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Selection-Enhanced Mutagenesis of *lac* Genes Is Due to Their Coamplification with *dinB* Encoding an Error-Prone DNA Polymerase

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ABSTRACT To test whether growth limitation induces mutations, Cairns and Foster constructed an *Escherichia coli* strain whose mutant *lac* allele provides 1–2% of normal ability to use lactose. This strain cannot grow on lactose, but produces ~50 Lac$^+$ revertant colonies per 10^8 plated cells over 5 days. About 80% of revertants carry a stable *lac*$^+$ mutation made by the error-prone DinB polymerase, which may be induced during growth limitation; 10% of Lac$^+$ revertants are stable but form without DinB; and the remaining 10% grow by amplifying their mutant *lac* allele and are unstably Lac$^+$. Induced DinB mutagenesis has been explained in two ways: (1) upregulation of *dinB* expression in nongrowing cells ("stress-induced mutagenesis") or (2) selected local overreplication of the *lac* and *dinB*$^+$ genes on lactose medium (selected amplification) in cells that are not dividing. Transcription of dinB is necessary but not sufficient for mutagenesis. Evidence is presented that DinB enhances reversion only when encoded somewhere on the F′*lac* plasmid that carries the mutant *lac* gene. A new model will propose that rare preexisting cells (1 in a 1000) have ~10 copies of the F′*lac* plasmid, providing them with enough energy to divide, mate, and overreplicate their F′*lac* plasmid under selective conditions. In these clones, repeated replication of F′*lac* in nondividing cells directs opportunities for *lac* reversion and increases the copy number of the *dinB*$^+$ gene. Amplification of *dinB*$^+$ increases the error rate of replication and increases the number of *lac*$^+$ revertants. Thus, reversion is enhanced in nondividing cells not by stress-induced mutagenesis, but by selected coamplification of the *dinB* and *lac* genes, both of which happen to lie on the F′*lac* plasmid.

KEYWORDS *dinB*; *Escherichia coli*; adaptive mutation; copy number variant; error-prone polymerase; gene amplification; lactose operon; local overreplication; mutagenesis; plasmid

IN 1943, Luria and Delbrück described evidence that strong selection detects bacterial mutants that arise prior to selection and cannot be stress-induced (Luria and Delbrück 1943). Others came to the same conclusion using similar stringent selection methods (Newcombe 1949; Lederberg and Lederberg 1952). These experiments showed clearly that the detected mutants were not stress-induced. However, the stringent selection conditions could only detect mutations that formed several generations before selection. These selections could not have detected stress-induced mutations. Thus, the question was left open, "Would less stringent conditions reveal mutations that are induced by growth limitation?"

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To address this question, Cairns and Foster constructed an *Escherichia coli* strain with a leaky *lac* mutation that just barely prevents growth on lactose. Any new mutation that increases *lac* function could allow immediate growth on lactose (Cairns *et al.* 1988; Cairns and Foster 1991). Using this selection, parallel cultures failed to show a fluctuation in revertant number. This result was taken as evidence that weak selection (unlike previous strong selections) could detect mutations that arise during selection. If any mutations form after selection is imposed, then it is possible that they are stress-induced (Cairns and Foster 1991).

The absence of fluctuation in revertant number was initially the strongest support for stress-induced mutagenesis. This finding argued against selection models, which propose that revertants are initiated by preexisting cells and might be expected to show fluctuation. This support for stress-induced mutagenesis disappeared once it was found that revertants are initiated by preexisting cells with an increased lac copy number. These cells form reversibly either by tandem duplication of *lac*

or by increases in copy number of the whole F'*lac* plasmid. In either case, the frequency of the copy variants is held at a high steady-state frequency by a balance between rates of copy increase and loss (Reams *et al.* 2010). The influences (rates of copy number increase and loss) that maintain this high steady state oppose the Luria–Delbruck fluctuation. That is, revertants in the Cairns–Foster system show no fluctuation, not because revertants form on the plate but because the preexisting initiator cells are immune to fluctuation. Later evidence demonstrated in a different way that Lac^+ revertants are initiated by cells that form prior to plating, even though these cells show no Luria–Delbruck fluctuation (Sano *et al.* 2014).

The behavior of the Cairns–Foster system has been widely interpreted as evidence that all cells possess evolved mechanisms that sense growth limitation and respond by increasing their general mutation rate (Torkelson *et al.* 1997), or might even direct mutations preferentially to sites that will improve growth (Foster and Cairns 1992). The error-prone DNA repair polymerase DinB has been proposed to be central to this mutagenic mechanism. The idea of stress-induced mutagenesis suggested by the Cairns–Foster system has been expanded to explain origins of cancer (Cairns 1998) and evolution without natural selection (Rosenberg 2001; Mittelman 2013). However, this idea seems unlikely in that it proposes an evolved mechanism for promotion of malignancy.

An alternative to stress-induced mutagenesis is natural selection, acting in subtle ways on preexisting variants. The Cairns–Foster system need not involve any evolved mutagenic mechanism. Instead, this system may include peculiarities that allow natural selection to operate on common preexisting variants and favor their conversion to revertants at a rate that gives the appearance of mutagenesis. Selection models propose that revertants are initiated by preexisting cells with multiple *lac* copies that arise during nonselective growth prior to plating. These cells are able to replicate their *lac* genes more than they replicate their chromosomes (Sano *et al.* 2014; Maisnier-Patin and Roth 2015, 2016; S. Maisnier-Patin and J. R. Roth, personal communication).

An early selection model proposed that plated cells with a tandem *lac* duplication can grow slowly on lactose and improve their growth by further amplifying their array of *lac* genes under selection (Hendrickson *et al.* 2002; Roth and Andersson 2004). General mutagenesis can occur in cells whose *lac* amplification includes the $dinB^+$ gene. The DinB protein (Pol IV) is an error-prone repair polymerase that can copy damaged templates and makes frequent errors when copying normal base sequences (Wagner *et al.* 1999; Ohmori *et al.* 2001). The gene for DinB just happens to be located 16 kb from *lac* on the F'*lac* plasmid (Kofoid *et al.* 2003). A newer model for the Cairns system (Maisnier-Patin and Roth 2016; S. Maisnier-Patin and J. R. Roth, personal communication) suggests that mutagenesis under selection requires increasing the copy number of the entire F'*lac* plasmid, which includes both the *lac* and *dinB* genes. In this model, preexisting initiator cells have a 10-fold increased *lac* copy number. These cells form during nonselective pregrowth and are held at a high steady-state frequency ($\sim$1 in a 1000) dictated by their rates of copy number increase and decrease (Reams *et al.* 2010; Sano *et al.* 2014). After plating, selection enhances reversion by allowing initiator cells to replicate their F'*lac* plasmid more than their chromosome, which provides more opportunities for reversion. Selection enables mutagenesis because the $dinB^+$ copy number increases while the *lac* target is replicated repeatedly. By selective overreplicating, F'*lac* (in excess of the chromosome) selection circumvents two major problems with stress-induced mutagenesis. One problem is theoretical, the other technical.

The theoretical problem with stress-induced general mutagenesis is that many, perhaps most, natural conditions that limit growth pose problems that cannot be solved by mutations (*e.g.*, lack of nutrients). In such cases, a mechanism for stress-induced mutagenesis would impose potentially disastrous costs with no hope of benefit. In the face of such costs, selection would favor destruction of the mechanism. This makes the idea of dedicated mechanisms for stress-induced mutagenesis seem unlikely. It is particularly difficult to understand how a dedicated mutagenesis mechanism could explain the origins of cancer. This idea requires an evolved mechanism that senses limitation of somatic cell growth and responds by causing uncontrolled cell division (cancer).

The technical problem with stress-induced mutagenesis is that revertants are initiated by highly reversible copy number variants, which do not show a Luria–Delbrück fluctuation test. Thus, the strongest support for stress-induced mutagenesis (absence of fluctuation) is eliminated by a technicality. The influences that maintain the steady-state plasmid copy number and the frequency of initiator cells serves to obscure fluctuation. The number of initiator cells does not change appreciably from one culture to the next, even though the frequency of those initiator cells is established before plating (Sano *et al.* 2014).

There are three models for reversion in the Cairns–Foster system which predict different effects of $dinB^+$ gene position on reversion under selection. The first is stress-induced mutagenesis in nongrowing cells. According to this original model, limitation of growth induces the SOS DNA-damage response and the stationary phase σ factor RpoS, which increase the transcription level of the *dinB* gene 10-fold and two- to threefold, respectively (Kim *et al.* 2001; McKenzie *et al.* 2000, 2001; Layton and Foster 2003; Lombardo *et al.* 2004; Galhardo *et al.* 2009). This model rejects early suggestions that plasmid transfer is critical for reversion (Galitski and Roth 1995; Radicella *et al.* 1995). Instead, it proposes that plasmid conjugation (plasmid transfer) functions make a single-strand nick at the plasmid transfer origin *oriT* (Foster and Trimarchi 1995a). This nick leads to a double-strand break whose repair is accomplished by the RecA-RecBCD pathway working with the DinB polymerase and causing mutagenesis (Ponder *et al.* 2005; He *et al.* 2006). Although DinB is known to be mutagenic and stress has been shown to increase DinB production (Courcelle *et al.* 2001; Kim *et al.* 2001; Layton and Foster 2003; Galhardo *et al.* 2009), this increase does not seem to be sufficient to explain *lac* reversion. The genomic position of the $dinB^+$ gene

influences the level of mutagenesis as described here. In the selection models below, the stress-induced upregulation of *dinB* provides a level of expression that is essential but not sufficient for mutagenesis. Mutagenesis is achieved by increasing the copy number of a properly positioned *dinB*$^+$ gene whose expression is upregulated by mainly the SOS response.

