Cyclic-di-GMP regulation promotes survival of a slow-replicating subpopulation of intracellular *Salmonella* Typhimurium

Erik Petersen[a], Erez Mills[a,1], and Samuel I. Miller[a,b,c,d,2]

[a]Department of Microbiology, University of Washington, Seattle, WA 98195; [b]Department of Immunology, University of Washington, Seattle, WA 98195; [c]Department of Genome Sciences, University of Washington, Seattle, WA 98195; and [d]Department of Medicine, University of Washington, Seattle, WA 98195

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***Salmonella* Typhimurium can invade and survive within macrophages where the bacterium encounters a range of host environmental conditions. Like many bacteria, *S.* Typhimurium rapidly responds to changing environments by the use of second messengers such as cyclic di-GMP (c-di-GMP). Here, we generate a fluorescent biosensor to measure c-di-GMP concentrations in thousands of individual bacteria during macrophage infection and to define the sensor enzymes important to c-di-GMP regulation. Three sensor phosphodiesterases were identified as critical to maintaining low c-di-GMP concentrations generated after initial phagocytosis by macrophages. Maintenance of low c-di-GMP concentrations by these phosphodiesterases was required to promote survival within macrophages and virulence for mice. Attenuation of *S.* Typhimurium virulence was due to overproduction of c-di-GMP–regulated cellulose, as deletion of the cellulose synthase machinery restored virulence to a strain lacking enzymatic activity of the three phosphodiesterases. We further identified that the cellulose-mediated reduction in survival was constrained to a slow-replicating persister population of *S.* Typhimurium induced within the macrophage intracellular environment. As utilization of glucose has been shown to be required for *S.* Typhimurium macrophage survival, one possible hypothesis is that this persister population requires the glucose redirected to the synthesis of cellulose to maintain a slow-replicating, metabolically active state.**

salmonella | cyclic di-GMP | biosensor | cellulose | persister

Cyclic di-guanosine monophosphate (c-di-GMP) is a bacterial second messenger involved in a variety of cellular processes including exopolysaccharide production, motility, and cell differentiation (1). The c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases (DGCs) and degraded by c-di-GMP–specific phosphodiesterases (PDEs) (2). These c-di-GMP–metabolizing enzymes (CMEs) are found in abundance within bacterial genomes, numbering near 100 in some species (3). Therefore, the c-di-GMP concentration (hereafter referred to as [c-di-GMP]) within an individual bacterium is the result of the combined activities of several different concurrently expressed enzymes and their activation state. These CMEs are often constitutively expressed at low levels and regulated through amino-terminal sensory domains that modulate enzymatic activity (4). This posttranslational regulation allows the bacterium to rapidly alter its [c-di-GMP] in response to changing environmental stimuli. Careful modulation of [c-di-GMP] is crucial, as c-di-GMP binds a host of effectors that include enzymes, riboswitches, structural components of protein complexes, and transcription factors (5). Enzymes and effectors combine to form a signaling network in response to environmental and intracellular cues. Resultant phenotypes arising from this response are critical for bacterial survival in a constantly changing environment.

Salmonella enterica serovar Typhimurium is an enteric pathogen that encodes 17 predicted CMEs: 6 DGCs, 9 PDEs, and 2 proteins with both enzymatic activities (6). Two well-studied c-di-GMP–binding effectors are encoded by the *S.* Typhimurium genome: the flagellar brake YcgR that inhibits motility (7) and the cellulose synthase BcsA that generates the extracellular polysaccharide cellulose (8, 9). The *S.* Typhimurium c-di-GMP pathway has been well studied in regard to established in vitro c-di-GMP–regulated phenotypes such as motility and biofilm formation (10–12). However, relatively little is known about the role of c-di-GMP in the context of eukaryotic cell infection—and even less is known about the specific CMEs and signals that regulate [c-di-GMP] in these conditions—making this an area requiring further study. We have previously utilized a c-di-GMP–binding fluorescence resonance energy transfer (FRET)-based biosensor to measure [c-di-GMP] of *S.* Typhimurium in vitro (6, 8, 13, 14). Here we modified the biosensor to measure the c-di-GMP of *S.* Typhimurium during intracellular infection of a macrophage. Coupled with fluorescence confocal microscopy, this biosensor allows real-time measurement of [c-di-GMP] in thousands of single bacteria to determine how [c-di-GMP] changes among the population of *S.* Typhimurium during intracellular survival.

Results

Generation of an mTFP–mKO2 c-di-GMP Biosensor to Overcome the Inherent Fluorescence of Eukaryotic Cells. A previously developed FRET-based c-di-GMP biosensor that utilized cyan and yellow fluorescent proteins (mCFP and mYFP, respectively) was

Significance

Cyclic di-GMP is a bacterial second messenger that transmits extracellular signals to the intracellular environment via sensor cyclic-di-GMP–metabolizing enzymes. Here a fluorescent biosensor is used to accurately measure cyclic-di-GMP concentrations in thousands of individual intracellular *Salmonella* Typhimurium. Furthermore, three enzymes that reduce cyclic-di-GMP concentrations were identified and shown to be essential for reduction of cyclic di-GMP, intracellular survival, and full virulence for mice. This was due to cyclic-di-GMP–mediated overproduction of cellulose that specifically affected a population of slowly replicating bacteria. These results further our knowledge of mechanisms of virulence and persistence of this important pathogen.

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Data deposition: Raw microscopy files and initial bacterial measurement values are available in the BioStudies database (www.ebi.ac.uk/biostudies) under accession number S-BSST238.

[1]Present address: Department of Animal Science, The Hebrew University of Jerusalem, 7610001 Rehovot, Israel.

[2]To whom correspondence should be addressed. Email: MillerSI@uw.edu.

