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Internalization of *Salmonella* by Macrophages Induces Formation of Nonreplicating Persisters

Sophie Helaine[*], **Angela M. Cheverton**[#], **Kathryn G. Watson**[#], **Laura M. Faure**, **Sophie A. Matthews**, and **David W. Holden**[*]
Section of Microbiology, Medical Research Council Centre for Molecular Bacteriology and Infection, Imperial College London, Armstrong Road, London SW7 2AZ, UK

[#] These authors contributed equally to this work.

Abstract

Many bacterial pathogens cause persistent infections despite repeated antibiotic exposure. Bacterial persisters are antibiotic-tolerant cells, but little is known about their growth status and the signals and pathways leading to their formation in infected tissues. We used fluorescent single-cell analysis to identify *Salmonella* persisters during infection. These were part of a nonreplicating population formed immediately after uptake by macrophages and were induced by vacuolar acidification and nutritional deprivation, conditions that also induce *Salmonella* virulence gene expression. The majority of 14 toxin-antitoxin modules contributed to intracellular persister formation. Some persisters resumed intracellular growth after phagocytosis by naïve macrophages. Thus, the vacuolar environment induces phenotypic heterogeneity, leading to either bacterial replication or the formation of nonreplicating persisters that could provide a reservoir for relapsing infection.

Many studies, mostly focusing on bacteria grown in laboratory media, have shown that persisters are an unstable, nongrowing, multidrug-tolerant subpopulation that result from phenotype switching (1–3). In contrast to antibiotic-resistant bacteria arising from heritable mutations, the progeny of persisters are mainly antibiotic-sensitive cells. Most studies on persistent infections are based on the assumption that a proportion of the bacterial population is nonreplicating (4); however, this consensus was challenged recently in studies that showed replicating antibiotic-tolerant mycobacteria (5, 6). Moreover, direct evidence for nongrowing persister bacteria in infected tissues is lacking.

Previously, we reported the use of a fluorescence dilution (FD) technique to study intracellular replication dynamics of *Salmonella* Typhimurium. FD can be used to monitor the extent of bacterial replication at the single-cell level by dilution of a preformed pool of fluorescent protein after its induction has been stopped. The technique reveals the presence of bacteria that maintained bright fluorescence during infection, indicating that they are nongrowing (7). We used FD to investigate whether nonreplicating *Salmonella* were persisters.

[*]Corresponding author. s.helaine@imperial.ac.uk (S.H.); d.holden@imperial.ac.uk (D.W.H.).

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Infection of susceptible mice by *S.* Typhimurium results in an acute typhoid-like disease characterized by intracellular proliferation of bacteria in organs including the Peyer's Patches (PP), mesenteric lymph nodes (MLNs), spleen, and liver. C57 Bl6 or BALB/c mice were inoculated per os (p.o.) with wild-type (WT) *S.* Typhimurium carrying a plasmid from which mCherry is produced constitutively (to provide a marker for bacterial cells) and green fluorescent protein (GFP) dilutes proportionally with bacterial cell division (8). At different time points, tissues were extracted, homogenized, and lysed. The released *Salmonella* cells were subjected to flow cytometry analysis (fig. S1). As early as 2 hours after inoculation, the majority of bacteria that were detected in the PP and MLNs had not undergone any replication (50 and 95%, respectively) (Fig. 1A). Nonreplicating bacteria were also found in these organs and in the spleen at later time points. To determine the viability of these nonreplicating cells, bacteria released from MLNs were incubated in Luria Bertani (LB) medium and analyzed by flow cytometry. Dilution of fluorescence (indicated by a decrease in bacterial fluorescence intensity) (Fig. 1B) revealed resumption of growth of the previously identified nonreplicating population. Regrowth of bacteria that had not undergone any replication in vivo was also observed with timelapse microscopy (Fig. 1C). To clear the infection and reveal persisters, mice were treated with enrofloxacin at 24 hours after inoculation. Five days later, all of the remaining bacteria (~10^2) extracted from MLNs [a known site of persistence (9)] had not undergone any replication (Fig. 1D), indicating an incomplete clearance of the non-replicating population. We transferred these nongrowing cells to LB medium and observed resumption of growth in the absence of antibiotic treatment (Fig. 1E), indicating that they were persisters.

