DinB Up-Regulation Is the Sole Role of the SOS Response In Stress-Induced Mutagenesis 

in Escherichia coli

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ABSTRACT

Stress-induced mutagenesis is a collection of mechanisms observed in bacterial, yeast and human cells in which adverse conditions provoke mutagenesis, often under the control of stress responses. Control of mutagenesis by stress responses may accelerate evolution specifically when cells are maladapted to their environments, i.e., are stressed. It is therefore important to understand how stress responses increase mutagenesis. In the Escherichia coli Lac assay, stress-induced point mutagenesis requires induction of at least two stress responses: the RpoS-controlled general/starvation-stress-response, and the SOS DNA-damage response, both of which upregulate DinB error-prone DNA polymerase, among other genes required for Lac mutagenesis. We show that up-regulation of DinB is the only aspect of the SOS response needed for stress-induced mutagenesis. We constructed two dinB(oC) (operator-constitutive) mutants. Both produce SOS-induced levels of DinB constitutively. We find that both dinB(oC) alleles fully suppress the phenotype of constitutively SOS-“off” lexA(Ind-) mutant cells, restoring normal levels of stress-induced mutagenesis. Thus, dinB is the only SOS gene induction of which is required for stress-induced point mutagenesis. Further, although spontaneous SOS induction has been observed to occur only in a small fraction of cells, upregulation of dinB by the dinB(oC) alleles in all cells does not promote a further increase in mutagenesis, implying that SOS induction of DinB, though necessary, is insufficient to differentiate cells into a hypermutable condition.
Genomic stability and mutation rates are tightly regulated features of all organisms. Understanding how cells regulate mutation rates has important implications for evolution, cancer progression and chemotherapy resistance, aging, and acquisition of antibiotic resistance and evasion of the immune system by pathogens, all processes driven by mutagenesis and all of which occur during stress.

Stress-induced mutagenesis is a group of related phenomena in which cells poorly adapted to their environment (i.e., stressed) increase mutation rates as part of a regulated stress response (reviewed by Galhardo et al. 2007). Abundant examples, particularly in microorganisms, now show the induction of specific pathways of mutagenesis in response to stresses. The types of genetic alteration induced by stress include base substitutions, small deletions and insertions, gross chromosomal rearrangements and copy-number variations, and movement of mobile elements. These various pathways require the functions of different sets of genes and proteins. Thus, there appear to be multiple molecular mechanisms of stress-inducible mutagenesis that operate in different organisms, cell types, and growth-inhibiting stress conditions.

However, a common theme in the many mechanisms of stress-inducible mutagenesis described to date is the requirement for the function of one or more cellular stress responses. Starvation-stress-induced mutagenesis in Bacillus subtilis requires the comK regulatory gene that controls the stress response that allows competence for natural transformation in response to starvation (Sung and Yasbin 2002). The RpoS-controlled general- or starvation-stress response is required for starvation-induced excisions of phage Mu in Escherichia coli (Gómez-Gómez et al. 1997), for base-substitution mutagenesis in aging E. coli colonies (Bjedov et al. 2003), for starvation-induced point mutations (Saumaa et al. 2002) and transpositions (Ilves et al. 2001).
in *Pseudomonas putida*, and for starvation-induced gene amplification (LOMBARDO *et al.* 2004) and frameshift mutagenesis (LAYTON and FOSTER 2003; LOMBARDO *et al.* 2004) in the *E. coli* Lac assay, described in more detail below. The SOS DNA-damage stress response is required for the stress-induced frameshift mutagenesis in the *E. coli* Lac assay discussed below, for *E. coli* mutagenesis in aging colonies (TADDEI *et al.* 1997), and for ciprofloxacin (antibiotic)-induced resistance mutagenesis (CIRZ *et al.* 2005), and in mutagenesis conferring resistance to bile salts in Salmonella (PRIETO *et al.* 2006). The stringent response to amino-acid starvation is required for a transcription-associated mutagenesis in *E. coli* that targets stringent-response-controlled genes (WRIGHT *et al.* 1999), and for amino-acid-starvation-induced mutagenesis in *B. subtilis* (RUDNER *et al.* 1999). Two different stress responses to hypoxia in human cancer cells also increase mutagenesis. One does so by specific down-regulation of mismatch-repair genes (BINDRA and GLAZER 2007; KOSHIJI *et al.* 2005; MIHAYLOVA *et al.* 2003). The other is postulated to promote genome rearrangement by its demonstrated down-regulation of RAD51 and BRCA1 functions required for high-fidelity repair of DNA double-strand breaks (BINDRA *et al.* 2004). These stress responses exert temporal control or restriction of mutagenesis, favoring genomic stability when cells/organisms are well adapted to their environments (not stressed) and increasing mutagenesis, potentially accelerating evolution, specifically during stress, when cells are maladapted to their environments. Except for the human examples, for few of these stress responses is it understood how the stress response up-regulates mutagenesis. We focus on that issue here for stress-induced mutagenesis in an *E. coli* model system.

Stress-induced mutagenesis is perhaps best understood in the *E. coli* model system. A widely used assay system uses a +1 frameshift allele of a *lacIZ* fusion gene located in the F’128 plasmid, and a deletion of the chromosomal *lac* genes (CAIRNS and FOSTER 1991). When these
cells are plated on lactose minimal medium, a few Lac\(^+\) revertant colonies are observed. Many of these arise from spontaneous generation-dependent mutations that occur during growth of the culture. Prolonged incubation of these plates results in the continuous accumulation of additional Lac\(^+\) revertants, which arise through two mechanisms, both different from the mechanisms that produce the generation-dependent mutants (reviewed by GALHARDO et al. 2007).

First, within the first few days, most of the Lac\(^+\) colonies are “point mutants” that possess a compensatory -1 frameshift mutation in the \(lacIZ\) gene (FOSTER and TRIMARCHI 1994; ROSENBERG et al. 1994). Cells that carry these mutations also carry increased numbers of secondary unselected mutations in other genomic regions, whereas most Lac\(^-\) cells starved on the same plates do not, indicating that a subpopulation of the cells undergoes genome-wide hypermutation (GODOY et al. 2000; ROSCHE and FOSTER 1999; TORKELSON et al. 1997). Therefore, a subset of the starved cells is experiencing increased mutagenesis when compared to the majority of the cells. We refer to this subpopulation as hypermutable hereafter. This hypermutable cell subpopulation appears to be important to the formation of most or all of the Lac\(^+\) stress-induced mutants (GONZALES et al. 2008). The hypermutable state is transient, ceasing after growth impairment is ended and growth resumes (GODOY et al. 2000; LONGERICH et al. 1995; ROSCHE and FOSTER 1999; ROSENBERG et al. 1998; TORKELSON et al. 1997).

Second, longer incubation also results in the formation of a significant proportion of \(lac-\) amplified colonies, in which the leaky \(lacIZ\) allele is amplified to 20-50 tandem copies, which produce sufficient enzyme activity to allow growth on lactose (HASTINGS et al. 2000; KUGELBERG et al. 2006; POWELL and WARTELL 2001; SLACK et al. 2006). In summary, \(E.\ coli\) cells may either increase point-mutation rates or undergo extensive genomic rearrangement in response to a growth-limiting environment.
Both of these processes require induction of the general or starvation stress response controlled by RpoS (LOMBARDO et al. 2004). Point mutagenesis, but not amplification, also requires induction of the SOS DNA-damage stress response (CAIRNS and FOSTER 1991; MCKENZIE et al. 2000; MCKENZIE et al. 2001). In this paper, we focus on the role of the SOS response in the mechanism of stress-induced point mutagenesis. See (HASTINGS 2007) for a review of the mechanisms of stress-induced amplification and genome rearrangement.