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unsuitable for measurements of bacteria within host cells due to the high blue-range background fluorescence of eukaryotic cells (6, 13, 15, 16). Therefore, an alternative c-di-GMP biosensor was engineered to optimize measurement of c-di-GMP within intracellular bacteria by exchanging mCFP for the brighter teal fluorescent protein (mTFP) (17) and mYFP for the more pH-stable kusabira orange variant 2 (mKO2) (18, 19). The c-di-GMP binding assays indicate that, at low [c-di-GMP], FRET is high (i.e., low mTFP/FRET ratio), and binding c-di-GMP results in a conformational change that decreases FRET fluorescence (i.e., high mTFP/FRET ratio) (Fig. 1), similar to the previous biosensor (13). While the mTFP–mKO2 biosensor is predicted to be stable to the intracellular environment, we sought to control for changes that may be occurring due to biosensor instability to ensure these were not mistaken for changing [c-di-GMP]. The c-di-GMP is bound within a conserved RXXXR motif (found at 114 and 118 within the full-length YcgR) of the PilZ domain (7). A nonbinding biosensor with both arginine residues mutated to alanine failed to bind c-di-GMP as measured by a change in mTFP/FRET fluorescence ratios. The use of this nonbinding biosensor as a control allowed for correction of native biosensor measurements, indicating that any change when bacteria are intracellular are not a result of fluorophore degradation. The biosensor's utility during intracellular survival was tested by infecting mouse bone marrow-derived macrophages (BMDMs) with *S.* Typhimurium expressing the mTFP–mKO2 c-di-GMP biosensor (*SI Appendix*, Fig. S1). Following gentamicin treatment to clear nonphagocytosed bacteria, live-cell microscopy was used to measure mTFP and FRET fluorescence levels of intracellular *S.* Typhimurium (Fig. 2*A*). The brightness of the mTFP/mKO2 biosensor overcame the background fluorescence of the eukaryotic cells and allowed for the determination of [c-di-GMP] of intracellular *S.* Typhimurium at single-cell resolution.

Intracellular *S.* Typhimurium c-di-GMP Concentrations Decrease Soon After Phagocytosis. To determine the baseline c-di-GMP changes occurring during macrophage survival, [c-di-GMP] of *S.* Typhimurium within BMDMs was measured at 1-h intervals between 1 h and 12 h (Fig. 2*B*). After controlling for a slight steady increase in mTFP/mKO2 ratios of the nonbinding biosensor attributed to biosensor degradation, average mTFP/nFRET ratio values of intracellular *S.* Typhimurium indicate that, at initial infection, *S.* Typhimurium exhibits raised [c-di-GMP]. By 2 h postinfection, [c-di-GMP] drops and remains at this low concentration for the next 4 h to 5 h. After 8 h of infection, average [c-di-GMP] begins to rise over the remaining course of the infection. Single-cell analysis of the intracellular bacteria identified whether each individual bacterium contained [c-di-GMP] sufficient to bind either of the two well-studied c-di-GMP–binding *S.* Typhimurium effectors, the flagellar brake YcgR that binds at 0.2 μM and the cellulose synthase BcsA that binds at 8.0 μM (Fig. 2*C*, red and blue lines, respectively) (8). Beginning at 8 h postinfection, high [c-di-GMP] bacteria begin to appear in greater numbers. This indicates that, while, initially, [c-di-GMP] is constrained at a low level, there is significant heterogeneity within intracellular *S.* Typhimurium [c-di-GMP] later during macrophage infection (Fig. 2*D*).

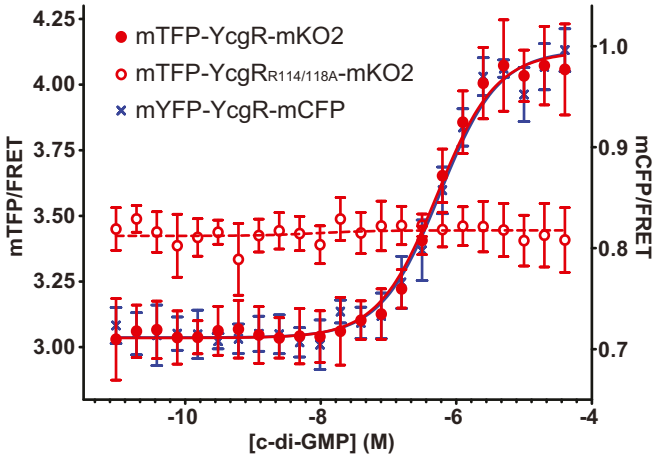

Fig. 1. The mTFP/mKO2 biosensor accurately measures c-di-GMP concentrations for intracellular bacteria. Purified biosensors were tested for change in mTFP/FRET ratios upon addition of c-di-GMP. Wild-type biosensor (n = 3) exhibits an increase in mTFP/FRET ratio upon c-di-GMP binding similar to previous mYFP–mCFP biosensor (n = 2), while mutation of two key c-di-GMP–binding residues (R114/118A) (n = 3) abrogates the mTFP/FRET ratio change.

Three PDEs Are Activated to Maintain Low c-di-GMP Concentrations in the Majority of Bacteria During Intracellular Survival. Seven hours represents a point in intracellular *S.* Typhimurium survival where bacteria are transitioning from an early, low c-di-GMP state to one in which higher [c-di-GMP] and increased diversity of [c-di-GMP] are present. To determine which c-di-GMP metabolizing proteins (CMEs) are responsible for maintaining this low [c-di-GMP] state, mutants in each CME were tested for [c-di-GMP] at 7 h postinfection (*SI Appendix*, Fig. S2). Deletion of the PDEs *stm2215* (*rtn*, *pdeN*, *STM14_2739*) and *stm2503* (*yfgF*, *pdeF*, *STM14_3068*) resulted in significantly higher [c-di-GMP] (Fig. 3*A* and *SI Appendix*, Fig. S3). Although deletion of the PDE *stm3615* (*yhjK*, *pdeK*, *STM14_4353*) resulted in a significant [c-di-GMP] increase only when compared directly to wild-type levels via Student's t test and not via ANOVA, this mutant was also included for further analysis. Mutation of the active site residues (EAL→AAA) in each protein phenocopied the full deletion phenotype. This indicates that the PDE activity of each protein is required for maintaining low [c-di-GMP], as deletion of a PDE is expected to raise [c-di-GMP]. These active site mutants were generated within a wild-type strain to ensure that the identified phenotype was the result of mutation of these PDEs and not of a secondary mutation elsewhere in the genome. A strain deleted for all three identified PDEs (3xKO) displayed much greater numbers of high [c-di-GMP] bacteria, indicating that each of these three PDEs is translated and actively maintaining low [c-di-GMP] during intracellular infection of *S.* Typhimurium.