In the mouse typhoid model, *Salmonella* proliferates in macrophages (10, 11). Internalization of *Salmonella* by resting or interferon-γ (IFN-γ)–activated bone marrow–derived macrophages (BMMs) from different mouse strains for 24 hours or 30-min periods produced a 100- to 1000-fold increase in the proportion of antibiotic-tolerant bacteria as compared with those already present in the inoculum (Fig. 2, A and B, and fig. S2). The progeny of BMM-derived bacteria that survived antibiotic exposure were as antibiotic-sensitive as the initial inoculum, indicating that they are bona fide persisters (Fig. 2A). Macrophage-induced persisters were tolerant to several antibiotics (Fig. 2C). FD was used to investigate the replication status of these bacteria. As was observed in mice, the BMM-derived persisters consisted of nonreplicating cells that formed before any bacterial division had occurred (as indicated by an absence of fluorescence dilution) (Fig. 2, D and E). These results contrast with those obtained with mycobacteria, in which persisters were shown to be replicating bacteria (5, 6).

We showed previously that intracellular nonreplicating *Salmonella* are composed of subpopulations of unresponsive and metabolically active bacteria whose numbers decline with time (7). To determine which of these subpopulations were persisters, we used a double-inducible fluorescent reporter (Fig. 3, A and B, and fig. S3). Bacteria preloaded with GFP were used to infect macrophages for up to 72 hours. Replicating bacteria gradually lost the GFP signal and were killed by exposure to cefotaxime. Nonreplicating bacteria retained green fluorescence, and their metabolic status was determined through addition of DsRed inducer (7). Under improved growth culture conditions (12), as many as 40% showed evidence of metabolic activity for the 72-hour observation period (Fig. 3B). This contrasted

with a rapid decrease in metabolic activity of antibiotic-tolerant *Salmonella* that formed in LB medium (Fig. 3B), a phenomenon that has been observed with *Escherichia coli* (13).

We released nonreplicating intracellular bacteria by lysis of infected macrophages and monitored subsequent growth in LB medium by FD. Regardless of how long they had been internalized, ~20% of the metabolically active subpopulation resumed growth within 2 hours after their release from macrophages, as indicated by concomitant dilution of green and red fluorescence (Fig. 3, A and B, and fig. S4). No re-growth of the metabolically inactive population was detected (Fig. 3A). Therefore, intracellular persisters are derived exclusively from the metabolically active subpopulation of nonreplicating *Salmonella*.

Nonreplicating bacteria recovered from macrophages after 24 hours were incubated with naïve macrophages. After re-phagocytosis, ~5% of the population that was metabolically active in the initial host cells resumed growth (Fig. 3C) and may thus be a source of relapsing infection (9). These results show that macrophages contain heterogeneous subpopulations of nonreplicating *Salmonella*, including cells that are (i) metabolically inactive (possibly dormant), (ii) metabolically active but which fail to regrow following their release from BMMs, (iii) competent for regrowth in LB medium, and (iv) competent for regrowth in naïve macrophages (Fig. 3D).

Next, we investigated processes required for macrophage-induced persister formation. Activation of BMMs with IFN-γ had little effect on the proportion of persisters (fig. S2). Exposure of *Salmonella* to BMMs that had been pretreated with Latrunculin B (Lat B) or Cytochalasin D (Cyt D) to prevent phagocytosis did not generate more persisters than the basal levels detected in LB medium. This indicated that internalization is required to stimulate macrophage-induced persister formation (Fig. 4A and fig. S5). There was no detectable decrease in macrophage-induced persister frequency obtained from *phox*$^{-/-}$ BMMs that are unable to produce reactive oxygen species (Fig. 4A). However, preventing *Salmonella*-containing vacuole (SCV) acidification with Bafilomycin A1 (Baf A1) caused a significant decrease in persister frequency (Fig. 4A and fig. S5). Transient exposure of *Salmonella* to acidified laboratory medium or serine hydroxamate (SHX) (to induce starvation) (14) both enhanced persister formation (Fig. 4B). These results indicate that acidification and probably nutritional deprivation within SCVs (15) contribute to formation of persisters (Fig. 4B).