DinB is the founding member of the most widespread subfamily of Y-family specialized DNA polymerases, with orthologues in bacteria, archaea, and eukaryotes, including humans (reviewed by NOHMI 2006). DinB/Pol IV can perform high-fidelity translesion DNA synthesis (TLS) across a number of different DNA lesion substrates (BJEDOV et al. 2007; JAROSZ et al. 2006; YUAN et al. 2008). Nevertheless, this enzyme shows a significant error rate when copying undamaged DNA templates (KOBAYASHI et al. 2002). Some mutations in DinB can abolish its TLS activity, without interfering with the mutator phenotype caused by overexpression of DinB, suggesting that mutagenesis and TLS are independent activities of Pol IV (GODOY et al. 2007).
85% of the stress-induced Lac\textsuperscript{+} point mutations generated in the non-growing cells arise in a DinB-dependent manner (McKENZIE et al. 2001).

The \textit{dinB} gene is under control of the SOS response, which up-regulates its transcription 10-fold (KIM et al. 2001). Additionally, the alternative sigma (transcription) factor \(\sigma^S\) (RpoS), responsible for the general-stress response, up-regulates \textit{dinB} expression transcriptionally by 2- to 3-fold upon entry in stationary phase (LAYTON and FOSTER 2003). Proteins such as Ppk (STUMPF and FOSTER 2005), the chaperones GroEL (LAYTON and FOSTER 2005), RecA and UmuD (GODOY et al. 2007) all seem to modulate DinB activity. An interesting \textit{in vivo} role of DinB is SOS untargeted mutagenesis of phage \(\lambda\) (KIM et al. 1997). In it, -1 frameshift mutations in runs of Gs are generated, similarly to the predominant mutations detected in the \textit{lac} gene during stress-induced mutagenesis (FOSTER and TRIMARCHI 1994; ROSENBERG et al. 1994). On the other hand, DinB has no effect on the spontaneous mutation rate in growing cells (KUBAN et al. 2004; McKENZIE et al. 2001; McKENZIE et al. 2003; WOLFF et al. 2004). DinB is implicated as the DNA polymerase that, only during the stress responses, makes DSB-repair-associated errors that become stress-induced point mutations (PONDER et al. 2005).

The role of the SOS response in controlling mutagenesis in the Lac assay is a complex issue because several SOS-controlled genes are required for the process. \textit{dinB}, \textit{recA}, \textit{ruvA} and \textit{ruvB} are all required for mutagenesis (CAIRNS and FOSTER 1991; FOSTER et al. 1996; HARRIS et al. 1994; HARRIS et al. 1996; HE et al. 2006; McKENZIE et al. 2001) and all are upregulated by SOS (COURCELLE et al. 2001). Also, the F-encoded psiB gene exerts a negative effect on mutagenesis in SOS-de-repressed cells (McKENZIE et al. 2000), and is thought to inhibit SOS induction and RecA (reviewed by COX 2007). We sought to determine whether the requirement for induction of the SOS response in stress-induced mutagenesis reflects a need for up-regulation.
solely of \( \text{dinB} \), or whether any other gene(s) are required at SOS-induced levels. We present evidence below that indicates first, that DinB is the only SOS-controlled gene required at induced levels for efficient stress-induced point mutagenesis, and second, that although SOS-induced levels of DinB are required, they are not sufficient to differentiate cells into a hypermutable condition.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media:** The bacterial strains used in this work are shown in Table 1. \( \text{dinB}(o^c) \) alleles were constructed as described below. Other strains were constructed using P1-mediated transduction as described (Miller 1992). Antibiotics were used as follows: ampicillin, 100 \( \mu \text{g/mL} \); chloramphenicol, 25 \( \mu \text{g/mL} \); tetracycline, 10 \( \mu \text{g/mL} \); kanamycin, 30 \( \mu \text{g/mL} \); and rifampicin, 40 \( \mu \text{g/mL} \). 5-bromo-4-chloro-3-indolyl-\( \beta \)-D-galactoside (X-gal) was at 40 \( \mu \text{g/mL} \). M9 minimal medium (Miller 1992) was supplemented with 10 \( \mu \text{g/mL} \) of vitamin B1 and either 0.1% glycerol or 0.1% lactose. Luria-Bertani-Herskowitz (LBH) medium is as per, e.g., (Torkelson et al. 1997).

The plasmids used in this study are listed in Table 2. Plasmids containing \( P_{\text{dinB}}\beta\text{lacZ} \) fusions used in beta-galactosidase assays for gene expression analysis were constructed by amplification of the \( \text{dinB} \) promoter (from bases -432 to -2 of \( \text{dinB} \)) with primers 5’ TCGGCTGAATTCTGTTCGACTCGCTCGATAAT 3’ and 5’ CGGTACAAGCTTGCTCACCTCTCAACACTGGT 3’, and cloning into the pFZY plasmid (Koop et al. 1987) using the EcoRI and HindIII sites introduced in the primers. The \( \text{dinB} \)
promoter was amplified from strain SMR4562 and cloned into pFZY to generate plasmid pPdinB, and amplified from strains SMR10308 and SMR10309 to generate the plasmids pPdinBOC1 and pPdinBOC2, respectively.

**Construction of the dinB(o5) alleles and strains bearing them:** We created each of two mutations predicted from previous work on other SOS genes (FRIEDBERG et al. 2005) to inactivate the predicted LexA-binding site in dinB (Figure 1A). The Lac assay strains carry two copies of dinB, one in the chromosome and one in F’128 (discussed in Results). The constructions required several steps as below. Primer sequences are given in Supplementary Material.

First we linked the cat selectable marker with dinB. We chose to put a selectable marker in the lafU (formerly known as mbhA) gene, which is present right upstream to the 5’ end of dinB. The FRTcatFRT cassette was amplified from pKD3 (DATSENKO and WANNER 2000) using primers CatupdinB-F and CatupdinB-R. The product was used to obtain SMR4562 recombinants containing the lafU::FRTcatFRT insertion (allele ∆lafU2::FRTcatFRT), using short-homology recombination as described (DATSENKO and WANNER 2000). One recombinant containing the ∆lafU2::FRTcatFRT in the F’ plasmid was selected. This strain (SMR10292) was used to amplify the ∆lafU2::FRTcatFRT-dinB′ region by PCR using primers CatupdinB-F and dinBcatnock-R. This product was used as a template for PCR-mediated site-directed mutagenesis, altering the dinB promoter.

Next we constructed a ∆lafU-dinB deletion strain to be used as a recipient for allelic replacement with the site-directed dinB-mutant genes linked to ∆lafU2::FRTcatFRT. We created a FC36-derivative containing a deletion encompassing the 3’ half of ∆lafU, and the whole dinB gene using primers kandinBchrom-F and DinBRCAT to amplify FRTKanFRT from pKD13
The products were used for short-homology recombination in the FC36 background, creating strain SMR10299. A similar deletion in the same region in the F' plasmid was created by short-homology recombination in SMR4562, using FRTcatFRT amplified from pKD3 with primers CatupdinB-F and DinBRCAT. Location of the deletion in the F' plasmid was confirmed by ability to conjugate the cat gene conferring chloramphenicol resistance. The cat gene was removed by FLP-mediated site-specific recombination using the pCP20 plasmid (DATSENKO and WANNER 2000). The resulting F'128 ΔlafU-dinB::FRT [allele Δ(lafU-dinB)2096(::FRT)] was mated into strain SMR10299, creating strain SMR10303 (SMR4562 Δ(lafU-dinB)2097(::FRTkanFRT) [F' Δ(lafU-dinB)2096(::FRT)]). This strain was used as a recipient for allelic replacement using the site-directed dinB mutants produced by PCR using the ΔlafU2::FRTcatFRT-dinB fragment as a template. The sequence of the promoter and coding sequence of the dinB gene from the KanR CamR recombinants was determined by PCR and DNA sequencing to ensure that the desired mutation was introduced, and that no other mutation in dinB was generated inadvertently by PCR. One recombinant containing the dinBo-21(o²) mutation (SMR10304) and one containing the dinBo-22(o²) mutation (SMR10306) were chosen. Those strains were used as a P1 donors of ΔlafU2::FRTcatFRT dinBo-21(o²) and ΔlafU2::FRTcatFRT dinBo-22(o²) respectively, to transduce the dinB(o²) alleles to all the genetic backgrounds of interest, including SMR4562, and strains FC231 and SMR868 carrying lexA3(Ind⁺).

