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Report

Failsafe Mechanisms Couple Division and DNA Replication in Bacteria

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Summary

The past 20 years have seen tremendous advances in our understanding of the mechanisms underlying bacterial cytokinesis, particularly the composition of the division machinery and the factors controlling its assembly [1]. At the same time, we understand very little about the relationship between cell division and other cell-cycle events in bacteria. Here we report that inhibiting division in *Bacillus subtilis* and *Staphylococcus aureus* quickly leads to an arrest in the initiation of new rounds of DNA replication, followed by a complete arrest in cell growth. Arrested cells are metabolically active but are unable to initiate new rounds of either DNA replication or division when shifted to permissive conditions. Inhibiting DNA replication results in entry into a similar quiescent state in which cells are unable to resume growth or division when returned to permissive conditions. Our data suggest the presence of two failsafe mechanisms: one linking division to the initiation of DNA replication and another linking the initiation of DNA replication to division. These findings contradict the prevailing view of the bacterial cell cycle as a series of coordinated but uncoupled events. Importantly, the terminal nature of the cell-cycle arrest validates the bacterial cell-cycle machinery as an effective target for antimicrobial development.

Results

An Extended Block in Division Leads to a Metabolically Active but Unrecoverable State

To determine the impact of an extended block in cell division on cell physiology, we took advantage of genetic and chemical methods to conditionally inhibit division. We utilized three *Bacillus subtilis* strains: one in which synthesis of the essential tubulin-like cell division protein FtsZ is dependent on a xylose-inducible promoter, P_{xyl}-*ftsZ* (*ftsZ*::*spc*, *thrC*::P_{xyl}-*ftsZ*, *xylA*::*tet*) [2]; one encoding a heat-sensitive allele of *ftsZ*, *ftsZts* (*ftsZ*::*ftsZ-gfp*) [3]; and one encoding a heat-sensitive allele of a protein required for septum formation, *divICts* (*divIC*::*divICts*) [4]. FtsZ assembles into a ring structure that serves as a scaffold for the division machinery at nascent septal sites [1]. DivIC is a membrane-anchored protein that contributes to the integrity of the cytokinetic ring [5]. Upon removal of inducer in P^{xyl}-*ftsZ*, FtsZ levels decreased quickly and were reduced to 3% at 5.2 mass doubling periods (MDPs) (Figure S1A available online). In contrast, both the *ftsZts* and *divICts* strains retained near wild-type levels of FtsZ following a division block (Figure S1A). In addition, we inhibited division in wild-type *Staphylococcus aureus* Newman, using PC190723, a derivative of 3-methoxybenzamide that inhibits FtsZ assembly [6, 7]. All four strains form FtsZ rings and septa under permissive conditions, although the *divICts* strain is somewhat impaired for division, even at 30°C (Figure S2).

Following a shift to nonpermissive conditions, cells continued to increase in mass at normal rates for several generations until growth arrested abruptly (Figures 1A–1D). For comparative purposes, we normalized growth rates between strains and culture conditions using exponential MDPs (Table S1). P_{xyl}-*ftsZ* and *ftsZts* increased in mass for ~5 MDPs, whereas *divICts* and PC190723-treated *S. aureus* grew for ~3 MDPs after the shift to nonpermissive conditions.

The growth arrest was irreversible in all strains. Shifting cultures to permissive conditions following the growth arrest failed to yield any significant increase in optical density (Figures 1A–1C). In addition, the colony-forming ability under permissive condition was reduced at least 100-fold in cells sampled at growth arrest and ~1,000-fold 7 MDPs after division inhibition (Figure 1E).

Based on the inability of cells blocked for division to recover, we termed the time of growth arrest as the *p*oint *o*f *n*o *r*eturn (PONR). The inability to recover following the shift to permissive conditions differentiates these cells from persisters, which are dormant variants that occur stochastically in wild-type populations and can divide and grow following the removal of an antibiotic [8].

