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Current Opinion in Microbiology

Studying antibiotic persistence *in vivo* using the model organism *Salmonella* Typhimurium

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Antibiotic persistence permits a subpopulation of susceptible bacteria to survive lethal concentrations of bactericidal antibiotics. This prolongs antibiotic therapy, promotes the evolution of antibiotic-resistant pathogen strains and can select for pathogen virulence within infected hosts. Here, we review the literature exploring antibiotic persistence *in vivo*, and describe the consequences of recalcitrant subpopulations, with a focus on studies using the model pathogen *Salmonella* Typhimurium. *In vitro* studies have established a concise set of features distinguishing true persisters from other forms of bacterial recalcitrance to bactericidal antibiotics. We discuss how animal infection models are useful for exploring these features *in vivo,* and describe how technical challenges can sometimes prevent the conclusive identification of true antibiotic persistence within infected hosts. We propose using two complementary working definitions for studying antibiotic persistence *in vivo*: the strict definition for studying the mechanisms of persister formation, and an operative definition for functional studies assessing the links between invasive virulence and persistence as well as the consequences for horizontal gene transfer, or the emergence of antibiotic-resistant mutants. This operative definition will enable further study of how antibiotic persisters arise *in vivo*, and of how surviving populations contribute to diverse downstream effects such as pathogen transmission, horizontal gene transfer and the evolution of virulence and antibiotic resistance. Ultimately, such studies will help to improve therapeutic control of antibiotic-recalcitrant populations.

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Current Opinion in Microbiology 2022, **70**:102224

This review comes from a themed issue on **Host-Microbe Interactions: Bacteria**

Edited by **Teresa Thurston** and **Sophie Helaine**

For complete overview of the section, please refer to the article collection, "Host-Microbe Interactions: Bacteria (February 2023)"

Available online 3rd November 2022

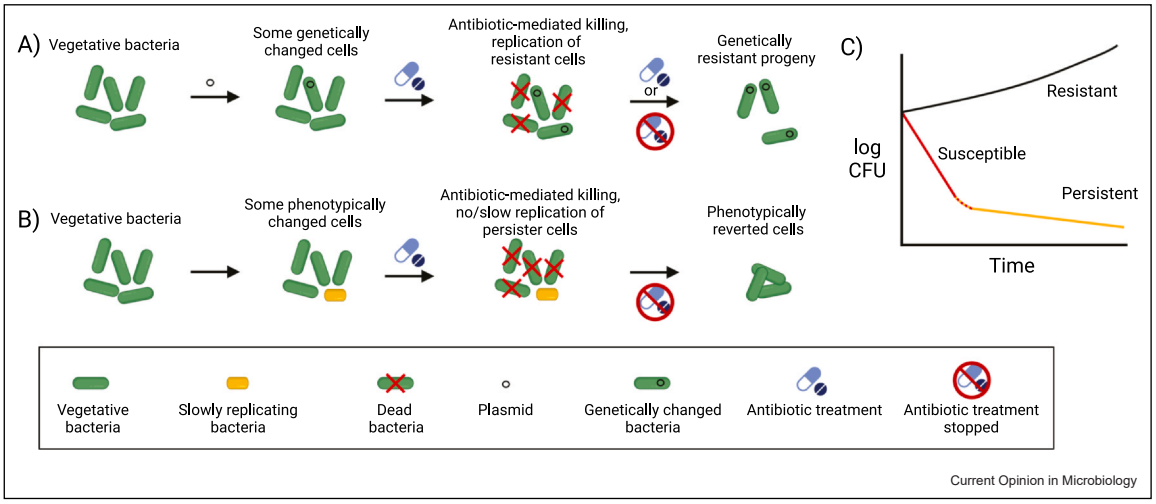
https://doi.org/10.1016/j.mib.2022.102224

Introduction to antibiotic persistence

Fundamentally different mechanisms can mediate bacterial survival of antibiotics. Antibiotic resistance is characterised by genetic changes conferring the ability to replicate under antibiotic pressure. Resistance is a heritable phenomenon, in that all descendants of a bacterium that acquires antibiotic resistance (e.g. by mutation) will similarly be resistant (Figure 1a). In contrast, antibiotic persistence describes a subpopulation of susceptible bacteria that express a phenotype allowing for the survival of bactericidal antibiotics over extended periods of time. Typically, these persisters replicate very slowly (if at all) and are therefore killed much more slowly than the susceptible majority of the population (Figure 1b). These persister cells can cause relapses once the antibiotic pressure has been lifted, and the descendants of these bacteria will be genetically and phenotypically identical to the parent strain [1].

Antibiotic persistence was first described [2,3] shortly after the development of early antibiotics and subsequent reports of antibiotic resistance [4,5]. Antibiotic persistence seems to be a general property of bacteria, given the plethora of species in which this phenomenon has been described (see [6]). To clearly delineate between antibiotic resistance, persistence and other forms of antibiotic survival, a consensus statement has provided specific definitions and recommended guidelines for their experimental study [7]. Several hallmark phenotypes of antibiotic persister formation are now well established. Biphasic killing curves of susceptible clonal populations are typically observed (Figure 1c), in which the susceptible majority of the population is killed rapidly, while persister cells are killed much more slowly over extended periods [8]. Isolation and re-culturing of these surviving cells, followed by re-exposure to the same dose of antibiotic, should lead to similar killing kinetics in repeated experiments [3,9]. Thus, persistence is attributed to a particular physiological state of the bacterium, which allows persister cells to survive lethal antibiotic pressure, as long as this state is maintained. While this is generally considered a phenotypic phenomenon rather than one mediated by genetic changes, the acquisition of certain genetic elements (e.g. toxin/anti-toxin genes) or mutations can alter the frequency of persisters in a bacterial population (see [10,11]). Thereby, bacteria can evolve towards an

Figure 1



Hallmarks of antibiotic resistance and persistence *in vitro.* **(a)** In an example of antibiotic resistance, a viable population of bacteria acquires a genetic element (here, a plasmid) encoding for an antibiotic-resistance mechanism. Treatment with a bactericidal antibiotic results in the elimination of the susceptible population, while those bacteria that have acquired a resistance mechanism survive, and can replicate either in the presence or absence of sustained antibiotic pressure. The progeny of the genetically resistant strain inherit this genetic resistance. **(b)** In antibiotic persistence, a phenotypic switch occurs due to starvation or another mechanism that induces slower replication, resulting in heterogeneity within an otherwise clonal population. When antibiotic pressure is applied, the susceptible majority is killed rapidly, while the slowly replicating subpopulation is killed far more slowly. Upon cessation of antibiotic treatment, phenotypic reversion and bacterial replication allows for the regrowth of a population that is genetically unchanged relative to the parental strain. **(c)** Typical killing kinetics observed for bacterial populations under antibiotic pressure *in vitro*. Resistant strains can replicate under antibiotic pressure. The majority of a susceptible population is killed rapidly, while persister cells are killed far more slowly. Adapted from [7].