Deletion of the yafNOP genes in the dinB operon was performed using short-homology recombination (DATSENKO and WANNER 2000) as follows. Strains SMR10292 (SMR4562 [F' ΔlafU2::FRTcatFRT]) and SMR10308 (SMR4562 [F' ΔlafU2::FRTcatFRT dinBo-21(o²)]) were used as recipients for deletion by transformation with a DNA fragment amplified from pKD13
with primers yafNwL and yafPwR. Homologous incorporation of this DNA fragment, which contains the FRTKanFRT marker, results in a deletion of the yafNOP genes. Kan^R recombinants were selected, and location of the marker in the F’ episome was confirmed both by ability to transfer the resistance during mating, and by co-transduction of Kan^R and Cam^R (present in the linked lafU2::FRTcatFRT in both strains). The strains resulting from deletion of yafNOP from the episomes of SMR10292 and SMR10308 were named SMR11023, and SMR11024 respectively. Both strains were used respectively as P1 donors to transfer the lafU2::FRTcatFRT ΔyafNOP::FRTKanFRT linkage and the lafU2::FRTcatFRT dinB-21(ơ) ΔyafNOP:FRT:KanFRT linkage into the FC231 background, creating strains SMR11026 and SMR11027.

**Beta-galactosidase assays:** Beta-galactosidase assays were performed in order to determine the relative expression of lacZ under the control of the different versions of the dinB promoter cloned into low copy plasmid pFZY (KOOP et al. 1987). Cells were grown in LBH medium until mid-log phase, and the levels of beta-galactosidase were determined in samples of the cultures as described (MILLER 1992).

**DinB Western blots:** For DinB detection on Western blots, stationary-phase cultures grown from single colonies in 5mL of M9 B1 glycerol medium for 48h, were harvested, and cells were suspended in sample loading/lysis buffer (62.5 mM Tris pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 0.5% β-mercaptoethanol), correcting for the OD_{600} of the terminal culture. For 1 mL of a culture at OD_{600} of 2 (measured at OD_{600} ≤ 1 with diluted samples), 100 µL of sample loading buffer was used. 20 µL of each sample were separated by electrophoresis on a SDS polyacrylamide gel (12.5%). Proteins were transferred to a Hybond-LFP PVDF membrane (Amersham Biosciences), and the membrane was probed with a
polyclonal DinB rabbit antiserum (Kim et al. 2001). A goat anti-rabbit secondary antibody conjugated to the Cy5 fluorescent dye (Amersham Biosciences) was used for detection of DinB, using the Typhoon scanner (Amersham Biosciences).

**Stress-induced mutagenesis assays:** Stress-induced lac reversion assays were performed as described (Harris et al. 1996) with four independent cultures of each strain. The proportion of Lac+ point mutants and lac-amplified colonies was determined by plating cells from 20 colonies of each culture for each day in which Lac+ colonies were counted (days 2 to 5) on LBH rifampicin X-gal plates. This allows the distinction between Lac+ point mutants (solid-blue colonies) and lac-amplified cells, given the lac-unstable sectoring-colony phenotype diagnostic of lac amplification (Hastings et al. 2000).

**Determination of the mutation sequences in the lac gene:** Lac+ point mutants from experiment day 5 were identified as described above and purified on LBH plates containing rifampicin and X-Gal. A 300-nucleotide region spanning the lac +1 allele was amplified by PCR using primers lacIL2 (5’ AGGCTATTCTGGTGCGGA 3’ and lacD2  (5’ GCCTCTTCGCTATTACGCCAGCT 3’). DNA sequencing was performed by Seqwright (Houston) using primer lacU (5’ ATATCCGCCTAGGCTTAACCACC 3’).

**RESULTS**

**Construction and characterization of the dinB(oε) alleles:** To test the hypothesis that dinB might be the sole SOS gene required at induced levels for stress-induced point mutagenesis, we constructed dinB mutants in which the transcriptional repression by LexA, the repressor
controlling the expression of the SOS regulon, is alleviated. This was achieved by site-directed mutagenesis of the dinB promoter, altering the binding site of the LexA repressor. These are used below to express dinB at SOS-induced levels in strains in which the rest of the SOS genes are repressed. The sequences of the operator-constitutive, dinB(o<sup>c</sup>), mutations constructed are shown in Figure 1A.

To test whether these mutations behave as bona fide operator-constitutive alleles, we fused the dinB promoter regions from the two dinB(o<sup>c</sup>) alleles to lacZ and measured the levels of beta-galactosidase expression from these P<sub>dinB</sub>lacZ fusions carried in a low copy plasmid (Figure 1B). Introduction of these plasmids into wild-type cells resulted in roughly 10-fold higher lacZ expression from both P<sub>dinB(o<sup>c</sup>)</sub>lacZ fusions, when compared with wild-type P<sub>dinB</sub>. This is in agreement with previous estimates of transcriptional induction of dinB during the SOS response (COURCELLE et al. 2001; KIM et al. 2001). lexA51(Def) cells have no functional LexA repressor, and show constitutive SOS expression (MOUNT 1977). We find that lacZ expression is increased in a lexA51(Def) strain, when driven by the wild-type dinB promoter, but see no significant increase with the dinB(o<sup>c</sup>) promoters, showing that levels of dinB transcription similar to that achieved by true SOS de-repression are achieved by the dinB(o<sup>c</sup>) mutations. The lexA51(Def) strain SMR5400 also carries a mutation in the sulA gene, which allows survival under constitutive SOS induction (MOUNT 1977), and a mutation in the F-encoded psiB gene, which has been shown to exert a negative effect on stress-induced mutagenesis (McKENZIE et al. 2000) probably by affecting SOS induction (reviewed by COX 2007).

In the Lac-assay strains such as FC40 and SMR4562, dinB is present both in the chromosome and in the F<sup>+</sup>128, at which locus it is more highly expressed (KIM et al. 2001). Introduction of both dinB(o<sup>c</sup>) alleles into the episomal dinB locus results in about 5-6-fold
increased DinB-protein levels in stationary-phase cells compared with an otherwise isogenic SMR4562 derivative, both in wild-type and \textit{lexA3}\textsuperscript{(Ind\textsuperscript{−})} backgrounds (Figure 2). This indicates that both \textit{dinB}\textsuperscript{(o\textsuperscript{c})} alleles are functional \textit{in vivo}, conferring an increased basal \textit{dinB} expression. Furthermore, both alleles confer levels of expression similar to those observed in \textit{lexA51}(Def) cells (Figure 2), at least in the growth conditions used by us in the stress-induced mutagenesis experiments (cells grown for 48h in M9 B1 glycerol minimal medium). It was noted before (Kim \textit{et al.} 2001) that expression of \textit{dinB} in the F'128 plasmid is higher than that from the chromosomal \textit{dinB}. Our finding that both \textit{dinB}\textsuperscript{(o\textsuperscript{c})} alleles, when present only in the episome, increase DinB to levels similar levels to that observed in the \textit{lexA51}(Def) strain (in which both the episomal and chromosomal copy are constitutively highly expressed), also implies that the episomal expression is more pronounced than chromosomal expression. To facilitate further strain construction and genetic analysis, we carried out the subsequent experiments in cells bearing a single \textit{dinB}\textsuperscript{(o\textsuperscript{c})} allele in the F’128 plasmid.