Cells Retain Membrane Integrity and Metabolic Activity after the PONR

To determine whether cells remain alive subsequent to the PONR, we assessed membrane integrity and metabolic levels in cells sampled before and after the growth arrest. We assessed membrane integrity using propidium iodide (PI), a nucleic acid binding agent that cannot pass through intact membranes [9]. Prior to growth arrest, P_{xyl}-*ftsZ* and *ftsZts* cells were refractory to PI staining (Figure 1F). At and even after the PONR, the majority of cells (~60%–100%, depending on the strain) remained impermeable to PI staining (Figure 1F). We observed 100% PI labeling for heat-killed and ethanol-killed cells (Figure S1B).

To assess metabolic activity, we employed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which measures nicotinamide adenine dinucleotide phosphate-dependent oxidoreductase activity [10]. MTT is reduced from its yellow, water-soluble form to purple insoluble formazan crystals in metabolically active cells. After resuspending the cells in DMSO, the optical density (OD_{550}) provides a direct measurement of cell metabolism [10].

MTT reduction indicates that cells inhibited for division are alive and metabolically active at growth arrest. At the PONR, MTT reduction rates in were 65%–100% of cells cultured under permissive conditions (Figure 1G). P_{xyl}-*ftsZ* and *ftsZts* strains

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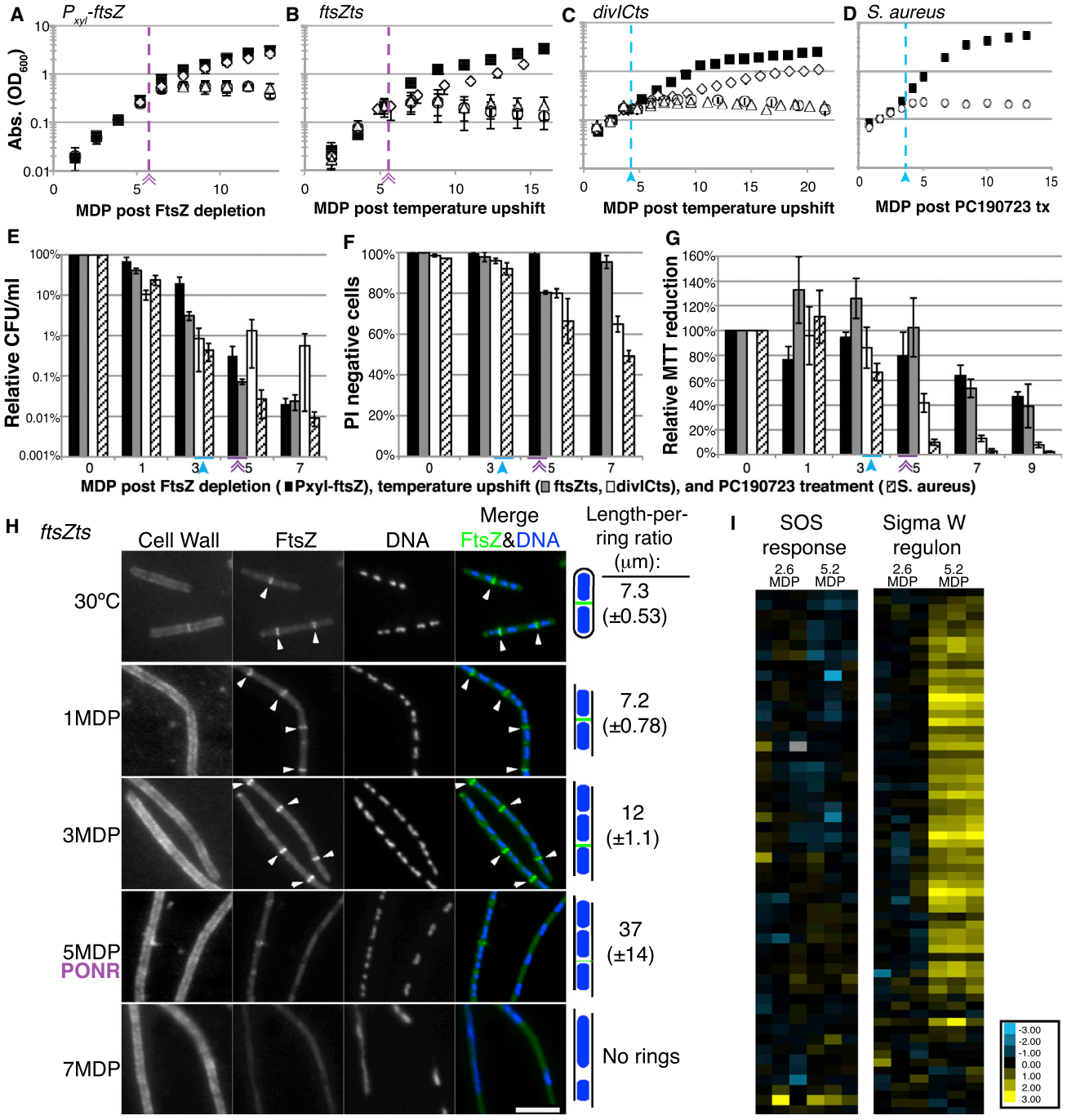