***S.* Typhimurium Survival During Macrophage and Mouse Infection Requires Full Phosphodiesterase Activity to Decrease Cellulose Production.** The ability to survive with BMDMs of the single PDE mutants as well as the triple deletion mutant were compared with wild-type *S.* Typhimurium to determine whether c-di-GMP dysregulation altered *S.* Typhimurium survival during macrophage infection (Fig. 3*B*). None of the individual PDE mutants showed more than a slight change in survival from wild-type levels during the course of infection. However, consistent with the finding that these three PDEs operate redundantly to lower [c-di-GMP], deletion of all three PDEs (3xKO) results in a significant loss of viable bacteria during macrophage infection. Each strain was then tested for competitive virulence versus wild-type *S.* Typhimurium during systemic mouse infection. Deletion of each individual PDE again resulted in a modest decrease in competitive fitness compared with wild type, while deletion of all three PDEs resulted in a much lower competitive index (Fig. 3*C*). These data suggest that not only are these three PDEs transcribed and enzymatically active during macrophage survival but also that *S.* Typhimurium uses these PDEs to lower [c-di-GMP] to improve virulence within a mouse model.

Under the high [c-di-GMP] found in the reduced-virulence triple PDE deletion mutant, both YcgR and BcsA effectors may be bound by c-di-GMP and acting to decrease competitive