Nonpathogenic *E. coli* K12 persisters are induced at low frequency in laboratory medium by class II toxin-antitoxin (TA) modules (16), and their formation was not enhanced by passage through BMMs (Fig. 4A). Activation of the stringent response through the ppGpp synthases RelA and SpoT leads to activation of the Lon protease (17, 18). Lon degrades antitoxins of Class II TA modules, releasing intrabacterial toxins that cause transient growth arrest. As a result, *E. coli lon* mutants produce low levels of persisters in vitro (18). In contrast, a *Salmonella lon* mutant had no defect in persister formation when grown in laboratory media (fig. S6, A and B). However, deletion of *Salmonella relA* and *spoT* or *lon* significantly reduced macrophage-induced persister numbers (Fig. 4C). Thus, the Lon-dependent persister induction pathway of *Salmonella* is specifically responsive to conditions encountered within macrophage vacuoles. Analysis of the *Salmonella* genome revealed 14

putative class II TA operons (19), all of which were up-regulated from 4- to 30-fold in a *relA*, *spoT*-dependent manner within 30 min of phagocytosis, which is in broad agreement with previous work (fig. S7A) (20). Transient acidification of the culture medium or exposure to SHX also activated the TA modules (fig. S7B).

Single-deletion mutant strains were obtained for each of the TA loci. Although the majority of mutants did not show any impairment of intracellular replication rates, they showed a 10 to 30% reduction in the proportion of nonreplicating bacteria in infected BMM as compared with the WT strain (Fig. 4D and fig. S8). Mutation of TA genes had an even greater effect on persister frequency (Fig. 4D and fig. S9). Thus, after phagocytic uptake 14 class II TA modules were activated by acidification and/or nutrient starvation within the SCV, leading to the rapid formation of nonreplicating persisters.

Because the *Salmonella shpAB* locus has been shown to encode a functional TA module (21), we investigated its contribution to persister formation during infection of mice. To do this, groups of 10 animals were inoculated p.o. with an equal mixture of WT and *shpAB* mutant strains. Animals were given enrofloxacin 24 hours later and for a further 5 days, at which time MLNs were extracted to recover persisters. The relative proportions of WT and *shpAB* mutant strains were determined by colony-forming units, and a competitive index (CI) value (12) of 0.85 ($P = 0.01$) was obtained. The reduced rate of persister formation by the mutant strain correlated with its enhanced net growth over the wild type in the absence of antibiotic [CI value of 1.83 ($P = 0.05$)]. Together, these experiments show that the *shpAB* TA locus contributes to the generation of persisters in the mouse model of typhoid fever.