Figure 1. An Extended Block in Cell Division Leads to Growth Arrest and Entry into a Permanent Quiescent State at the PONR

(A–D) Cell growth as measured by OD_{600} of P_{xyl}-*ftsZ* (A), *ftsZts* (B), *divICts* (C), and PC190723-treated *S. aureus* (D). Abs, absorbance.

(A–G) Purple lines/double arrow (P_{xyl}-*ftsZ, ftsZts*) and blue lines/closed arrow (*divICts*) delineate the PONR boundary. Shown is growth under permissive conditions (■), nonpermissive conditions (○), shifted to permissive conditions ~1-2 MDPs before the PONR (3.9 MDPs, P_{xyl}-*ftsZ*; 4.4 MDPs, *ftsZts*; 3 MDPs, *divICts*) (◇), or shifted to permissive conditions approximately one generation after the PONR (6.5 MDPs, P_{xyl}-*ftsZ*; 7 MDPs, *ftsZts*; 5 MDPs, *divICts*) (△). Error bars show SD, n = 3–5.

(E–G) Cells are metabolically active but unrecoverable following the PONR. Black bars, P_{xyl}-*ftsZ*; gray bars, *ftsZts*; white bars, *divICts*; dashed bars, *S. aureus*.

(E) Colony-forming ability (plating efficiency) was determined after culturing cells at nonpermissive conditions prior to plating cells under permissive conditions. CFU, colony-forming units.

(F) PI is a marker for loss of membrane integrity. PI-negative (live) cells were quantified after cells were cultured for the indicated number of generations under nonpermissive conditions.

(G) Percent MTT reduction serves as a direct readout of metabolic activity. Permissive levels were set to 100%.