\textit{dinB}\textsuperscript{(o\textsuperscript{c})} mutations restore stress-induced point mutagenesis in “SOS-off” strains:

Because DinB is a key player in stress-induced mutagenesis, we wanted to examine whether \textit{dinB} is the only gene required at SOS-induced levels for stress-induced point mutagenesis in the Lac assay. The SOS response is induced when DNA damage is sensed in the form of single-stranded DNA (reviewed by Friedberg \textit{et al.} 2005). RecA binds the single-strand DNA and becomes active as a co-protease that facilitates cleavage of the LexA repressor, resulting in up-regulation of the SOS genes, including \textit{dinB}. To determine whether \textit{dinB} upregulation constitutes the sole role of the SOS response in stress-induced point mutagenesis, we tested the effect of the \textit{dinB}\textsuperscript{(o\textsuperscript{c})} alleles on \textit{lac} reversion, both in wild-type and \textit{lexA3}(Ind\textsuperscript{−}) backgrounds. The \textit{lexA3}(Ind\textsuperscript{−}) mutation creates an uncleavable LexA/SOS repressor such that de-repression of the SOS
response genes during an SOS response is prevented (Mount et al. 1972). Previously, this allele was shown to cause reduced stress-induced point mutagenesis in the Lac assay (Cairns and Foster 1991; McKenzie et al. 2000; McKenzie et al. 2001), indicating that one or more SOS-controlled genes are needed at induced levels for efficient stress-induced mutagenesis. Representative results from single experiments with each of the two dinB(oC) alleles constructed are shown in Figure 3A and Figure 3B, and quantification of the stress-induced point mutagenesis rates from multiple experiments is shown in Figure 3C. Strikingly, either allele provides a complete suppression of the phenotype of the lexA3(Ind-) strain. These results show that the reduced stress-induced mutagenesis in a lexA3(Ind-) strain is caused specifically by the failure to up-regulate dinB, not any other gene in the LexA/SOS regulon. This finding places DinB as the central SOS-regulated protein in stress-induced mutagenesis, and indicates that up-regulation of other SOS genes, like recA, ruvA and ruvB beyond their constitutive levels of expression, is irrelevant.

SOS-induced levels of DinB are not sufficient to increase stress-induced point mutagenesis: We note that providing SOS-induced levels of DinB to all cells, with the dinB(oC) mutations, did not stimulate stress-induced mutagenesis above wild-type levels in the lexA3(Ind-) strain (Figure 3 and legend), even though normally, SOS is expected to be induced spontaneously in only about 1% of cells (Pennington and Rosenberg 2007). Neither did DinB overproduction increase mutagenesis at all in the wild-type genetic background (Figure 3 and legend). These results indicate that DinB upregulation by the SOS response, though required, is not sufficient to differentiate the mutating subpopulation—dinB(oC) appears not to make all cells in the population mutable. This might reflect either of two possible realities. First, in principle, it could have been possible that during stress-induced mutagenesis conditions, all cells are SOS-
induced such that providing an operator-constitutive \( \text{dinB} \) does not provide any more \( \text{DinB} \) protein than the population of cells already has, and so does not increase mutagenesis further. This is unlikely (discussed below). Second, and more likely, it could be that during stress-induced mutagenesis only a small fraction of cells is SOS induced, as is the case for growing cells in which \( \sim10^{-2} \) are (Pennington and Rosenberg 2007), but that in this cell subpopulation some other condition must be met to allow mutagenesis. For example, it is likely that possession of a DNA double-strand break at which the mutagenic repair occurs (Ponder et al. 2005), is also required such that \( \text{DinB} \) up-regulation alone is not sufficient.

**SOS induction of other genes in the \( \text{dinB} \) operon is irrelevant for stress-induced mutagenesis:** \( \text{dinB} \) is part of a four gene operon including \( \text{dinB}, \text{yafN}, \text{yafO} \) and \( \text{yafP} \) (McKenzie et al. 2003). The functions of the three \( \text{yaf} \) genes are unknown. The whole operon, including the three genes downstream of \( \text{dinB} \), is induced as part of the SOS response (Courcelle et al. 2001). Thus, in the experiments described above, all three \( \text{yaf} \) genes were also upregulated by the operator-constitutive mutations in the \( \text{dinB} \) promoter. We show that the restoration of mutability to SOS-off \( \text{lexA(Ind)} \) cells conferred by the \( \text{dinB(o\text{c})} \) mutations was not conferred by increased \( \text{yafNOP} \) expression, only by increased \( \text{dinB} \) expression, because it also occurred in strains carrying a deletion of the \( \text{yafNOP} \) genes in \( \text{cis} \) with (downstream of) the \( \text{dinBo-21(o\text{c})} \) mutation in F'128 (Figure 4). Although intact \( \text{yaf} \) genes are present in the chromosome of this strain, they will be repressed by the \( \text{lexA(Ind)} \)-encoded uncleavable LexA/SOS repressor, such that only \( \text{DinB} \) is produced at SOS-induced levels in this strain. Therefore, \( \text{dinB} \) is indeed the only gene of the SOS regulon upregulation of which is necessary for stress-induced mutagenesis in the Lac assay. These experiments do not rule out a role for the \( \text{yaf} \) genes (expressed at uninduced levels) in mutagenesis, a topic that will be addressed in a
A similar mechanism of stress-induced lac reversion in \textit{dinB}(o^c) as wild-type cells:

The results obtained show that the \textit{dinB}(o^c) alleles are able to rescue completely the mutagenesis-defective phenotype of \textit{lexA3} (Ind^−) (SOS-off) cells in stress-induced mutagenesis (Figure 3). This could result from restoration of the same stress-induced mutagenesis pathway and mechanism that operates in wild-type cells. Alternatively, it was possible that constitutive expression of \textit{dinB} might activate a different mutagenesis mechanism that coincidentally yielded similar mutant frequencies in the course of several days. We provide two lines of support for the first possibility—that the normal pathway and mechanism of stress-induced point mutagenesis was restored to \textit{lexA3} (Ind^−) (SOS-off) cells by the \textit{dinB}(o^c) mutations.

First, we find that the Lac-reversion-mutation sequences in \textit{lexA3} (Ind^−) \textit{dinB}(o^c) cells are indistinguishable from the characteristic point-mutation sequences seen normally in stress-induced point mutagenesis (in \textit{lexA}^+ \textit{dinB}^+ cells) (Figure 5A). The mutations are dominated by -1 deletions at mononucleotide repeats that occur preferentially in the same preferred hotspot sequences as observed in \textit{lexA}^+ \textit{dinB}^+ cells. This characteristic mutation sequence spectrum is highly specific and different from, for example, spontaneous reversions of this \textit{lac} allele during growth, which are more heterogeneous, including -1 deletions not at mononucleotide repeats and larger frameshift-reverting additions and deletions in about half the mutations (Foster and Trimarchi 1994; Rosenberg et al. 1994). These results imply that mutations occur via a similar or the same mechanism in both genetic backgrounds, supporting the idea that the rescue of the \textit{lexA3} (Ind^−) phenotype by the \textit{dinB}(o^c) alleles restored the same mutagenesis mechanism that normally operates in \textit{lexA}^+ \textit{dinB}^+ cells.
Second, a hallmark of stress-induced mutagenesis in the Lac assay is its requirement for homologous-recombinational double-strand-break-repair functions including \textit{recA}, \textit{recB}, and \textit{ruvAB} and \textit{ruvC} (FOSTER \textit{et al.} 1996; HARRIS \textit{et al.} 1994; HARRIS \textit{et al.} 1996; HE \textit{et al.} 2006), because the mutagenesis results from error-prone double-strand-break-repair events (PONDER \textit{et al.} 2005). Similarly, we find that deletion of \textit{ruvC} reduces stress-induced mutagenesis in \textit{lexA3}(Ind-) (SOS-off) cells carrying a \textit{dinB(o\textsuperscript{c})} allele (Figure 5B). Thus the mutagenesis restored to \textit{lexA3}(Ind-) cells by the \textit{dinB(o\textsuperscript{c})} mutation requires \textit{ruvC}. This supports the conclusion that a similar or the same recombination-dependent-mutagenesis pathway is operating in \textit{lexA3}(Ind-) \textit{dinB(o\textsuperscript{c})} cells as is normal in cells wild-type for \textit{lexA} and \textit{dinB}.

\textbf{DISCUSSION}

\textbf{How stress responses confer temporal regulation of mutagenesis.} The coupling of mutagenesis programs to cellular stress responses, observed in bacterial and eukaryotic cells (reviewed in Introduction and by GALHARDO \textit{et al.} 2007) provides a temporal regulation of mutagenesis, limiting mutagenesis to times of stress. This may potentially accelerate genetic change, and thus the ability to evolve, specifically when cells/organisms are maladapted to their environments, \textit{i.e.}, are stressed. Here we demonstrate that, in the case of the \textit{E. coli} Lac assay, the requirement for the SOS stress response can be deconvoluted to the need for induction of one specific gene, \textit{dinB}. A number of other stress responses have been shown to upregulate mutagenesis, such as the RpoS response in \textit{E. coli}, Salmonella and Pseudomonas, the stringent-response in \textit{E. coli} and \textit{B. subtilis}, the competence response of \textit{B. subtilis}, and two human
responses to hypoxic stress (see Introduction). All of these modulate the expression of tens to
hundreds of different genes. It is not yet known whether any other of these stress responses can
be narrowed down to relevant effects on one or a few genes.