The second model is selected tandem amplification of *lac* during growth under selection. In the first selection model, the *lac* region duplicates during nonselective growth prior to plating on lactose (Andersson *et al.* 1998; Hendrickson *et al.* 2002). After being plated, rare duplication-bearing cells grow slowly and improve their growth by expanding the tandem *lac* amplification. Cells whose *lac* duplication includes the nearby *dinB*$^+$ gene are expected to show an increased general mutation rate and produce stable revertants. By this model, all stable revertants arise from precursor cells with a selected tandem (*lac dinB*$^+$) amplification. This amplification is lost from cells that acquire a revertant *lac*$^+$ allele. In this model, a full yield of stable revertants requires a *dinB*$^+$ allele located close to *lac* on the F'*lac* plasmid.

The third model is when selected amplification of the entire F'*lac* plasmid overreplicates *lac* and *dinB*$^+$ with little cell division. In our current model for the Cairns–Foster system, selection favors increases in the copy number of the whole F'*lac* plasmid that coamplify *lac* and *dinB* (Maisnier-Patin and Roth 2015, 2016). In this model, upregulation of *dinB* gene transcription is necessary but not sufficient for mutagenesis under selection. Reversion requires that the expressed *dinB* gene be located anywhere on the F'*lac* plasmid, so it can be coamplified with *lac*. The *dinB*$^+$ gene does not need to be near *lac* within this plasmid. The chromosomal *dinB* gene does not contribute to reversion in either selection model. A detailed description of this model will be presented elsewhere (S. Maisnier-Patin and J. R. Roth, personal communication)

To decide among these three models, we moved the *dinB*$^+$ gene from its normal position (16-kb away from *lac* on the F'*lac* plasmid) to two other sites on that plasmid. One site is immediately adjacent to *lac* (180-bp away) and the other replaces *yebB* near the transfer origin *oriT*, diametrically opposite *lac* (115-kb away). We also inserted an additional *dinB*$^+$ gene into the chromosome. To be certain that the transplanted *dinB*$^+$ loci are functional, each repositioned allele was shown to relieve sensitivity to methyl methanesulfonate (MMS), a DNA alkylating agent that is toxic to strains lacking DinB (Bjedov *et al.* 2007; Benson *et al.* 2011). Results show that DinB provides MMS resistance regardless of its gene position. However, DinB stimulates *lac* reversion under selection (~10-fold) only when a functional *dinB*$^+$ allele is located somewhere on the F'*lac* plasmid, not necessarily near the *lac* locus.

The *dinB* position effect demonstrated here supports the idea (#3 above) that coamplification of *lac* and *dinB* genes under selection is required for the mutagenesis seen in the Cairns–Foster experiment. The critical directed mutagenesis appears to require increases in the copy number of the whole F'*lac* plasmid, rather than tandem amplification of the *dinB*–*lac* region within the plasmid.

Materials and Methods

Bacterial strains

All strains assayed for reversion data are derived from *E. coli* K-12. Intermediate strains used to manipulate plasmids are derived from *Salmonella enterica* (Serovar Typhimurium, LT2). The Cairns–Foster tester strain FC40 (TR7178) and scavenger strain FC29 (TR7177) were provided by Patricia Foster (Cairns and Foster 1991). The genotypes of all strains assayed for reversion and MMS resistance/sensitivity are listed in Table 1, with intermediate strains involved in mutant construction.

Strain construction

Deletions and insertions were constructed using recombineering techniques involving Red recombination functions of phage λ, as described by Court *et al.* (2002) and Thomason *et al.* (2014). Deletion junctions and inserted segments were sequenced in their entirety to verify the absence of accumulated point mutations. Transfer of deletions and insertions into isogenic backgrounds was accomplished by construction of plasmids by P22-mediated transduction in *Salmonella* and conjugation of these plasmids into *E. coli*. Chromosomal mutations were moved between strains by P1-mediated transduction.

The 16- and 17-kb plasmid deletions described in Figure 1 were constructed in a highly transformable *Salmonella* strain (TT24643) carrying the F'$_{128}$ plasmid from FC40 (or TR7178). A PCR product was amplified that includes the chloramphenicol resistance cassette (Cm^R) flanked by FRT sequences flanked by terminal F'$_{128}$ homologies, to direct the DNA insertion that replaces the deleted material (Datsenko and Wanner 2000). In strain TT27001 (strain #2), this FRT-Cm^R-FRT product replaced 16 kb between the normal *lac* and *dinB* genes of F'*lac* (strain #1). In strain TT27002 (strain #3), the FRT-Cm^R-FRT cassette replaced 17 kb, a region including the *dinB* gene and extending to the same point near the *lac* operon. These Cm^R-resistant deletions were transduced into *Salmonella* strain TT25414, carrying an F'$_{128}$ plasmid isogenic to that from FC40. A plasmid encoding flippase was then introduced and the FRT-Cm^R-FRT cassette was excised leaving a deletion with one FRT at the deletion junction point (Datsenko and Wanner 2000). These two constructed plasmids (with Cm^S 16 and 17 kb deletions) were transferred by conjugation into an F$^-$ *E. coli* strain isogenic to FC40 (TT26180). The resulting *E. coli* strains were used in the reversion tests described below.

To insert *dinB*$^+$ at a new site, the donor was the *Salmonella* strain (TT26997) with the 16-kb deletion and the FRT-Cm^R-FRT cassette near *dinB*$^+$ (strain #2 in Figure 1). A 2.4-kb fragment was amplified by PCR using the plasmid from strain #2 (Figure 1). The fragment included (Cm^R-FRT-*dinB*$^+$), but not the second FRT (see Figure 1). The primers for this amplification carried sequence homologous to the sites that would receive the (Cm^R-FRT-*dinB*$^+$) fragment. This amplified (Cm^R-FRT-*dinB*$^+$) fragment replaced the *yebB* gene in an F'$_{128}$ plasmid carrying the 17-kb *dinB* deletion (strain

Table 1 List of strains

Strain	Genotypes[a]
Strains derived from *E. coli* K12	
TR7178	*ara thiA* Rif^R Δ(*gpt lac*)$_5$/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) (was parent tester FC40 of Cairns and Foster (1991))
TR7177	*ara thiA* Rif^S Δ(*gpt lac*)$_5$/F′$_{128}$ *pro*$^+$ Δ(*lacIZ*) (was scavenger strain FC29 of Cairns and Foster (1991))
TT23663	*lac* ^{iq} *rrnB*3 Δ(*lacZ*)$_{4787}$ *hsdR*514 Δ(*araBAD*)$_{567}$ Δ(*rhaBAD*)$_{568}$ *rph*$^-$1/pKD78 *araC* P$_{BAD}$-λ red(*gam bet exo*) *repA*101(ts) *oriR*101(ts) *oriT*2 Cm^R
TT24669	*ara thiA* Rif^R Δ(*gpt lac*)$_5$/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) *dinB*62::Km^R(sw)
TT26180	*ara thiA* Rif^R Δ(*gpt lac*)$_5$/F$^-$
TT26908	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*67::Cm^R(sw)/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) *dinB*62::KmR(sw)
TT27001	*ara thiA* Rif^R Δ(*gpt lac*)$_5$/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-mbhA*)$_{2100}$::FRT
TT27002	*ara thiA* Rif^R Δ(*gpt lac*)$_5$/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-dinB*)$_{2101}$::FRT
TT27009	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*67::Cm^R(sw)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-mbhA*)$_{2100}$::FRT
TT27010	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*67::Cm^R(sw)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-dinB*)$_{2101}$::FRT
TT27279	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*67::Cm^R(sw)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) *yebB*12::(Cm^R *dinB*) Δ(*mhpR-dinB*)$_{2101}$::FRT
TT27280	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*67::Cm^R(sw)
TT27281	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*67::Cm^R(sw)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion)
TT27282	*ara thiA* Rif^R Δ(*gpt lac*)$_5$/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) *yebB12*::(Cm^R *dinB*) Δ(*mhpR-dinB*)$_{2101}$::FRT
TT27283	*lac* ^{iq} *rrnB*3 Δ(*lacZ*)$_{4787}$ *hsdR*514 Δ(*araBAD*)$_{567}$ Δ(*rhaBAD*)$_{568}$ *rph*$^-$1 *dinB*68::Tc^R(sw)/pKD46 *bla*(Ap^R) P$_{BAD}$-λ red(*gam bet exo*) *oriR101 repA101*(Ts) Datsenko and Wanner (2000)
TT27284	*lac* ^{iq} *rrnB*3 Δ(*lacZ*)$_{4787}$ *hsdR*514 Δ(*araBAD*)$_{567}$ Δ(*rhaBAD*)$_{568}$ *rph*$^-$1 *dinB*68::Tc^R(sw) *hisC*325::(Cm^R *dinB*$^+$)
TT27285	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *hisC*325::(Cm^R *dinB*$^+$)
TT27286	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *hisC*325::(Cm^R *dinB*$^+$)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion)
TT27289	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *hisC*326::Cm^R
TT27290	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *hisC*326::Cm^R/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion)
TT27291	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *hisC*326::Cm^R/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-dinB*)$_{2101}$::FRT
TT27292	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *hisC*325::(Cm^R *dinB*$^+$)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-dinB*)$_{2101}$::FRT
TT27293	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*68::Tc^R(sw)
TT27294	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*68::Tc^R(sw) *hisC*325::(Cm^R *dinB*)
TT27295	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*68::Tc^R(sw) *hisC*325::(Cm^R *dinB*)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion)
TT27296	*ara thiA* Rif^R Δ(*gpt lac*)$_5$ *dinB*68::Tc^R(sw) *hisC*325::(Cm^R *dinB*)/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-dinB*)$_{2101}$::FRT
Strains derived from *Salmonella enterica* Typhimurium LT2	
TT22971	*metA*22 *metE*551 *trpD*2 *ilv*$^-$452 *pro*$^-$(leaky) *leu*$^-$ *hsd*LT6 *hsd*SA29 *hsdB*$^-$ *strA*120/pKD46 *bla*(Ap) p$_{BAD}$ λ red
TT24643	*metA*22 *metE*551 *trpD*2 *ilv*$^-$452 *pro*$^-$(leaky) *leu*$^-$ *hsd*LT6 *hsd*SA29 *hsdB*$^-$ *strA*120/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) *mhpC*281::Tn10/pKD46 *bla*(Ap^R) P$_{BAD}$- red(*gam bet exo*) *oriR101 repA101*(Ts)
TT25414	*proAB*670::Sp^R(sw) Δ(*leu*)$_{21}$/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion)
TT26997	*proAB*670::Sp^R(sw) Δ(*leu*)$_{21}$/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-mbhA*)$_{2100}$::FRT-Cm^R-FRT (*DinB*$^+$ adjacent to *lac*)
TT26998	*proAB*670::Sp^R(sw) Δ(*leu*)$_{21}$/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-dinB*)$_{2101}$::FRT-Cm^R-FRT (*DinB* deleted)
TT26999	*proAB*670::Sp^R(sw) Δ(*leu*)$_{21}$/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-mbhA*)$_{2100}$::FRT
TT27000	*proAB*670::Sp^R(sw) Δ(*leu*)$_{21}$/F′$_{128}$ *pro*$^+$ *lac* ^{iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) Δ(*mhpR-dinB*)$_{2101}$::FRT (*DinB* deleted)
TT27278	*proAB*670::Sp^R(sw) Δ(*leu*)$_{21}$/F′$_{128}$ *pro*$^+$ *lac*^{Iq} *lacI33*(*fs*) *lacIZΩ*(Ω fusion) *yebB12*::(Cm^R *dinB*) Δ(*mhpR-dinB*)$_{2101}$::FRT

