct host pressure. For instance, when *P. aeruginosa* meets cell surfaces and divides, a combination of mechanical stimuli and the asymmetric distribution of c-di-GMP metabolic enzymes and effectors results in alternative envelope properties (flagellum or pili) and therefore distinct PRR-dependent host responses [59].

Finally, the generation of high and low performers regarding the infection process may be determined by the individual bacterial cell context. The cellular context spans spatial gradients (stressors, nutrients), lineage history, and local cell–cell interactions. Together, they create spatially regulated cell-to-cell variations in gene expression as documented for the induction of the SOS response [76], metabolic activities [76,77], and detoxification [78]. For instance, upon H₂O₂ oxidative stress exposure, a single *E. coli* bacterium can reduce by 30% the stressor in its vicinity by inducing scavenging enzymes. As a result, cells are heterogeneously exposed to H₂O₂ as the closest individuals protect those that are farther away [78]. This mechanism could explain the emergence of stress-induced persisters that form within the small colonies of *E. coli* [8] and *L. pneumophila* [19] during intravacuolar exponential growth.

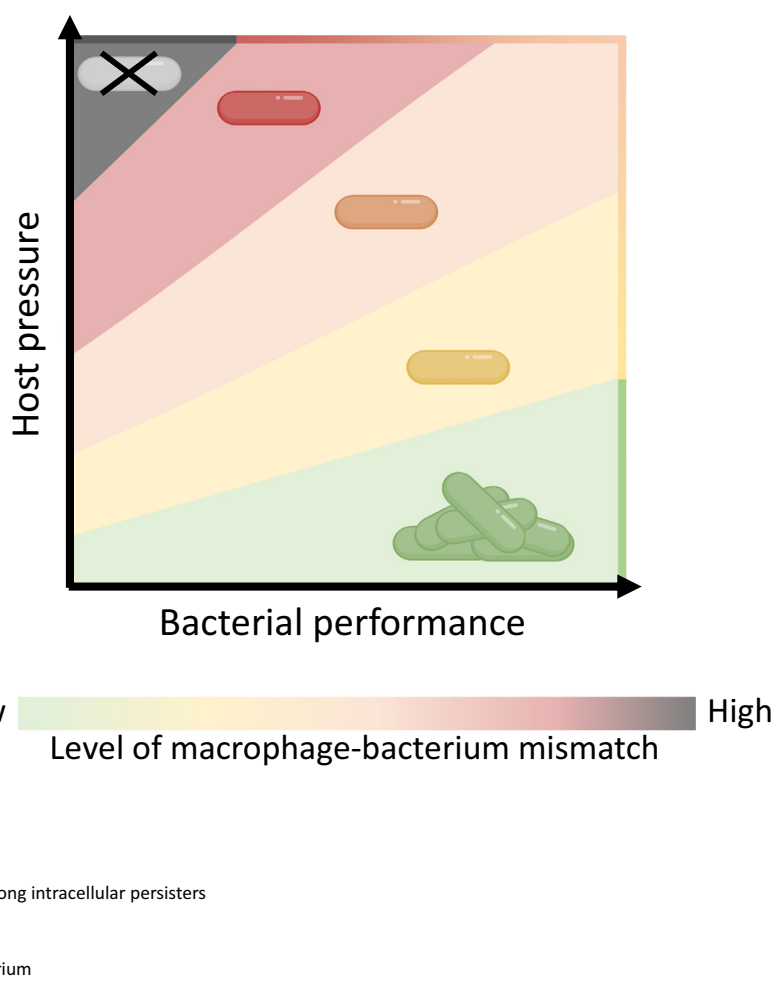
It is thus likely that every single bacterium possesses a preexisting program constraint by intrinsic and extrinsic cues that together determine its performance to avoid or counteract the host-derived antimicrobial activities. For a phagocytosed bacterium, this means differentiation or not into a stress-induced antibiotic persister.

Macrophage–bacterium mismatch and the physiological diversity of persisters

The persisters that form during infection are commonly detected by the small colony variants or the delayed regrowth they exhibit on agar plates [79] or by the biphasic killing kinetics observed with a broad range of antibiotics [1]. Such indirect detection methods are blind not only to the physiological status of the persisters but also to their individuality. This is also true for the global biochemical analyses that have been conducted predominantly on a collective population of persisters sorted by fluorescence-activated cell sorting (FACS). The notion that intracellular

persisters constitute a uniform group of stress-induced, drug-tolerant individuals has recently been called into question. Physiological diversity (i.e., dormancy depth, stress level/response) among coexisting isogenic intracellular persisters has been observed [4,6–8,10,11,80,81]. Divergent profiles of antibiotic recalcitrance and susceptibility or ability to exit the persister state have been documented [4,11,14,22,23,82]. Finally, in broth, it was demonstrated that the evolutionary trajectory of the persisters may itself be subject to extensive variations [70].