optimal size of the persister subpopulation. The size of the persister population is generally independent of the antibiotic dose (as long as the dose is well above the minimum inhibitory concentration), and persistence arises in response to a range of bactericidal antibiotics of different functional classes [7]. Persisters are hypothesised to form by two principal mechanisms. Firstly, the persister phenotype may be elicited in response to particular stimuli such as starvation or stress imposed by immune cells [7,8]. This type of regulated persister formation occurs in a range of conditions for which the bacterium has evolved to cope, while challenges outside this range provide a second route towards persister formation. Here, an overwhelming environmental challenge or a critical deviation from normal physiology (e.g. translational errors in transmembrane domains that dissipate the proton gradient, circumstantial loss of a critical metabolite, etc.) spontaneously halts bacterial growth until the normal physiology can be re-established [12••]. During this 'time of repair', the affected cells will be recalcitrant to killing by bactericidal antibiotics and are therefore persisters. Mechanistic studies exploring these differences are critical, as persister formation influences not only the kinetics of antibiotic-mediated killing, but also can contribute to the emergence of antibiotic-resistant strains both *in vitro* and *in vivo* [13–16•]. The various mechanisms contributing to the formation of antibiotic persisters, the clinical and evolutionary consequences and advances in therapeutic control of persisters have been extensively reviewed elsewhere [6,10,11,17].

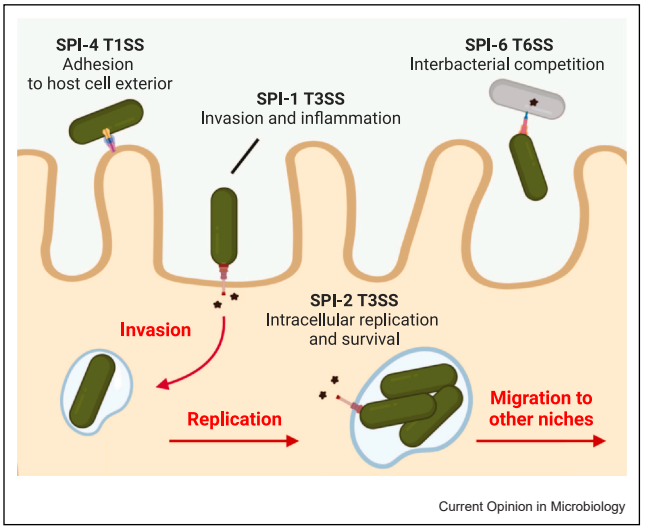
While most studies focused on antibiotic persistence have been performed *in vitro*, antibiotic persistence remains understudied *in vivo*. In part, this is attributable to the technical challenges that often prevent the unequivocal identification of true antibiotic persistence *in vivo* (as described in [7]). These studies are further complicated by the fact that bacterial subpopulations may also survive bactericidal antibiotics by mechanisms other than persistence, including poor antibiotic penetrance to the intracellular niche or overexpression of drug-efflux pumps. Thus, certain hallmarks of persister formation might be phenocopied by these alternative mechanisms, confounding the identification of true antibiotic persistence *in vivo*. Surmounting these technical challenges is important for research towards effective therapeutic applications, as well as for understanding the biological consequences of persister formation, including pathogen evolution and the spread of antibiotic-resistance plasmids. In this review, we summarise the use of *Salmonella* Typhimurium to explore how persisters form *in vivo*, and how these populations can be shaped by host–pathogen interactions.

Understanding *Salmonella* virulence using murine models

Antibiotic persistence *in vivo* is underpinned by the host–pathogen interactions that define the infection. *Salmonella* Typhimurium (hereafter *S.* Tm or *Salmonella*) has emerged as perhaps one of the most studied and best-understood bacterial pathogens. The discovery and characterisation of two distinct type-three secretion systems (T3SS) as principal virulence factors mediating *Salmonella* pathogenesis has precipitated several decades of research to elucidate how translocated bacterial effector proteins shape the host–pathogen interaction [18–20]. While much remains to be understood, it is well appreciated that the T3SS-1 (encoded on the genomic region termed *Salmonella* pathogenicity island 1 (SPI-1)) translocates effectors that mediate bacterial entry into non-phagocytic host cells [21,22], (reviewed in [23]), and thereby triggers the strong inflammatory response that facilitates prolonged gut infection and transmission to new hosts [24–28]). Separately, the T3SS-2 (encoded on the genomic region termed SPI-2) facilitates the intracellular survival of the bacterium, including bacterial replication within a modified vacuole, avoidance of host cellular defences and manipulation of host immune signalling [25,29–36]. The contributions of these secretion systems to *Salmonella* pathogenesis have been extensively reviewed elsewhere [37–39]. Additionally, *S.* Tm employs flagella to migrate towards the gut epithelium [40,41], a type-one secretion system encoded on SPI-4 to enhance adhesion to gut epithelial cells [42], while a type-six secretion system encoded on SPI-6 is used to outcompete resident microbiota and promote gut-luminal growth [43] (Figure 2). In combination, these virulence factors allow *S.* Tm to form intracellular reservoirs within host tissues, permitting replication and prolonged survival, and also providing a niche for persister formation.

