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Mutation as a Stress Response and the Regulation of Evolvability

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Abstract

Our concept of a stable genome is evolving to one in which genomes are plastic and responsive to environmental changes. Growing evidence shows that a variety of environmental stresses induce genomic instability in bacteria, yeast, and human cancer cells, generating occasional fitter mutants and potentially accelerating adaptive evolution. The emerging molecular mechanisms of stress-induced mutagenesis vary but share telling common components that underscore two common themes. The first is the regulation of mutagenesis in time by cellular stress responses, which promote random mutations specifically when cells are poorly adapted to their environments, *i.e.*, when they are stressed. A second theme is the possible restriction of random mutagenesis in genomic space, achieved *via* coupling of mutation-generating machinery to local events such as DNA-break repair or transcription. Such localization may minimize accumulation of deleterious mutations in the genomes of rare fitter mutants, and promote local concerted evolution. Although mutagenesis induced by stresses other than direct damage to DNA was previously controversial, evidence for the existence of various stress-induced mutagenesis programs is now overwhelming and widespread. Such mechanisms probably fuel evolution of microbial pathogenesis and antibiotic-resistance, and tumor progression and chemotherapy resistance, all of which occur under stress, driven by mutations. The emerging commonalities in stress-induced-mutation mechanisms provide hope for new therapeutic interventions for all of these processes.

Keywords

adaptive mutation; evolution; mutation rates; error-prone DNA polymerases; stress responses; recombination; DNA repair; SOS response; σ^S ; RpoS; cancer; genome instability; microbial pathogenesis; antibiotic resistance

INTRODUCTION

Evolution results from natural selection acting on variability in populations, which ultimately stems from mutations. In large populations of mostly asexual cells, such as bacteria, and also somatic cells such as in developing cancers, mutation is the most important driving force behind evolution. But how do mutations, and thus how does evolution, occur? Is acquisition of mutations constant and gradual, a ticking clock, independent of selective environments and inexorable, and is selection the panning by the environment for pre-existing genetic gold? Or does mutation, and by extension evolution, occur in bursts stimulated by changing selective environments? The former mutation mode

has been recognized since the elegant demonstrations of Luria and Delbrück (1943) and the Lederbergs (Lederberg and Lederberg, 1952) that *Escherichia coli* virus-resistant and antibiotic-resistant mutants can arise before exposure to virus and antibiotics, respectively. Elegant mathematics describe a constant process dependent on cell generations, and perhaps the result of inevitable errors in DNA replication (Lea and Coulson, 1949; Luria and Delbrück, 1943) (called spontaneous generation-dependent mutagenesis). However, changing environments are often stressful to maladapted cells and organisms, and a growing literature indicates that the same cellular stress responses long appreciated to shore-up damaged cellular hardware (other than DNA) can, surprisingly and importantly, also remodel genomic software (DNA) by increasing rates of random mutagenesis. Stress-inducible mutagenesis mechanisms can potentially accelerate adaptive evolution in populations specifically when organisms are maladapted to their environments, *i.e.*, when they are stressed, and then return genomes to low mutation rates in rare adapted mutants that thrive in the new environment and so are stressed no longer.

Stress-induced genomic instability has been studied in a variety of strains, organisms, stress conditions and circumstances, in various bacteria, yeast, and human cancer cells. Many kinds of genetic changes have been observed, including small (1 to few nucleotide) changes, deletions and insertions, gross chromosomal rearrangements and copy-number variations, and movement of mobile elements, all induced by stresses. Similarly, diversity is seen in the genetic and protein requirements, and other aspects of the molecular mechanisms of the stress-induced mutagenesis pathways. In this review, we will survey several cases/ experimental systems in which genomic instability appears to be inducible by stress and focus on evidence bearing on the molecular mechanisms of stress-inducible mutagenesis in each. We will see that unlike, *e.g.*, DNA replication and transcription, there is no single universal molecular mechanism of stress-inducible mutagenesis, but rather, it is a collection of mechanisms with similarities and differences. The good news is that there are several common components and broad themes evident in these mechanisms, which we shall highlight. For previous reviews of the history of ideas and early experiments in this field, see Foster (1999) and Rosenberg (2001).

In our view, the most interesting aspects of the emerging mechanisms of stress-induced mutagenesis are those either demonstrating or implying its regulation. We shall review many instances in which mutagenesis is coupled to cellular stress responses, which regulate mutagenesis in time. Stress responses restrict mutagenesis to times of stress, when, by definition, cells or organisms are poorly adapted to their environments. A second theme is potential restriction/localization of mutagenesis in genomic space *via* coupling of mutagenesis to localized processes, such as double-strand-break repair or transcription. This may localize mutagenesis within genomes, potentially reducing accumulation of non-adaptive mutations in rare adaptive mutants, and also potentially facilitating concerted evolution within genes or gene clusters.

The general picture across many strains, organisms and stress conditions is of diverse mechanisms with common elements and themes, which suggests multiple independent evolutions of stress-induced mutagenesis, that have probably been selected in changing environments, lost in static ones, and “re-invented” repeatedly.

Is There A Controversy?