[a] The letters (sw) following an insertion mutation indicate stand for "swap" and indicate that the inserted drug-resistance determinant replaces the coding sequence of the affected gene.

TT27002). The same *dinB*$^+$ region was also introduced into the *hisC* gene of the *E. coli* chromosome.

Testing MMS sensitivity

E. coli K12 strains with various *dinB* genotypes were grown overnight in 4 ml LB medium. On the day of the assay, two square LB plates, a control, and a 7.5 mM MMS plate were prepared. Drops (5 μl) of serial dilutions of each culture (10^{-4}, 10^{-5}, and 10^{-6}) were pipetted onto the LB plates with and without MMS. The LB control plate was incubated at 37° for ~8 hr and the MMS plate for ~12 hr. This difference in incubation times allowed colonies on both plates to grow to approximately the same size.

Lac reversion assays

Tester and scavenger cells (FC29) were pregrown overnight in NCE medium (Berkowitz *et al.* 1968) with added $MgSO_4$ (2 mM), glycerol (0.1%), and thiamin (50 μM). Cells were pelleted, washed, and resuspended in NCE medium. Each reversion plate [NCE, 0.1% lactose, and 25 mg/liter 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was plated with ~10^9 washed scavenger cells and incubated for 24 hr to remove contaminating carbon sources before plating ~10^8 tester cells. Viability was measured for each tester strain at day 0. The number of tester cells in the lawn was determined by taking at least six agar plugs from the selection plates. Cells from the plugs were suspended in minimal NCE (no citrate E) medium, diluted, and plated on LB plates containing X-Gal and Rifampicin. Reversion plates were incubated at 37° for 6 days and revertant colony number was scored daily. Each strain was assayed by testing > 10 independent cultures.

Testing stability of revertant lac$^+$ phenotypes

Revertant colonies appearing on day 5 (absent on day 4) were scored for the stability of their Lac$^+$ phenotype. Each new

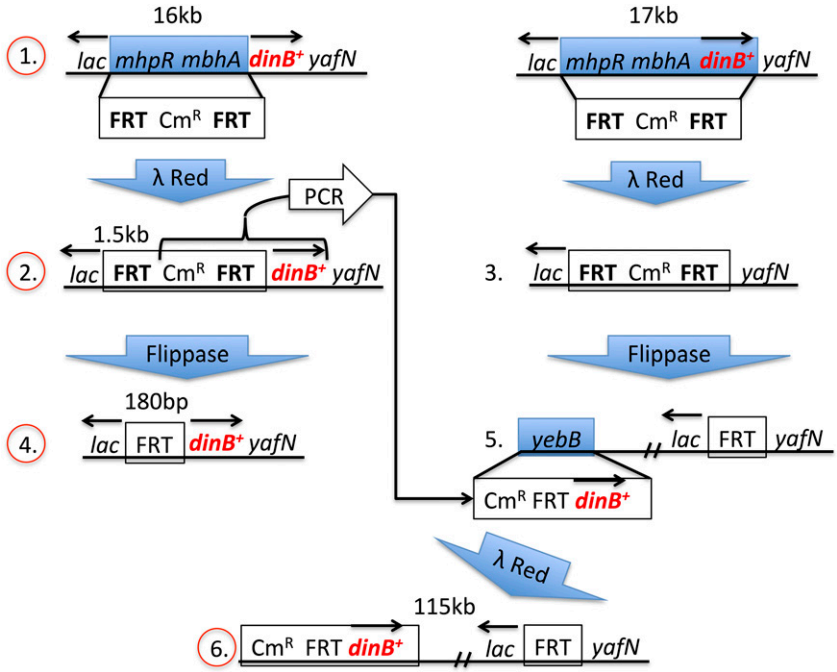

Figure 1 Deletions and insertions used to move *dinB* on the F′$_{128}$ plasmid. The two deletions described above (16 and 17 kb) were central to the strain constructions described later. Strain #1 is the original Cairns–Foster strain with *dinB*$^+$ located 16 kb from *lac* (TR7178). Strain #2 has a 1.5-kb sequence (FRT Cm^R FRT) replacing the 16 kb between *dinB* and *lac*. Strain #4, with the 16-kb deletion, has *dinB*$^+$ closest to *lac*, 180-bp away (TT27001). Strains #1, #2, #4, and #6 all show normal reversion under selection. The demonstrably functional *dinB*$^+$ allele near Cm^R in strain #2 was used as donor for inserting the (Cm^R-FRT-*dinB*$^+$) sequence into new sites. Strain #5 with the 17-kb *dinB* deletion (TT27002) received the (Cm^R-FRT-*dinB*$^+$) fragment in place of its plasmid *yebB* gene to produce strain #6 (TT27282). Cm^R, chloramphenicol resistance cassette.

colony was removed from the selection plate with a plug of agar. Cells were suspended in NCE and stored at 4°, before dilution and plating for single colonies on nutrient broth (Difco, Detroit, MI) plates containing rifampicin (50 mg/liter) to eliminate scavengers. These plates contained X-Gal (40 mg/liter) to distinguish unstable revertants (sectored blue "star" colonies) from stable revertants (solid blue colonies).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Moving the dinB$^+$ gene to different points in the genome

To test the three models described above, a functional *dinB*$^+$ gene was moved to various positions in the F′$_{128}$ *lac* plasmid and the chromosome of the Cairns–Foster tester strain. The general strategy (Figure 2) is based on a strain whose *dinB*$^+$ allele could be shown to support both reversion and MMS resistance. This strain was used as a donor of the moved *dinB*$^+$ locus (Cm^R-FRT-*dinB*$^+$). The DNA sequence of the transferred locus was shown to be identical with that of the functional donor.

To construct these strains, two deletion mutations were constructed in the F′*lac* plasmid of a *Salmonella* strain (see Figure 1 in *Materials and Methods*). One deletion (17 kb) removes the *dinB*$^+$ gene and the region between *dinB* and *lac*. The other deletion (16 kb) removes the same region but leaves the normal *dinB*$^+$ gene close to a chloramphenicol resistance determinant (Cm^R). This (Cm^R-FRT-*dinB*$^+$) region was PCR-amplified, and used to selectively insert a *dinB*$^+$ allele in place of the episomal *yebB* gene and the chromosomal *hisC* gene. The donor strain containing the 16-kb deletion produced the same number of Lac$^+$ revertants under selection before and after removal of the antibiotic resistance gene FRT-Cm^R-FRT (strains 2 and 4 in Figure 1) (data not shown). Both strains show the same resistance to MMS (see below). This demonstrated that the inserted Cm^R gene did not affect the function of the transferred *dinB*$^+$ allele (Cm^R-FRT-*dinB*$^+$). Plasmid mutations were combined to construct four plasmid types: the normal version with *dinB*$^+$ 16 kb from *lac*, a second with the 16-kb deletion (*dinB*$^+$ located 180 bp from *lac*), a third with the normal *dinB*$^+$ removed by the 17-kb deletion, and a fourth with both the 17-kb deletion and a *dinB*$^+$ insertion replacing the *yebB* gene 115 kb from *lac*. These four plasmids were transferred into *E. coli* strains whose chromosomes had various combinations of *dinB* alleles: no, one, or two copies. All of the final strains used in reversion tests shared a common *E. coli* genetic background.