Salmonella Typhimurium represents an excellent model organism for studying various aspects of host–pathogen interactions, owing particularly to the ease of culturing the bacterium, a range of well-established protocols for genetic manipulation and an abundance of available whole-genome sequence data. Further, a range of animal models of infection are well established, allowing for the study of different aspects of the disease. Oral infection allows for recapitulation of certain elements of gut-associated infections in humans and is greatly impacted by the resident microbiota, as well as O-antigen-specific secretory immunoglobulin A (IgA) responses against the invading pathogen [24,26,44–46]. Here, the pathogen requires virulence factors to efficiently breach the epithelial barrier and spread to the gut tissue, mesenteric lymph node and distal organs (Figure 2). In the streptomycin pre-treatment model, oral gavage of a high dose of streptomycin temporarily suppresses the gut microbiota, thus ablating colonisation resistance and promoting highly reproducible levels of *S.* Tm colonisation [24]. In contrast to the oral model, intravenous or intraperitoneal injection is typically performed without disrupting the gut microbiota, but also produces a systemic infection. This systemic infection is characterised by rapid colonisation of distal organs, including the liver and spleen, while gut tissues and the draining mesenteric lymph nodes are colonised with slower kinetics than in the streptomycin infection model [47,48]. These parenteral injection models allow for the robust establishment of tissue-lodged *S.* Tm populations that have not already been shaped by selective pressures in the gut (e.g. colonisation resistance imposed by gut microbiota, virulence deficiencies of T3SS-1 or T3SS-2 mutants, strong bottleneck selection imposed by the host environment, etc.) [49]. Commonly used inbred mouse lines can produce different severities of disease: the C57BL/6 or BALB/c lines are genetically susceptible and succumb rapidly to infection with *S.* Tm [50,51]. In contrast, genetically resistant 129/Sv or CBA mice (which have a functional *NRAMP1* gene [52]) more successfully control the infection [50,52–54], allowing for long-term experiments that study chronic infection,

Figure 2



Salmonella Typhimurium secretion systems support murine infection and thereby promote persister formation in host tissue compartments. Extracellular bacteria use the SPI-1 T3SS to translocate bacterial effector proteins in order to mediate uptake into non-phagocytic cells and promote immune signalling, leading to local inflammation in the gut. Intracellular bacteria use the SPI-2 T3SS to translocate effectors across the vacuolar membrane to mediate intracellular replication and counter host defences. The SPI-4 T1SS is used by extracellular bacteria to promote initial attachment to the exterior of gut epithelial cells, while the SPI-6 T6SS is used to compete against certain resident microbiota members in the gut and promote luminal growth and transmission. Collectively, these four secretion systems promote the full virulence of *S.* Tm *in vivo*, and are required for the intracellular niche establishment that permits persister formation.

adaptive immunity, within-host evolution or the effects of secretory IgA responses and how vaccination can prevent tissue invasion and persister formation [54–57•]. The availability of various genetically modified mouse lines, particularly in the C57BL/6 background, additionally allows for the study of host factors that shape the infection and persister formation. Thus, murine infection with *Salmonella* spp. provides an attractive and well-established model system for investigating host–pathogen interactions. Here, we describe how these animal models have been used to study antibiotic persistence *in vivo*, and consider how this phenomenon is shaped by differences in bacterial, host and therapeutic factors.

Antibiotic persistence in murine models of *Salmonella* infection

A range of early studies showed the failure to clear tissue-lodged *Salmonella* from infected mice is a common phenomenon in response to a range of bactericidal antibiotics, between different host genetic backgrounds and across different infection models. Ampicillin treatment of susceptible BALB/c mice infected intravenously was successful in suppressing *Salmonella* numbers in distal organs, but pathogen regrowth occurred after antibiotic therapy was stopped, leading to terminal infection [58]. Similarly, multiple studies showed that ciprofloxacin treatment could temporarily control *Salmonella* levels in a subcutaneous infection model, but elimination of tissue-lodged populations was not possible [59–61]. Mice infected by intraperitoneal injection were also protected from lethal infection during treatment with a range of antibiotics, including ofloxacin, ciprofloxacin, ampicillin, cefazolin and ceftazidime, but mice succumbed once antibiotic treatment was stopped, and tissue-lodged populations were not fully eliminated [62,63]. Oral infection recapitulating the natural route of infection similarly led to antibiotic-recalcitrant bacterial populations in the mesenteric lymph node, spleen and liver, and while symptoms and pathology could be temporarily controlled with ceftriaxone, ciprofloxacin or enrofloxacin, ultimately relapse occurred after treatment was stopped [64,65]. Thus, these early studies suggested that treatment with bactericidal antibiotics could temporarily control *Salmonella* during animal infection, but elimination of recalcitrant populations remained difficult to achieve. These observations are strikingly similar to the relapses reported in human infections, as seen in cases of classical diarrhoea caused by non-typhoidal *Salmonella* strains [66,67], as well as in complicated systemic infections described in early AIDS patients, who often suffered from life-threatening infections by *S.* Typhimurium before the advent of antiretroviral therapies [68,69]. These similarities suggest that antibiotic recalcitrance observed in mouse infection models may be more broadly applicable to clinical situations, and that these *in vivo* models are suitable for studying the underlying mechanisms.

With renewed attention given to the concept of antibiotic persistence as an explanation for antibiotic failure [1,70,71], and several key studies describing possible mechanisms that enable persistence *in vitro* [8,9,72], multiple groups turned to animal models of *Salmonella* infection to explore these concepts *in vivo*. Kaiser et al. [73] used the streptomycin pre-treatment model of *Salmonella* colitis [24] followed by oral ciprofloxacin treatment to further characterise the lymph node-resident population of *Salmonella* seen previously [65]. While the gut lumen was cleared within a few hours, surviving *S.* Tm cells were observed within the spleen and caecal lymph node. Biphasic killing of *S.* Tm in the lymph node occurred during ciprofloxacin treatment, and re-isolated surviving bacteria remained susceptible to ciprofloxacin *in vitro* — both of these phenotypes are considered hallmarks of antibiotic persisters [7]. Further, persisters isolated from the lymph node could be used to infect another animal, indicating that persisters were not impaired for virulence in subsequent infections. A combination of plasmid-dilution experiments and quantitative PCR (qPCR) to measure changes in neutral genomic tags provided evidence that these persister cells replicated slowly, while fluorescence microscopy and flow cytometry identified the host cells harbouring persisters as predominantly $CD11c^{+}$ dendritic cells [73]. Ultimately, persister levels could be reduced by manipulating numbers of these dendritic cells or by enhancing innate defences using lipopolysaccharide (LPS) or unmethylated cytosine–guanine dinucleotide (CpG) motifs as agonists in combination with ciprofloxacin. Together, these data provided important first insights into the dynamics and control of *S.* Tm persisters *in vivo*.

In parallel, Helaine et al. [74] used a fluorescence-dilution strategy to show that phagocytosis of *Salmonella* by macrophages induced the formation of a non-replicating or dormant-like population *in vitro*, and also observed these non-replicating cells in splenic macrophages recovered from infected BALB/c mice [74]. A later study extended these observations under antibiotic pressure, and found a non-replicating population of persisters within the mesenteric lymph node after five days of enrofloxacin treatment, which were able to regrow upon isolation and culture in LB medium [75]. *In vitro* experiments demonstrated that engulfment into macrophages greatly enriched the proportion of persister cells following cefotaxime treatment, relative to persisters already preformed in the inoculum, and that these persisters were non-replicating, similar to *in vivo* experiments [75]. Further experiments suggested these non-replicating persisters were metabolically active [75], while a later study would demonstrate that these same

cells also remained competent for SPI-2 T3SS effector secretion [76], providing a mechanism by which intracellular *S.* Tm persisters might continue to actively manipulate host cells *in vivo*, even during antibiotic treatment.

