Quick replication fork stop by overproduction of *Escherichia coli* DinB produces non-proliferative cells with an aberrant chromosome

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*Escherichia coli* dinB encodes the translesion DNA polymerase DinB, which can inhibit progression of replication forks in a dose-dependent manner, independent of exogenous DNA damage. We reported previously that overproduction of DinB from a multicopy dinB plasmid immediately abolished ongoing replication fork progression, and the cells rapidly and drastically lost colony-forming ability, although the mechanisms underlying this lethality by severe replication fork stress remained unclear. Here, we show that the reduced colony-forming ability in the dinB-overexpressing cells is independent of the specific toxin genes that trigger programmed bacterial cell death when replication is blocked by depletion of the dNTP pool. After DinB abolished replication fork progression and colony-forming ability, most of the cells were still viable, as judged by fluorescent dye staining, but contained irregularly shaped nucleoids in which chromosomal DNA was preferentially lost in the replication terminus region relative to the replication origin region. Flow cytometric analysis of the cells revealed chromosomal damage and the eventual appearance of cell populations with less than single-chromosome DNA content, reminiscent of sub-G1 cells with lethal DNA content produced during eukaryotic apoptosis. This reduced DNA content was not observed after replication fork progression was quickly stopped in temperature-sensitive dnaB helicase mutant cells at a non-permissive temperature. Thus, the quick replication stop provoked by excess DinB uniquely generates temporarily viable but non-reproductive cells possessing a fatally depleted chromosomal content, which may represent one of the possible fates of an *E. coli* cell whose replication is overwhelmingly compromised.

**Key words:** bacterial lethality, DNA polymerase IV, genomic instability, replication stress, Y-family DNA polymerase

**INTRODUCTION**

Movement of DNA replication forks can be impeded by depletion of the dNTP pool, various types of DNA damage, and protein-DNA complexes (Friedberg et al., 2006; Mirkin and Mirkin, 2007). Stalled replication forks cause DNA replication stress that threatens genome integrity and cell survival. Thus, perturbation of DNA replication in bacteria and eukaryotes activates complex cellular tolerance mechanisms to alleviate the potentially lethal consequences for the cells (Branzei and Foiani, 2010; Bichara et al., 2011). When replication fork arrest leads to irreparable genomic alteration, eukaryotes activate apoptosis to prevent the propagation of genetically abnormal cells (Su, 2006).

Blockage of *Escherichia coli* replication forks by DNA lesions triggers a damage tolerance system termed the SOS response, in which expression of chromosomal genes (the SOS regulon) is coordinately enhanced to ensure cell survival (Courcelle et al., 2001; Friedberg et al., 2006). In the SOS system, translesion synthesis (TLS) is one of the important pathways to rescue stalled replication forks (Goodman, 2002; Yang and Woodgate, 2007; Bichara et al., 2011). During TLS, a specialized DNA polymerase...
(Pol) directly copies the lesion site, with a relaxed fidelity of nucleotide incorporation, to allow the stalled fork to advance beyond the lesion site (Goodman, 2002; Nohmi, 2006; Fuchs and Fuji, 2007). In contrast to this protective response to replication stress, the E. coli cell’s fate has not been well elucidated when replication is extensively or irrevocably compromised. Programmed cell death has been found in E. coli and is induced under several stressful conditions including DNA damage (Gerdes et al., 2005; Ellenberg-Kulka et al., 2006; Yamaguchi et al., 2011). It has been proposed that replication fork arrest by depletion of the dNTP pool promotes cell death mediated through two specific toxin-antitoxin systems, mazEF and relBE (Sat et al., 2003; Godoy et al., 2006). MazF and RelE toxins that cleave mRNA are repressed by the presence of the specific antitoxins MazE and RelB, respectively (Yamaguchi and Inouye, 2011).