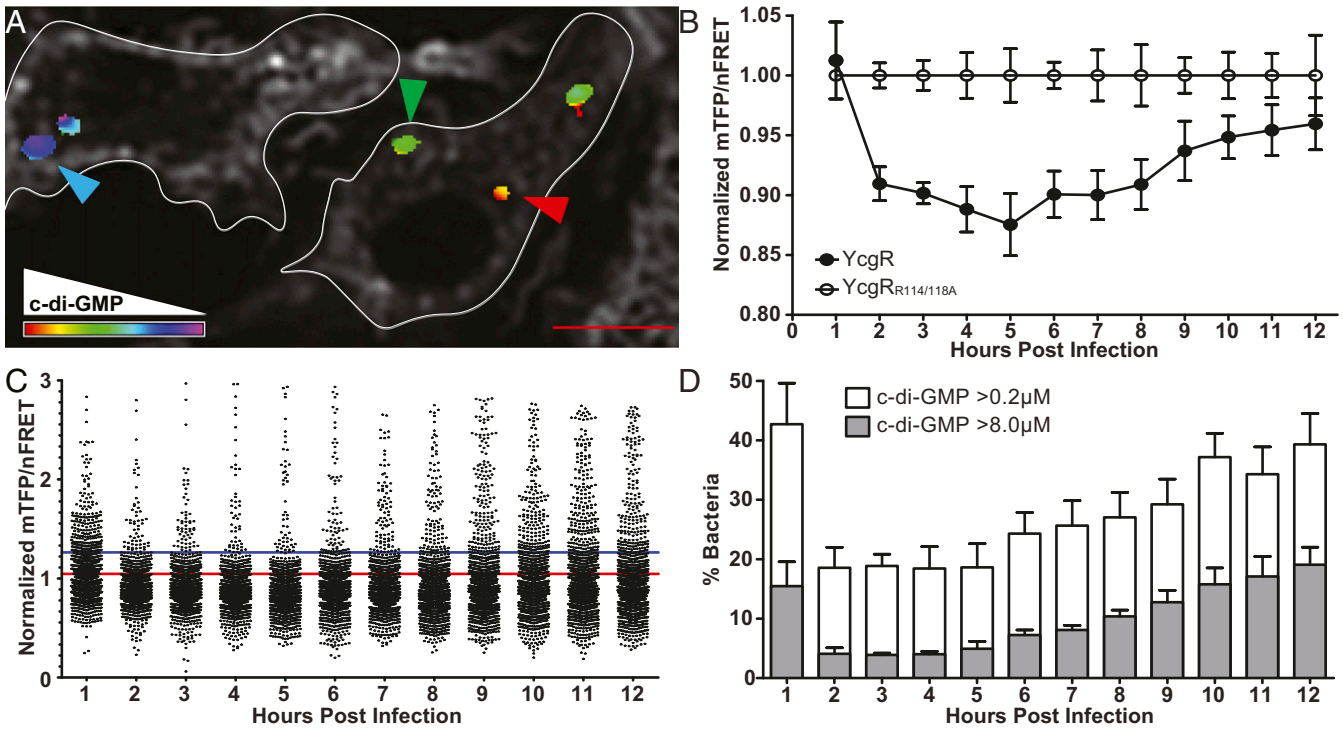

Fig. 2. Intracellular *S.* Typhimurium reduces c-di-GMP levels following initial phagocytosis. (*A*) BMDMs infected for 7 h with *S.* Typhimurium expressing the mTFP–mKO2 biosensor were stained with WGA-680 (white, with cell outline), and mTFP/nFRET ratios were calculated (rainbow). Arrows indicate high, medium, and low c-di-GMP concentration bacteria (red, green, and blue arrows, respectively). (Scale bar: 10 μm.) (*B*) Time course analysis of average mTFP/nFRET values of intracellular wild-type *S.* Typhimurium population ($n = 4$) normalized to YcgR$_{R114/118A}$ ($n = 3$) to correct for any potential biosensor instability. (*C*) Single-cell analysis of the intracellular *S.* Typhimurium population indicates that infection can be separated into three stages: initial infection where c-di-GMP levels are initially high, early survival where c-di-GMP levels drop between 2 h and 7 h postinfection, and late infection where the emergence of a high c-di-GMP subpopulation causes average values to rise during 8 h to 12 h postinfection ($n = 2{,}000$ random bacteria/time point). Red line = 0.2 μM c-di-GMP, and blue line = 8.0 μM c-di-GMP. (*D*) Quantification of the YcgR-bound (>0.2 μM c-di-GMP) and BcsA-bound (>8.0 μM c-di-GMP) subpopulations during intracellular survival indicate wild-type *S.* Typhimurium consists of a heterogeneous population, especially during later stages of survival.

virulence. Deleting either *ycgR* or *bcsA* would uncouple high [c-di-GMP] from the phenotypic output of the effector (reduced motility and cellulose production, respectively), allowing us to determine whether these effectors are responsible for the attenuation of the triple PDE deletion mutant. Mice were competitively infected with wild-type *S.* Typhimurium and the triple PDE mutant (3xKO) with

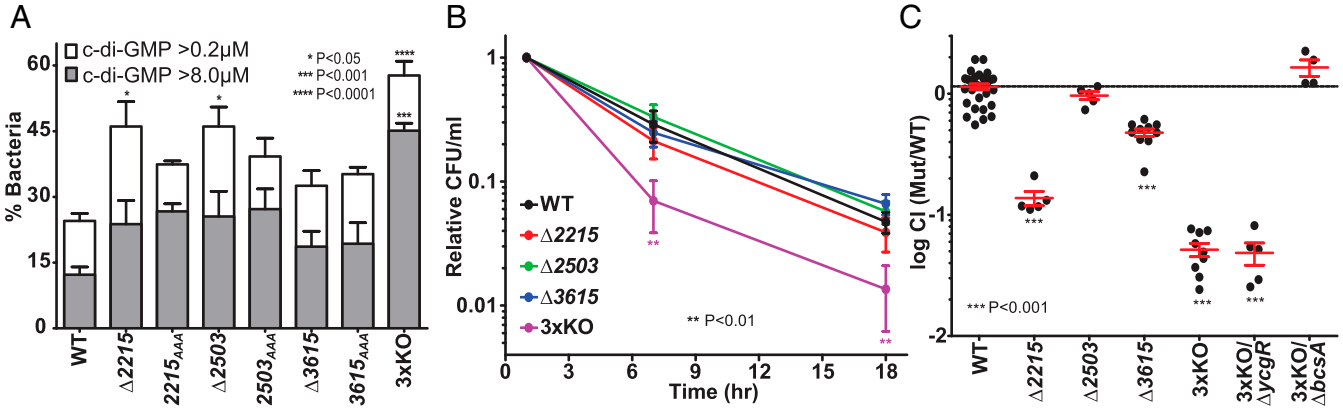

Fig. 3. Three PDEs maintain low c-di-GMP levels intracellularly to decrease virulence-attenuating cellulose overproduction. (*A*) Mutant analysis identified three c-di-GMP PDEs—STM2215, STM2503, and STM3615—that were required for low c-di-GMP levels at 7 h postinfection. Mutation of the EAL active site of each mutant (AAA) complemented the deletion phenotype. A triple deletion mutant (3xKO) displays much higher c-di-GMP levels, indicating these three enzymes act redundantly during intracellular survival. Statistics were calculated using ANOVA versus WT ($n = 10$ for WT, $n = 4$ for Δ*3615* and *3615*$_{AAA}$, and $n = 3$ for remainder). (*B*) Survival of single deletion mutants of each of the three PDEs, a triple deletion mutant (3xKO), and wild-type bacteria within BMDMs were determined at 1, 7, and 18 h postinfection. Samples ($n = 3$) were statistically analyzed by paired two-way ANOVA versus wild type on inoculum-adjusted log bacterial counts at each time point before normalization to the 1-h time point. (*C*) Mice were competitively infected with each mutant versus wild type, and bacterial splenic load was determined 2 d postinfection. While deletion of both *stm2215* and *stm3615* led to reduced competitive indices, loss of all three PDEs (3xKO) led to the highest reduction in virulence. Subsequent 3xKO strain deletion of the c-di-GMP effectors *ycgR* and *bcsA* indicated that attenuation in the 3xKO strain is due to increased cellulose synthesis in response to higher c-di-GMP levels. Statistical analysis was conducted by paired Student's *t* test on log values of raw splenic bacterial counts (*n* is displayed on graph).

further deletion of either *ycgR* or *bcsA* (Fig. 3*C*). While deletion of *ycgR* (3xKO/Δ*ycgR*) was insufficient to restore virulence within this mouse model, deletion of *bcsA* (3xKO/Δ*bcsA*) completely restored the ability of the triple PDE deletion mutant to survive at wild-type levels. Individual mutation of either the BcsA enzymatic active site or c-di-GMP binding site resulted in restored virulence (*SI Appendix*, Fig. S4), indicating that c-di-GMP–mediated cellulose synthesis was responsible for the virulence defect. The importance of cellulose overproduction to survival within macrophages was further shown by overexpressing a heterologous DGC during intracellular survival (*SI Appendix*, Fig. S5). These data indicate that *S.* Typhimurium encodes the PDEs STM2215, STM2503, and STM3615 to reduce [c-di-GMP] during intracellular survival, in part, to prevent the detrimental overproduction of cellulose.