Overall, these results reveal several important characteristics of nonreplicating intracellular *Salmonella*. First, all persisters that were detected, both in mice and macrophages, formed within minutes upon infection and failed to replicate. Second, nonreplicating bacteria displayed unexpected phenotypic heterogeneity (Fig. 3D). Third, in contrast to nonpathogenic *E. coli*, the *Salmonella lon*–dependent persister induction pathway responded specifically to the host cell environment. The acidified and nutritionally poor vacuolar environment is also required for the expression of many *Salmonella* virulence genes (22), which are required for bacterial replication in macrophages (7). Thus, the vacuolar environment stimulates some bacterial cells to initiate replication and others to enter a nonreplicating state in which they are more likely to survive long-term host-induced damage and exposure to antibiotics (fig. S10). The generation of such cell-to-cell heterogeneity is likely to provide a strong selective advantage to *Salmonella* and other bacterial pathogens that encounter the antimicrobial activities of macrophages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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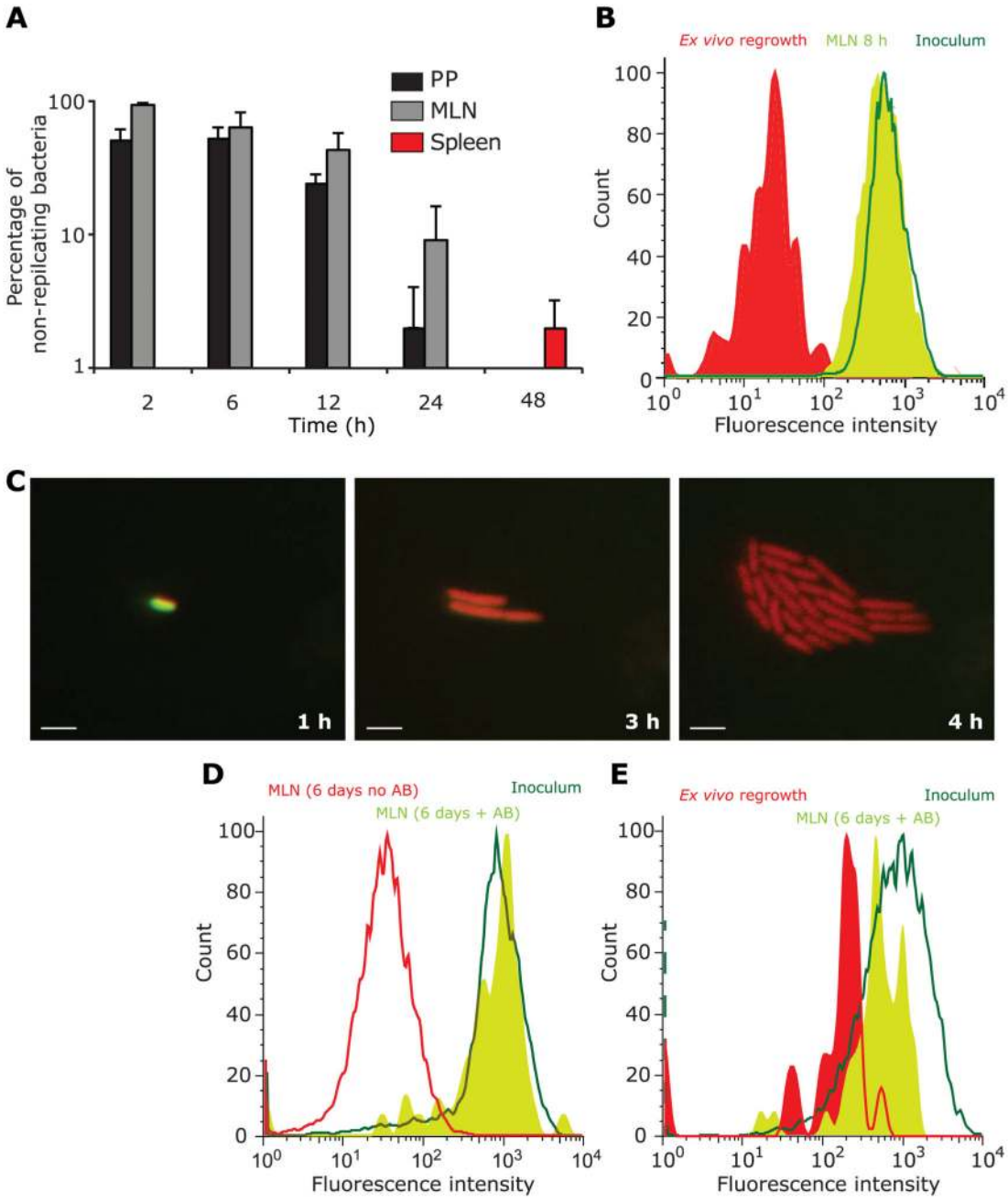

Fig. 1. Nonreplicating *Salmonella* persisters in immune organs.