One of the DNA polymerases for TLS in E. coli is Pol IV (Wagner et al., 1999), which is encoded by dinB in the SOS regulon (Ohmori et al., 1995; Courcelle et al., 2001) and called DinB hereafter. DinB functions in TLS over N2-deoxyguanine adducts in vivo (Napolitano et al., 2000; Shen et al., 2002; Jarosz et al., 2006). Together with Pol V, DinB belongs to the Y-family DNA polymerases (Ohmori et al., 2001; Jarosz et al., 2007) and is universally conserved in all large phylogenetic groups such as archaean Dpo IV and eukaryotic Pol κ (Gerlach et al., 1999; Ogi et al., 1999; Boudsocq et al., 2001). Under non-SOS conditions, the intracellular level of E. coli DinB is 250 molecules per cell (Kim et al., 2001). In a SOS-induced cell, enhanced expression of dinB causes the number of DinB molecules to rise ten-fold, to 2500 molecules per cell (Kim et al., 2001). Since these amounts of DinB are far in excess of the number of replication forks in the cell during log phase (2–8 forks; Skarstad et al., 1986), other biological functions have been proposed for DinB. A newly discovered dose-dependent function of E. coli DinB is to inhibit DNA replication by targeting the replicative Pol III in vitro (Furukohri et al., 2008; Indiani et al., 2009; Wagner et al., 2009). Consistent with this biochemical activity, ectopic overexpression of dinB impedes replication fork progression in a dose-dependent manner in vivo (Uchida et al., 2008; Indiani et al., 2009; Mori et al., 2012). Although blockage of replication fork progression readily induces the SOS response to protect the cell from replication stress (Friedberg et al., 2006), the SOS response is not significantly activated by DinB-mediated fork inhibition, suggesting a unique mechanism for fork arrest (Mori et al., 2012).

We have previously reported that replication fork progression was inhibited after DinB was overproduced from a dinB construct, under the control of the PBAD promoter (PBAD-dinB), that was inserted ectopically into the chromosome (Uchida et al., 2008; Mori et al., 2012). The DNA synthesis rate was reduced to about half of that in the wild-type strain when the intracellular DinB level was 2- to 8-fold higher than that in SOS-induced cells, and synthesis eventually ceased 60 min after arabinose addition. Growth of the E. coli cells stopped immediately after arabinose addition, although cell viability, assessed by measuring colony-forming units (CFU), was maintained until 60 min. On the other hand, when DinB was rapidly overproduced from PBAD-dinB on a multi-copy plasmid, replication fork progression was quickly and completely abolished within 15 min after arabinose addition, and the cells showed an almost complete loss in CFU after 60 min, whereas protein synthesis was barely suppressed (Uchida et al., 2008). The decrease of CFU was not an artifact of DinB protein overproduction per se (Uchida et al., 2008). As an example of bacterial lethality in response to extensive replication stress, we here analyzed the fate of cells carrying the PBAD-dinB plasmid after the quick replication stop. No single deletion among known toxic genes including mazF and relE protected the dinB-overexpressing cells from a fast reduction in CFU. Nucleoids (bacterial chromosomal DNA structures) in these cells contained damaged DNA lacking the replication terminus region more frequently than the replication origin region, indicating genomic instability. The dinB-overexpressing cells eventually exhibited less than one chromosome per cell, a level not observed in cells with an inactive DnaB helicase, in which replication also stops quickly (Carl, 1970) but without a severe reduction of CFU. These observations suggest that E. coli cells respond to a quick replication block by excess DinB by a mechanism that generates an incomplete chromosome and leads to an irreversible loss of proliferation activity. This may be one possible outcome of multiple responses in cells undergoing severe genotoxic stress.

**MATERIALS AND METHODS**

**Media and chemicals** LB medium was prepared as described (Sambrook and Russell, 2001). E salt is 1x Vogel and Bonner’s synthetic E medium (Vogel and Bonner, 1956), and ECA growth medium is E salt with supplements as described (Uchida et al., 2008). Fluorescent dyes other than 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Japan) were from Invitrogen, USA.