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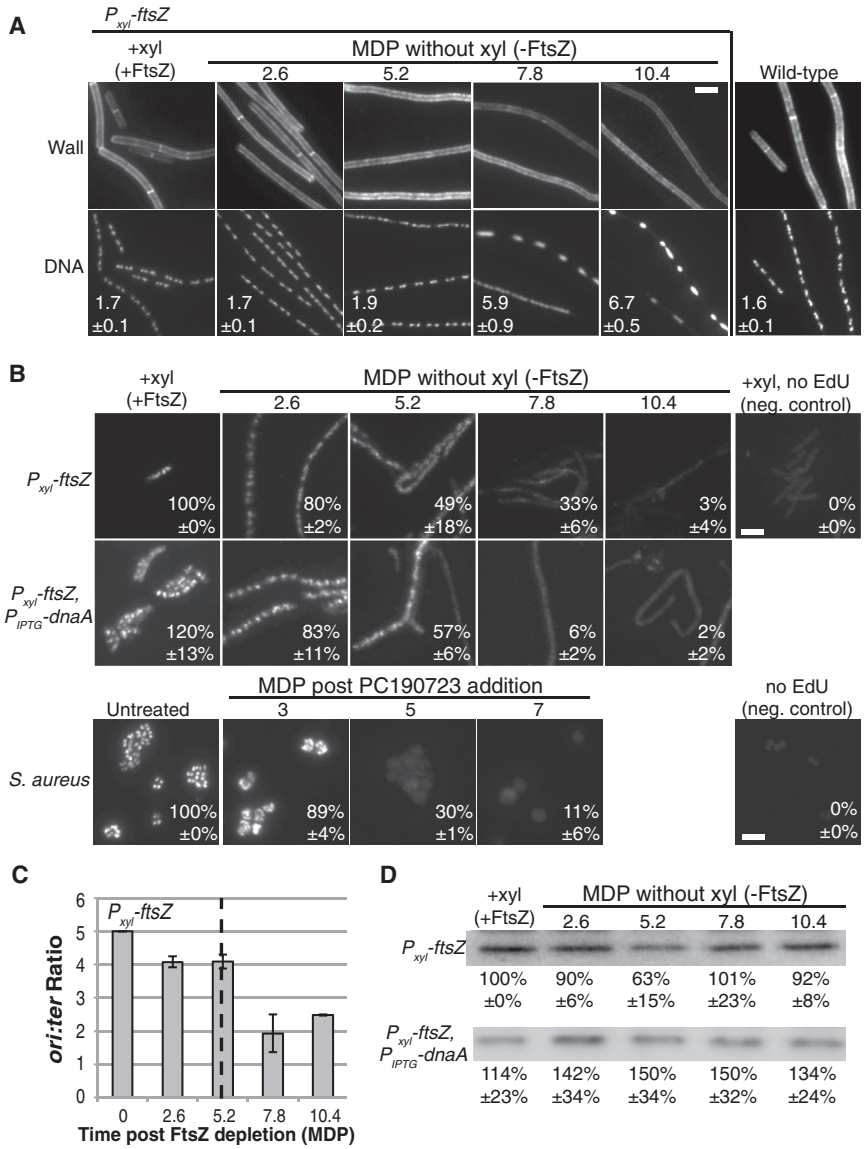

Figure 2. Inhibiting Cell Division Triggers an Arrest in DNA Replication

(A) Wheat germ agglutinin-labeled cell wall and DAPI-labeled DNA in wild-type and P_{xyl}-*ftsZ* cells. The average length per nucleoid ratio ± SD in micrometers are shown (n = 3, ~200 cells scored per replicate). Scale bar, 5 μm.

(B) Newly replicated DNA labeled with EdU. The fluorescence of induced P_{xyl}-*ftsZ* or untreated *S. aureus* was set to 100%. Mean ± SD of fluorescence is shown (n = 3, ~100 cells/replicate). Scale bar, 5 μm. neg., negative.

(C) Marker frequency analysis of the origin to terminus (*ori*:*ter*) ratios of P_{xyl}-*ftsZ* after depleting FtsZ (error bars show SD, n = 3).

(D) Quantitative immunoblots of DnaA in P_{xyl}-*ftsZ* and P_{xyl}-*ftsZ*, P_{IPTG}-*dnaA* cells depleted for FtsZ. The mean ± SD is shown below a representative blot. Induced P_{xyl}-*ftsZ* (+xyl) was set to 100%, and n = 3.

See also Figure S3.

exhibited a gradual decline in metabolic activity at the PONR, At 9 MDPs, they exhibited ~40% of the activity of cells at permissive conditions (Figure 1G). *divICts* and *S. aureus* declined rapidly in MTT reduction after the PONR, with MTT reduction rates falling to <20% of cells at permissive conditions (Figure 1G). Heat-killed cells had 0%–4% of permissive activity (Figure S1C). Consistent with high levels of metabolic activity, ^{35}S-methionine labeling indicated that bulk protein synthesis remains robust in P_{xyl}-*ftsZ* cells up to 9 MDPs following FtsZ depletion (Figure S1D).

We speculate that the increased sensitivity of *divICts* and *S. aureus* strains following a division block is due to physiological differences between these bacteria and P_{xyl}-*ftsZ* and *ftsZts* strains, specifically regarding membrane integrity. *divICts* mutants grow slowly and are somewhat filamentous, even under permissive conditions, whereas *S. aureus* exhibits an extremely rapid increase in diameter following inhibition of division (Figures S2C and S2G and Table S1).