A functional dinB$^+$ allele provides resistance to MMS, regardless of genomic position

In testing the effect of the *dinB*$^+$ gene position on reversion, it is critical that the relocated gene retains wild-type function. The *dinB*$^+$ gene encodes an error-prone bypass polymerase that is able to copy a damaged template that blocks progression of normal replication forks (Ohmori *et al.* 2001). While the DinB repair polymerase can replicate a damaged strand, it is prone to making mistakes that can contribute to general mutagenesis during reversion (Kim *et al.* 1997). DinB provides resistance to DNA alkylating agents such as MMS, which was the basis of a test for DinB functionality devised

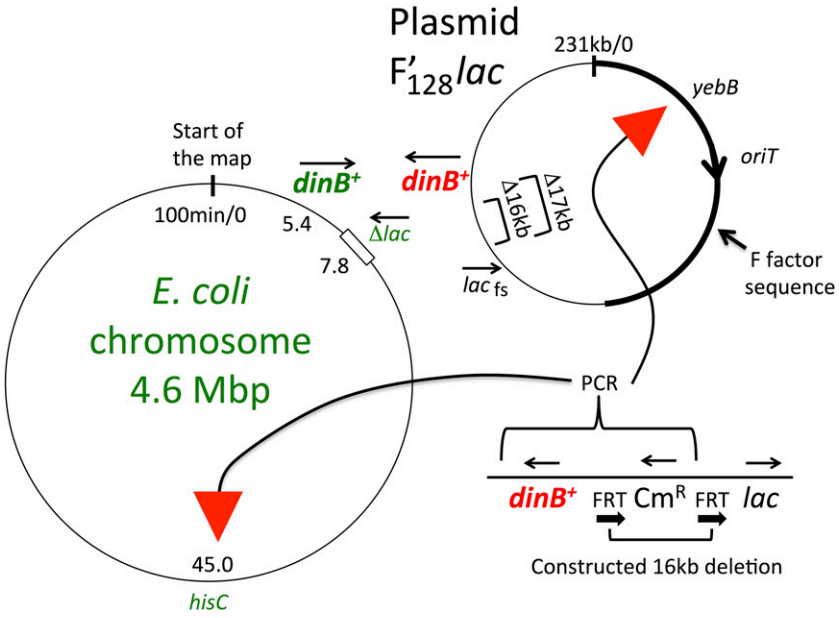

Figure 2 The relevant loci on chromosome and plasmid. This figure depicts the genotype of the standard Cairns–Foster strain (TR7178) and the changes introduced to test the effect of *dinB*$^+$ position on reversion. A heavy line indicates the portion of the F'*lac* plasmid derived from the original F plasmid; the rest of the plasmid is derived from the chromosome of *E. coli* (Kofoid *et al.* 2003). The orientation of the *lac* and *dinB* genes is reversed by the way in which the plasmid is excised from the chromosome. Two deletions (16 and 17 kb) share an endpoint near *lac* on the plasmid, but extend different distances to either leave or remove the *dinB*$^+$ gene. The insert is a PCR fragment from the 16-kb deletion strain, as described in Figure 1. The *yebB* gene is located at a maximum distance from *lac* in the tester F'$_{128}$*lac* plasmid. To make the isogenic set of strains used for reversion, these insertions and deletions were assembled into isogenic combinations by either transduction or conjugation. Cm^R, chloramphenicol resistance cassette.

by Bjedov *et al.* (2007) and Benson *et al.* (2011) (see *Materials and Methods*).

Some of the constructed strains described above have *dinB*$^+$ at positions that do not support reversion. Therefore, it was critical to show that the genes at these sites were functional and had not been damaged in the strain construction process. The *dinB*$^+$ genes of all of the tested strains were sequenced to show that no mutations had occurred during PCR and linear transformation, and all strains were tested for their resistance to MMS.

The parent Cairns–Foster tester strain is shown in Figure 3, line 2. Isogenic derivatives with no functional *dinB* gene are sensitive to MMS (lines 7, 9, 10, and 13). Any single copy of the *dinB*$^+$ gene is sufficient to provide resistance to MMS (lines 1, 3, 4, 8, 11, 12, and 14). Strains with multiple *dinB* copies do not show increased resistance (compare lines 1, 2, and 18). The genomic position of the *dinB*$^+$ gene does not affect the ability to provide MMS resistance. That is, strains with a single chromosomal *dinB* gene are resistant regardless of the position of their functional *dinB* allele (compare lines 1 and 14). Strains with *dinB*$^+$ on the F'*lac* plasmid show the same resistance to MMS regardless of whether *dinB* is located near *lac* (line 11) or far from *lac* within the *yebB* gene (line 12). Surprisingly, while all strains lacking a functional *dinB* gene are sensitive to MMS, this sensitivity is increased by the presence of an F'*lac* plasmid (compare lines 7 and 13 to lines 9 and 10). We suspect that some gene on the F'*lac* plasmid may either enhance MMS import or reduce SOS expression. A candidate is the *psi* gene, which minimizes SOS induction following a plasmid transfer (Bailone *et al.* 1988).

In summary, these tests show that a *dinB*$^+$ allele at its normal chromosomal position (lines 1 and 3) has a functionality that is indistinguishable from that of the same allele inserted within the *hisC* gene (line 16) or at any of three sites on the F'*lac* plasmid, adjacent to *lac* (line 11), 16-kb away from *lac* (line 8), or 115-kb away from *lac* (line 12).

DinB is an error-prone translesion DNA polymerase that provides MMS resistance, but does not contribute to basal mutation rates in normal growing cells (Kuban *et al.* 2004). In the absence of DNA damage, DinB becomes mutagenic only when strongly overexpressed from a multi-copy plasmid (Kim *et al.* 1997; Wagner *et al.* 1999). The key to understanding the Cairns–Foster system is to determine how DinB expression is elevated sufficiently during selection to increase mutation rates. Previous tests showed that reversion under selection requires the global positive regulator RpoS and inactivation of the SOS repressor protein LexA (McKenzie *et al.* 2001; Layton and Foster 2003; Lombardo *et al.* 2004; Foster 2005). These proteins are known to regulate multiple genes including *dinB* (Friedberg *et al.* 2006; Battesti *et al.* 2011). The results below show that the transcription increases mediated by the RpoS and SOS responses are not sufficient for mutagenesis, because reversion occurs only when a normal *dinB*$^+$ gene is located somewhere on the F'*lac* plasmid, where it can be selectively coamplified with *lac*.

Reversion under selection requires a functional dinB$^+$ *allele on the F'lac plasmid*

In the original Cairns–Foster strain, one copy of *dinB*$^+$ is located in the chromosome and another on the F'*lac* plasmid ~16-kb away from *lac* (see Figure 2). As seen in Figure 4, removal of the *dinB*$^+$ gene from the plasmid reduced the revertant yield fivefold, even in a strain with a normal chromosomal *dinB*$^+$

The bottom of Figure 4 shows that the 10-fold excess of scavenger cells plated with testers prevents lawn growth. Thus, revertant colonies developed in a population that grows very little. It also shows that starved cells do not lose viability in the absence of DinB.

The essential role of the plasmid *dinB*$^+$ gene was also tested directly by eliminating the chromosomal *dinB*$^+$ allele.

Line (Strain #)	DinB Genotype: Chrom.	DinB Genotype: F'_{128}	Treatment: Control 10^{-4}	Control 10^{-5}	Control 10^{-6}	7.5mM MMS 10^{-4}	7.5mM MMS 10^{-5}	7.5mM MMS 10^{-6}	Phenotype: Resistance to MMS
1 (TT26180)	+	no F′							R
2 (TR7178)	+	+							R
3 (TT24669)	+	− (Δ*dinB*::Km^R)							R
4 (TT27002)	+	− (Δ*dinB*::FRT)							R
5 (TT27001)	+	+ (180bp from *lac*)							R
6 (TT27282)	+	− (Δ*dinB*::FRT) + (*yebB*::*dinB*$^+$)							R
7 (TT27280)	− (Δ*dinB*::Cm^R)	no F′							S
8 (TT27281)	− (Δ*dinB*::Cm^R)	+							R
9 (TT26908)	− (Δ*dinB*::Cm^R)	− (Δ*dinB*::Km^R)							S
10 (TT27010)	− (Δ*dinB*::Cm^R)	− (Δ*dinB*::FRT)							S
11 (TT27009)	− (Δ*dinB*::Cm^R)	+ (180bp from *lac*)							R
12 (TT27279)	− (Δ*dinB*::Cm^R)	− (Δ*dinB*::FRT) + (*yebB*::*dinB*$^+$)							R
13 (TT27293)	− (Δ*dinB*::Tc^R)	no F′							S
14 (TT27294)	− (Δ*dinB*::Tc^R) + (*hisC*::*dinB*$^+$)	no F′							R
15 (TT27295)	− (Δ*dinB*::Tc^R) + (*hisC*::*dinB*$^+$)	+							R
16 (TT27296)	− (Δ*dinB*::Tc^R) + (*hisC*::*dinB*$^+$)	− (Δ*dinB*::FRT)							R
17 (TT27285)	+ + (*hisC*::*dinB*$^+$)	no F′							R
18 (TT27286)	+ + (*hisC*::*dinB*$^+$)	+							R
19 (TT27292)	+ + (*hisC*::*dinB*$^+$)	− (Δ*dinB*::FRT)							R

Figure 3 MMS sensitivity of strains with *dinB*$^+$ at various positions. Strains were plated on LB plates with or without 7.5 mM MMS by spotting 5 μl droplets of cultures that had been diluted 10^{-4}, 10^{-5}, or 10^{-6} fold. The first column from the left denotes line numbers referenced in the text as well as strain numbers (genotypes described in Table 1). The second and third columns describe *dinB* genotypes in the chromosome (Chrom.) and the F'_{128} plasmid, using "+" to indicate *dinB*$^+$ and "−" to indicate a *dinB* deletion. For the "+," annotations in parentheses indicate where a new *dinB* allele was inserted, and no annotation means that the *dinB* gene is in its original position on the chromosome or F'_{128} episome. For the "−," annotations indicate what was inserted to replace the *dinB* gene. All strains tested for resistance to MMS were isogenic to *E. coli* K-12. The fourth and fifth columns show the effects of the MMS treatment on cell viability and growth compared to a control. The last column on the right indicates whether the strain is sensitive (S) or resistant (R) to MMS.