**Construction of bacterial strains** All E. coli K12 strains, plasmids and oligonucleotides used are listed in supplementary Table S1. Replacement of the chromosomal toxin genes of MK7010 (Uchida et al., 2008) by the kanamycin resistance gene (kan) was performed by Pl(vir)-mediated transduction (Miller, 1972), with selected strains from the Keio collection as donors (Baba et al., 2006), followed by elimination of kan by recombination at the flippase recognition target (FRT) with the flippase enzyme (Datsenko and Wanner, 2000). Chromo-
somal yafNOP genes were deleted using the chromosomal targeting method with Red recombinase (Datsenko and Wanner, 2000). The FRT-flanked kan gene of plasmid pKD13 was amplified by PCR with primers 2006M1 and 2006M2 and used to replace the region spanning the yafN and yafP genes of BW25113 carrying plasmid pKD46. The kan gene of the resulting MK6729 strain was transferred to MK7010 by P1 transduction and eliminated with the flippase enzyme to obtain MK6913. Strain MK6320 was constructed for microscopic analysis of the ori and ter regions. A tandem insertion containing the chimeric resistance gene (cam) at the chromosomal yjaR locus was transferred, together with the FRT-flanked chloramphenicol resistance gene (cam) from MKG271 (Cui et al., 2007), into MK7010 by P1-mediated transduction. After the FRT-flanked cam of the transductants was deleted by flippase, the lacI and tetR operator arrays were sequentially transferred from MKG296 and MKG206 (Cui et al., 2007) with the gentamicin resistance gene and cam, respectively, by P1 transduction. The resulting strain MK6320 carries the tetO array at the replication origin region (15 kb counterclockwise from oriC) and the lacO array at the replication termination region (20 kb counterclockwise from the dif site), which are visualized respectively by two repressor-fluorescent fusion proteins, TetR-EYFP (enhanced yellow fluorescent protein) and LacI-ECFP (enhanced cyan fluorescent protein), expressed from the arabinose promoter (P_\text{BAD}).

**Cell growth** Cells transformed with the pBAD-HisA vector or the dinB-expression plasmid pDB10 (Uchida et al., 2008) were routinely grown at 37°C in ECA medium containing 50 μg ml\(^{-1}\) ampicillin, as described previously (Uchida et al., 2008). When the cultures reached OD\(_{600}\) = 0.1–0.2, L(+)-arabinose was added (designated as time zero) to a final concentration of 0.2% (w/v) to induce dinB expression from the P_\text{BAD} promoter, and incubation was continued at 37°C. For temperature-sensitive strains, cells were grown first at 30°C to an OD\(_{600}\) of 0.1–0.2 and then either at 39°C for 1 h. The ethanol-fixed samples were spread on a poly-L-lysine-coated glass slide. The presence of the ori and ter regions was judged by the presence of EYFP and ECFP foci, respectively, under a fluorescence microscope.

**Chromosomal DNA amount per cell** Cells were stained with SYTO 16 and served as the standard for DNA content values of one and two chromosomes per cell (Pennington and Rosenberg, 2007). To visualize nucleoids, DAPI (final concentration of 2 μg ml\(^{-1}\)) was added to the cell suspension. The cells were spread on a poly-L-lysine-coated glass slide. For phase-contrast and fluorescence microscopy, cells were visualized with a 100x objective on a DMRE-HC fluorescence microscope (Leica Microsystems, Germany) with appropriate filters and a cooled digital CCD camera (1300Y; Roper Scientific, Germany). Images were captured and analyzed by MetaMorph software (Universal Imaging, USA) and processed by Photoshop software (Adobe, USA).

**Membrane integrity and nucleoids** Cells were suspended in 1 ml of saline (0.85% NaCl) to final OD\(_{600}\) = 1.0 and stained simultaneously with SYTO 9 and propidium iodide (PI) dyes using the Live/Dead BacLight bacterial viability detection kit (Invitrogen), and then mounted on a 1% agarose pad on a glass slide to evaluate viability with a fluorescence microscope. For DAPI staining, cells were suspended in 1 ml of ice-cold LB medium to OD\(_{600}\) = 1.0 and incubated with 10 ml of 80% methanol at room temperature for 1 h. The cell samples were stained with DAPI solution (5 μg ml\(^{-1}\)) and SYTO 16 (Invitrogen) for 1 h at room temperature and then mounted on a glass slide. For DAPI staining, cells were stained with SYTO 16 and served as the standard for DNA content values of one and two chromosomes per cell (Pennington and Rosenberg, 2007). Wild-type cells (MK7010) were grown in LB medium over-
RESULTS AND DISCUSSION

