Phenotypic Variation of *Salmonella* in Host Tissues Delays Eradication by Antimicrobial Chemotherapy

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SUMMARY

Antibiotic therapy often fails to eliminate a fraction of transiently refractory bacteria, causing relapses and chronic infections. Multiple mechanisms can induce such persisters with high antimicrobial tolerance in vitro, but their in vivo relevance remains unclear. Using a fluorescent growth rate reporter, we detected extensive phenotypic variation of *Salmonella* in host tissues. This included slow-growing subsets as well as well-nourished fast-growing subsets driving disease progression. Monitoring of *Salmonella* growth and survival during chemotherapy revealed that antibiotic killing correlated with single-cell division rates. Nondividing *Salmonella* survived best but were rare, limiting their impact. Instead, most survivors originated from abundant moderately growing, partially tolerant *Salmonella*. These data demonstrate that host tissues diversify pathogen physiology, with major consequences for disease progression and control.

INTRODUCTION

Treatment of infections with appropriate antibiotics rapidly reduces bacterial burden, but often fails to eliminate a fraction of refractory cells that can cause relapses and chronic infections (Balaban et al., 2013; Kint et al., 2012; Lewis, 2012). Recalcitrant cells are phenotypic variants that transiently tolerate extraordinary levels of antibiotics but remain genetically drug sensitive. Such so-called persisters can be induced in vitro by diverse mechanisms (Balaban et al., 2013; Kint et al., 2012; Lewis, 2012). Persisters may originate from rare stochastic nondividing subpopulations (Balaban et al., 2004; Maisonneuve et al., 2013), but high tolerance can also occur in actively growing pathogen subsets (Nguyen et al., 2011; Orman and Brynildsen, 2013; Wakamoto et al., 2013).

Only a few studies have analyzed mechanisms that cause pathogen antimicrobial tolerance in infected host tissues. Subsets of *Mycobacterium marinum* in infected fish larvae express high levels of drug efflux pumps conferring antimicrobial tolerance (Adams et al., 2011), whereas minor nondividing *Salmonella* subsets can persist in infected mice during early-onset/high-dose antimicrobial chemotherapy (Helaine et al., 2014; Kaiser et al., 2014). The extent of pathogen phenotypic variation and its impact on treatment efficacy under clinically relevant conditions is still unclear (Balaban et al., 2013).

To determine pathogen growth variation in vivo, we devised a single-cell growth reporter and applied it to a mouse typhoid fever model. The results revealed distinct *Salmonella* subsets with divergent division rates in spleen and other tissues. Analysis of ex vivo purified subsets suggested that differential host nutrient supply contributed to this heterogeneity. To assess antimicrobial tolerance of the various subsets, we monitored *Salmonella* division and killing during fluoroquinolone therapy under clinically relevant conditions. Slow-growing *Salmonella* survived best after each dose but, surprisingly, overall eradication was delayed primarily by abundant subsets of moderately growing *Salmonella* with partial tolerance. These results provide a new paradigm for pathogen variation in host tissues and its impact on antimicrobial chemotherapy.

RESULTS

Construction and Characterization of a TIMERbac Growth Rate Indicator

The DsRed S197T variant called TIMER spontaneously changes fluorescence color from green to green/orange (Terskikh et al., 2000). This results from a branched maturation pathway with rapid emergence of green fluorophores and delayed formation of orange fluorophores. Fluorescence resonance energy transfer (FRET) from green to orange fluorophores in mixed green/orange TIMER tetramers increasingly quenches green fluorescence and further enhances orange fluorescence (Strack et al., 2010).

DsRed variants are very stable against proteolysis (Verkhusha et al., 2003). In nonproliferating cells, both fast green and slowly
maturing orange TIMER molecules should thus accumulate over time, yielding green/orange fluorescence (Figure 1A). In contrast, growing cells dilute both forms with each cell division. Fast-maturing green TIMER molecules should emerge earlier, at a more concentrated stage, compared to slowly maturing orange TIMER molecules, resulting in a dominant green fluorescence (Figure 1A). TIMER color might thus serve as a growth rate reporter.

To test this hypothesis, we exchanged serine 197 for threonine in a DsRed variant with high yields in bacteria (Sörensen et al., 2003) and expressed the resulting TIMERbac in Salmonella enterica serovar Typhimurium SL1344. Salmonella with constitutive TIMERbac expression grew as orange colonies (Figure 1B). Freshly induced TIMERbac formed green fluorophores (emission peak at 503 nm), followed by orange fluorophores (peak at 587 nm) (Figure 1C). Orange TIMERbac molecules had a bimodal excitation spectrum with peaks at 483 and 561 nm (Figure 1C), consistent with FRET within mixed green/orange TIMER tetramers. Maturation kinetics depended on oxygen partial pressure (Figure 1D), as expected (Strack et al., 2010). This oxygen dependency represents an important caveat for using TIMER as a growth rate reporter in environments with inhomogeneous oxygenation (see Discussion).