**Roles of SOS in other stress-induced-mutagenesis mechanisms.** The SOS response is
a major upregulator of mutagenesis during stress conditions but may not function identically in
each case. For example, the SOS response is required for phage lambda untargeted mutagenesis
(UTM) ([ICHIKAwa-Ryo and KONDO 1975]), stress-induced point mutagenesis in *E. coli* in the
Lac assay ([McKENZIE et al. 2000]), ciprofloxacin (antibiotic)-resistance mutagenesis induced by
exposure ciprofloxacin ([CIRZ et al. 2005]), mutagenesis in aging colonies in a laboratory *E. coli*
strain ([TADDeI et al. 1995]), and bile-resistance mutagenesis in Salmonella ([PRIETO et al. 2006]).
Although DinB is required for lambda untargeted mutagenesis ([KIM et al. 1997]), and Salmonella
bile-resistance mutation ([PRIETO et al. 2006]), it is not yet known whether the SOS requirement in
either case is based on DinB upregulation. In the ciprofloxacin-induced mutagenesis, the SOS-
controlled DNA polymerases DinB, Pol II and Pol V are all required, and also double-strand-
break-repair genes including SOS-regulated *recA*, *ruvA* and *ruvB* ([CIRZ et al. 2005]). Part of this
mutation pathway’s requirement for SOS is likely to be for production of DNA Pol V ([CIRZ et al.
2005]), because this polymerase is required for the mutagenesis, and is not produced at all
without an SOS response (reviewed by [NOHMI 2006]). It is not yet known whether upregulation
of any, all, or none of the other two SOS-controlled DNA polymerases additionally account for
the requirement for an SOS response for ciprofloxacin-induced mutagenesis. Conversely, in the
Lac assay, we measure frameshift reversion, which DinB promotes but Pols II and V do not,
whereas the ciprofloxacin-induced mutations are base substitutions, which all three SOS
polymerases promote ([CIRZ and ROMESBERG 2007]). Cirz and Romesberg ([CIRZ and ROMESBERG
2007) have pointed out that the mutagenesis pathway in the Lac system might be identical to that in ciprofloxacin-resistance mutagenesis and might also require Pols II and V if base substitution mutations were assayed. In a different assay system, mutagenesis in aging colonies required and SOS response, did not require DinB, and required Pol I instead (TADDEI et al. 1997). Therefore, in the mutagenesis mechanism operating during that stress, the SOS requirement must be for some other function. Thus, though the SOS response is required for multiple examples of stress-inducible mutagenesis, its means of promoting mutagenesis in at least some of these different stress circumstances is different.

**SOS induction of DinB is necessary but not sufficient for stress-induced mutability and for differentiation of a hypermutable cell subpopulation.** Several lines of evidence indicate that although SOS-induction of DinB is necessary, it is not sufficient for creating the transient mutable state in which most Lac reversions occur (GONZALES et al. 2008), and support a model in which at least three events must occur: (1) a double-strand-break and its repair; (2) SOS-induction; (3) induction of the RpoS stationary-phase/general stress-response. The concerted induction of these stress responses in cells bearing double-stranded DNA ends (DSEs) is proposed to differentiate the hypermutable subpopulation, as depicted in Figure 6A. It has been shown recently that DNA damage itself can induce the RpoS regulon (MERRIKH et al. 2009), so it could contribute at least part to the induction of this regulon in the hypermutable subpopulation cells.

The first evidence for this model comes from experiments in which DNA double-strand breaks were delivered to the DNA by expression of the I-SceI double-strand endonuclease in vivo (PONDER et al. 2005). DinB-dependent stress-induced mutagenesis was stimulated over 1000-fold near the DSBs, but only weakly (3-fold) in a another DNA molecule (with no DSB) in
the same cell. SOS is induced robustly by I-SceI-induced DSBs (PENNINGTON and ROSENBERG 2007). Therefore, these results indicate that the SOS-mediated DinB upregulation caused by I-SceI-mediated DNA cleavage, was not sufficient for mutagenesis. A DSB was also required locally. The mutations appear to occur in acts of error-prone DSB repair (PONDER et al. 2005). Figure 6B shows a model for such mutagenic double-strand-break repair occurring in stressed cells.

Second, RpoS is required for stress-induced Lac reversion (LAYTON and FOSTER 2003; LOMBARDO et al. 2004). Moreover, in the study using I-SceI-induced DSBs (PONDER et al. 2005), the DinB-dependent DSB-associated mutagenesis was provoked only in cells that were either in stationary phase or expressing the RpoS stationary-phase/general stress-response transcriptional activator inappropriately during log phase. Again, because the SOS response is induced efficiently by I-SceI-mediated double-strand breakage (PENNINGTON and ROSENBERG 2007), this implies that repair of a DSB under the influence of the SOS response is also not sufficient for DinB-dependent mutagenesis; RpoS must also be induced.

Finally, in growing cells, only about 1% of cells are SOS-induced spontaneously (PENNINGTON and ROSENBERG 2007), and in the present study we observed that making every cell in the population experience SOS-induced levels of DinB production, using the \( \text{dinB}(o^c) \) alleles, did not increase stress-induced mutagenesis in otherwise wild-type cells (Figure 3). This could mean either of two things: (1) that unlike growing cells, in stationary phase, all of the cells are already SOS induced, and so the \( \text{dinB}(o^c) \) alleles did not change the number of cells expressing DinB or the DinB levels in most cells. This possibility is unlikely given the large increase in DinB levels we observed with the \( \text{dinB}(o^c) \) alleles measured in stationary-phase cells (Figure 2). (2) More plausibly, the data imply that even though the \( \text{dinB}(o^c) \) alleles confer SOS-
induced levels of DinB to all cells, this is not sufficient for mutagenesis. These data support the model in which DNA double-strand breaks and their repair, an RpoS response, and an SOS response (now shown to act solely via DinB production) are all required for stress-induced point mutagenesis (Figure 6A and B). We note that if all cells had become hypermutable when DinB was overproduced in all cells, mutation rate would have been higher than normal because the hypermutable cells would no longer be a small subpopulation. This was not observed (Figures 3, 4).

**Role of SOS/DinB in a hypermutable cell subpopulation.** We previously suggested a model for the origin of the hypermutable cell subpopulation that appears to underlie most double-strand-break-repair-associated stress-induced point mutagenesis (GONZALES et al. 2008), based on the three requirements for stress-induced mutability discussed above: a genomic DSB/DSE (and its repair), an SOS response, and an RpoS response (GALHARDO et al. 2007). The simultaneous occurrence of these three events is proposed to differentiate the hypermutating cells. It is unknown what fraction of the cells in a stationary population experience an SOS response, but about 1% of the cells in log-phase cultures display spontaneous SOS induction, about 60% of those (~6 x 10^{-3}) due to a spontaneous DSB/DSE (PENNINGTON and ROSENBERG 2007). When these cells enter the stationary phase, RpoS is likely to be induced in all of them (HENGGE-ARONIS 2002). Thus, if the numbers for growing cells held, then the ~6 x 10^{-3} of cells with a DSB and an SOS response would become the HMS when RpoS induction occurred in the whole population in stationary phase (Figure 6A). We can now refine this model to note that the sole component of the SOS response required would be DinB upregulation.

The additional requirement for RpoS, to license the use of DinB in error-prone DSB/DSE repair (shown by PONDER et al. 2005), could either be based solely on RpoS upregulation of
DinB, or on RpoS-controlled expression of other factors that permit DinB use (Figure 6B). The SOS and RpoS responses increase DinB expression roughly 10-fold and 2- to 3-fold, respectively (KIM et al. 2001; LAYTON and FOSTER 2003). The identities of potential DinB-licensing factors in the RpoS regulon are not yet known. This control would provide a restriction of the mutagenesis to periods of stress, and only to those few cells with a DSB/DSE. The restriction of mutagenesis to a cell subpopulation may allow clonal populations to hedge their bets during adaptation to changing environments, both conserving the original genome sequence, which is well adapted to the previous environment and useful if resources become available again suddenly, while simultaneously exploring the new adaptive landscape in the subpopulation.