The multicopy plasmid pDB10 was constructed by cloning wild-type dinB in the pBAD-HisA vector so that the gene is overexpressed under the control of the tightly regulated PBad promoter, as described previously (Uchida et al., 2008). When arabinose was added to a culture of wild-type MK7010 cells carrying pDB10 (MK7010/pDB10), progression of replication forks was quickly abolished. Judging from the severe reduction in CFU, dinB overexpression was lethal to the cells (Uchida et al., 2008). This severely reduced CFU of the MK7010/pDB10 cells was not due simply to protein overproduction per se, since cells overproducing the carboxyl-terminal half of DinB retained CFU, or to prolonged inhibition of DNA synthesis, because overproduced DinB was susceptible to protein degradation (Uchida et al., 2008).

**MazF and RelE toxins are not involved in lethality of dinB-overexpressing cells** Thymine starvation of thymine-auxotrophic cells (thyA) halts DNA synthesis due to substrate starvation, and the cells lose viability, a phenomenon that is called thymineless death (TLD). It has been suggested that this lethality is mediated by the MazF toxin, which triggers an intrinsic death program in bacteria (Sat et al., 2003). Like TLD, hydroxyurea (HU) treatment depletes dNTP pools by inhibiting ribonucleotide reductase and induces lethality (Davies et al., 2009). Activation of the mazF and relE toxin genes contributes to the promotion of HU-induced cell death (Godoy et al., 2006; Davies et al., 2009). To examine if the MazF and RelE toxins are involved in the reduction of CFU that follows dinB-mediated fork arrest, we constructed MK7010/pDB10 derivatives that harbor a deletion of either mazF or relE and determined whether deletion of either toxin gene could alleviate the fatal effects of dinB overexpression. CFU were determined for cultures of the wild-type MK7010, MK6743 (ΔmazF) and MK6751 ( ΔrelE) carrying either pBAD-HisA vector or pDB10 (Fig. 1, A and B). In the presence of arabinose, CFU of each cell carrying vector increased logarithmically on the LB plates, (A) mazF+ and ΔmazF, (B) relE+ and ΔrelE, and (C) ΔyafQ and ΔyafNOP. Symbols are MK7010/pBAD-HisA (open circles) and MK7010/pDB10 (closed circles), MK6743/pBAD-HisA (closed triangles) and MK6743/pDB10 (open triangles), MK6751/pBAD-HisA (closed squares) and MK6751/pDB10 (open squares), and MK6913/pBAD-HisA (closed diamonds) and MK6913/pDB10 (open diamonds).

E. coli K-12 contains at least 36 putative toxin genes (Yamaguchi and Inouye, 2011). In addition to mazF and relE, no single deletion of other known type II toxin genes (chpB, hipA, yoeB and yafQ) in MK7010/pDB10 was able to overcome the lethality of DinB overproduction (data
not shown). dinB constitutes an operon together with the recently discovered toxin-antitoxin module yafNO (Singletary et al., 2009) and yafP, which encodes a putative acetyltransferase that is proposed to work together with dinB in recovery from genotoxic shock (Gutierrez et al., 2011). Deletion of yafNOP from the dinB operon also had no effect on the reduction in CFU (Fig. 1C). Therefore, none of seven discrete toxin genes triggers the reduced CFU in dinB-overexpressing MK7010/pDB10 cells, suggesting that programmed cell death is not the cause of lethality in cells overexpressing dinB. However, we cannot exclude the possibility that another toxin gene(s) operates in cell death pathways in the DinB-mediated fork block.