Inhibiting Cell Division Triggers an Arrest in DNA Replication

Although cells remained intact and metabolically active, DNA staining revealed a marked impact on chromosome number and morphology following an extended block in division. As reported previously, nucleoids (discrete DNA-containing units) display normal morphology and segregation up to the initial arrest in growth [11–13] (Figures 2A and S2). After the arrest, however, we observed a clear deterioration in both the size and position of nucleoids, culminating in bright discontinuous structures (Figures 2A and S2). The nucleoid morphology differences following the PONR most likely result from cells at different stages of the cell cycle at the onset of the division block.

Consistent with the abnormal nucleoid morphology, we find that DNA replication is inhibited in cells subjected to a prolonged block in division (Figure 2B). Labeling P_{xyl}-*ftsZ* cells with the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) [14, 15], revealed an ~20% reduction in DNA replication at 2.6 MDPs after FtsZ depletion, an ~60% reduction at 5.2 MDPs, and replication was essentially nonexistent after 10.4 MDPs (Figure 2B). *S. aureus* treated with PC190723 also exhibited a decline in EdU labeling that is detectable before the PONR (Figure 2B).

(H) Cells are refractile to FtsZ assembly after the PONR. *ftsZts* cells were cultured under nonpermissive conditions for 1, 3, 5, and 7 MDPs prior to a shift to permissive conditions for 1 MDP. Length-per-ring ratios were calculated by dividing total cell length by the number of FtsZ rings. Approximately 200 wild-type cell lengths were scored per replicate. n = 3–4. SD is shown in parentheses. Arrows indicate FtsZ rings. Scale bar, 5 μm.

(I) Relative expression of genes in the SOS response or the Sigma W regulon. Transcript levels for three replicates are represented as the fold change for FtsZ-depleted cells compared with FtsZ-positive cells.

See also Figures S1 and S2 and Table S2.

DNA Replication Is Blocked at Initiation following Division Inhibition

The gradual reduction in EdU incorporation following division inhibition suggested that cells were blocked at initiation, the earliest stage in DNA replication. Defects in initiation allow ongoing replication forks to complete but block new rounds of replication [11]. Fast-growing *B. subtilis* cultured in nutrient-rich medium undergo multifork replication, with up to 16 replication forks proceeding simultaneously, and can complete approximately three rounds of chromosome segregation in the absence of new initiation events [16].

To confirm that DNA replication was blocked at initiation, we employed a marker frequency analysis using quantitative PCR to amplify origin and terminus proximal sequences and calculate the ratio of the origin to terminus DNA (*ori*:*ter*) [17]. Therefore, inhibiting replication at initiation but permitting completion of ongoing replication forks leads to a reduction in the *ori*:*ter* ratio.

In support of the idea that replication initiation is coupled to the successful completion of cytokinesis [18], we observed a steady decline in the *ori*:*ter* ratio in P_{xyl}-*ftsZ* cells cultured in the absence of inducer. Marker frequency analysis revealed a reduction in the *ori*:*ter* ratio from 5:1 to 4:1 at the PONR (5.2 MDPs) (Figure 2C). The *ori*:*ter* ratio continued to decline after the PONR approaching 2:1 at 7.8 MDPs (Figure 2C). The failure to reach a 1:1 *ori*:*ter* ratio (the expected outcome of a complete initiation arrest) suggests the presence of a secondary arrest in elongation after the PONR. Localization of cyan fluorescent protein fusion to Spo0J, which binds near the origin of replication, further supports a block at initiation in FtsZ-depleted cells (Figure S3C).

We hypothesized that the defect in replication initiation might be due to a reduction in the highly conserved initiation regulator DnaA [19]. However, although we observed a transient drop in DnaA concentration to ~60% of wild-type levels at the PONR, a modest overproduction of DnaA (~1.3–1.5 times wild-type levels) was insufficient to bypass the replication arrest in FtsZ-depleted cells (Figures 2B and 2D). The dip in DnaA levels is most likely the result of autorepression in response to a transient increase in the ratio of DnaA to *oriC* DNA at growth arrest [17, 20].

FtsZ Rings Are Unable to Form after the PONR

The block in DNA replication together with the unrecoverable nature of cells subsequent to the PONR suggested the onset of a terminal cell-cycle arrest. To confirm this possibility, we examined the ability of cells cultured past the PONR to support the assembly of FtsZ rings at nascent division sites following a shift to permissive temperatures.