During continuous growth in chemostats, TIMERbac-Salmonella had green/orange fluorescence ratios that correlated with division rates and were robust against cell-to-cell variations in protein content and cell size (Figure 1E). Green/orange ratios depended on oxygen tension (Figure 1F), as expected based on maturation kinetics (Figure 1D). After switching cultures from one division rate to another, color changed with response times of several hours (Figure 1G), consistent with slow TIMERbac maturation and dilution as decisive processes determining color.

**TIMERbac-Salmonella Fluorescence in Macrophage Cell Cultures**

In cell-culture macrophage infections, Salmonella have diverse intracellular growth rates, including a large nondividing subset (Abshire and Neidhardt, 1993; Helaine et al., 2010) (Figure 2A). Consistent with this finding, intracellular TIMERbac-Salmonella showed varying green/orange ratios including a prominent subset with low green/orange ratios, indicative of poor replication (Figure 2B, Stat. phase). Live-cell imaging confirmed green fluorescence of growing Salmonella and orange fluorescence of nondividing Salmonella (Movies S1 and S2 available online).
Interestsingly, growth heterogeneity depended on the inoculum. Large nondividing subsets occurred after infection with Salmonella from stationary cultures (as used in previous studies), possibly reflecting the extensive cell-to-cell variation in stress sensitivity in such cultures (Ryall et al., 2012). In contrast, exponentially grown Salmonella showed more homogeneous intracellular growth, with few nondividing bacteria both in the fluorescence dilution (Helaine et al., 2010) and TIMERbac approaches (Figures 2A and 2B; Movies S3 and S4). The close agreement with fluorescence dilution and video microscopy confirms the utility of TIMERbac as a reporter for intracellular Salmonella growth.

**TIMERbac-Salmonella Fluorescence in Mice**

To evaluate TIMERbac in vivo, we infected genetically susceptible BALB/c mice, a model for human typhoid fever (Tsolis et al., 2011). TIMERbac-Salmonella retained full virulence (spleen colony-forming units [CFUs] 4 days after intravenous [i.v.] infection with some 1,000 CFUs: SL1344, [1.3 ± 0.3] × 10³; Salmonella/pBR322_TIMER, [1.6 ± 0.2] × 10⁶; Salmonella/pSC101_TIMER, [1.2 ± 0.4] × 10⁶). Salmonella stably maintained functional episomal TIMERbac expression cassettes (all colonies recovered on nonselective medium were orange). Flow cytometry of detergent-treated spleen homogenates revealed released green/orange Salmonella with baseline separation from autofluorescent host debris for episomal, but not single-copy, chromosomal expression cassettes (Figures 3A and Figure S1). We used pBR322_TIMER, yielding bright fluorescence in most subsequent experiments.

During initial adaptation to host conditions, Salmonella show substantial nondividing subsets (Helaine et al., 2014) before reaching optimal growth rates at 24–48 hr postinfection (p.i.) (Mastroeni et al., 2011). Consistent with these observations, TIMERbac-Salmonella subsets with low green/orange ratios were present at day 1 p.i. but declined to stable low levels thereafter (Figure 3B, left; Figure S2). Color distributions of adapted Salmonella resembled macrophage cell cultures infected with exponentially grown Salmonella (Figures 2B and S2A).

Comparison of in vivo TIMERbac fluorescence (Figure 3B, left) with chemostat cultures under in vivo-like conditions (5% oxygen, acidic pH, limiting nutrients) (Figure 1F) suggested an average in vivo division rate of 0.14 ± 0.02 hr⁻¹, in excellent agreement with plasmid dilution data (0.15 ± 0.005 hr⁻¹; see below) and generation times of 6–8 hr in mouse spleen (Becker et al., 2006; Grant et al., 2008). In contrast, the SPI-2 mutant Salmonella ssrB poorly colonized spleen and had low green/orange ratios at day 4 p.i. (Figure 3B, right), consistent with marginal growth of SPI-2 mutants from day 2 p.i. (Grant et al., 2012; Shea et al., 1999). Other Salmonella mutants had intermediate TIMERbac colors (Figure 3C). Color-based growth rate estimates correlated with data from competitive infections (Becker et al., 2006; Steeb et al., 2013) (Figure 3D). This is remarkable, because competitive infections reflect Salmonella growth and killing over several days of infection, whereas TIMERbac reports on current growth and provides no information on killed nonfluorescent Salmonella (Burton et al., 2014).