Alongside these studies, Claudi et al. established a fluorescence-based reporter that allowed for the discrimination between fast- and slow-growing *S.* Tm *in vivo* [77]. Fluorescence microscopy and flow cytometry revealed the presence of distinct subpopulations of phagocytosed *S.* Tm *in vitro*, and similarly provided evidence for subsets of intracellular *S.* Tm in the spleen of infected mice. These subsets appeared to have different growth rates, distinct proteomes and responded differently to enrofloxacin treatment (i.e. fast-growing cells were more readily killed than slow-growing cells). Importantly, the authors demonstrate that growth of intracellular *S.* Tm in the spleen had resumed within 24 hours of enrofloxacin treatment, suggesting that the treatment schedule used here [77] and elsewhere [75] may not be effective at sustained control of tissue-lodged *Salmonella* (i.e. regrowth of persisters may begin between doses of antibiotics). Interestingly, the authors conclude that a moderately replicating and moderately persisting population ultimately outcompetes bacteria that replicate quickly or slowly, and suggest that these 'intermediate' persisters ultimately come to dominate the surviving populations [77]. Further, this study found that oral infections of C57BL/6 mice without streptomycin pre-treatment did not yield many viable persisters in the mesenteric lymph node following sustained enrofloxacin treatment (in contrast to [75]), while streptomycin pre-treated mice given high doses of ciprofloxacin showed high levels of poorly replicating *S.* Tm persisters in the mesenteric lymph node (in support of [73]). Thus, conclusions regarding persister formation *in vivo* may not always be generalisable between studies due to differences in experimental design (e.g. route of infection, the levels of microbiota-mediated colonisation resistance, varying antibiotic treatments, etc.). Reproducing phenotypes in various models of infection and with different antibiotic regimens might help to identify the generalisable principles of antibiotic persistence.

In later studies, Rossi et al. [78] investigated persister formation after intravenous infection of susceptible C57BL/6 mice with either *S.* Tm wild type (WT) or a Δ*aroC* mutant, which has a reduced replication rate due to deficiency in aromatic amino acid biosynthesis [79]. Intravenous application of ampicillin or ciprofloxacin reduced *S.* Tm WT loads in the liver and spleen in a biphasic manner, while *S.* Tm Δ*aroC* was killed more gradually [78]. Further, *S.* Tm WT was able to begin replicating after cessation of antibiotic treatment, while the attenuated Δ*aroC* strain continued to decline slowly in number. These kinetics confirmed that killing *in vivo* in the liver and spleen is biphasic, similar to killing reported previously in the mesenteric lymph node [73], and added further support to the link between growth rate and antibiotic-mediated killing rate [77]. In contrast, this study showed surprisingly poor killing in the mesenteric lymph node for both WT and attenuated *S.* Tm [78], but did seem nonetheless effective at controlling these populations, as WT bacteria were able to re-initiate replication in the mesenteric lymph node after antibiotic treatment was stopped. This may suggest that intravenous infection leads to a smaller but more recalcitrant persister population in the lymph node than oral infection, for which larger populations exist but of which the majority remain susceptible to antibiotic treatment [73,77]. Using genomic tags and qPCR, the authors show that the population within the lymph node was distinct from relapsing populations in the liver and spleen [78], indicating that intravenous infection with *S.* Tm produces a small lymph node-resident population that arises separately to those in the distal organs, and is extremely recalcitrant to antibiotic treatment, perhaps due to a local environment that selects or enriches persisters more effectively than in the liver or spleen. In a follow-up study [80•], these authors use these genomic tag distribution data to develop a mathematical model, which described how non-replicating subpopulations in the lymph node and spleen endure ampicillin treatment and ultimately repopulate these sites during relapsing infection. Of broader interest, the authors provide a table summarising key features of experimental design for six major studies exploring antibiotic persisters during *Salmonella* infection *in vivo* [80], which highlights some important differences in methodology that may give rise to differences in persister formation.

More recently, attention has been given to how cellular niches *in vivo* might shape persister formation, and how these niches might be manipulated experimentally. Li et al. [81••] orally infected susceptible BALB/c mice (without streptomycin pre-treatment) and waited until symptoms appeared to start a 10-day regimen of enrofloxacin or ceftriaxone. *S.* Tm loads in the spleen decreased at a gradual rate, and regrowth occurred after treatment was stopped, but this could be again reduced by further antibiotic treatment. The authors developed a serial two-photon tomography approach to image antibiotic persisters in the spleen of infected mice, and showed that most *S.* Tm had been cleared from the red pulp and marginal zones, while the proportion of surviving bacteria was considerably higher in the white pulp zone. Using a fluorescence-based reporter to measure growth rates (as in [77]), bacteria in the white pulp zone were shown to replicate more slowly, but nonetheless continued replicating even under enrofloxacin pressure [81••]. Intriguingly, the failure to clear these survivors was attributed to declining local inflammation

characterised by fewer neutrophils and monocytes, rather than antibiotic penetrance or replication arrest. In support of this, clearance of persisters was promoted during co-infection with an enrofloxacin-resistant strain that could continue to drive inflammatory responses during antibiotic treatment, and similarly stimulation of neutrophils *via* immunotherapy increased clearance of bacteria. Together, these data demonstrate the impact of the host microenvironment on clearance of bacteria under antibiotic pressure *in vivo*, which has otherwise remained underappreciated. Modulation of immune cell populations to reduce persister reservoirs had been shown previously [73], and Kanvatirth et al. extended these observations to experimentally increase or decrease persister populations *via* immunomodulatory agents [82•]. Here, intravenous infection of susceptible C57BL/6 mice with a green fluorescent protein (GFP)-expressing *S.* Tm strain allowed for identification of splenic immune cells bearing persisters following ciprofloxacin treatment. Dendritic cells ($CD11b^{+}CD11c^{hi}$) and macrophages ($CD11b^{+}CD11c^{lo}$) were reported to harbour the most significant proportion of GFP^{+} *S.* Tm following four days of antibiotic treatment. Depletion of these cell types *via* clodronate liposomes reduced the numbers of viable persisters, while depletion of neutrophils ($Ly6G^{+}CD11b^{+}$) *via* anti-Gr-1 antibody seemed to increase persister numbers, suggesting that the availability of certain host niches can impact persister formation.