**Regulation of DinB mutator activity.** In many other assay systems in which DinB-dependent mutagenesis has been observed, stress responses other than or in addition to SOS are required. In Salmonella bile-induced resistance mutagenesis, which is DinB-dependent, the SOS and RpoS responses are required (J. Casadesus, personal communication and PRIETO et al. 2006). In *B. subtilis* starvation-associated mutagenesis, the ComK competence stress response is required for the DinB-dependent mutagenesis (SUNG and YASBIN 2002). In *E. coli*, beta-lactam antibiotics induce *dinB* transcription independently of SOS (PEREZ-CAPILLA et al. 2005). *P. putida* DinB-dependent stress-induced mutagenesis requires RpoS (SAUMAA et al. 2002). It is not known whether any stress response other than SOS is required for DinB-dependent ciprofloxacin-induced resistance mutagenesis (CIRZ et al. 2005). Thus, it is plausible that DinB-dependent mutagenesis might usually require more than one stress-response input to occur. Although effects of DinB in SOS mutagenesis of *E. coli* (KUBAN et al. 2006) have been observed, it is not known whether the DinB-dependent mutations may have arisen in cells also induced for RpoS or another stress response simultaneously.
What factors modulate DinB mutator activity? As a translesion DNA polymerase that inserts bases opposite several otherwise-replication-blocking DNA adducts, DinB performs this role mostly in a high-fidelity fashion (BJEDOV et al. 2007; GODOY et al. 2007; JAROSZ et al. 2006). This, and the existence of dinB mutations that separate translesion from mutagenic functions (GODOY et al. 2007), imply that mutator activity occurs during synthesis that is not part of translesion synthesis (though during DSB-repair, PONDER et al. 2005). Alternatively, DinB mutator activity could be taking place at sites of yet unidentified DNA lesions.

A recent study implicated the SOS-induced UmuD protein as a candidate to inhibit DinB mutagenic potential during SOS induction (GODOY et al. 2007). UmuD is produced only during an SOS response (COURCELLE et al. 2001). Consequently, no UmuD is expected to be present in lexA(Ind−) dinB(o−) cells, but stress-induced mutation rates were similar to those in wild-type cells (Figures 3 and 4). Therefore, UmuD appears not to inhibit DinB in its role in stress-induced point mutagenesis. Other levels of control are likely to exist.

One case of strongly DinB-dependent mutagenesis thought to occur with only one stress-response input in physiological conditions is SOS-mediated untargeted mutagenesis of phage λ (KIM et al. 1997). In those conditions, mutagenesis of the phage DNA is heavily dependent on DinB, presumably relying on the physiological levels of dinB expression achieved in vivo during SOS induction. It is interesting to note that extensive double-strand-end-initiated recombination between the multiple copies of the phage DNA is expected to occur during a lytic cycle (THALER and STAHL 1988). Those might be the sites of mutagenic action of DinB. Nevertheless, it is not known whether other factors, lambda- or host-encoded, might also play a role in that process.

Another example is the recent finding of increased DinB-dependent mutagenesis in cells lacking the ClpXP protease (AL MAMUN and HUMAYUN 2009). RpoS does not seem to be involved in the
observed mutagenesis. However, the lack of a major protease is likely to have many pleiotropic effects, and there remains the possibility that other responses are triggered in those cells, which may foster DinB activity.

**Evolution of stress-induced mutagenesis pathways.** The occurrence of many different molecular mechanisms of stress-inducible mutagenesis [reviewed in the Introduction, here, and by (Galhardo et al. 2007)], and even of mechanisms by which a single stress response such as SOS promotes mutagenesis in different stress circumstances suggests that these mutagenesis programs have evolved many times at least somewhat independently. This is also suggested by a survey of starvation-stress-inducible mutability in 787 E. coli natural isolates (Bjedov et al. 2003). Those authors found that whereas more than 80% of the natural isolates displayed stress-inducible mutator activity, the ability to do so was correlated well with ecological niche and poorly with strain phylogeny, suggesting multiple recent evolutions of the stress-inducible mutagenesis pathways. We have suggested that stress-inducible mutagenesis mechanisms are both somewhat varied and recently acquired because they confer a benefit to cells that is under periodic (alternating positive-then-negative) second-order selection (Galhardo et al. 2007). That is, these pathways are useful and selected in changing environments in which adaptation is promoted by mutability and responsiveness, and superfluous and perhaps costly in static environments. Despite the variability and potential multiple origins, the basic themes of regulation of mutability temporally by stress responses, and potentially spatially in genomes (Galhardo et al. 2007) are widespread and appear to be potentially important evolutionary strategies.
We thank Kenneth Hu for comments on the manuscript. This work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (18201010) (TN), and U.S. National Institutes of Health Grants R01-GM-64022 (PJH), and R01-GM53158 (SMR). RSG was supported in part by a postdoctoral fellowship from the Pew Latin American Fellows Program.

LITERATURE CITED


COURCELLE, J., A. KHODURSKY, B. PETER, P. O. BROWN and P. C. HANAWALT, 2001 Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient Escherichia coli. Genetics 158: 41-64.


**TABLE 1**

**Bacterial strains used in this study**

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<th>Name</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
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<td>FC40</td>
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<td>(CAIRNS and FOSTER 1991)</td>
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<tr>
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<td>FC40 lexA3(Ind&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>(CAIRNS and FOSTER 1991)</td>
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<td>SMR868</td>
<td>FC40 lexA3(Ind&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>(MCKENZIE et al. 2000)</td>
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<td>Identical to FC40, independent construction</td>
<td>(MCKENZIE et al. 2000)</td>
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<td>SMR5400</td>
<td>SMR4562 sulA211 lexA51(Def) ΔpsiB::cat</td>
<td>(MCKENZIE et al. 2000)</td>
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<td>SMR9436</td>
<td>SMR4562 ΔruvC::FRTKanFRT</td>
<td>(MAGNER et al. 2007)</td>
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<td>(MCKENZIE et al. 2001)</td>
</tr>
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dinBo-22(o^c)]

SMR10308 SMR4562 [F’ lafU2::FRTcatFRT dinBo-21(o^c)]

SMR10309 SMR4562 [F’ lafU2::FRTcatFRT dinBo-22(o^c)]

SMR10310 SMR868 [F’ lafU2::FRTcatFRT dinBo-21(o^c)]

SMR10311 SMR868 [F’ lafU2::FRTcatFRT dinBo-22(o^c)]

SMR10314 SMR868 [F’ lafU2::FRTcatFRT]

SMR10760 FC231 [F’ lafU2::FRTcatFRT]

SMR10761 FC231 [F’ lafU2::FRTcatFRT dinBo-21(o^c)]

SMR10762 FC231 [F’ lafU2::FRTcatFRT dinBo-22(o^c)]

SMR10766 SMR4562 ΔruvC::FRTKanFRT [F’ lafU2::FRTcatFRT]

SMR10767 FC231 ΔruvC::FRTKanFRT [F’ lafU2::FRTcatFRT dinBo-21(o^c)]

SMR10768 FC231 ΔruvC::FRTKanFRT [F’ lafU2::FRTcatFRT]

SMR10838 SMR4562 [pPdinB]

SMR10839 SMR4562 [pPdinBOC1]

SMR10840 SMR4562 [pPdinBOC2]

This study

SMR4562 X P1(SMR10304)

SMR4562 X P1(SMR10306)

SMR868 X P1(SMR10304)

SMR868 X P1(SMR10306)

SMR868 X P1(SMR10292)

FC231 X P1(SMR10292)

FC231 X P1(SMR10304)

FC231 X P1(SMR10306)

SMR10292 X P1(SMR9436)

SMR10761 X P1(SMR9436)

SMR10760 X P1(SMR9436)