In Figure 4, it can be seen that removal of *dinB*$^+$ from the chromosome has no effect on revertant number, whether or not a functional allele is located on the F′*lac* plasmid. That is, a chromosomal *dinB*$^+$ copy does not provide mutagenesis to strains lacking any other *dinB*$^+$ allele, and removing the chromosomal *dinB*$^+$ allele does not reduce mutagenesis in a strain carrying *dinB*$^+$ on the F′*lac* plasmid. Thus, the *dinB*$^+$ copy on the plasmid is both necessary and sufficient for reversion under selection. This same conclusion was reached earlier for the *Salmonella* version of the Cairns–Foster system (Slechta *et al.* 2002a, 2003). Two studies have previously addressed *dinB*$^+$ position effects in *E. coli*. One of these studies supports the

conclusion drawn here (Kim *et al.* 2001) and the other contradicts it (McKenzie *et al.* 2001). These results will be discussed later.

The plasmid dinB⁺ gene stimulates reversion regardless of its position on the F'lac plasmid

The two selection models for the Cairns system make different predictions regarding the effect of the *dinB*$^+$ gene on reversion under selection. Figure 5A describes the reversion behavior of three strains with the *dinB*$^+$ allele at different sites in the F'*lac* plasmid and a control strain whose plasmid lacks a *dinB*$^+$ gene. The top label in Figure 5A indicates the standard Cairns–Foster tester strain (TR7178) with *dinB*$^+$ located 16 kb from *lac* (strain #1 in Figure 1). The second label indicates strain (TT27001), whose plasmid has the 16-kb deletion, which places the *dinB*$^+$ promoter ~180 bp from the divergent *lac* promoter: strain #4 in Figure 1. The third strain (TT27282) carries a plasmid with the 17-kb deletion and a copy of *dinB*$^+$ inserted at *yebB* 115 kb from *lac*: strain #6 in Figure 1. These three *dinB*$^+$ strains show equivalent high yields of Lac$^+$ revertants, 57–70 Lac$^+$ colonies at day 6, compared to a control strain (TT27002) whose plasmid lacks the *dinB* allele. Strain #2 in Figure 1, which was used as donor for all transplanted *dinB*$^+$ alleles and has *dinB*$^+$ located 1.5 kb from *lac*, showed reversion behavior indistinguishable from the three *dinB*$^+$ strains described in Figure 5A. The control strain (TT27002) lacks *dinB*$^+$ on the F' plasmid and shows four- to fivefold fewer cumulative Lac$^+$ revertants at day 5. All the strains in Figure 5A have a chromosomal *dinB*$^+$ gene, but the same results were obtained for strains carrying the same F'*lac* plasmids and a chromosomal *dinB* deletion mutant (data not shown).

The absence of *dinB* on the plasmid decreased the number of Lac+ revertant colonies by around fivefold, as seen in Figure 4 and Figure 5A. The remaining revertants (20% of that in the Cairns' strain) are of two equally abundant types (~10% of each type) (Figure 5B). One residual type (42 and 45%) is unstable and forms by tandem *lac* amplification without need for reversion of DinB. The second residual type (58 and 55%) is stably Lac$^+$ and forms by a sequence change that occurs by local overreplication of *lac* without benefit of DinB. The original Cairns' strain (TR7178) with a chromosomal and plasmid *dinB*$^+$ gene produces 14% unstable and 86% stable revertants (scored on day 5). Thus, DinB has no effect on the number of unstable revertants, but a *dinB*$^+$ allele on the F'*lac* plasmid stimulates stable revertants eightfold. At the same time, a chromosomal *dinB*$^+$ gene does not affect the number of either revertant type.

The position of dinB⁺ on F'lac does not affect the frequency of unstable revertants

In the Cairns–Foster system, ~10% of Lac$^+$ revertants are due to a tandem amplification of the mutant *lac* allele within the F'*lac* plasmid (Slechta *et al.* 2002b; Hastings *et al.* 2004; Kugelberg *et al.* 2006). The sequenced duplication endpoints lie in the immediate vicinity of the *lac* region, with many

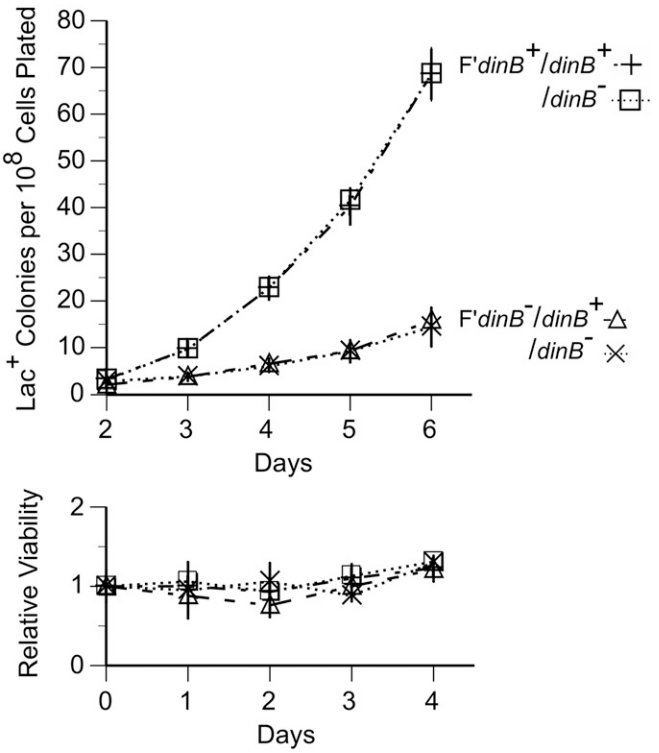

Figure 4 Effects of *dinB*$^+$ gene position on reversion under selection. Isogenic strains were tested for accumulation of revertants under selection. Four isogenic strains have *dinB*$^+$ alleles at various genomic positions. The top two strains above have a *dinB*$^+$ allele on F'*lac*, and a chromosome with either a *dinB*$^+$ allele (TR7178) or a *dinB* deletion formed by drug cassette replacement (TT27281). The bottom two strains have a *F'lac* plasmid whose *dinB gene was* removed by a 17-kb deletion. Their chromosome has either a functional *dinB*$^+$ allele (TT27002) or a *dinB* deletion made by drug cassette replacement (TT27010). The lower graph shows the lawn populations, which were assessed by removing agar plugs from random spots on the selection plate. The results are presented for each strain individually and expressed relative to the cell number at the time of plating.

endpoints falling within the *dinB–lac* region that was removed by the 16-kb deletion discussed here. The tandem amplification model suggested that mutagenesis was caused by selected tandem coamplification of the *dinB*$^+$ and *lac* genes (Slechta *et al.* 2003). Short amplifications that include *dinB* and *lac* were suggested to amplify highly, increasing the mutation rate and giving rise to stable revertant cells. Amplifications that do not include *dinB* or include a segment that is costly to amplify lead to unstable revertants. According to this model, the frequency of unstable revertants might increase if one brought *dinB*$^+$ closer to *lac* such that a higher fraction of *lac* duplications could include *dinB*$^+$.

As seen in Figure 5B, the frequency of unstable revertants is not affected by the position of the *dinB*$^+$ gene on the F'*lac* plasmid. Of the revertants, 10–14% are unstably Lac$^+$ regardless of the distance between *dinB* and *lac* (180 bp, or 16 or 115 kb). This suggests that the process leading to stable revertants does not require previous tandem coamplification of *dinB* and *lac*, as suggested by the tandem amplification model.