Recent work by Balaban and colleagues, inspired by the models of **aging** in glassy systems, suggests that gradual stress leads a single bacterium to a regulated response. Furthermore, this response causes a growth-arrested state from which the bacteria emerge in a fast and uniform manner (no aging). On the contrary, acute stress results in a disrupted state where the bacteria are driven away from their adaptative potential; that is, the inability of genetic networks to deal with the stress leads to slow and heterogeneous recovery dynamics [83]. This resembles the



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Figure 3. Heterogeneity of intracellular persisters. The level of mismatch between the strength of the host defense mechanisms (host pressure) and individual bacterial preparedness to cope with or soften them (bacterial performance) may explain the coexistence of intracellular proliferative bacteria (green) and stress-induced antibiotic persisters of different nature (e.g., dormancy depth; yellow to red). When the mismatch is too high, bacterial death is inevitable (depicted in gray, black cross). Color code is indicated.

dynamics observed during macrophage infection by numerous pathogens following phagocytosis. On the one hand, a fraction of the engulfed bacteria resumes growth quickly, constituting the reservoir of proliferative and antibiotic susceptible individuals. On the other hand, some individuals exhibit a slow and heterogeneous recovery and are considered as a heterogeneous subpopulation of intracellular persisters. Following the perspective of this opinion, this dynamic may reflect the level of mismatch between the strength of the host antimicrobial activities and the preparedness of individual bacteria to cope with or soften them (Figure 3).

Concluding remarks and future perspectives

Amid the apparent uniformity within both a clonal bacterial population and the macrophage host, only a subset of intracellular individuals specialize into nonreplicating antibiotic persisters. Years of research have established a prominent role for bacterial exposure to stress and the associated bacterial response. Nevertheless, what underlies the heterogeneity in the host pressure or in the bacterial proficiency in tackling it? This opinion integrates recent research lines to address this persistent question. First, the functional heterogeneity of macrophages may cause invading bacteria to be exposed to different stressors as well as different stress intensities (Figure 1). Second, bacterial phenotypic heterogeneity can produce multiple individuals with different vulnerabilities to the host defense program. In either case, the unfavorable setting promotes the emergence of a fraction of stress-induced persisters (Figure 2). During infection, the formation of persisters is unlikely to be restricted to a single scenario but rather involves a combination of many. Therefore, the extent of mismatch between macrophage permissiveness and bacterial preparedness to invade may explain the broad spectrum of bacterial subpopulations and the documented diversity among intracellular persisters (Figure 3). Future research should concentrate on unraveling the intricate interplay between macrophage functional heterogeneity and bacterial phenotypic diversity, shedding light on the mechanisms driving different exposure to stressors or varying stress intensities and persister formation. Understanding how stress-induced persisters emerge during infection from both macrophage and bacterial perspectives and subsequently exploring clinical relevance will be crucial for developing strategies to target and eliminate the intracellular subpopulation of persisters (see Outstanding questions). The underlying molecular mechanisms are likely highly diverse, and a single-cell level analysis is essential to unveil and untangle them. Unfortunately, the nuanced dynamics causing the emergence of intracellular stress-induced nonreplicating antibiotic persisters may also complicate the identification of a common mediator with high therapeutic potential.

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Declaration of interests

The authors declare no competing interests.

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Outstanding questions

Could the observed severity in clinical outcomes be attributed to the extent of functional heterogeneity among macrophages?

What specific factors within macrophages induce heterogeneous stress exposure/intensity and varying bacterial responses?

Is there heterogeneity in the macrophage nonoxidative defense mechanisms, and does this heterogeneity contribute to intracellular persister formation?

Can macrophages introduce cellular state information into their decision-making process, leading to extensive cell-to-cell variations in functionalities? Further research is required to explore the impact of internalized pathogens on macrophage heterogeneity and the mechanisms by which macrophages integrate intrinsic and extrinsic cues to determine their spectrum of activities.

In alignment with the aging concept, could scenarios involving a gradient of stress exposure/intensity and varying degrees of failure in stress management elucidate the dynamics of pathogen intracellular growth, encompassing processes such as persister formation and growth resumption?

Bacteria exhibiting overperformance in stress response within permissive macrophages may be maladapted. Could this lead to the development of a distinct type of antibiotic persisters not genuinely subjected to host-derived stressors?

Throughout the infection process, in accordance with the concept of bacterial community behavior, what tasks or niches favor the thriving of intracellular antibiotic persisters? This knowledge could guide the development of targeted interventions.

Do bacterial persisters and growers cooperate when coexisting within the same cell?

During infection or antibiotic chemotherapy, are the proportions of low and high performers altered or fine-tuned? Identifying such constraints through environmental and clinical isolates, as well as within-

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