Overproduction of Cellulose Reduces Survival of Slow-Replicating Intracellular *S.* Typhimurium. One hypothesis for the attenuation of cellulose-overproducing strains was that removal of glucose as an important carbon source through generation and secretion of an extracellular polymer has an effect on bacterial replication. Therefore, replication rates of intracellular *S.* Typhimurium were measured using a technique utilizing two fluorescent proteins, one constitutively expressed (mCherry) and the other expressed via an inducible promoter whose induction can be terminated after infection (GFP) (20). While we anticipated an overall reduction in replication rates in the triple PDE mutant, instead, a cellulose-dependent decrease in survival of primarily the slowly replicating bacterial subpopulation was observed (high GFP/mCherry ratio) (Fig. 4*A*). Distribution of wild-type bacteria into three equal replication rate populations (fast, moderate, and slow) displays a significant decrease in survival of the triple PDE-deleted slow-replicating population (Fig. 4*B*). These data suggest that cellulose overproduction predominantly inhibits the slow-replicating intracellular *S.* Typhimurium subpopulation. While the mechanism of attenuation as a result of cellulose overproduction is unknown, the death of these bacteria would be consistent with the results of both macrophage survival assays and competition within a mouse model of *S.* Typhimurium infection.

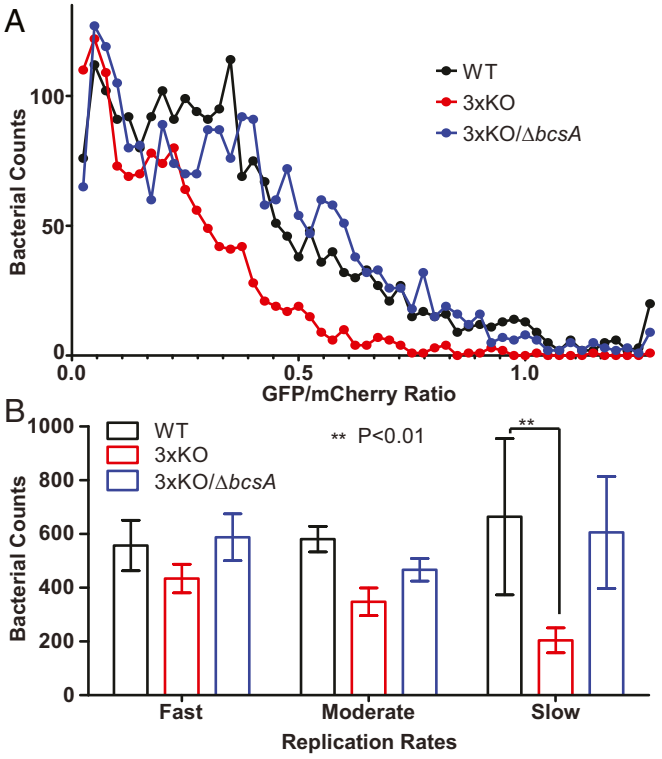

Fig. 4. Cellulose production reduces survival of slow-replicating intracellular *S.* Typhimurium. *S.* Typhimurium strains possessing the pFCcGi fluorescence dilution plasmid were used to measure replication rate at 7 h postinfection where increased replication rate leads to lower GFP fluorescence (and therefore lower GFP/mCherry ratios). (*A*) Deletion of the three identified PDEs (3xKO) results in decreased survival of slow-replicating bacteria that can be alleviated via deletion of *bcsA* (3xKO/Δ*bcsA*). Shown is a representative experiment from six different experiments conducted from different BMDMs on different days. (*B*) Enumeration of bacteria with subpopulations representing fast-replicating (<0.15 GFP/mCherry), moderate-replicating (0.15 to 0.35 GFP/mCherry), and slow-replicating (>0.35 GFP/mCherry) bacteria indicates that slow-replicating bacteria are predominantly excluded from the triple PDE mutant strain, indicating that cellulose overproduction is causing these bacteria to decrease in number. Statistics were calculated via two-way paired ANOVA between strains and subpopulations ($n = 6$).

Discussion

The c-di-GMP is a second messenger primarily used by bacteria to rapidly respond to a changing environment and adapt accordingly without transcription or translation (21). Intracellular *S.* Typhimurium is exposed to a variety of stimuli during invasion, survival, and replication with a host cell, but, to date, little was known about how c-di-GMP is regulated during this important phase of infection. Analysis of *S.* Typhimurium intracellular c-di-GMP kinetics within macrophages indicates a dynamic response to these changing environments. Intracellular *S.* Typhimurium initially raises [c-di-GMP], potentially to halt flagellar-mediated motility. Rich culture-grown *S.* Typhimurium predominantly expresses flagella (22) and lower [c-di-GMP] through the coordinate expression of the constitutively active PDE YhjH within the flagellar cascade (8), conferring motility on these bacteria. This initial increase in c-di-GMP is potentially a mechanism to halt flagellar motility through c-di-GMP binding to the flagellar brake YcgR (7). Subsequent reduction in [c-di-GMP] during the establishment of an intracellular lifestyle coincides with down-regulation in flagellar synthesis (23) via PhoPQ in response to the acidified vacuole (24). At later points during macrophage survival, *S.* Typhimurium generates a high [c-di-GMP] subpopulation resulting in significant [c-di-GMP] heterogeneity within the intracellular population. While heterogeneity in [c-di-GMP] has been observed within in vitro grown bacteria (13), this has now been observed within an in vivo condition. Unlike bacteria like *Caulobacter crescentus* (13) and *Pseudomonas aeruginosa* (14) for which the mechanisms behind this heterogeneity have been determined to be organelle-mediated partitioning of CMEs at cell division, the mechanism behind generation of c-di-GMP heterogeneity within *S.* Typhimurium is unknown. As several bacterial species generate c-di-GMP heterogeneity following cell division, this may occur as the bacteria divide within macrophages to generate a phenotypic heterogeneity that better positions the bacteria for survival either within the changing macrophage phagosome or after release into the host environment.