Collectively, these principal studies provided fundamental insights into the mechanisms and dynamics of *Salmonella* persister formation *in vivo*, establishing in particular that certain hallmarks of persister formation were observable *in vivo*, that the replication rate was closely linked to bacterial killing kinetics and that certain characteristics of the host–pathogen interaction (particularly intracellular stresses and the local immune response) shaped the formation of antibiotic persisters. Thus, later studies were well positioned to begin exploring the broader consequences of persister formation.

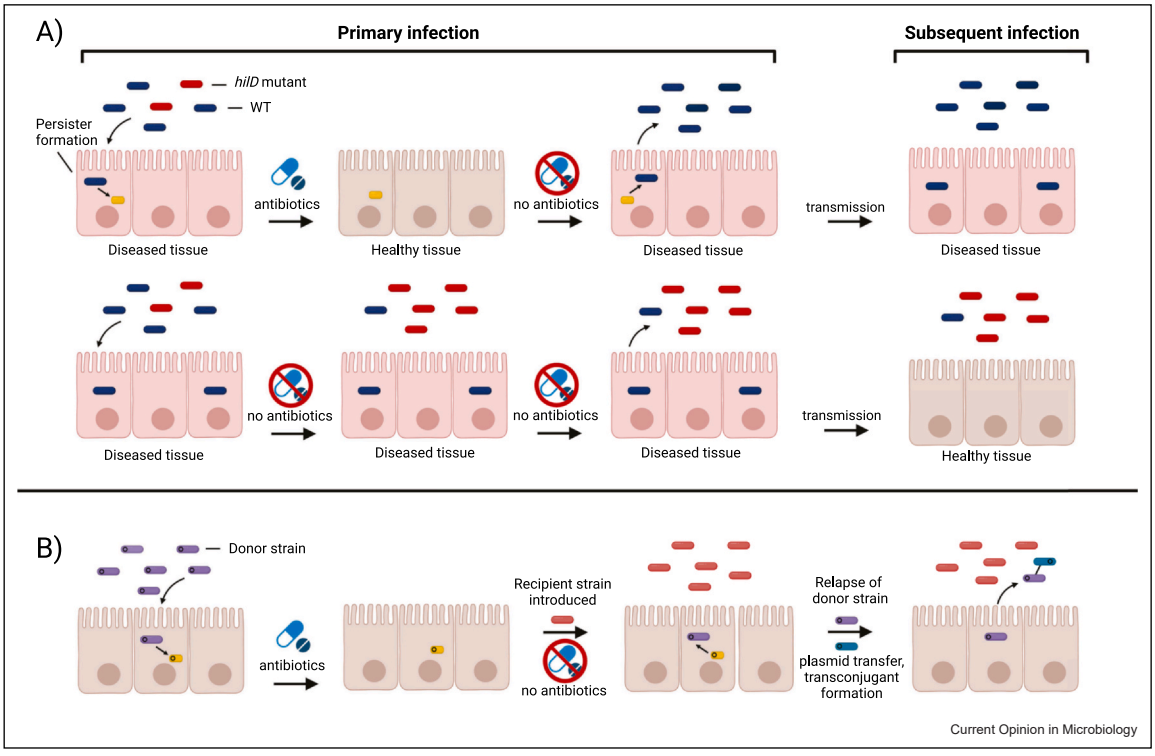
Biological consequences of antibiotic persistence *in vivo*

Research focused on the broader consequences of persister formation for pathogen evolution, host responses and the spread of antibiotic resistance has built on the fundamental observations described above. In experiments exploring how antibiotic treatment might favour the maintenance of virulence, Diard et al. [83] observed that ciprofloxacin treatment could promote the virulence of *S.* Tm populations in the gut, which can otherwise be overcome with mutants deficient for *hilD* expression that arise during oral infection of mice (Figure 3a). As *hilD* encodes a master regulator of SPI-1 expression, *hilD* mutants are deficient for expression of the SPI-1 T3SS, lack the ability to invade into the gut tissue, are attenuated at triggering gut inflammation and are impaired in transmission to new hosts [84,85]. Here, Diard et al [83] showed that *S.* Tm WT (but not the *hilD* mutants) could form persisters in the caecal tissue and that these could reseed the gut lumen to ultimately be excreted through faeces and infect a new host, while *hilD* mutants were eliminated from the gut during antibiotic treatment. This difference was attributed to invasion of caecal tissue, as a multi-mutant strain deficient for four SPI-1 effectors that mediate host cell invasion was similarly cleared from the mouse. Thus, the authors used an established model of *Salmonella* persister formation *in vivo* to make an evolutionary argument about the maintenance of virulence, but also provided key early evidence that virulence was tightly linked to potential for antibiotic persister formation.

Meanwhile, Dolowschiak et al. [86] studied how the acutely infected gut tissue recovered during therapy with bactericidal antibiotics. Oral ciprofloxacin therapy cleared *S.* Tm WT from the gut lumen within a few hours and prevented pathogen spread to the spleen, while persisters remained in the caecal tissue and in the mesenteric lymph node. While enteropathy was resolved within ten days, transcriptional profiling of the caecal tissue detected a pronounced pro-inflammatory gene expression programme, which partially depended on IFN-γ and remained even after resolution of mucosal histopathology at the macroscopic level. The degree to which surviving bacteria contributed to this prolonged inflammatory state was not assessed, however. Nonetheless, this model may help to decipher why antibiotic treatment is generally ineffective at controlling acute disease symptoms in non-typhoidal *Salmonella* infections, and why relapses of gut colonisation are commonplace after therapy with bactericidal antibiotics [67].