**Coexistence of Salmonella Subsets with Divergent Division Rates in Mice**

TIMERbac-Salmonella had broad color distributions in infected spleen (Figure 3B, left) compared to chemostat cultures (Figure 1E). Ex vivo sorting and reanalysis confirmed the presence of Salmonella subsets with genuine color differences (Figure 4A). Reinjection of sorted subsets into naive mice yielded superimposable color distributions in spleen 4 days later (Figure 4A), suggesting phenotypic rather than heritable variation. TIMERbac-Salmonella resided in spleen red pulp, as expected (Burton
et al., 2014), and showed divergent colors in confocal microscopy, consistent with flow cytometry data. Salmonella within the same infected host cell had similar colors whereas neighboring infected cells often contained Salmonella with different colors (Figures 4B and S3A), suggesting a potential impact of individual host cells rather than regional factors. Salmonella colors did not correlate with Salmonella load (Figure S3B) or host cell type (Figure S3C). Further work might explore additional, potentially relevant, host cell properties.

The broad color distribution could indicate divergent Salmonella growth rates. We tested this hypothesis with the thermosensitive plasmid pVE6007 conferring resistance to chloramphenicol (CAMR) (Maguin et al., 1992). pVE6007 cannot replicate at 37°C and is thus transmitted in vivo to only one of the two daughter cells at each division, resulting in progressive dilution of plasmid-harboring CAMR cells. Comparison of the TIMERbac−Salmonella/pVE6007 inoculum and spleen homogenates at 48 p.i. revealed a pVE6007 dilution factor (input frequency/output frequency of CAMR clones) of 145 ± 17, equivalent to log₂(145) = 7.2 ± 0.2 Salmonella divisions within 48 hr. This is equivalent to an average growth rate of 0.15 ± 0.005 hr⁻¹, consistent with our previous data (0.16 ± 0.03 hr⁻¹; Becker et al., 2006). Importantly, TIMERbac−Salmonella subsets sorted according to their green/orange ratios contained diverging proportions of CAMR clones, indicative of differential plasmid dilution and division numbers.

Figure 3. Fluorescence of TIMERbac−Salmonella in Infected Mouse Spleen
(A) Flow cytometry of an infected mouse spleen homogenate (for gating, see Figure S1). Similar observations were made for more than 25 infected mice.
(B) Fluorescence of wild-type (WT) TIMERbac−Salmonella (left panel) and TIMERbac−Salmonella ssrB deficient for expression of the SPI-2-associated type III secretion system. Green/orange fluorescence ratios for Salmonella in vitro cultures at 5% O₂ and various division rates (Figure 1E) are shown in blue for comparison. Median green/orange ratios for replicates from individual mice are shown in Figure 6C.
(C) Fluorescence colors of various attenuated TIMERbac−Salmonella strains in infected spleen at day 4 postinfection. Median green/orange ratios for replicates from individual mice are shown in Figure 6C.
(D) Comparison of TIMER-based growth rate estimates with data from competitive infections (CIs, competitive indices). Data represent averages ± SD for one to five TIMER replicates and three to six CI values from individual mice (r, Spearman’s rank-order correlation coefficient). See also Figures S1 and S2.
Salmonella subsets with distinct growth rates coexist in infected spleen, and (2) TIMERbac can be used to detect and purify these subsets.

We calculated division rate distributions based on in vivo TIMER colors and calibration data from chemostats at 5% O2 (Figure 1F) (that yielded reasonable average growth rate estimates; see above). The results suggested extensive Salmonella division rate heterogeneity with a full width at half maximum (FWHM) spanning a 5-fold range (0.04–0.19 hr\(^{-1}\); Figure 5B, blue line), compared to commonly studied in vitro conditions (our chemostat cultures, FWHM 1.3- to 1.5-fold range; microfluidic devices, FWHM <2-fold ranges for Escherichia coli and Mycobacterium smegmatis; Aldridge et al., 2012; Wakamoto et al., 2013).

Similar color and division rate distributions were obtained for low-level TIMERbac expression from pSC101 TIMER (Figure S4; FWHM, 0.05–0.20 hr\(^{-1}\)), confirming that the approach is robust against differences in TIMER protein quantities. TIMERbac-Salmonella in genetically resistant 129/Sv mice had an even broader distribution (FWHM, 0.01–0.13 hr\(^{-1}\)) and lower division rates (median rate, 0.063 ± 0.004 hr\(^{-1}\) versus 0.133 ± 0.001 hr\(^{-1}\) for BALB/c mice, p < 0.0001; Figure S4B). This was consistent with slower Salmonella growth as the primary cause of dramatically lower tissue loads (Figure S4B, inset) in resistant compared to genetically susceptible mice (Benjamin et al., 1990).