We identified three PDEs that each contributed to low [c-di-GMP] during early macrophage survival. Each PDE contains periplasmic or inner membrane domains that could be involved in sensing the intracellular environment the bacteria encounter within a phagosome (25). This suggests that *S.* Typhimurium encodes several mechanisms to reduce [c-di-GMP] during intracellular growth, and that these three PDEs are all expressed intracellularly and activated by the bacterium's intracellular environment to degrade c-di-GMP. One such signal sensed by intracellular *S.* Typhimurium CMEs may be ATP levels. The virulence protein MgtC has been shown to restrict ATP synthesis during growth under intracellular-mimicking conditions (26). An *mgtC* mutant with high ATP levels results in increased [c-di-GMP], resulting in attenuation via cellulose overproduction, as also seen with the PDE triple mutant. This suggests that high

ATP levels are acting to either inhibit a PDE or activate a DGC. The redundant nature of the identified PDEs indicates that it is likely that the bacteria evolved to respond to multiple specific signals present in the complex unique environment within macrophages, and not just ATP concentrations.

The identification of three redundant PDEs responsible for reducing [c-di-GMP] indicates that *S.* Typhimurium has evolved under a strong pressure to maintain low [c-di-GMP] during this stage of intracellular survival. This is further evidenced by the decreased competitive fitness of the triple PDE mutant compared with wild-type *S.* Typhimurium due to c-di-GMP–mediated cellulose overproduction. *S.* Typhimurium-produced cellulose is an exopolysaccharide polymer of β-1,4-linked D-glucose monomers that can be postsynthetically modified with phosphoethanolamine (27). One potential mechanism of bacterial attenuation by cellulose could be due to a decrease in available glucose as an energy/carbon source. Utilization of glucose via the glycolytic pathway is required for *S.* Typhimurium survival within macrophages, indicating that glucose is an important substrate in vivo (28). While attenuation of glycolytic mutants is severe during macrophage survival (3% of wild type), epithelial cell infection is much less restrictive (60% of wild type) (29). Similarly, an *mgtC* mutant is highly attenuated in macrophage survival due to cellulose overproduction (26), while it replicates to wild-type levels within epithelial cells (30). As cellulose production would remove glucose monomers from the bacterial cytosol in the form of an inaccessible extracellular polymer, depletion of this important carbon/energy source may result in decreased bacterial survival within macrophages.

However, glucose limitation is only one possible hypothesis for the role of cellulose during *S.* Typhimurium virulence. *S.* Typhimurium requires the type III secretion system located on pathogenicity island 2 (SPI2) to survive within macrophages (31), exopolysaccharide production has been shown to prevent the passage of exogenous type VI secretion systems (32), and electron microscopy of cellulose overproducing *S.* Typhimurium displays a rigid layer of cellulose (33). Therefore, it is possible that cellulose production provides either a physical barrier to the SPI2 secretion system or increases the physical distance between the bacterium and vacuolar membrane so as to reduce effector secretion. Alternatively, glucose limitation may lead to down-regulated transcription of SPI2 or other virulence factors that leads to attenuation. Whether via glucose limitation or another mechanism, it is clear that cellulose overproduction is detrimental to the survival of intracellular *S.* Typhimurium.

Data presented here also suggest that overproduction of cellulose specifically decreases survival of the population of slow-replicating bacteria. These slow-growing bacteria are of particular interest, as their decreased growth rate renders them more tolerant of both host antimicrobial attack and antibiotic challenge (34, 35). This provides a subpopulation that can resist early treatment attempts and provide a chronic infectious reservoir. Whether this slow-replicating population is generated via self-induced toxicity (35–37) or in response to a subset of vacuoles with increased hostility (38), we hypothesize that these slow-replicating bacteria are undergoing increased stress and/or lower amounts of necessary glucose that results in their slowed replication. If these slow-replicating intracellular *S.* Typhimurium are induced to overproduce cellulose, glucose limitation may allow the macrophage to control their survival either due to sacrifice of bacterial defense mechanisms or failure to maintain a metabolic state necessary for their future ability to survive and replicate.

In this manuscript, we have generated an updated FRET-based c-di-GMP biosensor that allowed us to both measure the intracellular kinetics of [c-di-GMP] during intracellular growth of *S.* Typhimurium and identify three PDEs that are required to maintain low [c-di-GMP] following phagocytosis. As this biosensor is predicted to be brighter and more stable than previous versions, the potential applications are extensive. The c-di-GMP regulatory pathways are encoded by several intracellular bacterial pathogens—including members of the *Yersinia* (39), *Mycobacterium* (40), and *Brucella* (41) families—as well as intracellular symbiotic bacteria such as *Rhizobium* (42). The role of c-di-GMP during intracellular survival is not well understood in these bacteria, and the technology we have developed could be applicable. Further, this biosensor may aid the understanding of c-di-GMP regulation in a variety of conditions in which alternative measurements methods may not have been feasible, as in the interaction of bacteria with animal cell and plant surfaces that might have an inherent fluorescent background or in methods like flow cytometry where bacterial particles require a baseline brightness for measurement. In each case, the tool developed here should lead to a broader understanding of c-di-GMP regulation, the environmental signals sensed by bacteria that induce a phenotypic alteration, and the population diversity of [c-di-GMP] in bacteria.

Materials and Methods

For more detailed material and method information, see *SI Appendix*.

Generation of the mTFP–mKO2 c-di-GMP Biosensor and in Vitro Binding Assay. The mTFP–mKO2 biosensor was generated from the mYFP–mCFP 6xHis tag c-di-GMP biosensor (13) via cloning of the indicated fluorophore sequence to either side of the *ycgR* coding sequence. Arginine residues at 114 and 118 of the YcgR protein sequence were mutated to alanine residues to generate the nonbinding mutant. Purified biosensors were incubated with increasing concentrations of c-di-GMP within a black 96-well plate (13) and recorded for mTFP and FRET fluorescence.