Elsewhere in the literature, much consideration had been given to the idea that antibiotic persistence might give rise to antibiotic resistance (reviewed in [6,10,11]). Bakkeren et al. [15••] extended this concept by establishing that tissue-lodged *S.* Tm persisters could migrate to the gut and participate in horizontal gene transfer, effectively transferring resistance plasmids to viable recipient bacteria *in vivo* (Figure 3b). In the streptomycin model of oral infection, *S.* Tm (strain SL1344) bearing a modified P2 plasmid (i.e. the pCol1B9 Incl1 plasmid) was cleared from the gut lumen with oral ciprofloxacin, but tissue-lodged persisters were ultimately able to reseed the gut and participate in plasmid conjugation to a recipient *S.* Tm (strain 14028), thus forming resistant transconjugant populations. Complementary intravenous infection experiments attributed this to tissue-lodged persisters and not gut-luminal survivors. Tissue-associated persisters formed in the spleen and liver following intraperitoneal

Figure 3



Examples of biological consequences of antibiotic persistence *in vivo*. **(a)** In the inflamed gut, non-invasive *hilD* mutants emerge by random mutation, benefit from local inflammation caused by WT *S.* Tm and due to the cost of expressing the SPI-1 T3SS, these cheater/defector cells ultimately overcome the WT population. Upper panel — invasive *S.* Typhimurium (WT) forms tissue-lodged reservoirs that allow for survival during bactericidal antibiotic exposure, while invasion-deficient *hilD* mutants are cleared from the gut. When antibiotic treatment is stopped, WT *S.* Tm can relapse into the gut lumen, spread to new hosts and cause disease. Lower panel — in the absence of antibiotics, the non-invasive *hilD* mutants remain prominent in the gut lumen. After transmission to a new host, the *hilD* mutant-dominated population is unable to cause disease. Thus, antibiotic pressure selects for the maintenance of co-operative virulence in populations of *S.* Tm in the gut. **(b)** *S.* Tm bearing a conjugative plasmid invades the mucosa and establishes tissue-lodged reservoirs. Antibiotic treatment clears the gut-luminal population but fails to clear the tissue-lodged persisters. After antibiotics are stopped, a viable recipient strain is introduced to the gut lumen. Relapsing *S.* Tm migrating from the tissue back into the gut lumen initiates plasmid conjugation, giving rise to a transconjugant population. Thus, persisters can represent a reservoir for the storage of genetic elements, which are transferable in future contexts.
(a) Adapted from [83,85]. **(b)** Adapted from [15•• ,87••].

administration of ceftriaxone, but following cessation of antibiotics, *S.* Tm WT could migrate to the gut and conjugate a resistance plasmid to the recipient strain. Several supplementary data presented here are of broad interest, including the plating of various organs from the infected mouse to establish the primary locations of *S.* Tm persisters during intravenous infection (principally the liver and spleen), and also a description of the antibiotic- resistance profiles of several strains of *S.* Tm in response to a range of antibiotics commonly used in research and the clinic. Further experiments and mathematical modelling established the rate of gut reseeding as the limiting factor in transconjugant formation, and indeed vaccination of mice reduced persister reservoirs (i.e. by preventing tissue invasion) and limited plasmid transfer in the gut. Finally, the authors showed that a clinically relevant pESBL15 plasmid (encoding resistance *via* extended-spectrum beta-lactamase) could also be efficiently transferred in the gut, suggesting these persister cells may contribute to the spread of antibiotic-resistance plasmids in animals or humans.

Two studies extended these observations to further describe how antibiotic persisters can drive antibiotic resistance *via* plasmid conjugation. Bakkeren et al. [87••] used intravenous injection to establish a tissue-lodged population of *S.* Tm 14028 (lacking P2 and thus a natural recipient), then used streptomycin pre-treatment and oral gavage of *E. coli* bearing a modified P2 to transiently colonise the gut and observed conjugation of the resistance plasmid into the recipient *S.* Tm in the gut. This transconjugant *S.* Tm could then re-colonise tissue and form persisters in response to ciprofloxacin treatment, while the *E. coli* donor population was eradicated from the gut. Subsequent introduction of a third strain (SL1344 ΔP2) by oral gavage allowed for further plasmid

transfer to the new recipient, in which the tissue-lodged persisters could migrate back to the gut once more and participate in further rounds of plasmid transfer, conjugating the resistance plasmid to the secondary transconjugants. Thus, persisters allow for the transfer of a resistance plasmid between a primary donor and a secondary transconjugant (*via* a primary transconjugant) that never co-localised spatially or temporarily, suggesting that persister formation can powerfully extend the range of opportunities for indirect interactions between viable donor–recipient partners in horizontal gene transfer. More recently, Gaissmaier et al. [88] showed that reseeded *S.* Tm persisters can also transfer the mobilisable P3 plasmid (the pRSF1010 IncQ plasmid, which encodes for streptomycin resistance), in a manner dependent on the P2-conjugative machinery. Intraperitoneal injection of *S.* Tm SL1344 $P3^{SmR}$ followed by gavage of the *S.* Tm 14028 recipient strain led to the rapid emergence of streptomycin-resistant transconjugants in the faeces. To explore if antibiotic persisters could also conjugate P3, intraperitoneal injection of ceftriaxone was used to control tissue-lodged populations of the donor SL1344. Also, in this case, some plasmid-containing *S.* Tm cells survived in the host tissue and subsequent reseeding into the gut enabled transfer of P3 to the recipient 14028 strain. Thus, persisters can also conjugate mobilisable plasmids *via* conjugation machinery encoded on other plasmids, broadening the possible range of transferrable plasmids. This study provides a further example of how persisters can transfer naturally occurring resistance plasmids (as in [15••]), with implications for the conjugation and maintenance of resistance plasmids in natural contexts.