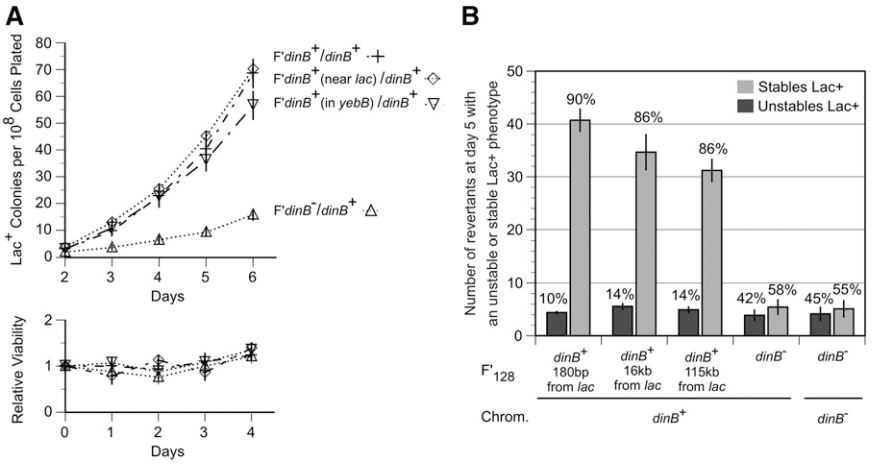

Figure 5 Effects of *dinB* position on reversion and on fraction of unstable revertants on day 5. (A) Four strains compared with a *dinB*+ allele at various positions in the F'*lac* plasmid. All strains have a *dinB*+ allele at the normal position in the chromosome (Chrom.). The strain (TR7178) has *dinB*+ located 16 kb from *lac* on the F'*lac* plasmid. The second strain (TT27001) has *dinB*+ moved nearer to *lac* (180-bp away). The third strain (TT27282) lacks the normal 17-kb *dinB* region but carries a functional *dinB*+ inserted at the opposite side of the F'*lac* plasmid, within the *yebB* gene (see Figure 2). The fourth strain (TT27002) has a plasmid with no *dinB*+ allele. The lower graph shows lawn cell population, which was assessed by removing plugs from random spots on the selection plate. The results are presented for each strain individually relative to the cell number at the time of plating. (B) The number of Lac+ revertants at day 5 and the percentage of revertants with an unstable or stable Lac+ phenotype for each strain of (A) (TT27001; TR7178; TT27282; and TT27002), and also strain TT27010 with *dinB* deleted from both the F'*lac* plasmid and the chromosome. The stability phenotype was assessed by restreaking Lac+ colonies at day 5 from the lactose plates onto rich medium with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, as described in the *Materials and Methods*.

However, the result is consistent with selective amplification of the whole plasmid (model #3). As seen in Figure 5B, regardless of the position of *dinB*+ on the plasmid, 86–90% of total revertants are stable (10–14% are unstable). According to the whole-plasmid amplification model, unstable revertants are initiated by preexisting cells whose plasmid acquired an internal *lac* duplication before its plasmid copy number increased. Reducing the distance between *dinB* and *lac* does not seem to alter the likelihood of an unstable revertant. This suggests that unstable revertants are initiated prior to selection by cells with multiple copies of a plasmid with any of a wide variety of internal *lac* duplication types, such that removal of the *dinB–lac* spacer region does not significantly alter the overall likelihood of a *lac* duplication. Thus, stable and unstable revertants arise by a related series of events that proceed independently. With unstable revertants, each act of plasmid transfer between siblings under selection stimulates the unequal recombination events that allow amplification expansion. Tandem amplification of the preexisting duplication improves growth ability and leads to an unstable revertant, while DinB function and mutagenesis are required only for stable revertants.

An extra ectopic chromosomal dinB+ gene does not compensate for lack of dinB+ on F'lac

The need for a functional *dinB*+ gene on the F'*lac* plasmid might be explained by the fact that the F' *lac* plasmid has a slightly higher (1–2) copy number than that of the chromosome. If plasmid copy number explained this, one might expect that any defect caused by removing *dinB*+ from the F'*lac* plasmid would be corrected by adding an extra ectopic *dinB*+ allele to the chromosome. To test this, an extra *dinB*+ copy was inserted into the chromosomal *hisC* gene. The strain with only this inserted *dinB*+ allele is MMS-resistant (see Figure 1, lines 14 and 16). Strains with either or both chromosomal *dinB*+ alleles were tested for reversion with or without a functional *dinB*+ gene on the F'*lac* plasmid. Results are shown in Figure 6. Strains with a functional *dinB*+ allele on the F'*lac* plasmid all showed the same high revertant yield regardless of whether the chromosome carried the normal *dinB*+ allele, the allele inserted at *hisC*, or both of these chromosomal alleles. Recall that lack of *dinB*+ in the chromosome has no effect on reversion (Figure 4). Strains lacking *dinB*+ on the F'*lac* showed low reversion, whether their chromosome carried the standard *dinB*+ allele, the *dinB*+ allele inserted at *hisC*, or both. Thus, increasing the dosage of a chromosomal *dinB*+ gene does not compensate for lack of a plasmid *dinB*+ gene. This result differs from that of McKenzie *et al.* (2001), who found that addition of an ectopic chromosomal allele did compensate for lack of *dinB*+ on the plasmid. We suspect that their results reflect a problem in strain construction, which we discuss later.

Discussion

Evidence is presented that the stable *lac*+ revertants appearing under selection in the Cairns–Foster system depend on the presence of a functional *dinB*+ allele on the F'*lac* plasmid for their formation. These results support our current selection model, in which reversion and mutagenesis require selective amplification of the entire F'*lac* plasmid with its included *lac* and *dinB*+ genes (Maisnier-Patin and Roth 2015). Stress-induced mutagenesis models propose that *dinB* gene transcription is increased during growth limitation (by RpoS-mediated induction and LexA-mediated derepression) and that this increase is important to the reversion process (McKenzie *et al.* 2001; Layton and Foster 2003; Lombardo *et al.* 2004; Foster 2005; Galhardo *et al.* 2009). While these expression increases have experimental support, they cannot be the full explanation, since they do not explain the *dinB*+ position effect. That is, a *dinB*+ allele located in the chromosome should be induced just as well as one on F'*lac* plasmid.

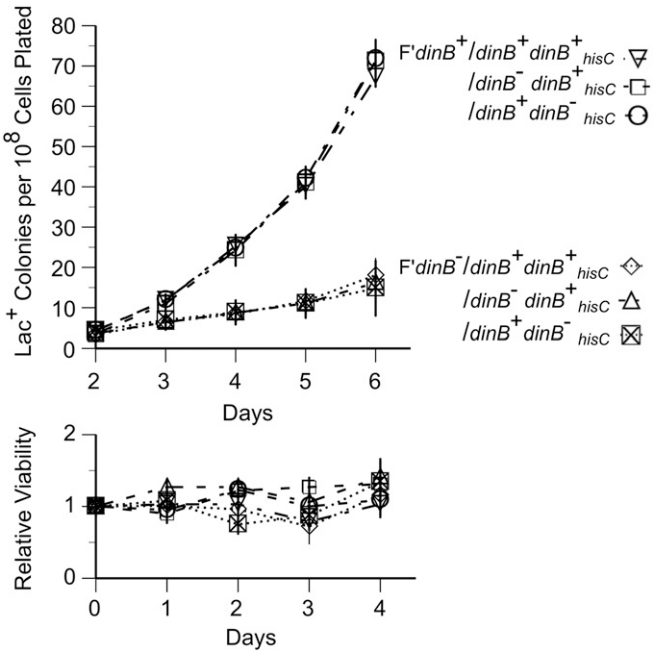

Figure 6 Contribution to reversion of ectopic *dinB*+copies. The top three strains all carry an F'*lac* plasmid with a functional *dinB*+ gene. The first strain (TT27286) also has a *dinB*+ allele at its normal chromosomal location and an additional copy inserted into the chromosomal *hisC* gene. The second (TT27295) has only the *dinB*+ allele inserted into the *hisC* gene. The third strain (TT27290) carries the normal *dinB*+ and a Cm+ determinant inserted in *hisC* without a *dinB*+. Lower strains (TT27292, TT27296, and TT27291) all have an F'*lac* with a *dinB* deletion. These strains have one, the other, or both of the chromosomal *dinB*+ alleles. The lower graph shows the lawn population, which was assessed by removing plugs from random spots on the selection plate. The results are presented for each strain individually relative to the cell number at day 0. Lack of lawn growth shows that revertant accumulation is not a simple result of population growth.

A *dinB*+ allele at any genomic position should produce active DinB protein that has access *in trans* to the entire genome. Contrary to stress-induced mutagenesis models, the chromosomal *dinB* allele makes no contribution and reversion is enhanced under selection only when a *dinB*+ allele is located somewhere on the F'*lac* plasmid. We propose below that *dinB* induction may contribute to the DinB level but provides a level that is insufficient to cause mutagenesis unless the *dinB*+ allele is amplified under selection.

The tandem amplification model predicts that, contrary to the data reported here, *dinB*+ and *lac* must be close together on the F'*lac* plasmid so that they can both be included in a small region of the F'plasmid that amplifies in tandem under selection. Selection drives expansion of the tandem array including the (*dinB* Cm^R *lac*) sequence, and improves growth by adding more *lac* copies while it stimulates the reversion rate with more *dinB*+ copies (Hendrickson *et al.* 2002; Slechta *et al.* 2003; Roth *et al.* 2006). The results reported here show that reversion requires *dinB*+ to be somewhere on the F'*lac* plasmid but not necessarily close to *lac*. That is, the same numbers of revertants are seen when *dinB*+ is immediately adjacent to *lac*, or 16- or 115-kb away from *lac* (the maximum distance possible on the 230-kb plasmid). Thus, the results reported here argue against our previous tandem duplication model.

The results described here are consistent with a model in which revertants are initiated by preexisting cells with ~10 extra copies of the whole plasmid (Sano *et al.* 2014). These cells arise during nonselective growth prior to plating on lactose. Of the plated population (10^8 cells), only these initiator cells (10^5) have enough energy to divide and overreplicate their F'*lac* plasmid under selection. Mating between sibling cells initiates rolling circle plasmid replication, which enhances reversion by repeatedly copying the mutant *lac* allele in the presence of a mutagenic excess of DinB protein. This model predicts that mutagenesis and enhanced reversion under selection depends on a *dinB*+ allele located somewhere on the F'*lac* plasmid, but not necessarily near *lac* (Maisnier-Patin and Roth 2015, 2016; S. Maisnier-Patin and J. R. Roth, personal communication). The unstable Lac+ revertants are thought to arise from initiator cells whose plasmids have an internal *lac* duplication. Mating between siblings of these initiators stimulates unequal recombination events that allows selective expansion of the tandem array and cell growth without reversion.