(**A**) The average percentage of nonreplicating bacteria in the PP, MLNs, and spleens of mice, detected with flow cytometry by use of FD at different times after inoculation. Data represent the mean ± SEM and were analyzed by using a Student's *t* test. $^{**}P < 0.01$. (**B**) Representative FD profiles of bacteria recovered from MLNs of five mice, 8 hours after infection (light green) and 16 hours after inoculation in LB (red) (number of bacteria $\geq 10^4$), showing regrowth of nonreplicating bacteria isolated from MLNs. (**C**) Regrowth of a nonreplicating bacterium recovered from MLNs 24 hours after inoculation on LB agar. Scale

bar, 2 μm. (**D** and **E**) FD profiles of bacteria recovered 6 days after inoculation from MLNs of 10 mice, treated (light green) or not (red) with enrofloxacin, and 12 hours after inoculation in LB medium (red) (number of bacteria $\geq 10^2$) showing regrowth of nonreplicating bacteria that survived antibiotic treatment (AB) of animals.

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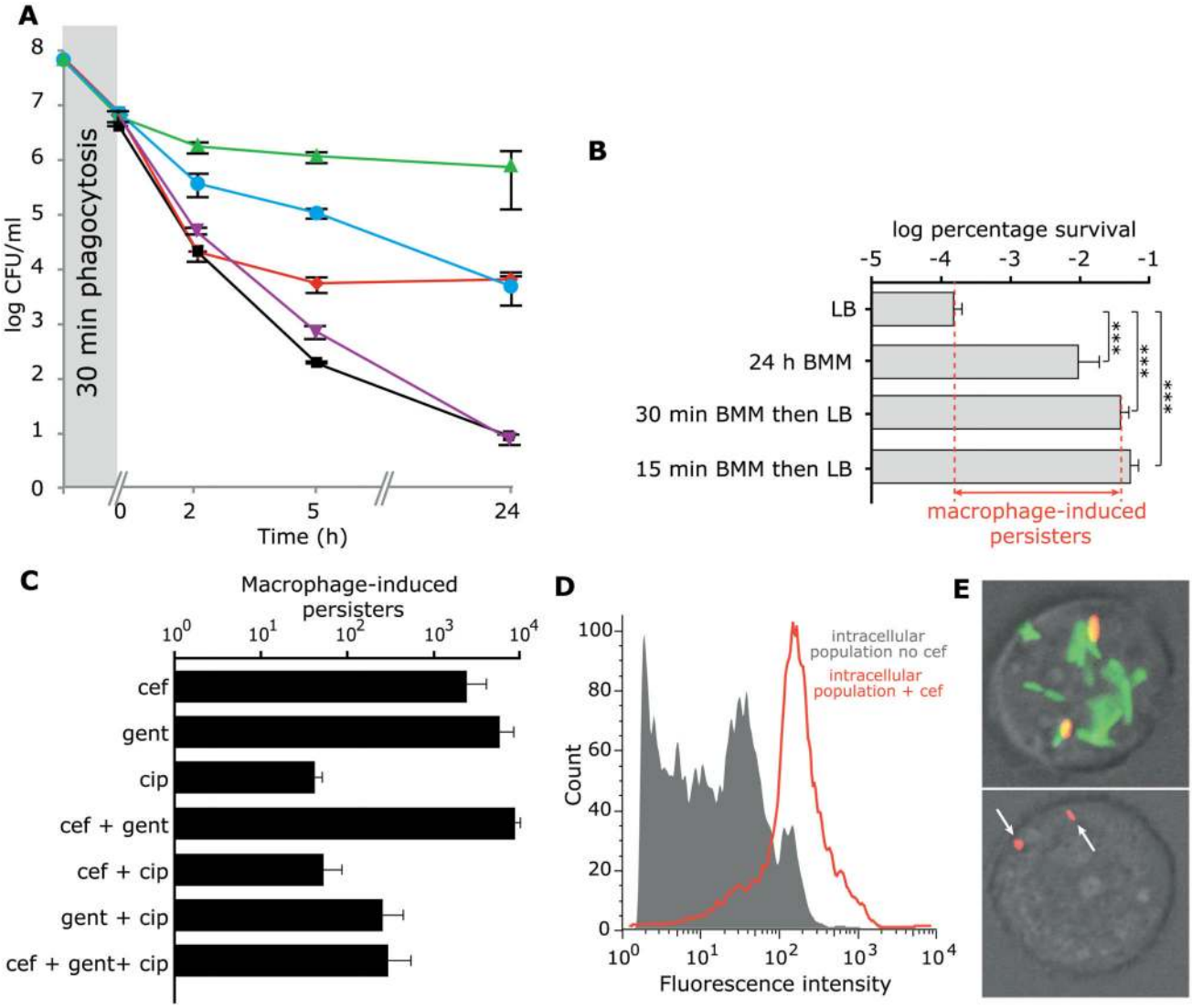

Fig. 2. Rapidformation of non-replicating persisters in macrophages.