Calculated TIMERbac-based division rates for sorted fractions a–d correlated with respective plasmid dilution data (Figure 5B, inset). The lower TIMERbac-based values for slow-to-medium fractions a–c suggest that some Salmonella initially proliferated with substantial plasmid dilution but subsequently slowed down, resulting in low green/orange TIMER ratios. Indeed, Salmonella with division rates above the median were predicted to generate some 90% of the daily new Salmonella daughter cells (Figure 5B), but many of these new, initially green daughter cells likely slowed down and turned more orange, because overall color distributions remained stable at increasing loads over several days of infection (Figure S2).

Salmonella Subsets with Different Growth Rates Have Distinct Proteomes

Proteome analysis of sorted subsets (Figure 6A; Table S1) revealed higher levels of ribosomal proteins in green Salmonella, consistent with faster growth (Schaechter et al., 1958), but more starvation sigma factor (σ\(^S\))-dependent proteins in orange Salmonella, implying slower growth/carbon starvation/ATP shortage (Peterson et al., 2012). The transcription factor CRP appeared to be more active in the orange subset (p = 0.011), suggesting carbon limitation (You et al., 2013). Enzymes RelA, SpoT, and GppA involved in (p)pGpp metabolism, and (p)pGpp-activated proteins (Ramachandran et al., 2012), were also more abundant in the orange Salmonella subset (p = 0.0036), suggesting elevated (p)pGpp levels in response to amino acid starvation, other nutrient limitations, and/or heat and oxidative stress (Potrykus and Cashel, 2008). Nutrient limitation regulating Salmonella growth would be consistent with our previous observations (Steeb et al., 2013).
Orange cells contained more entericidin B, the toxin component of the ecnAB toxin/antitoxin (TA) module (Bishop et al., 1998) (Figure 6A). Toxins can arrest bacterial growth, in particular when antitoxin degradation is stimulated by elevated (p)pGpp levels (Maisonneuve et al., 2013), but Salmonella ecnB had wild-type green/orange ratios (Figure 6B), arguing against a major impact of entericidin B. Salmonella ecnB shpAB phD-doc (TA D3) defective for two additional TA modules influencing Salmonella growth and persistence in macrophage cell cultures (Helaine et al., 2014) had unaffected TIMER bac colors (Figures 6B and 6C) and almost normal virulence (competitive index versus wild-type at day 5 postoral infection: 0.8 ± 0.4, p = 0.03). We did not detect other Salmonella toxins (De la Cruz et al., 2013) (Table S1). Efflux pumps AcrAB and MacAB had similar abundance in both subsets (ratios 0.96–1.19), whereas other efflux systems including IceT (the ortholog of a mycobacterial system involved in antimicrobial tolerance; Adams et al., 2011) were not detected.

Green cells contained more IgaA (internal growth attenuator A), which inhibits detrimental activation of the RcsD/RcsC/RcsB signaling system (Domínguez-Bernal et al., 2004) (Figure 6A). Orange Salmonella might contain inadequate IgaA amounts, resulting in elevated RcsC activity and slow growth. However, Salmonella rcsC had slightly orange-shifted color distributions (Figure 6B), consistent with minor attenuation of such mutants (Domínguez-Bernal et al., 2004). This is the opposite of what one would expect if insufficient IgaA-RcsC interactions impaired growth of orange Salmonella.

Members of oxygen-dependent FNR (Fink et al., 2007) and ArcA (Evans et al., 2011) regulons were similarly abundant (p = 0.42 and p = 0.49, respectively), suggesting similar oxygen tension around green and orange Salmonella. This is important, because inhomogeneous oxygenation could confound TIMER as a growth rate reporter (see Discussion). Many other metabolic enzymes were differentially abundant (Figure 6A; Figure S5A; Table S1; an interactive map with descriptions of all associated enzymatic reactions is available at http://www.biozentrum.unibas.ch/personal/bumann/Claudi_et_al/TIMER.html). A small number of pathways showed consistent differences for several enzymes (Figure 6D).