**dinB-overexpressing cells contain irregular nucleoids** To gain a better insight into lethality of the dinB-overexpressing cells upon immediate blockage of DNA replication fork progression, we investigated microscopically the MK7010/pDB10 and MK7010/vector cells after incubation for 3 h in the presence of arabinose. E. coli cells often show a filamentous cell shape because replication fork inhibition results in a cell division block through sulA expression in the SOS response (Friedberg et al., 2006). Phase-contrast images of MK7010/pDB10 show that the cells elongated up to several-fold relative to MK7010/vector (compare Fig. 2, A–C with Fig. 2, D–F), suggesting some cellular stress. However, unlike SOS-induced cells, the cell shape of MK7010/pDB10 was not filamentous, which confirms cytologically the lack of strong SOS induction in DinB-mediated fork arrest (Mori et al., 2012).

The dinB-overexpressing cells were stained with mixtures of the nucleic acid-binding fluorescent dyes SYTO 9 and PI. Since the green-fluorescing SYTO 9 generally labels all bacteria in a population having either intact or damaged membranes, while the red-fluorescing PI penetrates only bacteria having damaged membranes (dead cells), dead and living cells can be distinguished. Whereas programmed cell death elicited by toxin genes produces PI-stained cells (Godoy et al., 2006), the MK7010/pDB10 cells, with almost no colony formation (Fig. 1), were barely stained by PI, in common with MK7010/vector cells that grew normally (Fig. 2, B and E). This shows that MK7010/pDB10 retains intact membranes (Fig. 2, D and E). Protein synthesis in MK7010/pDB10 was hardly affected even 3 h after arabinose was added (Uchida et al., 2008). These observations indicate that the MK7010/pDB10 cells are still alive, even though their reproductive capacity is almost completely abolished. This state probably thus reflects cell stasis rather than death.

The MK7010/pDB10 cells were fixed with methanol and stained with the nucleic acid-binding fluorescent dye DAPI. DAPI-stained cells were photographed under simultaneous phase contrast and DAPI fluorescence exposure, to detect nucleoid and cell shape at the same time (Fig. 2, C and F). Nucleoid-lacking cells (cells lacking bacterial chromosomes) were not produced extensively in either MK7010/pDB10 or MK7010/vector. In exponen-

![Fig. 2. Fluorescence staining of dinB-overexpressing cells. MK7010/vector (A–C) and MK7010/pDB10 cells (D–F) were incubated in ECA medium containing 0.2% arabinose for 3 h. (A, B, D, E) Live/dead staining. After the cells were stained simultaneously with SYTO 9 (green) and PI (red) dyes, they were observed using a fluorescence microscope with appropriate filters for SYTO 9 (A and D) or PI (B and E) to evaluate cell viability based on membrane integrity. Fluorescence images were overlaid on images of cell shapes detected with a phase-contrast microscope. (C, F) DAPI staining. Methanol-fixed cells were stained with DAPI solution (5 μg ml⁻¹). Cell shapes and nucleoids were observed simultaneously with a combination of phase-contrast and DAPI fluorescence microscopes. Representative images are shown for (C) MK7010/vector and (F) MK7010/pDB10. The intracellular DAPI signals are white. Black and white bars represent 15 and 5 μm, respectively.](image-url)
entially growing MK7010/vector, nucleoids were situated in the center of the cells (Fig. 2C). In contrast, the nucleoids in MK7010/pDB10 were more dispersed in the cytosol, and enclosed irregular compact regions, some of which seemed to be detached from each other (Fig. 2F). From these results, we concluded that chromosomal DNA was retained but might be damaged in most of the dinB-overexpressing cells.

dinB-overexpressing cells are damaged by chromosomal region-specific DNA loss We integrated genes expressing TetR-EYFP and LacI-ECFP fusion proteins tandemly from the P<sub>BAD</sub> promoter into genomic DNA (Lau et al., 2003). Tandem copies of the tetO and lacO operators were then inserted at positions close to the chromosomal replication origin oriC and terminus ter of MK7010, respectively (Lau et al., 2003). The fluorescent proteins were used as probes for the oriC and ter regions in the resulting strain, MK6320, to assess the integrity of aberrantly organized nucleoids. After exponentially growing MK6320 cells harboring either pDB10 or vector were induced to synthesize the fluorescent repressor proteins for 1.5 or 3 h by the addition of arabinose, fluorescent foci in the cells were monitored. It should be noted that the MK6320/pDB10 cells overproduce DinB together with the fluorescent proteins following arabinose addition.