This study

This study

This study
SMR10841  SMR5400 [pPdinB]  This study
SMR10842  SMR5400 [pPdinBOC1]  This study
SMR10843  SMR5400 [pPdinBOC2]  This study
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SMR11024  SMR4562 [F’  lafU2::FRTcatFRT  ΔyafNOP::FRTKanFRT  dinBo-21(o^c))]  This study
SMR11026  FC231 [F’  lafU2::FRTcatFRT  ΔyafNOP::FRTKanFRT]  FC231 X P1(SMR11023)
SMR11027  FC231 [F’  lafU2::FRTcatFRT  ΔyafNOP::FRTKanFRT  dinBo-21(o^c))]  FC231 X P1(SMR11024)
**TABLE 2**

**Plasmids used in this study**

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<th>Description and source</th>
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<td>pFZY</td>
<td>Low-copy plasmid with multicloning site abutting a promoterless lacZ (KOOP <em>et al.</em> 1987)</td>
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<td>pPdinB</td>
<td>bases -432 to -2 of <em>dinB</em> from strain SMR4562 cloned into pFZY, producing a ( P_{dinB}lacZ ) fusion</td>
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<td>pPdinBOC1</td>
<td>bases -432 to -2 of <em>dinB</em> from strain SMR10308 cloned into pFZY, producing a ( P_{dinBo-21(o)}lacZ ) fusion</td>
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<td>pPdinBOC2</td>
<td>bases -432 to -2 of <em>dinB</em> from strain SMR10309 cloned into pFZY, producing a ( P_{dinBo-22(o)}lacZ ) fusion</td>
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FIGURE LEGENDS

FIGURE 1.—Construction and characterization of two dinB(o<sup>c</sup>) alleles. (A) Location of
the operator-constitutive mutations in the dinB promoter. The SOS operator (from FERNANDEZ
DE HENESTROSA et al. 2000) is shown in gray shading, and the mutations introduced in each of
the alleles are marked in bold and Italics. The beginning of the dinB ORF is shown in bold. (B)
Activity of the dinB promoter in transcriptional fusions with lacZ, measured in both wild-type
(SMR4562) and its LexA-defective (null), lexA51(Def), derivative strain SMR5400, in which
SOS is constitutively highly induced. The strains from left to right are SMR10838, SMR10839,
SMR10840, SMR10841, SMR10842 and SMR10843. P<sub>dinB</sub> indicates the wild-type dinB
promoter present in plasmid pPdinB, PO<sub>o<sup>c1</sup></sub> indicates the dinB<sub>o<sup>c1</sup></sub>(o<sup>c</sup>) promoter contained in
plasmid pPdinBOC1, and PO<sub>o<sup>c2</sup></sub> indicates the dinB<sub>o<sup>c2</sup></sub>(o<sup>c</sup>) promoter contained in plasmid
pPdinBOC2. Mean ± one standard error of the mean (SEM) for 3 independent determinations.

FIGURE 2.—DinB Western blots. Stationary-phase cells grown in M9 B1 glycerol
medium were harvested and analyzed using a rabbit polyclonal DinB antiserum as described
(MATERIAL AND METHODS). Values shown represent the average DinB protein levels
relative to wild-type determined in three independent experiments ± SEM. Similar results were
obtained with Western blots performed with a DinB monoclonal antibody. Strains are: dinB,
SMR5889; dinB<sup>+</sup>, SMR10292; dinBo-21(o<sup>c</sup>), SMR10308; dinBo-22(o<sup>c</sup>), SMR10309; lexA3(Ind<sup>+</sup>), SMR10760; lexA3(Ind<sup>−</sup>) dinBo-21(o<sup>c</sup>), SMR10761; lexA3(Ind<sup>−</sup>) dinBo-22(o<sup>c</sup>), SMR10762;
lexA(Def), SMR5400. 

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FIGURE 3.—Two *dinB* operator-constitutive alleles restore stress-induced Lac point mutagenesis to SOS-off *lexA*(Ind′) cells. (A) Effect of the *dinBo-21(o^c)* allele in stress-induced mutagenesis, representative experiment. (B) Effect of the *dinBo-22(o^c)* allele in stress-induced mutagenesis, representative experiment. Note that for both alleles, the stress-induced point mutagenesis-defective phenotype of *lexA3*(Ind′) cells is fully suppressed, however overproduction of DinB with these alleles does not stimulate mutagenesis above wild-type levels. Data represent means ± SEM for 4 cultures. Strains are: *dinB^+ lexA^+*, SMR10292 (closed diamonds); *lexA*(Ind′), SMR10760 (open circles); *dinBo-21(o^c)*, SMR10308 (closed circles in panel A); *dinBo-22(o^c)*, SMR10309 (closed circles in panel B); *lexA*(Ind′) *dinBo-21(o^c)*, SMR10761 (open squares in panel A); *lexA*(Ind′) *dinBo-22(o^c)*, SMR10672 (open squares in panel B). (C) Quantification of stress-induced point-mutation rates from six independent experiments each with all genotypes done in parallel. Strains were as above except that three experiments were performed in the *lexA3*(Ind′) strains listed above, whereas an additional three experiments were performed, with similar results, in an independently constructed, identical set of *lexA3*(Ind′) strains: *lexA*(Ind′), SMR10314; *lexA*(Ind′) *dinBo-21(o^c)* SMR10310; *lexA*(Ind′) *dinBo-22(o^c)*, SMR10311. Rates represent the increase of Lac^+ point mutant colonies per day observed between days 3 and 5 of each experiment. Means ± one SEM are shown. *P*-values were obtained for pair-wise comparisons by non-parametric Mann–Whitney Rank Sum test using the SYSTAT 11 statistics software by SYSTAT Software, Inc, and are as follows. The mutation rate of *dinB^+* is not different from those of *dinBo-21(o^c)* (*P* = 0.699), *dinBo-22(o^c)* (*P* = 0.699), *lexA*(Ind′) *dinBo-21(o^c)* (*P* = 0.818), or *lexA*(Ind′) *dinBo-22(o^c)* (*P* = 0.18), but is significantly different from the rate of *lexA*(Ind′) (*P* = 0.002), and the *lexA*(Ind′) rate differs from those of *lexA*(Ind′) *dinBo-21(o^c)* (*P* = 0.002) and *lexA*(Ind′) *dinBo-22(o^c)* (*P* = 0.002).
FIGURE 4.—Stress-induced-mutagenesis proficiency in SOS-“off”lexA3(Ind-) cells with a dinB(oc) mutation does not require the yafNOP genes. (A) Representative experiment showing that deletion of the yafNOP genes in cis with dinBo-21(oc) does not affect the ability of this promoter mutation to rescue the phenotype of SOS-“off”lexA3(Ind-) cells. (B) Quantification of stress-induced point-mutation rates (calculated as in Figure 3) from four independent experiments. Means ± SEM are shown. P-values (calculated as in Figure 3) are as follows. For the yaf+ background, the dinB+ rate is not different from the rates observed with dinBo-21(oc) (P = 0.886) or lexA(Ind-) dinBo-21(oc) (P = 0.686) but differs from that of lexA(Ind-) (P = 0.029), and the rate of lexA(Ind-) differs from that of lexA(Ind-) dinBo-21(oc) (P = 0.029). Similarly for the yaf- background, the dinB+ rate is not significantly different from the rates of dinBo-21(oc) (P= 0.114) or lexA(Ind-) dinBo-21(oc) (P= 0.886), but differs from the lexA(Ind-) rate (P= 0.029), and the lexA(Ind-) rate differs from that of lexA(Ind-) dinBo-21(oc) (P = 0.029). Strains are dinB+, SMR10292; lexA(Ind_), SMR10760; dinBo-21(oc), SMR10308; lexA(Ind_) dinBo-21(oc), SMR10761; yaf dinB+ lexA+, SMR11023 (closed diamonds); yaf dinBo-21(oc), SMR11024 (open triangles); yaf lexA(Ind_), SMR11026 (open circles); yaf lexA(Ind_) dinBo-21(oc), SMR11027 (closed squares).