This new model resurrects the importance of plasmid transfer in the reversion process, which was suggested early in the history of the Cairns system (Galitski and Roth 1995; Radicella *et al.* 1995; Peters *et al.* 1996). Mating had been rejected previously because only ~4% of Lac+ revertants had experienced plasmid transfer, based on reconstruction experiments using mixtures of genetically marked tester strains (Foster and Trimarchi 1995b). The new model suggests that mating is indeed rare between random plated cells, which are starving under selection, but is frequent between the siblings of the initiator cells, which both have the energy to support mating. Independently plated initiator cells are rare enough (1/1000 cells) that they seldom mate on the plate. Supporters of stress-induced mutagenesis propose that mating is unimportant and that plasmid conjugation functions contribute only by introducing a single-strand nick at the plasmid transfer origin (*oriT*). It is proposed that this nick becomes a double-strand break whose repair becomes mutagenic in the presence of DinB (Foster and Trimarchi 1995a; Ponder *et al.* 2005). This model does not explain why double-strand breaks in the chromosome are so inefficient at causing mutagenesis or how the effects of repairing a break at *oriT* can extend to the *lac* locus, which is located over 100-kb away from *oriT* on the F'*lac* plasmid (Shee *et al.* 2011a,b). Stress-induced mutagenesis models do not explain the *dinB* position effects described here.

The idea of a *dinB*+ position effect was tested previously for the Cairns–Foster system in both *Salmonella* and *E. coli*. In *Salmonella*, reversion clearly depended on having a *dinB*+ gene on the F'_{128} plasmid (Slechta *et al.* 2002a, 2003). The first test in *E. coli* supported the same conclusion: *lac* reversion required *dinB*+ on the F'*lac* plasmid and was not affected by the chromosomal *dinB* allele (Kim *et al.* 2001). This

experiment was done as a Luria–Delbrück fluctuation test rather than a time-dependent accumulation of revertants on solid medium. Parallel cultures of the Cairns–Foster tester strain were plated without scavenger cells on lactose, allowing some growth under selection. Revertant (Lac^+) colonies were counted on day 2 in the belief that they resulted from events occurring prior to plating. However, the revertant number in the several cultures after day 2 showed a Poisson distribution, instead of a Luria–Delbrück distribution. This observed distribution was like that seen for revertant accumulation in the Cairns–Foster system. The Poisson distribution suggested that revertants either form on the selection plate (Cairns and Foster 1991) or are initiated by preexisting copy number variants that are not subject to Luria–Delbrück fluctuation (Sano *et al.* 2014). In this *E. coli* position-effect test, revertant number was increased by a *lexA*(Def) mutation, which causes constitutive expression of *dinB* by SOS derepression, and was reduced by removal of the $dinB^+$ from the F'*lac* plasmid (Kim *et al.* 2001). Revertant number was not reduced by removal of the chromosomal $dinB^+$ allele.

A different conclusion was drawn when the $dinB^+$ position effect was addressed by McKenzie *et al.* (2001). In this experiment, removal of *dinB* from either the plasmid or chromosome caused a strong reduction in the number of revertants in the homozygous ($dinB^-/dinB^-$) strain. The role of the chromosomal $dinB^+$ allele was tested in an unusual way. Instead of repairing the *chromosomal dinB* mutation allele or moving the plasmid to a $dinB^+$ recipient, an ectopic $dinB^+$ allele was added to the chromosome of the $dinB^-/dinB^-$ homozygote. This addition restored reversion. The $dinB^+$ allele was added to the chromosome at the insertion site of phage λ, near the galactose operon. The insertion method was not described, and the expression level of the ectopic allele was not tested. We suspect that the unusual result reported was due to the proximity of $dinB^+$ to the galactose operon. Expression of the galactose operon is essential to reversion in the Cairns system since growth requires use of the galactose released by the splitting of lactose (Andersson *et al.* 1998). It seems possible that derepression of the *gal* operon enhances expression of an adjacent $dinB^+$ gene, or that selection for growth on lactose favors amplification of the chromosomal *gal-dinB* region during reversion under selection. It is also possible that the inserted *dinB* fragment included a drug-resistance determinant that amplified with $dinB^+$ during growth of strains on an antibiotic.

Enhanced lac reversion under selection in the Cairns–Foster system is not evidence for a general phenomenon of stress-induced mutagenesis

Results presented here suggest that the error-prone DinB polymerase enhances reversion in the Cairns system only if its structural gene is located somewhere on the F'*lac* plasmid and can be selectively amplified with *lac* using plasmid conjugation functions, which are essential for reversion. The colocation of these genes is a peculiarity of the F'*lac* plasmid, a laboratory construction that fuses a chromosomal *lac* fragment with the conjugative F plasmid. The *dinB* and *lac* genes were brought close together when this plasmid formed by recombination between chromosomal REP sequences (Kofoid *et al.* 2003). (REP indicates a "repeated extragenic palindromic" element) Enhanced reversion of the *lac* mutation also depends on the conjugation functions of the F' *lac dinB* plasmid (Galitski and Roth 1995; Radicella *et al.* 1995; Peters *et al.* 1996) and is not seen for a *lac* mutation located in the chromosome (Foster and Trimarchi 1995a; Radicella *et al.* 1995). These observations suggested that DinB mutagenesis during *lac* reversion is not due to an evolved global mechanism for creating mutations in response to stress, but is rather an artifact of a particular system that happens to juxtapose the $dinB^+$ and *lac* alleles on a plasmid whose conjugation functions can support repeated rolling circle replication of the whole plasmid in cells that divide very little.

Evidence has been presented that reversion requires positive regulation of *dinB* by the stationary phase transcription factor RpoS (Layton and Foster 2003; Lombardo *et al.* 2004) and derepression of *dinB* by the SOS regulatory protein LexA (McKenzie *et al.* 2001; Foster 2005; Galhardo *et al.* 2009). These observations have been interpreted as evidence for an intricate control mechanism that regulates mutation rates during growth limitation. While these transcription controls do increase *dinB* expression, the results presented here suggest that this increase is not sufficient to explain mutagenesis in the Cairns–Foster system. We suggest that $RpoS^-$ and Lex^{Ind} mutations reduce reversion because they lower the transcription level of DinB. The observed mutagenesis requires that the $dinB^+$ gene must not only be transcribed, but must also be amplified under selection. The transcriptional controls of DinB seem likely to have evolved to manage DNA damage repair rather than variation in mutation rates. The critical variation in DinB expression is supplied by selective coamplification of $dinB^+$ with *lac*. This amplification can only be effective if there is some expression of the $dinB^+$ gene.

Summary: the take-home lesson of the Cairns–Foster system

The Cairns–Foster system has been pursued for over 25 years because it appeared to show evidence of an iconoclastic possibility: that cells might have mechanisms to increase mutation rates in response to growth limitation (stress-induced mutagenesis) (Torkelson *et al.* 1997) or might even be able to direct mutations preferentially to sites that improve growth (Foster and Cairns 1992). It now seems more likely that the behavior of this system reveals the power of natural selection to detect common variants with small effects that enhance a cell's ability to replicate a growth-limiting gene. The key to the reversion process is the high frequency of plasmid copy number variants (1 in a 1000 cells) that arise before plating on lactose (Sano *et al.* 2014). Under selection, these cells can divide and transfer the mutant F'*lac* plasmid between siblings to stimulate repeated rolling circle plasmid replication. This model will be explained in detail

(S. Maisnier-Patin and J. R. Roth, personal communication). Understanding how selection works in this system has required the unraveling of multiple arcane aspects of a complicated biological situation. It seems likely that many other cases of apparent stress-induced mutagenesis will be similarly explicable without regulated mutability once one unravels the system-specific peculiarities exploited by selection.

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Literature Cited

Andersson, D. I., E. S. Slechta, and J. R. Roth, 1998 Evidence that gene amplification underlies adaptive mutability of the bacterial lac operon. Science 282: 1133–1135.

Bailone, A., A. Bäckman, S. Sommer, J. Célérier, M. M. Bagdasarian *et al.*, 1988 PsiB polypeptide prevents activation of RecA protein in *Escherichia coli*. Mol. Gen. Genet. 214: 389–395.

Battesti, A., N. Majdalani, and S. Gottesman, 2011 The RpoS-mediated general stress response in *Escherichia coli*. Annu. Rev. Microbiol. 65: 189–213.

Benson, R. W., M. D. Norton, I. Lin, W. S. Du Comb, and V. G. Godoy, 2011 An active site aromatic triad in *Escherichia coli* DNA Pol IV coordinates cell survival and mutagenesis in different DNA damaging agents. PLoS One 6: e19944.

Berkowitz, D., J. M. Hushon, H. J. Whitfield, J. R. Roth, and B. N. Ames, 1968 Procedures for identifying nonsense mutations. J. Bacteriol. 96: 215–220.

Bjedov, I., C. N. Dasgupta, D. Slade, S. Le Blastier, M. Selva *et al.*, 2007 Involvement of *Escherichia coli* DNA polymerase IV in tolerance of cytotoxic alkylating DNA lesions *in vivo*. Genetics 176: 1431–1440.