Snapshot photos of multiple cells were taken with a digital camera mounted on a fluorescence microscope. At both time points, about 40% of MK6320/vector cells had 2 ori and 1 ter foci (Fig. 3, A and C). The ori signal was detected in 88 and 98% of the cells 1.5 and 3 h after arabinose addition, respectively (Fig. 3, B and C). As with ori, the ter signal was also observed in most of the cells: 72% at 1.5 h and 88% at 3 h. The profile of ori and ter foci in MK6320/pDB10 cells was different from that in MK6320/vector cells. At 1.5 h, only 17% of MK6320/pDB10 had 2 ori and 1 ter foci, whereas 23% and 30% had 1 ori and 2 ori without ter foci, respectively (Fig. 3, A and C). MK6320/pDB10 virtually lost CFU 1 h after arabinose addition (Fig. 1), but retained the oriC signal comparable to that of MK6320/vector: 81% at 1.5 h and 73% at 3 h (Fig. 3B). In contrast, 27% and 8.4% of MK6320/pDB10 carried the ter signal at these respective time points. These results show that the ter but not the oriC signals were markedly under-represented in the irregular nucleoids of the dinB-overexpressing cells.

Detection of the ter signal might have been preferentially impeded due to inhibitory effects of excess DinB on the ability of the fluorescent protein LacI-ECFP to bind to the lacO operator array and form foci. However, considering the sequence-independent nature of the DNA-binding activity of DinB (Grüz et al., 2001; Furukohi et al., 2008), it is not plausible that DinB would block only the binding of LacI-ECFP to ter. Since we detected the ter foci in 27% of the dinB-overexpressing cells 1.5 h after arabinose addition, it also seems unlikely that excess DinB suppresses expression of the lacI-cfp gene to result in the apparent absence of the ter region. Hence, dinB-overexpressing cells specifically lost ter-proximal DNA, which indicates genomic instability after a quick block of the replication fork by DinB. Given this conclusion, the ratio of oriC to ter should be imbalanced in these cells. In fact, the gene dosage near oriC relative to that near ter was slightly enhanced in genomic profiles previously determined using a microarray for MK7010/pDB10 even 30 min after arabinose addition (Uchida et al., 2008). Interestingly, ectopic overproduction of the human ortholog of DinB, Pol κ, blocks replication fork progression (Pillaire et al., 2007) and induces genomic instability in hamster cells (Bavoux et al., 2005), as shown here for DinB in E. coli.