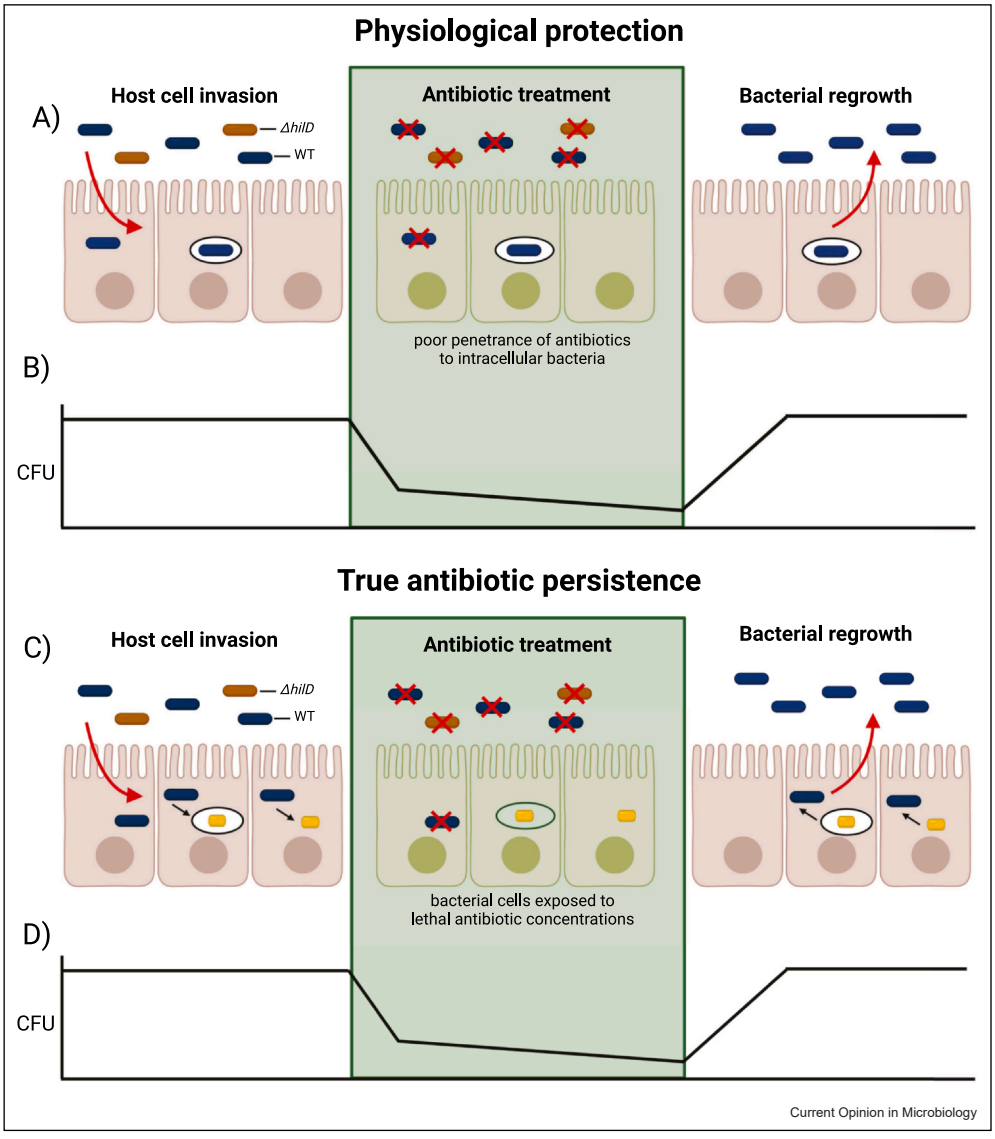
Collectively, these studies exploring the biological consequences of antibiotic persistence have demonstrated that persisters contribute to horizontal gene transfer, to the maintenance of virulent genotypes and to prolonged immunopathology in the infected gut. Further work might explore how horizontal gene transfer (particularly plasmid conjugation) under antibiotic pressure contributes to antibiotic resistance, particularly in livestock animals in agricultural contexts. Given that persisters are well established as reservoirs for conjugative plasmids, it is possible that tissue-lodged persisters may similarly act as a reservoir for temperate bacteriophages, which may provide the opportunity for later lysis and further interactions with other bacterial hosts. Indeed, it might be argued that persisters represent a niche for the storage of various genetic elements that can subsequently interact with viable hosts in other spatial or temporal contexts. Finally, given that active translocation of SPI-2 effectors by intracellular persisters has been reported *in vitro* [76], it may be that persisters have the opportunity to continue a subtle programme of immune manipulation that favours the undermining of host cells. Future work should consider how virulence factors, particularly the SPI-2 T3SS, influence persister dynamics within the infected host. Overall, persisters that form during therapy with bactericidal antibiotics likely have profound effects on pathogen evolution towards virulence or antibiotic resistance, and further work is needed to explore these hypotheses.

Towards functional definitions of antibiotic persistence *in vivo*

A considered review of the literature describing formation of *Salmonella* persisters during murine infection raises several possibilities as to why these bacteria can survive lethal doses of antibiotics *in vivo*. While it is overwhelmingly clear that antibiotic regimens are effective at controlling bacterial replication so long as treatment continues, total clearance of bacterial niches seems rare, and infections relapse once treatment is stopped. Two alternative scenarios are possible: one in which *S.* Tm forms true antibiotic persisters as is reported *in vitro*, and a second in which tissue-lodged persisters are protected by physiological factors that prevent the effective penetrance of antibiotics or decrease susceptibility by mechanisms other than persistence (such as niche-induced overexpression of drug-efflux pumps).

Broadly, the study of antibiotic persisters described here encompasses two separate but overlapping endeavours: one in which the mechanisms underpinning antibiotic persister formation are the focus, and another in which the consequences of antibiotic persisters are more relevant. In studies focused on the mechanisms of antibiotic persistence, it is important to firstly demonstrate the hallmark phenotypes of antibiotic persistence to establish if true persisters form *in vivo*, and then to explore how this phenotype is achieved [7]. As discussed above, this can be done by observing biphasic killing curves in the organ of interest, or by the assessment of growth rates of the surviving populations (e.g. by genetic barcoding combined with mathematical modelling, or by fluorescent dilution assays or similar growth rate reporters). This has already been achieved with reasonable precision in studies exploring ciprofloxacin- or enrofloxacin-treated C57BL/6 mice during oral infection with *S.* Tm [73,76,77]. Future studies building on this foundation are thus well placed to explore the mechanisms underpinning initial formation of persister populations, the influence of the immune system on controlling persisters and the consequences for pathogen evolution towards resistance or virulence. However, in spite of considerable technical effort, it remains to be established if all antibiotic-surviving bacterial cells represent true antibiotic persisters in accordance with the established *in vitro* criteria. Future advances in fluorescent reporter assays or single-cell analytics will help to more conclusively distinguish between true persister formation and alternative survival mechanisms *in vivo*.

Figure 4



Alternative hypotheses for bacterial survival under antibiotic pressure *in vivo*, using WT and invasion-deficient *Salmonella* Typhimurium as an example. **(a)** Intracellular bacteria are physiologically protected due to poor penetrance of antibiotics. Invasive *S.* Tm (i.e. WT but not *hilD* mutant) enters the gut tissue and establishes a replicative niche. Antibiotics kill gut-luminal populations and extravacuolar bacteria, while *S.* Tm residing within the vacuole is not exposed to bactericidal concentrations and thus survives. After antibiotic treatment is stopped, these bacteria can escape from the host cell and re-initiate new infectious cycles. **(c)** Intracellular bacteria are exposed to bactericidal concentrations of antibiotics, but survive due to true antibiotic persistence. Invasion of host cells is necessary to form the persister state, and as such, WT but not *hilD* mutants form intracellular persisters. Bacteria nonetheless encounter lethal doses of antibiotics within the cell, but survive due to very low rates of replication. Cessation of antibiotics allows for the relapse of bacterial infection. **(b)**, **(d)**, Killing kinetics of tissue-lodged bacteria during bactericidal antibiotic treatment will look similar for both physiological protection (as in **(a)**) and for true antibiotic persistence (as in **(c)**).
Adapted from [15••].