Histidine biosynthesis enzymes were more abundant in orange Salmonella, suggesting a potentially limiting host histidine.
Indeed, Salmonella purH degradation (Figure 6D), suggesting a diverging purine supply. Histidine biosynthesis is inactive in strain SL1344 due to a dysfunctional hisG<sup>Pro69</sup> allele (Hoiseth and Stocker, 1981; Richardson et al., 2011), but restoring functional hisG<sup>Leu69</sup> had no significant effect on spleen colonization (spleen CFU increase in 4 days: SL1344, 2.8 ± 0.2 log; SL1344 hisG<sup>Leu69</sup>, 3.1 ± 0.3 log; p = 0.55) or green/orange ratios (Figure 6C). These data indicate sufficient histidine supply for all subsets of auxotrophic SL1344, although orange cells might encounter lower levels that induce biosynthesis enzymes.

Green Salmonella contained more transporters and enzymes for utilization of sialic acid and galactose (Figure 6D) but Salmonella nanT had indistinguishable color distributions (Figure 6C), consistent with full virulence of these mutants (Steeb et al., 2013). Green subsets might be exposed to levels above the catabolism induction threshold for these carbohydrates, but other nutrients are apparently more relevant for growth.

Green Salmonella contained slightly less enzymes for purine nucleoside biosynthesis but more enzymes for purine nucleoside degradation (Figure 6D), suggesting a diverging purine supply. Indeed, Salmonella purH, which depends on purine supplement-

Figure 6. Properties of Salmonella Subsets
(A) Proteome comparison of sorted green and orange TIMER<sup>bac</sup>-Salmonella subsets (see Table S1 for the full data set). Ribosomal proteins were more abundant in green subpopulations (**p < 0.0001; two-tailed Wilcoxon signed-rank test), whereas σ<sup>D</sup>-dependent proteins were more abundant in orange subpopulations (**p < 0.0001). Many metabolic enzymes had differential abundance ratios.
(B) Color distributions of parental strain SL1344 and various mutants in spleen. Similar data were obtained for another rcsC, another TA Δ3, two srrB, four purH, and four SL1344 replicates.
(C) Median green/orange ratios for various Salmonella strains (each symbol represents one mouse).
(D) Abundance ratios of enzymes involved in biosynthesis of histidine (His) or purines (Pur), or degradation of Pur, sialic acid (Sial), or galactose (Gal) (**p < 0.01; ***p < 0.001; two-tailed t test on log-transformed data).

Divergent Antimicrobial Tolerance in Salmonella Subsets with Different Growth Rates
To investigate the impact of antimicrobial chemotherapy on differentially growing Salmonella, we used the fluoroquinolone enrofloxacin that is effective in the mouse typhoid fever model (Griffin et al., 2011; Helaine et al., 2014). Fluoroquinolones are the antibiotic class of choice for human typhoid fever (Sánchez-Vargas et al., 2011).

Treatment of TIMER<sup>bac</sup>-Salmonella chemostat cultures with 5 mg l<sup>-1</sup> enrofloxacin caused some 90% drop in CFUs within 1 hr but fluorescent cell counts remained stable (Figure S6A), indicating that dead Salmonella initially retained fluorescent TIMER<sup>bac</sup>. This was consistent with fluoroquinolone-mediated killing by double-strand DNA breaks (Hooper, 2001) without immediate lysis. Green/orange ratios of (mostly dead) Salmonella did not change during 1 hr treatment (Figure S6A, inset), consistent with slow TIMER<sup>bac</sup> response times (Figure 1G).
In i.v. infected mice, treatment with 0.2 mg enrofloxacin reduced spleen CFUs by 93% ± 3%, but fluorescent counts and TIMERbac color remained stable for at least 1 hr (Figure S6B), as in vitro. Slowly responding TIMERbac thus still reports on pre-treatment growth rates of both live and dead Salmonella, without confounding effects by later antibiotic-induced growth alterations. This enabled us to investigate the impact of Salmonella growth heterogeneity at the onset of treatment on antimicrobial efficacy. Specifically, we sorted Salmonella subsets with different colors at 1 hr after treatment and determined their viability by comparing plating and flow cytometry counts (CFUs per sorted fluorescent Salmonella). There was a strict correlation between killing and color/pretreatment growth rate (Figures S6C–S6E), consistent with in vitro data (Eng et al., 1991; Tuomanen et al., 1986). Fast-growing subsets were extensively killed, whereas slow-growing/nondividing Salmonella tolerated treatment better, resulting in their overrepresentation among survivors. Nevertheless, most survivors originated from abundant Salmonella with moderate pretreatment growth rates and intermediate antimicrobial tolerance.