(**A**) Cefotaxime (cef) survival kinetics of *Salmonella* grown in LB medium (black), BMMs (blue), LB medium after 30-min internalization (red), compared with survival of bacteria in BMM without cef treatment (green). Progeny of persisters (24 hours time-point, red kinetics) were subjected to a second exposure to cef in LB medium (purple). (**B**) Log percentage survival after 24 hours of cef treatment of LB medium–grown bacteria, or bacteria within BMM for 24 hours, 30 min, or 15 min followed by transfer to LB medium. Formation of macrophage-induced persisters was measured as the fold-increase in persister levels quantified in bacterial populations either grown exclusively in LB medium or in LB medium after 30-min internalization in BMM. Data were analyzed by using a Student's *t* test. ***$P < 0.001$. (**C**) Macrophage-induced persister formation measured after exposure to cef, gentamicin (gent), ciprofloxacin (cip), or different antibiotic combinations. Replication status of intracellular bacteria, by using FD and detected by (**D**) flow cytometry before (gray area) or after (red line) 16 hours exposure to cef, added 18 hours after uptake; or (**E**) live-

imaging microscopy before (top) and after (bottom) 12 hours exposure to cef, added 18 hours after uptake. The bacteria that remained after cef exposure were non-replicating [(D), red line, and (E), white arrows]. Data represent the mean ± SEM.

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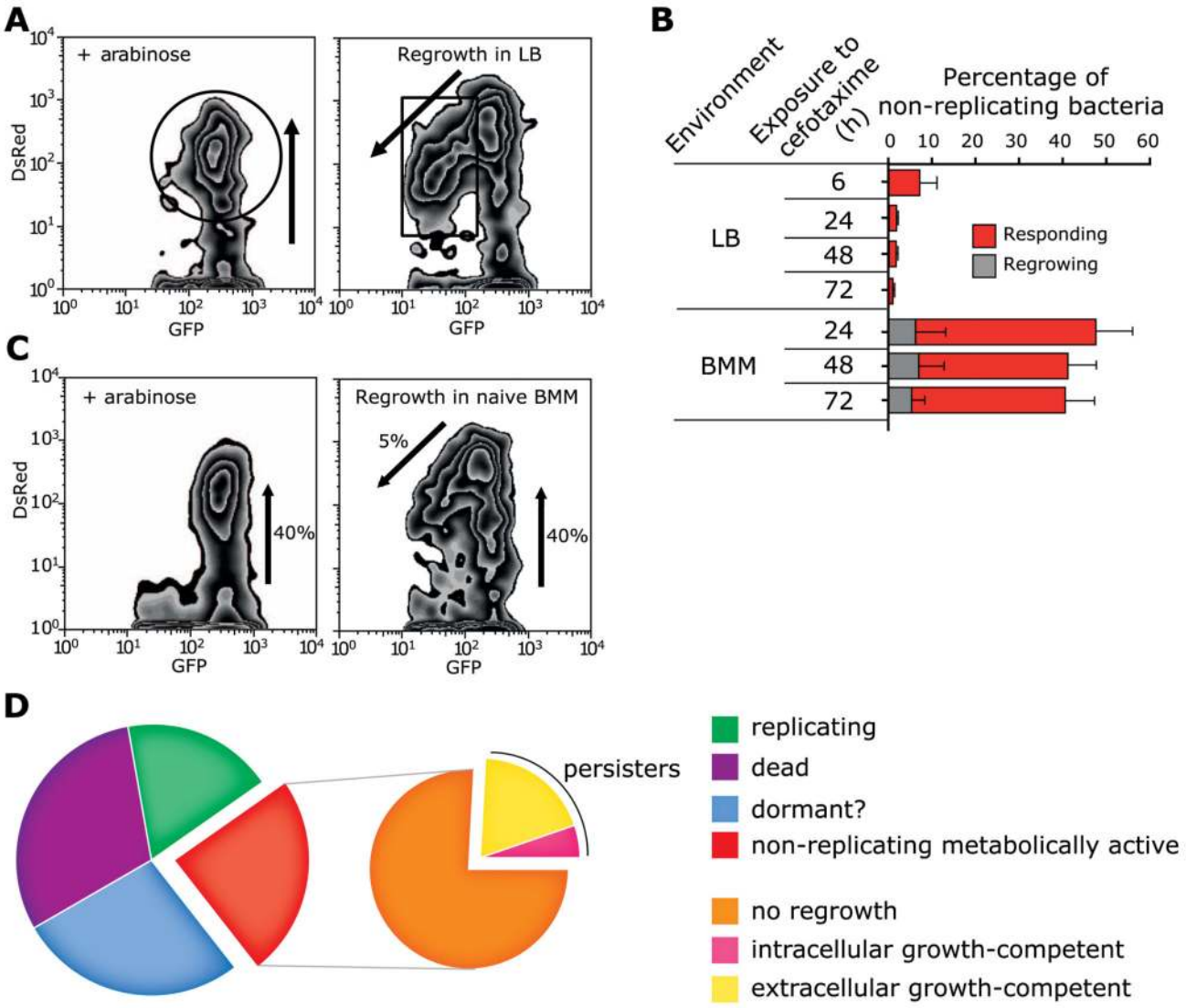