Consistent with a terminal cell-cycle arrest, the PONR served as a sharp boundary in the ability to form FtsZ rings when shifted to permissive conditions (Figures 1H and S2). In *ftsZts* cells, extant FtsZ rings were stable under restrictive conditions, and new FtsZ rings assembled readily in cells downshifted to permissive conditions prior to the PONR (1 MDP or 3 MDPs) (Figures 1H and S2A). However, FtsZ rings failed to form in *ftsZts* cells downshifted after the PONR (7 MDPs) (Figures 1H and S2B). The timing of the division arrest rules out delocalization of the MinCD division inhibitor as FtsZ rings form readily in cells downshifted prior to the PONR, when MinCD should already be delocalized [21].

P_{xyl}-*ftsZ* and *divICts* cells cultured past the PONR were similarly incapable of forming new FtsZ rings (Figures S2C–S2F). DivIC is required for steps in division subsequent to FtsZ assembly, reinforcing the idea that a general block in division is sufficient to trigger the PONR.

An Extended Block in DNA Replication Initiation Leads to Entry into a Quiescent but Unrecoverable State

The onset of the replication arrest ~3 MDPs prior to the PONR suggested that inhibition of DNA replication, rather than the division block, might be the proximal trigger for entry into the quiescent state. To test this possibility, we employed a strain encoding a conditional mutation in *dnaB*, the replicative helicase coloader [22]. *dnaB134ts* (*dnaBts*) mutants can initiate new rounds of DNA replication at the permissive temperature of 30°C but are unable to do so upon a shift to the nonpermissive temperature of 45°C [22].

As observed in cells subjected to a prolonged block in division, inhibiting initiation resulted in a growth arrest and entry into a nonrecoverable state. *dnaBts* cells increased in mass for ~3 MDPs after a shift to 45°C before growth arrested abruptly (Figure 3A). Downshifting *dnaBts* cells to 30°C prior to the growth arrest resulted in almost full recovery (as measured by OD_{600}). However, mutants were unable to recover when shifted to permissive conditions after 5 MDPs at 45°C (Figure 3A). The colony-forming ability was impaired similarly after growth arrest in *dnaBts* cells cultured at 45°C (Figure 3B).

Despite their unrecoverable nature, ~97%–100% of *dnaBts* cells cultured at 45°C retained intact membranes well past growth arrest (Figure 3B). *dnaBts* cells were also metabolically active after growth arrest, retaining MTT reduction rates of 60% after 12 MDPs at nonpermissive conditions (Figure 3C).

A Prolonged Block in DNA Replication Initiation Triggers a Terminal Arrest in Division

Consistent with a terminal cell-cycle arrest, *dnaBts* cells subjected to a prolonged block in DNA replication were unable to form FtsZ rings (Figures 3D and S4A). As has been observed in other DNA replication mutants, *dnaBts* cells were elongated and contained single FtsZ rings adjacent to the medially positioned nucleoids 3 MDPs after the shift to nonpermissive conditions (Figure 3D) [23–26]. Although *dnaBts* cells cultured at 45°C were able to form FtsZ rings at growth arrest (3 MDPs), they were unable to do so 2 MDPs later, even when shifted to permissive conditions (Figures 3D and S4A). FtsZ levels remained unchanged over the course of the experiment (Figure S4B). A loss-of-function mutation in the gene encoding Noc, implicated in coordinating chromosome segregation and division [27], had no impact on the onset of growth arrest or the ability of cells to recover after the PONR (data not shown).