To investigate antimicrobial efficacy under more clinically relevant conditions, we infected mice via the natural oral route and waited until disease signs appeared before starting treatment according to the recommended schedule (daily intraperitoneal [i.p.] doses of 0.1 mg enrofloxacin equivalent to about 5 mg/kg body weight; http://www.animalhealth.bayer.com/5176.0.html). CFU counts in spleen, mesenteric lymph nodes, and Peyer’s patches declined during treatment and disease signs improved (Figure 7A) but, even after 5 days of treatment, some mice still harbored residual Salmonella and/or showed weak disease signs, reproducing effective but slow fluoroquinolone therapy of severe human cases of invasive salmonellosis (Sanchez-Vargas et al., 2011).

TIMERbac colors in spleen, mesenteric lymph nodes, and Peyer’s patches at onset of treatment resembled data for spleen of i.v. infected mice (Figure S2). This was consistent with similar net growth (about 1 log per day) in spleen regardless of oral or i.v. inoculation (Barat et al., 2012; Bumann, 2002; Cheminay et al., 2004). Data were more surprising for Peyer’s patches and mesenteric lymph nodes, in which net growth stops a few days postoral infection (Figure S7; data were derived from Bumann, 2002). In these organs, active growth (as suggested by high green/orange ratios) might be counterbalanced by equally fast killing/dissemination to other organs. Differential oxygen availability might also affect TIMERbac color in these organs. More work is required to clarify this issue.

In spleen, each enrofloxacin dose killed 88%–92% of all Salmonella, whereas TIMERbac content and color remained again stable for at least 1 hr (Figure S6B). One day after the first enrofloxacin dose, many Salmonella had moderate-to-high division rates, suggesting that most survivors had resumed growth (Figure 7B), consistent with enrofloxacin tissue concentrations dropping below the minimal inhibitory concentration between doses (http://www.animalhealth.bayer.com/5176.0.html). In addition, a new subset emerged with high orange fluorescence, low green/orange ratios (Figure 7C, red box), and low colony-forming capability (87% ± 3% dead), which might represent Salmonella with blocked division but residual expression of accumulating TIMERbac.

After each enrofloxacin dose, initially fast-growing Salmonella survived poorly, whereas nondividing/slow-growing Salmonella subsets (<0.04 hr⁻¹; less than 1 division per day) survived best (Figure 7D). However, the largest number of survivors originated from an abundant Salmonella subset with moderate growth rates (1–3.4 divisions per day) and partial tolerance (Figures 7D and 7E). Survivors again generated a dominant subset of moderately growing Salmonella at the time of the next enrofloxacin dose 1 day later (Figure 7E). Repeating cycles of extensive killing/partial tolerance and resumption of growth resulted in only slow Salmonella clearance and dominance of moderately growing Salmonella subsets throughout therapy.

For comparison, we investigated two other Salmonella infection/treatment models. In one model (Helaine et al., 2014), mice are orally infected with 2 × 10¹⁰ CFUs Salmonella and continuously treated with 2 g l⁻¹ enrofloxacin in drinking water from day 1 p.i. (mice drink 1–1.5 ml/10 g body weight per day). After 5 days of treatment, a few fluorescent Salmonella persist in mesenteric lymph nodes (Helaine et al., 2014). We detected fluorescent Salmonella in this model, but our attempts to demonstrate their viability/colony-forming capability were unsuccessful in three independent experiments, even when using preenrichment in liquid lysogeny broth medium and prolonged plate incubation. Poor recovery of live Salmonella under similar treatment conditions has previously been reported (Griffin et al., 2011).

In another model (Kaiser et al., 2014), mice are pretreated with streptomycin to partially deplete endogenous gut microbiota and then orally infected with Salmonella. From day 1 p.i., mice are treated twice daily with 62 mg/kg ciprofloxacin. This therapy eradicates Salmonella from most organs, but some Salmonella with low proliferation rates persist in mesenteric lymph nodes over many days of treatment. In this case, we successfully recovered viable colony-forming Salmonella from mesenteric lymph nodes before and after ciprofloxacin treatment, as expected.

Before ciprofloxacin treatment, a surprisingly large proportion of TIMERbac-Salmonella had low green/orange ratios, indicative of poor growth in mesenteric lymph nodes (but not in spleen; Figure S2). This subset might reflect initial growth arrest of hundreds of Salmonella that continuously arrive from the densely colonized gut lumen (Diehl et al., 2013; Kaiser et al., 2013). New arrivals might require extensive adaptations to tissue microenvironments (Becker et al., 2006; Rollenhagen and Bumann, 2006) before starting replication.