Fig. 3. Metabolic status of persisters.

(**A**) Analysis of metabolic activity (left) and regrowth (right) of persisters, analyzed by means of flow cytometry. (Left) Intracellular antibiotic-tolerant bacteria retained high GFP intensity because of a lack of replication, and their metabolic activity was revealed by appearance of DsRed fluorescence upon addition of arabinose (circle). (Right) After release from macrophages and transfer to LB medium, regrowth of some bacteria was revealed through concomitant dilution of DsRed and GFP fluorescence (rectangle). (**B**) Quantification of proportion of nonreplicating bacteria showing metabolic activity upon addition of inducer (production of DsRed fluorescence; red bars) and resuming growth (indicated by fluorescence dilution; gray bars), from LB medium-grown or intracellular nonreplicating cells in activated BMM over time. Data represent the mean ± SEM. (**C**) Representative example of quantification of proportion of intracellular non-replicating bacteria, after 24 hours within activated BMM, showing (left) metabolic activity revealed by

appearance of DsRed upon addition of arabinose and (right) quantification of growth resumption of metabolically active bacteria within newly infected BMM. (**D**) Chart showing relative proportions and heterogeneity of different *Salmonella* subpopulations in BMM. Thick arrows in (A) and (C) indicate direction of fluorescence shift, either representing production of DsRed upon arabinose addition (vertical arrows) or dilution of DsRed and GFP fluorescence during regrowth (oblique arrow).