Microarray Analysis of FtsZ-Depleted Cells Suggests that Division Is Coordinated with DNA Replication via a Posttranscriptional Mechanism

Microarray analysis of P_{xyl}-*ftsZ* cells prior to and at the PONR suggests that both the DNA replication arrest and entry into the quiescent state are controlled via a posttranscriptional mechanism. Comparison of gene expression in FtsZ-depleted cells and mock-depleted cells revealed that the overwhelming majority of genes maintained constant expression levels, similar to what has been reported in *E. coli* [28]. We did not observe an induction of genes known to play a role in cell-cycle regulation, nor did we see an induction of the SOS response, which controls the cell-cycle arrest associated with DNA damage (Figure 1I) [29]. Importantly, we did not observe a

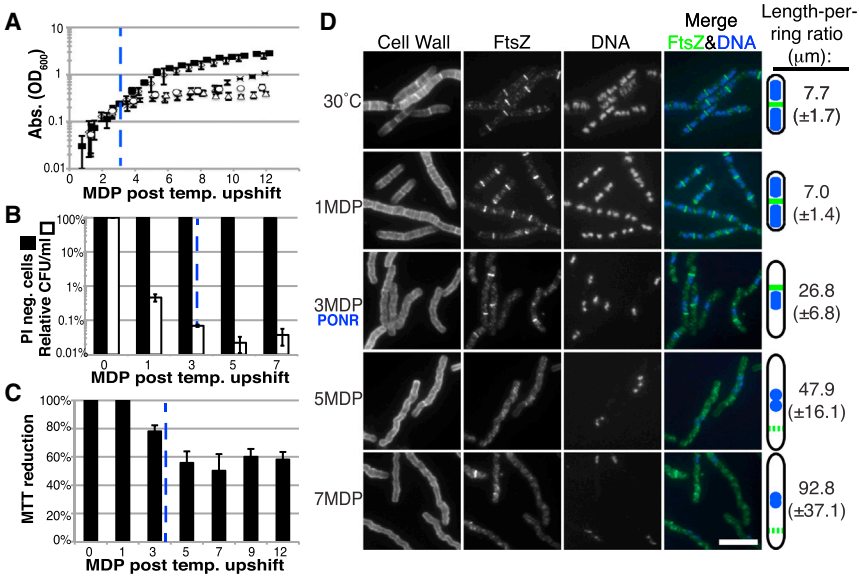

Figure 3. An Extended Block in DNA Replication Initiation Results in Terminal Cell-Cycle Arrest

(A) Cell growth of *dnaB134ts* under permissive conditions (■), nonpermissive conditions (○), shifted to permissive conditions at 1 MDP (◇), shifted to permissive conditions approximately at 3 MDPs (Xs), or shifted to permissive conditions at 5 MDPs (△). Error bars show SD, and n = 3. The dashed blue line indicates the PONR. Temp, temperature.

(B) PI-negative (live) cells and colony-forming ability (plating efficiency) was determined after culturing cells under nonpermissive conditions for the indicated MDPs (PI) and prior to plating cells at permissive conditions (CFU). Error bars show SD, and n = 3. The dashed blue line indicates the PONR.

(C) MTT reduction levels indicate metabolic activity of cells blocked for initiation. Error bars show SD, and n = 3. The dashed blue line indicates the PONR.

(D) Cells were cultured under nonpermissive conditions for 1, 3, 5, and 7 MDPs. Length-per-ring ratios were calculated as in Figure 1. n = 3, and SD is shown in parentheses. Scale bar, 5 μm.

See also Figure S4.

significant induction of stationary phase-specific gene expression, consistent with the PONR being a distinct physiological state.

We observed upregulation of the Sigma W regulon, a set of genes involved in the response to a variety of cell envelope stresses, including antibiotic challenge and osmotic shock [30]. However, deletion of the transcription factor controlling this regulon, Sigma W, had only a modest impact on the growth and plating efficiency of P_{xyl}-*ftsZ* cells cultured past the PONR (Figures 1I, S1E, and S1F and Table S2). Additional upregulated transcripts at 5.2 MDPs included the flagellar component *motA*, two genes involved in mannitol phosphorylation (*mtlA* and *mtlD*) two putative phage genes (*ydjG* and *yqaL*), and several putative and hypothetical proteins (Table S2).

Discussion

Our findings support a model in which inhibiting division for an extended period of time (~5 MDPs in rich medium) triggers entry into a quiescent state characterized by a terminal cell-cycle arrest. Although a link between division and DNA replication in bacteria has been proposed [18], our data are the first to demonstrate a direct connection between these two cell-cycle processes. Importantly, we observed a significant reduction in DNA replication prior to the PONR, with complete arrest occurring several generations after the PONR.