dinB overexpression produces cells with less than a single chromosomal DNA To confirm the loss of DNA from the chromosome, the DNA content of the cells was directly monitored with a flow cytometer. The temperature-sensitive dnaA<sub>46</sub> strain SMR8375 was used as a reference for cells having a DNA content of one and two chromosomes, since it cannot initiate DNA replication at the nonpermissive temperature of 39°C but continues to divide until most cells carry only one chromosome (Pennington and Rosenberg, 2007). As expected, there were two peaks in flow cytometry histograms of DNA in dnaA<sub>46</sub> cells that had been grown at 39°C (gray peaks in Figs. 4 and 5). We confirmed that these peaks represent cells with one and two chromosomes, respectively, since the latter one coincided with the lower-DNA-content peak of cells grown to the stationary phase at which cells with two and four chromosomes are accumulated (data not shown; Steen and Boye, 1980). MK7010/vector and MK7010/pDB10 cells were grown exponentially in ECA medium, and DNA content per cell was monitored by flow cytometry at 1-h intervals, with time zero defined as when arabinose was added to the culture (Fig. 4). Because most growing cells have two or more chromosomes, flow cytometric profiles of both cell populations at time zero showed broad distributions of DNA content between two and four chromosome equivalents. During the next 3 h of incubation, the DNA content of MK7010/vector gradually converged into a single peak representing two chromosomes per cell (Fig. 4A). This shows that the exponentially growing MK7010/vector cells sustained DNA replication and reached stationary phase in 3 h. Even when MK7010/vector was further incubated until 5 h, DNA content less than 2 chromosomes per cell was not detected (data not shown). In contrast to the vector control, MK7010/pDB10 cells, in which DNA replication had ceased, displayed a broad peak at 1.5 chromosomes per cell 1 h after arabinose addition (Fig. 4B), indicating apparent chromosomal damage at the time when most of
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Fig. 3. Chromosomal origin and terminus regions of dinB-overexpressing cells. MK8320 cells carrying either pBAD-HisA vector or pDB10 were grown in ECA medium containing 0.2% arabinose at 37°C for 1.5 or 3 h. The cells were stained by DAPI, and labeled near the ori and ter sites with the fluorescent fusion proteins TetR-EYFP and LacI-ECFP, respectively. (A) Representative images of cells carrying either vector or pDB10. Images were taken with filters specific for red (oriC; left) and green (ter; center) fluorescent foci, and merged (right). (B) Percentage of cells with either ori or ter foci. The bar graphs show the relative numbers (%) of cells having ori (black), ter (gray) or no foci (white) among nucleoid-containing cells. The numbers of observed cell images were 496 and 1132 for MK8320/vector 1.5 and 3 h, and 624 and 586 for MK8320/pDB10 1.5 and 3 h after arabinose addition, respectively. (C) Percentage of cells with different ori and ter foci. Observed cell images were the same as those in B.
the cells failed to form colonies (Fig. 1). As shown in Fig.
3, ter-proximal DNA is highly depleted in nucleoids of
these MK7010/pDB10 cells. Altogether, these observa-
tions suggest that damaged chromosomes lacking the ter
region were responsible for the reduction in CFU dis-
played by the dinB-overexpressing cells.
In the subsequent 2 h of incubation, the MK7010/pDB10 culture produced doomed cells carrying less than one chromosome each (Fig. 4B), reminiscent of the sub-G1 cells with hypodiploid DNA content that are produced during apoptosis of diploid eukaryotic cells (Friedberg et al., 2006). The DNA content of MK7010/pDB10 cells, with an average of three chromosomes at time zero, was apparently reduced to half (1.5 chromosomes per cell) and then to a quarter (0.75 chromosomes per cell). However, the doomed cells do not appear to be the primary explanation for the reduction of CFU, because they arose 2 h after DinB had completely abolished CFU (Figs. 1 and 4). Rather, the fate of the cells may be a consequence of an irreversible loss of cell proliferation activity.

We next examined whether dysfunction of replication fork enzymes, which also cause replication fork block independently of DNA damage, produces the abnormal chromosome content. The hexameric form of DnaB functions as the replicative helicase of the E. coli replisome (O’Donnell, 2006). The replication fork of temperature-sensitive dnaB8 cells is also blocked immediately after a shift to the restrictive temperature of 42°C (Carl, 1970; Saluja and Godson, 1995). Unlike dinB overexpression, the exponentially growing dnaB8 cells did not significantly lose CFU 3 h after a temperature shift to 42°C (Fig. 5A). Moreover, the cells displayed approximately the same broad flow cytometric profile of DNA as that at time zero (Fig. 5B). This represents blockade of on-going replication forks at random chromosomal positions. However, in contrast to dinB overproduction, DNA content less than 1 chromosomes per cell was not detected. Therefore, the quick replication stop does not necessarily result in the fatally depleted chromosome content. This indicates that the production of the lethal chromosome content is specific to the rapid fork block by DinB.

How might dinB overexpression cause aberrant chromosomal DNA in MK7010/pDB10 cells? One possible explanation is DNA degradation at stalled replication forks. When the replication fork is inhibited, DNA becomes more prone to breakage (Dillingham and Kowalczykowski, 2008; Branzei and Foiani, 2010). Double-stranded breaks are generated under dNTP starvation (Guarino et al., 2007), and chromosomal DNA is lost during TLD (Fonville et al., 2010). In the lethal dinB-overexpressing cells, the total amount of 3H-thymine-labeled DNA was maintained (Uchida et al., 2008). Therefore, it is unlikely that massive DNA degradation is the principal cause of reduced genome content. Furthermore, no detectable decrease in the total DNA amount was observed, even when overproduction of a catalytically defective mutant DinB (DinB-D8A) immediately blocked DNA replication and rapidly reduced CFU (Uchida et al., 2008). This eliminates the possibility that DNA synthesis catalyzed by excess wild-type DinB offsets substantial DNA loss and results in apparently constant amounts of DNA. However, we cannot exclude the possibility that a small amount of DNA was lost at replication forks arrested by DinB.