FIGURE 5.—Stress-induced mutagenesis in SOS-“off”lexA3(Ind-) cells with dinB(oc) alleles occurs via a similar mechanism to normal stress-induced mutagenesis in the Lac assay. (A) Sequences of Lac+ mutations are the same in lexA3(Ind_) dinB(oc) strains and lexA+ dinB+ cells. Both sets of reversion mutations are nearly all -1 deletions in mononucleotide repeats. The positions of the -1 deletions observed in the lexA3(Ind_) dinBo-21(oc) and lexA3(Ind_) dinBo-
22(o\(^5\)) strains (SMR10761 and SMR10762) are shown as circles, and the position of the -1 deletions observed in *lexA*\(^+\) *dinB*\(^+\) cells [data from (FOSTER and TRIMARCHI 1994; ROSENBERG *et al.* 1994)] are marked as Xs. The region shown is part of the *lacIZ* fusion gene present in these strains. Compensatory frameshift mutations in a 130 nt region between the two out-of-frame stop codons (boxed) can restore gene function. (B) Stress-induced mutagenesis promoted by the *dinBo-21*(o\(^5\)) allele in the *lexA*3(Ind\(^-\)) background requires RuvC. A representative experiment performed with four independent cultures of each strain is shown. Means ± SEM. This result was repeated twice. Strains are: *dinB*\(^+\) *lex*\(^+\), SMR10292 (open diamonds); *dinBo-21*(o\(^5\)), SMR10308 (closed squares); *lexA*(Ind\(^-\)) *dinBo-21*(o\(^5\)), SMR10761 (closed triangles); *lexA*(Ind\(^-\)), SMR10760 (open circles); *ruvC*, SMR10766 (open triangles); *lexA*(Ind\(^-\)) *ruvC*, SMR10768 (open squares); *lexA*(Ind\(^-\)) *ruvC dinBo-21*(o\(^5\)), SMR10767 (closed diamonds).

**FIGURE 6:** Models for the role of SOS induction and DinB in differentiation of a hypermutable cell subpopulation and double-strand-break-repair-associated mutagenesis during stress. (A) Venn-diagram model of cell subpopulations that overlap to produce a transiently hypermutable cell subpopulation (HMS). In this model (modified from GALHARDO *et al.* 2007), differentiation of a HMS (green) is proposed to occur when three conditions are met: (1) induction of the SOS response in cells bearing (2) a double-strand break or double-strand DNA end (DSE) (blue) and (3) induction of the RpoS regulon (yellow) by suboptimal growth conditions. During stress, all cells induce RpoS and, we suggest, the fraction of cells bearing a DSE and SOS induction [~6 x 10\(^{-3}\) of growing cells (PENNINGTON and ROSENBERG 2007)] might remain roughly constant. In this model, the *dinB*(o\(^5\)) mutations, which upregulate DinB in all cells, would not increase HMS size because the DSEs are not available in all cells. (B)
Mutagenic double-strand-break repair during stress. PONDER et al. (2005) showed that repair of a double-strand break is a high-fidelity, non-mutagenic process in unstressed cells (left half) but switches to a mutagenic mode during stress under the control of the RpoS general stress response (right side). This process requires a double-strand end (DSE) and its repair; induction of the SOS response (MCKENZIE et al. 2000), which we show here is solely to provide DinB up-regulation, and induction of RpoS regulon (PONDER et al. 2005). Some as yet unknown function regulated by the RpoS response licenses the use of DinB in those conditions. This function could be the documented increase in DinB expression (LAYTON and FOSTER 2003) or the induction of another regulatory factor, or a combination of both. ssDNA, single-strand DNA; HR, homologous recombination; X’s, DNA polymerase errors/mutations; parallel lines, double-stranded DNA; dashed lines, newly synthesized DNA strands.
Figure 1

A

wild-type dinB promoter
TGAAATCACTGTATACTTTACCAGTGTTGAGAGGTGAGCAATGC\text{GTA}

dinBo-21(oc)
TGAAATCAG\text{G}TGTTGAGAGGTGAGCAATGC\text{GTA}

dinBo-22(oc)
TGAAATCACTGTATACTTTACC\text{CT}TGTTGAGAGGTGAGCAATGC\text{GTA}

B

\begin{center}
\begin{tabular}{c c c c}
\hline
 & \(P_{dinB}\) & \(PO^{c1}\) & \(PO^{c2}\) & \(P_{dinB}\) & \(PO^{c1}\) & \(PO^{c2}\) \\
\hline
\(lexA^+\) & \\
\(lexA\text{(Def)}\) & \\
\hline
\end{tabular}
\end{center}
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<td>dinB</td>
<td>dinB (1)</td>
<td>dinBo-21 (o)</td>
<td>(5.7 ± 0.5)</td>
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<tr>
<td></td>
<td></td>
<td>dinBo-22 (o)</td>
<td>(5.2 ± 0.5)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>lexAnd (1.4 ± 0.1)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>lexAnd: dinBo-21 (o)</td>
<td>(5.6 ± 0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>lexAnd: dinBo-22 (o)</td>
<td>(5.4 ± 0.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>lexADef (4.8 ± 0.3)</td>
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Figure 2
Figure 3

(A) Graph showing the growth of Lac+ point-mutant colonies per $10^8$ cells over 5 days for different genotypes: $\text{dinBo-21(c)}$, $\text{dinB}^+\text{lexA}^+$, $\text{lexAln}^+\text{dinBo-21(c)}$, and $\text{lexAln}^+$.

(B) Graph showing the growth of Lac+ point-mutant colonies per $10^8$ cells over 5 days for different genotypes: $\text{lexAln}^+\text{dinBo-22(c)}$, $\text{dinB}^+\text{lexA}^+$, $\text{dinB}^+\text{lexA}^+$, and $\text{lexAln}^+$.

(C) Bar graph comparing the rate of Lac+ point-mutant colonies per $10^8$ cells/day for different genotypes: $\text{dinB}^+$, $\text{dinBo-21(c)}$, $\text{dinBo-22(c)}$, $\text{lexAln}$, $\text{lexAln}^+\text{dinBo-21(c)}$, $\text{lexAln}^+\text{dinBo-22(c)}$.
Figure 4

A

![Graph showing the number of Lac^+ point-mutant colonies over days for different genotypes. The genotypes include yaf lexAInd^+ dinBo-21(o^c), yaf din^+ lex^+, yaf dinBo-21(o^c), and yaf lexAInd^-.](image)

B

![Bar graph showing the number of Lac^+ point-mutant colonies per 10^8 cells per day for different genotypes. The genotypes include dinB^+, lex^+, lexAInd^+, dinBo-21(o^c), lexAInd^-, and lexAInd^-.](image)
Figure 5

A

-1 Stop

cgctgaaGGGcaatcagctttgCCCCgtctcactgtgaa

agAAAAACCaccctGGcgCCCaatacgcaaaaccgcctctcccc

gcgctttGGCGattcattaatgcagctggacgacaGGTTT

+1 Stop

cccgaacatcgcccttgacgcacat

• mutations generated in the lexAlnd- dinB+ strains

x mutations generated in the dinB+ strain

B

![Graph showing Lac+ point-mutant colonies/10^8 cells over time.](image)

- lexAlnd- dinBo-21(o^c)
- lexAlnd- dinBo-21(o^c)
- dinBo-21(o^c)
- dinB+ lexA+
- ruvC; lexAlnd- ruvC;
- lexAlnd- ruvC dinBo-21(o^c)
Stress triggers RpoS induction in all cells

Figure 6

**A**

Optimal growth

- RpoS-induced (unknown fraction)
- SOS-induced (1% of cells; 60% of that have a DSE)

**B**

ssDNA break

- Replication fork collapse
- DSE
- SOS induction
- DSE Repair by HR

Optimal Growth

High-fidelity repair synthesis

Stress

- Increased dinB expression and/or licensing use of DinB in DSB repair

RpoS

Stress-induced point mutations

**Hypermutable subpopulation**

= Cells with DSE and SOS- and RpoS-induction

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[Diagram A: Optimal growth with arrows to stress leading to RpoS induction.]

[Diagram B: Diagram showing ssDNA break, replication fork collapse, DSE, SOS induction, DSE repair by HR, and the effects of stress and RpoS induction on point mutations.]