***S.* Typhimurium Infection and Fluorescence Microscopy of BMDMs.** Primary macrophages on coverslips were infected with stationary-phase *S.* Typhimurium cultures (multiplicity of infection = 20) expressing the biosensors, then followed 30 min later by gentamicin killing of remaining extracellular bacteria (*SI Appendix*, Fig. S1). Before microscopy, live samples were stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 680 to label the BMDM outer membrane. Samples were then live-imaged via confocal microscopy. All raw microscopy files, including those for the fluorescent dilution experiment described below, are available in the BioStudies database (www.ebi.ac.uk/biostudies) under accession number S-BSST238 (43). To quantify [c-di-GMP], individual fluorescent channel images were background-subtracted within ImageJ and measured for fluorescence intensity using the ImageJ plug-in MicrobeJ (44). FRET fluorescence values were normalized to account for bleed-through of mTFP fluorescence into the FRET channel (nFRET). The mTFP/nFRET ratios were determined for each bacterium, and bacteria with nFRET or mTFP fluorescence values outside of the linear range for the camera (1,000 to 40,000) were excluded from analysis. For average displayed values in Fig. 2*B*, outlier bacteria falling outside 5 SEs from the mean were removed from analysis to avoid large values from unduly influencing the average; these values were not omitted in further subpopulation and statistical calculations. To calculate the percentage of bacteria predicted to contain bound YcgR (>0.2 μM c-di-GMP) and bound BcsA (>8.0 μM c-di-GMP), the average value of bacteria expressing the nonbinding mTFP–mKO2 biosensor at each time point was used with the in vitro c-di-GMP binding curve to determine mTFP/nFRET values that correspond to 0.2 and 8.0 μM c-di-GMP. Mutant values of each subpopulation at 7 h postinfection were statistically compared with wild-type values using ANOVA. BMDMs were infected with the individual strains for quantification of bacterial survival, and the number of colony-forming units (CFU) were measured at 1, 7, and 18 h postinfection. Inoculums were plated for counting to determine the true inoculum, and all future CFU measurements were adjusted to these values. At each indicated time point, infected BMDMs were washed, lysed with 0.01% Triton X-100 in PBS, and plated to LB agar plates for counting. Statistical analysis was conducted on inoculum-adjusted log CFU values versus wild type via either two-way ANOVA at all time points (Fig. 3*B*) or one-way ANOVA on 18-h samples (*SI Appendix*, Fig. S5*A*).

Competitive i.p. Infection of BALB/c Mice. BALB/c mice (Jackson Labs) were housed in the Department of Comparative Medicine at the University of Washington according to Institutional Animal Care and Use Committee approved protocols. Wild-type *S.* Typhimurium and each mutant to be tested were competitively infected within BALB/c mice ($n = 5$ for each experiment) at a final dose of 1×10^5 total CFU (5×10^4 CFU per strain). Inoculums were plated to determine the true ratio of wild type to mutant (or wild type to wild type). At 2 d postinfection, competitive indices were

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determined by the ratio of mutant:wild-type splenic inoculum-adjusted CFU counts. Statistical analysis was conducted by paired Student's *t* test for inoculum-adjusted log CFU counts of each mutant versus the wild-type counts from the same spleen.

Measurement of Intracellular Replication Rate via Fluorescence Dilution. BMDMs infected with *S.* Typhimurium strains encoding the fluorescence dilution plasmid pFCcGi (20) (Addgene #59324) were measured via fluorescent microscopy for GFP/mCherry values of bacteria (n = 1,000 to 3,000 bacteria per sample) with mCherry values within the linear range of measurement and GFP values below the level of saturation. Bacteria were separated into fast-, moderate-, and slow-replicating populations based on the arbitrary GFP/mCherry values of less than 0.15, 0.15 to 0.35, and greater than 0.35 in such a way that wild-type bacteria were approximately split into thirds for each group. Strains and subpopulations were statistically compared to each other by two-way paired ANOVA versus wild type.