This orange Salmonella subset survived ciprofloxacin treatment best (Figure 7F) and, because of its abundance before treatment, also dominated among survivors (Figure 7F, inset). One day later, most Salmonella had not yet resumed growth, based on the low proportion of green Salmonella subsets (Figures 7G and 7H). This might be the consequence of growth arrest due to continuously high ciprofloxacin levels (Kaiser et al., 2014). Together, these data support the proposed major role of nondividing Salmonella in this streptomycin-pretreatment model (Kaiser et al., 2014).

DISCUSSION

Successful antimicrobial chemotherapy of infections might depend on pathogen growth patterns. Testing this hypothesis
Figure 7. Impact of Antibiotic Therapy on Salmonella Subpopulations

(A) Salmonella tissue loads during therapy with daily doses of 0.1 mg enrofloxacin. Spleen colonization levels before start of therapy (b; gray) were estimated based on flow cytometry counts. Each symbol represents one mouse. To avoid delays in TIMER flow cytometry, we prepared only the most distal small intestinal Peyer’s patch (Last PP) (mLN, mesenteric lymph nodes). Average disease scores ± SEM of between 20 (dose 1) and 3 (dose 5) mice from three independent experiments are also shown.

(B) Division rate distributions at various time points during multidose therapy. Pooled data for two to five mice per time point are shown.

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requires monitoring of pathogen growth and survival during disease and treatment. Sequence tags and nonreplicating plasmids/phages (Grant et al., 2008; Kaiser et al., 2014) reveal pathogen growth, clearance, and spreading between organs at the population level but lack single-cell resolution. Fluorescence dilution (Hélaïne et al., 2010; Roostalu et al., 2008) is the first single-cell method for assessing pathogen growth, but is mostly suitable for early infection stages because of decreasing signal to background and does not necessarily reveal current growth (cells that stopped dividing after initial rapid growth, or that started to proliferate after an initial lag phase, are indistinguishable).

We developed a complementary approach based on the TIMER protein that changes fluorescence color over time. TIMER reports on single-cell growth rates at various stages of disease. TIMER is blind to short-term fluctuations, but can resolve growth changes occurring over several hours (about one in vivo generation time). TIMER color also depends on oxygen tension. This is an important caveat in environments with inhomogeneous oxygenation, where green TIMER fluorescence might reflect fast growth, poor oxygenation, or a combination of both parameters. Further developments decreasing TIMER oxygen sensitivity are thus highly desirable.

However, TIMER can be used as a growth rate reporter in rather homogeneously oxygenated tissues such as spleen red pulp. Oxygen tension data for mouse spleen range from 2% to 9% (Braun et al., 2001; Caldwell et al., 2001; Giraudue et al., 2012), averaging over white pulp with limited blood flow and red pulp with strong circulation of arterial blood and low oxygen consumption (Vaupel et al., 1989). Live Salmonella reside exclusively in red pulp in close vicinity to erythrocytes (Burton et al., 2014), suggesting similar oxygen availability for various subsets, consistent with homogeneous levels of Salmonella oxygen-regulated proteins. Most importantly, TIMER-based Salmonella growth estimates closely correlated with an independent approach, plasmid dilution, confirming the utility of TIMER as an in vivo division rate reporter under these conditions.

TIMER revealed extensive phenotypic variation in Salmonella single-cell growth rates. Rapidly growing subsets dominated overall Salmonella proliferation and disease progression. These Salmonella might have superior access to nutrients such as purines, carbohydrates, and amino acids, but other yet-unidentified factors likely influence growth as well.

To assess the impact of Salmonella growth on therapy, we treated infected mice with a potent fluoroquinolone following recommended dosing regimens. Resolution of disease signs and Salmonella eradication required many doses, similar to severe cases of human typhoid fever. Delayed eradication can cause serious problems in critically ill patients and promote resistance development, a major concern in medicine today (Laxminarayan et al., 2013).

In our model, slow eradication was the consequence of Salmonella antimicrobial tolerance, with a strong dependency on pretreatment single-cell growth rates. Fast-growing Salmonella survived poorly, whereas nondividing/slow-growing Salmonella survived best but were rare, limiting their impact. Instead, Salmonella with moderate growth rates (one to four divisions per day) and intermediate levels of tolerance dominated throughout therapy. More effective targeting of this large subset could substantially accelerate therapy. Pathogen physiology and antimicrobial action at moderate growth rates might thus deserve more research efforts in addition to the current focus on rich in vitro cultures with vigorously growing, drug-sensitive cells and rare nondividing, highly tolerant cells.