Cairns, J., 1998 Mutation and cancer: the antecedents to our studies of adaptive mutation. Genetics 148: 1433–1440.

Cairns, J., and P. L. Foster, 1991 Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics 128: 695–701.

Cairns, J., J. Overbaugh, and S. Miller, 1988 The origin of mutants. Nature 335: 142–145.

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.

Court, D. L., J. A. Sawitzke, and L. C. Thomason, 2002 Genetic engineering using homologous recombination. Annu. Rev. Genet. 36: 361–388.

Datsenko, K. A., and B. L. Wanner, 2000 One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97: 6640–6645.

Foster, P. L., 2005 Stress responses and genetic variation in bacteria. Mutat. Res. 569: 3–11.

Foster, P. L., and J. Cairns, 1992 Mechanisms of directed mutation. Genetics 131: 783–789.

Foster, P. L., and J. M. Trimarchi, 1995a Adaptive reversion of an episomal frameshift mutation in *Escherichia coli* requires conjugal functions but not actual conjugation. Proc. Natl. Acad. Sci. USA 92: 5487–5490.

Foster, P. L., and J. M. Trimarchi, 1995b Conjugation is not required for adaptive reversion of an episomal frameshift mutation in *Escherichia coli*. J. Bacteriol. 177: 6670–6671.

Friedberg, E. C., G. C. Walker, W. Siede, R. D. Wood, A. Schultz *et al.*, 2006 *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.

Galhardo, R. S., R. Do, M. Yamada, E. C. Friedberg, P. J. Hastings *et al.*, 2009 DinB upregulation is the sole role of the SOS response in stress-induced mutagenesis in *Escherichia coli*. Genetics 182: 55–68.

Galitski, T., and J. R. Roth, 1995 Evidence that F plasmid transfer replication underlies apparent adaptive mutation. Science 268: 421–423.

Hastings, P. J., A. Slack, J. F. Petrosino, and S. M. Rosenberg, 2004 Adaptive amplification and point mutation are independent mechanisms: evidence for various stress-inducible mutation mechanisms. PLoS Biol. 2: e399.

He, A. S., P. R. Rohatgi, M. N. Hersh, and S. M. Rosenberg, 2006 Roles of *E. coli* double-strand-break-repair proteins in stress-induced mutation. DNA Repair (Amst.) 5: 258–273.

Hendrickson, H., E. S. Slechta, U. Bergthorsson, D. I. Andersson, and J. R. Roth, 2002 Amplification-mutagenesis: evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification. Proc. Natl. Acad. Sci. USA 99: 2164–2169.

Kim, S. R., G. Maenhaut-Michel, M. Yamada, Y. Yamamoto, K. Matsui *et al.*, 1997 Multiple pathways for SOS-induced mutagenesis in *Escherichia coli*: an overexpression of *dinB/dinP* results in strongly enhancing mutagenesis in the absence of any exogenous treatment to damage DNA. Proc. Natl. Acad. Sci. USA 94: 13792–13797.

Kim, S. R., K. Matsui, M. Yamada, P. Gruz, and T. Nohmi, 2001 Roles of chromosomal and episomal dinB genes encoding DNA pol IV in targeted and untargeted mutagenesis in Escherichia coli. Mol. Genet. Genomics 266: 207–215.

Kofoid, E., U. Bergthorsson, E. S. Slechta, and J. R. Roth, 2003 Formation of an F′ plasmid by recombination between imperfectly repeated chromosomal Rep sequences: a closer look at an old friend (F′(128) *pro lac*). J. Bacteriol. 185: 660–663.

Kuban, W., P. Jonczyk, D. Gawel, K. Malanowska, R. M. Schaaper *et al.*, 2004 Role of *Escherichia coli* DNA polymerase IV in in vivo replication fidelity. J. Bacteriol. 186: 4802–4807.

Kugelberg, E., E. Kofoid, A. B. Reams, D. I. Andersson, and J. R. Roth, 2006 Multiple pathways of selected gene amplification during adaptive mutation. Proc. Natl. Acad. Sci. USA 103: 17319–17324.

Layton, J. C., and P. L. Foster, 2003 Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in *Escherichia coli*. Mol. Microbiol. 50: 549–561.

Lederberg, J., and E. M. Lederberg, 1952 Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63: 399–406.

Lombardo, M. J., I. Aponyi, and S. M. Rosenberg, 2004 General stress response regulator RpoS in adaptive mutation and amplification in *Escherichia coli*. Genetics 166: 669–680.

Luria, S. E., and M. Delbrück, 1943 Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28: 491–511.

Maisnier-Patin, S., and J. R. Roth, 2015 The origin of mutants under selection: how natural selection mimics mutagenesis (adaptive mutation), pp. 97–115 in *Microbial Evolution*, edited by H. Ochman. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Maisnier-Patin, S., and J. R. Roth, 2016 The adaptive mutation controversy, pp. 26–36 in *Encyclopedia of Evolutionary Biology*, edited by R. M. Kliman. Academic Press, Oxford.

McKenzie, G. J., R. S. Harris, P. L. Lee, and S. M. Rosenberg, 2000 The SOS response regulates adaptive mutation. Proc. Natl. Acad. Sci. USA 97: 6646–6651.

McKenzie, G. J., P. Lee, M.-J. Lombardo, P. Hastings, and S. Rosenberg, 2001 SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Mol. Cell 7: 571–579.

Mittelman, D. (Editor), 2013 *Stress-Induced Mutagenesis*. Springer Science+Business Media, New York, NY.

Newcombe, H. B., 1949 Origin of bacterial variants. Nature 164: 150–151.

Ohmori, H., E. C. Friedberg, R. P. Fuchs, M. F. Goodman, F. Hanaoka *et al.*, 2001 The Y-family of DNA polymerases. Mol. Cell 8: 7–8.

Peters, J. E., I. M. Bartoszyk, S. Dheer, and S. A. Benson, 1996 Redundant homosexual F transfer facilitates selection-induced reversion of plasmid mutations. J. Bacteriol. 178: 3037–3043.

Ponder, R. G., N. C. Fonville, and S. M. Rosenberg, 2005 A switch from high-fidelity to error-prone DNA double-strand break repair underlies stress-induced mutation. Mol. Cell 19: 791–804.

Radicella, J. P., P. U. Park, and M. S. Fox, 1995 Adaptive mutation in *Escherichia coli*: a role for conjugation. Science 268: 418–420.

Reams, A. B., E. Kofoid, M. Savageau, and J. R. Roth, 2010 Duplication frequency in a population of *Salmonella enterica* rapidly approaches steady state with or without recombination. Genetics 184: 1077–1094.

Rosenberg, S. M., 2001 Evolving responsively: adaptive mutation. Nat. Rev. Genet. 2: 504–515.

Roth, J. R., and D. I. Andersson, 2004 Adaptive mutation: how growth under selection stimulates Lac$^+$ reversion by increasing target copy number. J. Bacteriol. 186: 4855–4860.

Roth, J. R., E. Kugelberg, A. B. Reams, E. Kofoid, and D. I. Andersson, 2006 Origin of mutations under selection: the adaptive mutation controversy. Annu. Rev. Microbiol. 60: 477–501.

Sano, E., S. Maisnier-Patin, J. Aboubechara, S. Quiñones-Soto, and J. R. Roth, 2014 Plasmid copy number underlies adaptive mutability in bacteria. Genetics 198: 919–933.

Shee, C., J. L. Gibson, M. C. Darrow, C. Gonzalez, and S. M. Rosenberg, 2011a Impact of a stress-inducible switch to mutagenic repair of DNA breaks on mutation in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 108: 13659–13664.

Shee, C., R. Ponder, J. L. Gibson, and S. M. Rosenberg, 2011b What limits the efficiency of double-strand break-dependent stress-induced mutation in Escherichia coli? J. Mol. Microbiol. Biotechnol. 21: 8–19.

Slechta, E. S., J. Harold, D. I. Andersson, and J. R. Roth, 2002a The effect of genomic position on reversion of a *lac* frameshift mutation (lacIZ33) during non-lethal selection (adaptive mutation). Mol. Microbiol. 44: 1017–1032.

Slechta, E. S., J. Liu, D. I. Andersson, and J. R. Roth, 2002b Evidence that selected amplification of a bacterial *lac* frameshift allele stimulates Lac$^+$ reversion (adaptive mutation) with or without general hypermutability. Genetics 161: 945–956.

Slechta, E. S., K. L. Bunny, E. Kugelberg, E. Kofoid, D. I. Andersson *et al.*, 2003 Adaptive mutation: general mutagenesis is not a programmed response to stress but results from rare coamplification of *dinB* with *lac*. Proc. Natl. Acad. Sci. USA 100: 12847–12852.

Thomason, L. C., J. A. Sawitzke, X. Li, N. Costantino, and D. L. Court, 2014 Recombineering: genetic engineering in bacteria using homologous recombination. Curr. Protoc. Mol. Biol. 106: 1.16.1–1.16.39.

Torkelson, J., R. S. Harris, M. J. Lombardo, J. Nagendran, C. Thulin *et al.*, 1997 Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation. EMBO J. 16: 3303–3311.

Wagner, J., P. Gruz, S. R. Kim, M. Yamada, K. Matsui *et al.*, 1999 The *dinB* gene encodes a novel *E. coli* DNA polymerase, DNA pol IV, involved in mutagenesis. Mol. Cell 4: 281–286.

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