Cell

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

Beatrice Claudi,[1,5] Petra Spröte,[1,4,5] Anna Chirkova,[1,5] Nicolas Personnic,[1,5] Janine Zankl,[2] Nura Schürmann,[1] Alexander Schmidt,[3] and Dirk Bumann[1,*]
[1]Focal Area Infection Biology
[2]FACS Core Facility
[3]Proteomics Core Facility
Biozentrum, University of Basel, 4056 Basel, Switzerland
[4]BASF SE, 67056 Ludwigshafen, Germany
[5]Co-first author
*Correspondence: dirk.bumann@unibas.ch
http://dx.doi.org/10.1016/j.cell.2014.06.045

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 TIMER^{bac} 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

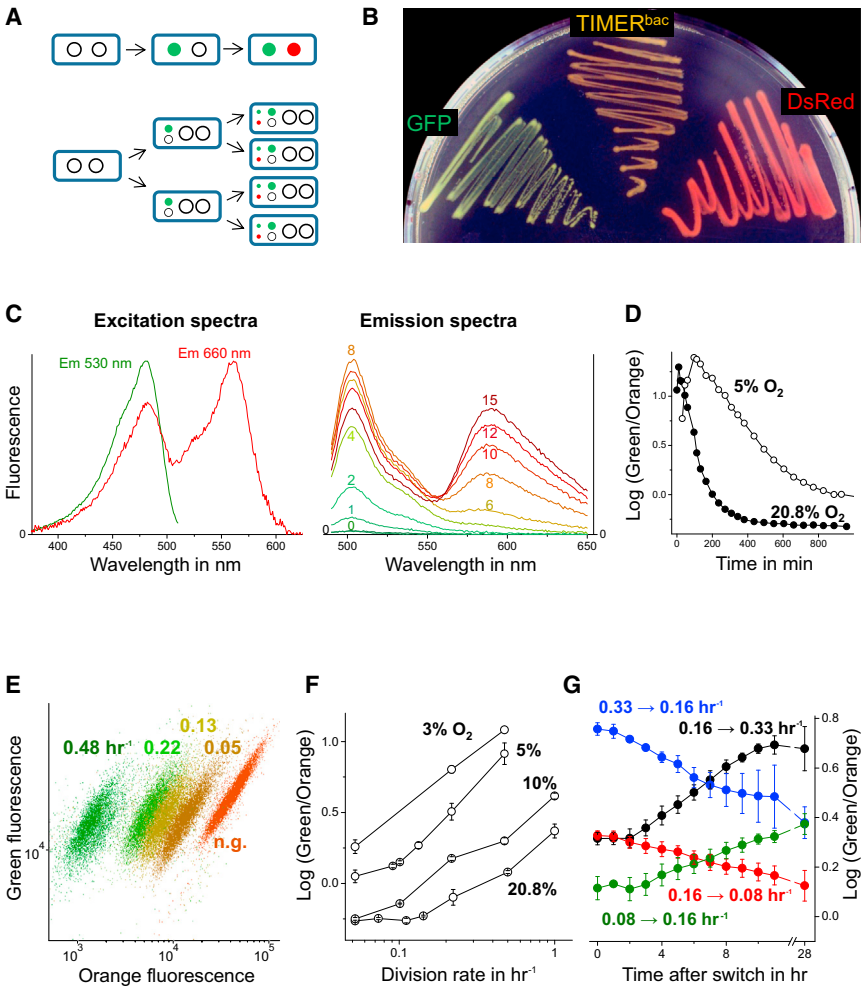

Figure 1. TIMER^{bac} Fluorescence Properties

(A) Schematic representation of TIMER fluorescence in nongrowing (upper panel) or actively growing (lower panel) cells. In nongrowing cells, both rapidly maturing green and slowly maturing orange TIMER molecules can accumulate. In dividing cells, rapidly maturing green molecules dominate over orange molecules that are diluted by cell division before maturation.

(B) Agar plate with *Salmonella* constitutively expressing GFP, TIMER^{bac}, or DsRed.

(C) Fluorescence spectra of TIMER^{bac}-*Salmonella* 18 hr after induction of TIMER^{bac} expression (excitation spectra; left) or at various time intervals during maturation at 37°C, 5% O_2, and pH 5.0 (emission spectra; right) (numbers show time points in hours). Similar data were obtained in two independent experiments.

(D) Fluorescence maturation kinetics in TIMER^{bac}-*Salmonella* at two different oxygen concentrations calculated from spectral data such as shown in (C).

(E) Flow cytometry of *Salmonella* with constitutive expression of TIMER^{bac} at defined division rates in chemostats maintained at 5% O_2 (n.g., nongrowing culture). Similar data were obtained in three independent experiments.

(F) TIMER^{bac}-*Salmonella* fluorescence colors at different division rates and oxygen concentrations. Combined data from four experiments are shown (averages ± SD of two to four reactors for each data point).

(G) TIMER^{bac}-*Salmonella* fluorescence dynamics after switching division rates in chemostats (averages ± SD for two or three reactors from one experiment).

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 TIMER^{bac} in *Salmonella enterica* serovar Typhimurium SL1344. *Salmonella* with constitutive TIMER^{bac} expression grew as orange colonies (Figure 1B). Freshly induced TIMER^{bac} formed green fluorophores (emission peak at 503 nm), followed by orange fluorophores (peak at 587 nm) (Figure 1C). Orange TIMER^{bac} 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, TIMER^{bac}-*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 TIMER^{bac} maturation and dilution as decisive processes determining color.

TIMER^{bac}-*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 TIMER^{bac}-*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).