In our disease/treatment model, we tried to mimic common clinical settings where patients with established infections and clear disease signs are treated with safe antibiotic doses. In other models (Hélaïne et al., 2014; Kaiser et al., 2014), mice are treated with high antibiotic doses already at early infection stages. Under these conditions, tissues harbor many maladapted, nondividing Salmonella with high antimicrobial tolerance at the onset of therapy, and surviving Salmonella cannot resume growth because of steady high antibiotic levels. As a consequence, long-term persisters are mostly nondividing Salmonella, in contrast to our conditions.

For many infectious diseases, pathogen eradication with antibiotics is slow compared to standard in vitro cultures. Pathogen subsets with moderate growth and partial antimicrobial tolerance might be involved in some cases, as shown here for Salmonella. In other cases, nondondiving/dormant subpopulations with high tolerance or a growth-independent tolerance mechanism might be more relevant. Single-cell approaches as developed in this study might help to clarify these important issues as a basis for more efficacious therapies.

**EXPERIMENTAL PROCEDURES**

**Microbiology and Molecular Biology**

Salmonella strains were constructed and cultured as described in Extended Experimental Procedures. Fluorescence spectra were recorded in a

(C) TIMER fluorescence 1 hr or 1 day after the first enrofloxacin dose. The red box indicates an antibiotic-induced new subset. Similar observations were made for 14 mice in five independent experiments.

(D) Salmonella survival (CFUs per sorted TIMERFACS–Salmonella cell) in subsets with different division rates from mice treated with one to three doses of enrofloxacin (averages ± SD for two to five mice from two independent experiments; one-way repeated-measures ANOVA with posttest for linear trend).

(E) Proportions of live slow (s: <0.04 hr−1), moderate (m), and fast (f: >0.14 hr−1) Salmonella cells before or 1 hr after (“survivors”) daily enrofloxacin doses (averages ± SD of two to five mice per time point). At doses 4 and 5, low Salmonella loads prevented sorting-based analysis of survivor distributions.

(F) Survival of Salmonella with various TIMERFACS colors 1 hr after an oral dose of 1.3 mg ciprofloxacin in mesenteric lymph nodes in the streptomycin-pretreatment model (averages ± SD for three mice from two independent experiments; one-way repeated-measures ANOVA with posttest for linear trend). The inset shows the proportions of Salmonella with log10(green/orange) <−0.055 among survivors in our typhoid fever/enrofloxacin model (En) and the streptomycin/ciprofloxacin model (CIP).

(G) Fluorescence of Salmonella in mesenteric lymph nodes 1 hr or 1 day after an oral dose of 1.3 mg ciprofloxacin.

(H) Color distributions for three mice from one experiment are shown. See also Figures S6 and S7.
fluorescence plate reader (Synergy H4; BioTek). Fluorescence colors at various growth rates were determined in a defined low-pH medium in chemostats.

**Cell-Culture Infections**

Nontumorigenic monocyte-derived Maf-DKO macrophages (Aziz et al., 2009) or primary bone-marrow-derived macrophages were infected with exponential or stationary Salmonella cultures. After 30 min, wells were washed and incubated with medium containing 100 μg ml⁻¹ gentamicin for 60 min, followed by 16 μg ml⁻¹ gentamicin.

**Mouse Infections**

All animal experiments were approved (license 2239; Kantoneses Veterinärmhalt Basel-Stadt) and performed according to local guidelines (Tierschutz-Verordnung, Basel-Stadt) and the Swiss animal protection law (Tierschutz-Gesetz). Mice were infected i.v. with 400–2,000 CFUs or orally with 10⁷ or 2 CFU per mouse. Mice were monitored for disease signs and treated as described in Extended Experimental Procedures.

**Flow Cytometry and Proteomics**

Tissue homogenates were treated with Triton X-100 to liberate intracellular Salmonella as described (Steeb et al., 2013). Samples were maintained on ice and measured between 1 and 3 hr after tissue homogenization. Samples were analyzed by flow cytometry (Fortessa; BD Biosciences) as shown in Figure S1. Samples were sorted according to green/orange ratio using an Aria III (BD Biosciences). Sorted samples were subjected to proteomics as described (Steeb et al., 2013).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, one table, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.06.045.

**AUTHOR CONTRIBUTIONS**

D.B. conceived the study. B.C., P.S., A.C., N.S., and D.B. designed the experiments. B.C. performed in vivo experiments. P.S. constructed and characterized TIMERbac. A.C. performed chemostat experiments. N.P. performed cell-culture experiments. J.Z. sorted samples and measured TIMER matura. N.S. performed immunohistochemistry. A.S. performed proteome analyses. D.B. wrote the paper with input from all authors.

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