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1. Tamayo R, Pratt JT, Camilli A (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol* 61:131–148.
2. Hengge R (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 7: 263–273.
3. Römling U, Galperin MY, Gomelsky M (2013) Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 77:1–52.
4. Römling U, Simm R (2009) Prevailing concepts of c-di-GMP signaling. *Contrib Microbiol* 16:161–181.
5. Chou SH, Galperin MY (2016) Diversity of cyclic Di-GMP-binding proteins and mechanisms. *J Bacteriol* 198:32–46.
6. Mills E, Petersen E, Kulasekara BR, Miller SI (2015) A direct screen for c-di-GMP modulators reveals a *Salmonella* Typhimurium periplasmic L-arginine-sensing pathway. *Sci Signal* 8:ra57.
7. Ryjenkov DA, Simm R, Römling U, Gomelsky M (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: The PilZ domain protein YcgR controls motility in enterobacteria. *J Biol Chem* 281:30310–30314.
8. Pultz IS, et al. (2012) The response threshold of *Salmonella* PilZ domain proteins is determined by their binding affinities for c-di-GMP. *Mol Microbiol* 86:1424–1440.
9. Römling U, Galperin MY (2015) Bacterial cellulose biosynthesis: Diversity of operons, subunits, products, and functions. *Trends Microbiol* 23:545–557.
10. García B, et al. (2004) Role of the GGDEF protein family in *Salmonella* cellulose biosynthesis and biofilm formation. *Mol Microbiol* 54:264–277.
11. Kader A, Simm R, Gerstel U, Morr M, Römling U (2006) Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* 60:602–616.
12. Le Guyon S, Simm R, Rehn M, Römling U (2015) Dissecting the cyclic di-guanylate monophosphate signalling network regulating motility in *Salmonella enterica* serovar Typhimurium. *Environ Microbiol* 17:4818.
13. Christen M, et al. (2010) Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division. *Science* 328:1295–1297.
14. Kulasekara BR, et al. (2013) c-di-GMP heterogeneity is generated by the chemotaxis machinery to regulate flagellar motility. *eLife* 2:e01402.
15. Croce AC, Bottiroli G (2014) Autofluorescence spectroscopy and imaging: A tool for biomedical research and diagnosis. *Eur J Histochem* 58:2461.
16. Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA (2010) Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol Rev* 90:1103–1163.
17. Ai HW, Henderson JN, Remington SJ, Campbell RE (2006) Directed evolution of a monomeric, bright and photostable version of *Clavularia* cyan fluorescent protein: Structural characterization and applications in fluorescence imaging. *Biochem J* 400: 531–540.
18. Sakaue-Sawano A, et al. (2008) Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132:487–498.
19. Sun Y, et al. (2009) Characterization of an orange acceptor fluorescent protein for sensitized spectral fluorescence resonance energy transfer microscopy using a white-light laser. *J Biomed Opt* 14:054009.
20. Helaine S, et al. (2010) Dynamics of intracellular bacterial replication at the single cell level. *Proc Natl Acad Sci USA* 107:3746–3751.
21. Jenal U, Reinders A, Lori C (2017) Cyclic di-GMP: Second messenger extraordinaire. *Nat Rev Microbiol* 15:271–284.
22. Wada T, Tanabe Y, Kutsukake K (2011) FliZ acts as a repressor of the ydiV gene, which encodes an anti-FlhD4C2 factor of the flagellar regulon in *Salmonella enterica* serovar typhimurium. *J Bacteriol* 193:5191–5198.
23. Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JC (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* 47:103–118.
24. Adams P, et al. (2001) Proteomic detection of PhoPQ- and acid-mediated repression of *Salmonella* motility. *Proteomics* 1:597–607.
25. Galperin MY, Nikolskaya AN, Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* 203:11–21.
26. Pontes MH, Lee EJ, Choi J, Groisman EA (2015) *Salmonella* promotes virulence by repressing cellulose production. *Proc Natl Acad Sci USA* 112:5183–5188.
27. Thongsomboon W, et al. (2018) Phosphoethanolamine cellulose: A naturally produced chemically modified cellulose. *Science* 359:334–338.
28. Bowden SD, Rowley G, Hinton JC, Thompson A (2009) Glucose and glycolysis are required for the successful infection of macrophages and mice by *Salmonella enterica* serovar typhimurium. *Infect Immun* 77:3117–3126.
29. Bowden SD, et al. (2014) Nutritional and metabolic requirements for the infection of HeLa cells by *Salmonella enterica* serovar Typhimurium. *PLoS One* 9:e96266.
30. Smith RL, Kaczmarek MT, Kucharski LM, Maguire ME (1998) Magnesium transport in *Salmonella* typhimurium: Regulation of mgtA and mgtCB during invasion of epithelial and macrophage cells. *Microbiology* 144:1835–1843.
31. Kuhle V, Hensel M (2004) Cellular microbiology of intracellular *Salmonella enterica*: Functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. *Cell Mol Life Sci* 61:2812–2826.
32. Toska J, Ho BT, Mekalanos JJ (2018) Exopolysaccharide protects *Vibrio cholerae* from exogenous attacks by the type 6 secretion system. *Proc Natl Acad Sci USA* 115: 7997–8002.
33. Echeverz M, et al. (2017) Lack of the PGA exopolysaccharide in *Salmonella* as an adaptive trait for survival in the host. *PLoS Genet* 13:e1006816.
34. Abshire KZ, Neidhardt FC (1993) Growth rate paradox of *Salmonella* typhimurium within host macrophages. *J Bacteriol* 175:3744–3748.
35. Helaine S, et al. (2014) Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. *Science* 343:204–208.
36. Rycroft JA, et al. (2018) Activity of acetyltransferase toxins involved in *Salmonella* persister formation during macrophage infection. *Nat Commun* 9:1993.
37. Cheverton AM, et al. (2016) A *Salmonella* toxin promotes persister formation through acetylation of tRNA. *Mol Cell* 63:86–96.
38. Holden DW (2002) Trafficking of the *Salmonella* vacuole in macrophages. *Traffic* 3: 161–169.
39. Ren GX, Fan S, Guo XP, Chen S, Sun YC (2016) Differential regulation of c-di-GMP metabolic enzymes by environmental signals modulates biofilm formation in *Yersinia pestis*. *Front Microbiol* 7:821.
40. Sharma IM, Prakash S, Dhanaraman T, Chatterji D (2014) Characterization of a dual-active enzyme, DcpA, involved in cyclic diguanosine monophosphate turnover in *Mycobacterium smegmatis*. *Microbiology* 160:2304–2318.
41. Petersen E, Chaudhuri P, Gourley C, Harms J, Splitter G (2011) *Brucella melitensis* cyclic di-GMP phosphodiesterase BpdA controls expression of flagellar genes. *J Bacteriol* 193:5683–5691.
42. Pérez-Mendoza D, et al. (2014) Responses to elevated c-di-GMP levels in mutualistic and pathogenic plant-interacting bacteria. *PLoS One* 9:e91645.
43. Petersen E, Mills E, Miller SI (2019) Cyclic-di-GMP regulation promotes survival of a slow-replicating subpopulation of intracellular Salmonella Typhimurium. BioStudies. Available at www.ebi.ac.uk/biostudies/studies/S-BSST238. Deposited February 21, 2019.
44. Ducret A, Quardokus EM, Brun YV (2016) MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nat Microbiol* 1:16077.