A second possible explanation for the occurrence of an abnormal chromosome following dinB overexpression is the so-called guillotine effect that is often observed during cell division in chromosome partitioning mutants of E. coli (Niki et al., 1991). Cellular responses to replication fork inhibition are complex and do not invariably result in a strong induction of the SOS response (Bernard et al., 2010). Because of the negligible SOS response to DinB-mediated fork inhibition (Mori et al., 2012), dinB-overexpressing cells may form a premature septum when replication is blocked, leading to a guillotine break of nucleoids. However, we observed few cells containing a guillotined nucleoid bisected by a division septum (data not shown). Furthermore, ectopic induction of SOS did not rescue the lethality of DinB overproduction (data not shown). Thus, loss of the SOS response is probably not a direct cause of the aberrant chromosome.

The third possibility is that DNA breakage might occur if the cell tried to divide an unresolved chromosome having an arrested replication fork (Fonville et al., 2010). This would transmit either a ter-less or a ter-containing chromosome into each daughter cell. If DNA breakage happened near the terminus, ter of the latter chromosome might also disappear as a result of limited DNA degradation from broken DNA ends. This model is supported by the progressive appearance of cells containing 1.5 and 0.75 chromosomes in the absence of DNA replication (Fig. 4B). In this case, excess DinB may disrupt cellular regulatory systems in which cell division and chromosome segregation are coordinated to faithfully transmit intact chromosomes into daughter cells. An abnormal cell cycle in the presence of an incompletely replicated chromosome might prevent the propagation of genetically damaged cells. Further investigations of dinB-overexpressing cells at the molecular level are required to evaluate this possibility.

**Concluding remarks** Overexpression of plasmid-borne $\text{P}_{\text{BAD}}\cdot\text{dinB}$ immediately halts replication fork progression and rapidly reduces CFU (Fig. 1; Uchida et al., 2008). This reduced CFU does not appear to reflect cell death triggered by toxin genes, including $\text{mazF}$ and $\text{relE}$, which account for lethality in dNTP-starved cells (Fig. 1). The $\text{dinB}$-overexpressing cells are static but contain a damaged chromosome, which probably causes the lethality (Figs. 2 and 3). We also found that cells in which a replication fork that was quickly blocked by excess DinB, but not by dysfunction of the replicative DnaB helicase, eventually contained less than a single chromosome per cell, possibly through an unusual cell cycle without new DNA synthesis (Figs. 4 and 5). E. coli cells probably therefore respond to replication blockage caused by excess DinB.

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using a mechanism which is distinct from that underlying fork arrest caused by dNTP starvation or a dysfunctional replication apparatus. In *E. coli*, extensive replication blockage may produce cell stasis and finally a non-functional genome. This might represent a process that irreversibly abolishes the proliferation activity of bacterial cells that have suffered severe replication stress.

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Note added in proof:
Recently, Walker’s group has reported that 8-oxo-guanine (8-oxo-dG) in the nucleotide pool underlies much of the cell death caused by DinB overproduction and bactericidal antibiotics (Foti J. J. et al. (2012) Science, 336, 315–319). Their data suggest a model in which the elevated levels of DinB incorporate a limited amount of 8-oxo-dGTP and cause lethality most likely due to double-strand DNA breaks generated by an incomplete action of base excision repair systems at closely spaced 8-oxo-dG lesions. However, the polymerase activity of DinB is not absolutely required for the rapid decline in colony-forming ability of our dinB-overexpressing cells (Uchida et al., 2008). Thus, in our experimental system, an enhanced usage of 8-oxo-dGTP by excess DinB is not sufficient to explain the severe lethality of these cells.

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