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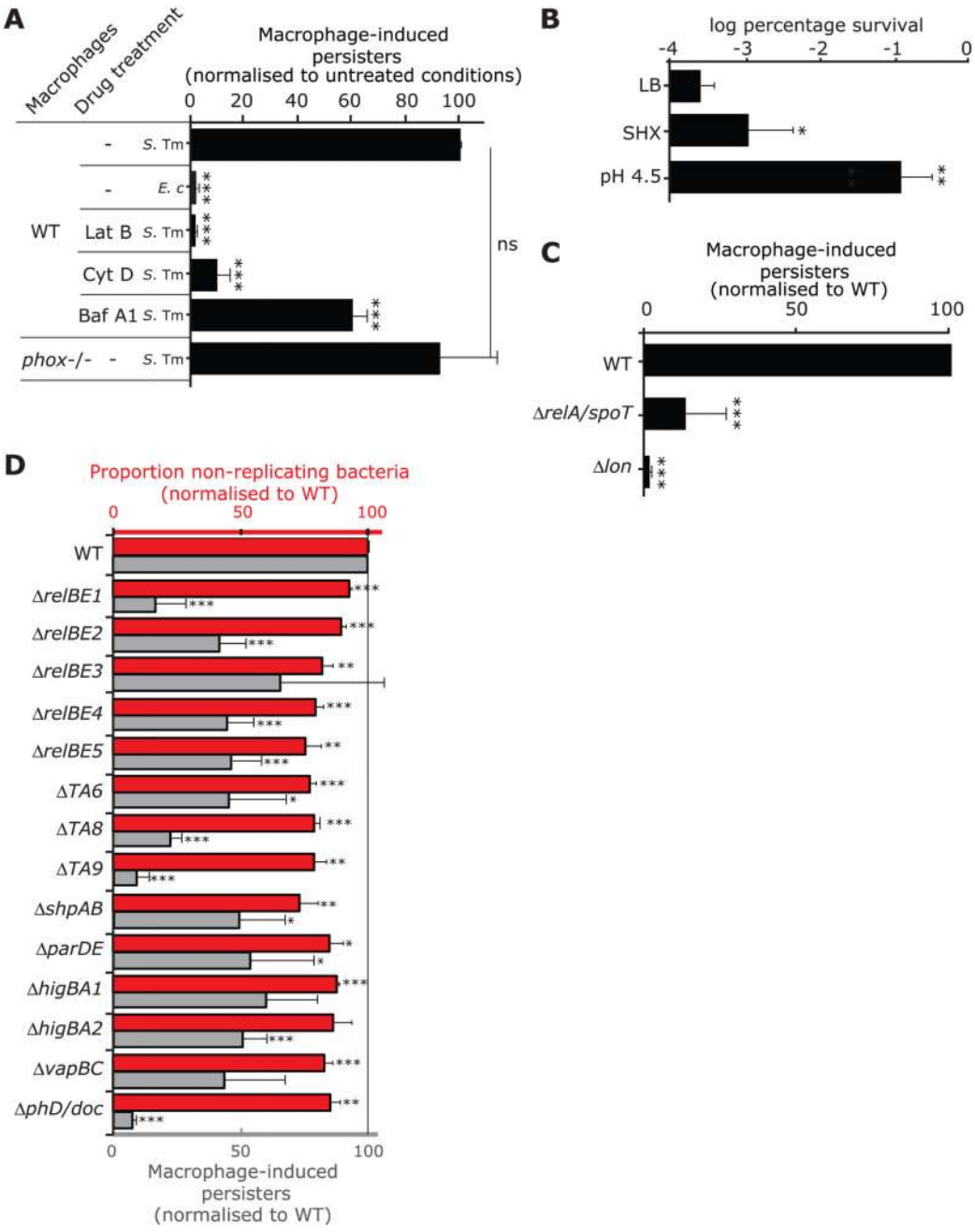

Fig. 4. TA modules are required for formation of macrophage-induced nonreplicating persisters in response to vacuolar stimuli.

(**A**) Formation of macrophage-induced persisters of *Salmonella* (*S.* Tm) or *E. coli* (*E. c*) caused by 30-min internalization in BMM, WT BMM treated with Lat B, Cyt D or Baf A1, or *phox*$^{-/-}$ BMM. (**B**) Log percentage survival after 24 hours of cef treatment of LB medium–grown bacteria pre-exposed to SHX or pH shift from 7 to 4.5 for 30 min before transfer to LB/cef and compared with untreated bacteria. (**C**) Formation of macrophage-induced persisters of WT, Δ*relA*Δ*spoT*, and Δ*lon* mutant strains caused by 30-min

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internalization in BMM. (**D**) Quantification of the proportion of nonreplicating intracellular bacteria after 18 hours of BMM infection (red bars), and the formation of macrophage-induced persisters caused by 30-min internalization in BMM (gray bars), of WT or single–TA module mutant strains. Data represent the mean ± SEM and were analyzed through comparison with WT *Salmonella* [(A), (C), and (D)] or to LB medium (B) by using a Student's *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant.

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