Failsafe Mechanisms Couple Cell Division and DNA Replication in Bacteria

Based on these data, we propose that there is a failsafe mechanism that inhibits DNA replication in the absence of division (Figure 4). Coupling division to replication ensures that bacterial cells initiate only one round of replication per mass doubling period, regardless of whether or not they are undergoing multifork replication. Our finding that an extended block in DNA replication leads to an arrest in new rounds of division supports the presence of a second failsafe mechanism that prevents division in the absence of DNA replication. In function, if not in form, these mechanisms are akin to the cell-cycle checkpoints that ensure the orderly progression through the eukaryotic cell cycle. Such failsafe mechanisms ensure that cells defective in cell division or DNA replication are removed from the growing population.

We favor the idea that the terminal cell-cycle arrest observed in cells subjected to an extended block in cell division or DNA replication is a consequence of an infinite loop (a "vicious cell cycle") in which cells that cannot divide cannot initiate DNA replication and cells that cannot initiate replication cannot divide (Figure 4). The earlier onset of the growth arrest in *dnaBts* cells suggests that the block in DNA replication might be the proximal cause of the PONR following the division block.

The Cell Division Machinery Is an Ideal Target for Antibiotic Development

In addition to providing fundamental and unforeseen insights into our understanding of the bacterial cell cycle, our findings validate the bacterial cell division machinery as an ideal target for the development of new antibiotics. Not only does inhibiting division prevent the production and proliferation of viable daughter cells, but the concurrent arrest in DNA replication serves as a barrier to the acquisition of antibiotic-resistant mutations. Most importantly, the terminal cell-cycle arrest at the PONR ensures that treated cells cannot recover after the drug has been metabolized.

Accession Numbers

The GEO Series accession number for the microarray data reported in this paper is GSE56753.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.07.055.

Author Contributions

H.A.A. and A.K. performed the research. J.D.S. and N.A.S. contributed reagents (PC190723). H.A.A., A.K., J.D.W., and P.A.L. designed the research and analyzed the data. H.A.A. and P.A.L. wrote the paper.

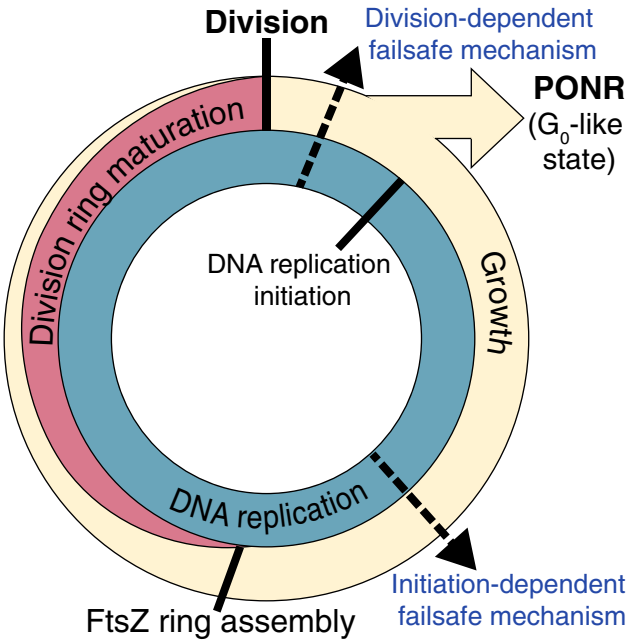

Figure 4. Failsafe Mechanisms Couple Division and DNA Replication in the Bacterial Cell Cycle

DNA replication (blue) and growth (beige) are ongoing, and the division ring is present for approximately the second half of each cell cycle. A division-dependent failsafe mechanism ensures that one DNA replication initiation occurs for each division. Failure to divide leads to a block in replication initiation followed by entry into a terminal cell-cycle arrest at the PONR, equivalent to a eukaryotic G_0 state. A second initiation-dependent failsafe mechanism ensures that cells impaired in the initiation of DNA replication do not assemble division rings. The dashed arrows indicate hypothetical localizations of the failsafe mechanisms.

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