The studies of the consequences of persister formation (e.g. for evolution of virulence or horizontal gene transfer) will similarly benefit from improved tools to identify persisters *in vivo*. Here, distinguishing between true antibiotic persisters and otherwise antibiotic-recalcitrant bacteria is less critical, particularly in cases where any type of antibiotic survival will promote these consequences. The similarities and differences between true antibiotic persistence and other mechanisms of antibiotic recalcitrance (described here as physiological protection) are illustrated in Figure 4, using the example of WT *S.* Tm competition versus *hilD* mutants, as discussed above. Invasion-competent WT *S.* Tm cells will enter into the host tissue, while *hilD* mutants cannot. Thereby, the need for tissue invasion will enrich for WT *S.* Tm in the host tissue. These WT cells (but not the

hilD mutant) will have a chance to survive treatments with bactericidal antibiotics in the host tissue and cause relapses after cessation of antibiotic treatment. Importantly, this selection for the virulent WT *S.* Tm will occur, regardless if the antibiotic-surviving subpopulations in the tissue are true persisters or if they are surviving by other means (e.g. poor antibiotic penetrance into a host tissue lesion harbouring intracellular bacteria) (Figure 4). In either case, it should be possible to study the consequences of antibiotic survival, without the strict requirement of discriminating between these two different survival mechanisms. The true role of persisters can be established, once suitable *in vivo* techniques become available.

Based on this, we propose two complementary definitions that broadly enable the study of both the mechanisms and the consequences of antibiotic persistence *in vivo*. Firstly, we propose a looser operational definition of phenotypic antibiotic survival *in vivo*. Here, several broader criteria should apply: bactericidal antibiotic concentrations (i.e. the dose given to the mouse) should be greatly in excess of the minimum inhibitory concentration, biphasic killing kinetics should be observed, which are presumably attributable to the rapid elimination of susceptible bacteria and the delayed killing of the persister subpopulation; bacterial infections should relapse *in vivo* some time after antibiotic treatment is stopped (determined by plating and counting colony forming units (CFU)); and re-isolated surviving bacteria should demonstrate the same level of susceptibility to bactericidal antibiotics as for the parental strain. This permissive definition should be broadly suitable to the study of the biological consequences of antibiotic survivors *in vivo*, regardless of whether these phenotypes arise from physiologically protected bacteria or true antibiotic persisters (Figure 4). In contrast, the restrictive definition of antibiotic persistence is pivotal for those studies that seek to explore how true antibiotic persisters may arise *in vivo*; here, more rigorous methods are critical to establish whether lethal doses of bactericidal antibiotics truly penetrate to tissue-lodged bacteria, to ensure that sufficient antibiotic concentrations are sustained between treatments, and to explore how the persister phenotype is established and maintained at the molecular level (note that these true persisters will also satisfy all of the criteria for the looser definition of antibiotic survival). The technical challenges implicit here are largely concerns for the future, but are nonetheless necessary to reconcile the burgeoning field of *in vitro* antibiotic persistence with the developing *in vivo* literature, with the ultimate goal of informing the development of effective therapeutics for clinical use or strategies for preventing the rise of virulence or antibiotic resistance.

Conclusions and perspectives

The use of animal models of *S.* Tm infection has played a critical role in establishing basic principles of antibiotic persistence *in vivo*, and has become a platform for more elegant experiments exploring the biological consequences of persistence, such as the promotion of antibiotic resistance, the maintenance of virulence phenotypes and the role of host pressures on persistent niches. Refining these models holds great promise for exploring unresolved questions, particularly regarding the penetrance of antibiotics into intracellular niches, and the possibility of therapeutically manipulating persister niches by controlling the local host environment. The establishment and maintenance of the intracellular niche seems to be critical to the ability of *S.* Tm to survive antibiotic treatment, and so it will be particularly important to explore how virulence factors contribute to persister formation, particularly the SPI-1 and SPI-2 T3SS and their cognate repertoires of effector proteins. It is interesting to reflect that the evolutionary pressures that have produced virulence factors capable of maintaining and transmitting *S.* Tm within and between hosts have also enabled the recalcitrance of these bacteria to modern antibiotic treatment.

The recent consensus statement [7] on antibiotic persistence research called for the alignment of experimental methodology for *in vitro* experiments, and we suggest that a similar degree of co-ordination and attention to variation between experimental protocols would benefit advances in understanding antibiotic persistence *in vivo*. We propose a loose operational definition for studying various consequences of phenotypic antibiotic survival *in vivo* while also reserving a strict definition for true antibiotic persistence. Ultimately, while *in vitro* experiments offer great insight into the mechanisms and properties of antibiotic persistence, we have already seen examples of how this understanding can fail to translate to the study of persisters *in vivo*. Thus, studies using well-established animal models will play an essential role in efforts to understand and manipulate persisters in order to address the challenges arising from bacterial populations that survive antibiotic treatment.

We envision that studies linking virulence to antibiotic persistence during *Salmonella* infection might lead to the description of general principles of persister formation *in vivo*. More broadly, it seems likely that antibiotic persistence will arise *in vivo* for any pathogen that has some mechanism for forming and maintaining a niche within the host, be that intracellular pathogens that manipulate host cell processes to form a permissive niche, or extracellular bacteria that rely on biofilm formation or other factors to promote adherence and colonisation. Future work will explore how these virulence phenotypes enable true antibiotic persistence or otherwise physiological protection of bacteria, though we do not exclude the possibility that both situations may arise (indeed, both cases might arise within an individual host

concurrently). Studying how different pathogens use their associated virulence factors to achieve persister formation will help to elucidate the general principles of antibiotic persistence *in vivo*, and will be useful in developing treatments aimed at eliminating these bacterial populations.

Conflict of interest statement

Nothing declared.

Data Availability

No data were used for the research described in the article.

Acknowledgements

Work in the lab of WDH is generously funded by the Swiss National Science Foundation, Switzerland (NRP 72 407240_167121, 310030B_173338, 310030_192567, Swiss National Centre of Competence in Research (NCCR) Microbiomes, Switzerland, 51NF40_180575); the COL_RES Marie Sklodowska-Curie Innovative Training Network (ITN), European Union; and the Gebert Rüf Foundation, Switzerland (GRS-060/18). The authors thank Dr. Erik Bakkeren and Dr. Yassine Cherrak for their comments on draft versions of this review. Figures created with BioRender.com.

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