It has been argued (Roth *et al.*, 2006) that the existence of stress-inducible mutagenesis mechanisms is controversial. In our view, there is currently no real controversy. We shall touch on a specific mathematical model proposed to account for mutagenesis without stress-inducibility in one *E. coli* model (the Lac assay), and note several lines of experimental evidence that have superseded the mathematical model, demonstrated control of

mutagenesis by stress responses, and provided a clear picture of a molecular mechanism of stress-induced mutagenesis. We compare this with several other strains, stresses, and organisms, illustrating the commonalities.

The Important Questions

In our view, the most important current questions in this field are not whether stress-induced mutagenesis occurs—the evidence for this being substantial and widespread—but rather what are the specific molecular mechanisms of stress-induced mutagenesis in many different experimental and natural systems/circumstances, organisms, and stresses? What are the common themes and components in these mechanisms, and what do they indicate about how evolution works? Can, and how might, such mechanisms be selected? We suggest that understanding of the common components in mechanisms of stress-induced mutagenesis will allow the design of anti-evolution drugs that will short-circuit mutagenesis responses that drive evolution of antibiotic resistance, microbial pathogenesis strategies, tumor progression, radiation- and chemotherapy-resistance mechanisms, all of which are driven by mutation and selection under stress.

MUTAGENESIS AND EVOLVABILITY

Constitutive Mutators Win in Competitive Environments

The concept of evolvability relates to the intrinsic capacity of organisms to evolve, and reflects the extent of genetic variability in populations (Sniegowski and Murphy, 2006). Most mutations in well adapted organisms are deleterious or neutral, and consequently low, constant mutation rates are thought to be advantageous in the long term. Although generation-dependent mutation rates are mostly low and remarkably constant between different organisms (Drake *et al.*, 1998), bacterial mutants with constitutively high mutation rates (constitutive mutators) are fitter in competition experiments with non-mutators. This is true whether the mutation rate is elevated by defects in mismatch repair (MMR) (Table 1) (Gibson *et al.*, 1970), the post-replicative error-correction system (Kunkel and Erie, 2005), or by transposon mobility (Chao *et al.*, 1983), in competition between bacterial strains colonizing mice (Giraud *et al.*, 2001), in repetitive rounds of selection in the lab (Mao *et al.*, 1997), and is predicted by computer modeling (Taddei *et al.*, 1997b) in which mutators increase the fitness of populations during the adaptation period, then burden the adapted cells with deleterious mutations after adaptation. In agreement with this idea, constitutive mutator mutants, most of which are MMR-defective, arise spontaneously and are selected in long-term cultures (Sniegowski *et al.*, 1997), and are found much more often than expected in natural populations of pathogenic (LeClerc *et al.*, 1996) and commensal (Matic *et al.*, 1997) *E. coli* and *Salmonella enterica*, constituting up to 1% of individuals, and can be as prevalent as 20% of pathogenic *Pseudomonas* isolated from the lungs of patients undergoing antibiotic therapy (Blazquez, 2003). The mutator alleles “hitchhike” along with favorable mutations that they generate and so are selected indirectly (“second-order” selection).

Genomic analyses suggest that there has been alternating selection for and against the functions of MMR genes during evolution (“periodic” selection) (Denamur *et al.*, 2000); the MMR gene sequences are highly mosaic, within species, showing sequence characteristics of multiple species, indicating that they have been subject to multiple rounds of loss and reacquisition through horizontal transfer. This fits with the idea that higher mutation rates are selected in some, presumably variable, environments, but eventually become disadvantageous in well-adapted populations, where reacquisition of MMR capability is then selected.

These observations of selective sweeps driven by constitutive mutator mutants illustrate the point that selection favors high mutation rates in competitive environments, and imply that

elevated mutation rate is under alternating (periodic) positive and negative selection. Stress-inducible mutagenesis allows the alternation between mutator and stable states to occur controlled by stress responses. Computer modeling and studies of natural isolates described below lead to similar conclusions concerning selection for this, we will suggest, somewhat more refined evolutionary strategy.

STRESS-INDUCED MUTAGENESIS IN NATURAL BACTERIAL POPULATIONS: WIDESPREAD AND SELECTED

Like constitutive mutators, strains that increase mutation rate transiently during stress are found in natural environments, but appear to be far more abundant than constitutive mutators. Among 787 natural isolates of *E. coli* from a wide range of habitats worldwide, an astonishing >80% exhibited stress-inducible mutagenesis (Bjedov *et al.*, 2003) (Figure 1). The authors analyzed mutations arising in bacterial colonies aged for 1 or 7 days. Rifampicin-resistant mutants increase in frequency among cells in the colony, which grows little after 1 day. Because the rifampicin-resistant mutants, which carry base-substitution mutations in the gene encoding RNA polymerase, have no growth advantage in these colonies (demonstrated with rigorous control experiments), this indicates an increase in rates of random mutagenesis in the old, stressed colonies. This finding was confirmed with other mutation assays. Several important observations and conclusions come from this study:

First, the capacity for stress-inducible mutagenesis is prevalent in nature.

Second, the magnitude of stress-inducible mutability varied from a few-fold to more than a thousand-fold in the positive strains (Figure 1), revealing variability in the factors modulating and mechanisms of stress-induced mutagenesis in the different strains.

Third, the strengths of the stress-inducible mutagenesis phenotypes are negatively correlated with constitutive, generation-dependent mutation rates. This implies that strains adapt to changing environments *via either* a constitutive *or* a stress-inducible mutator pathway (suggested previously, Rosenberg *et al.*, 1998) implying that, like constitutive mutator phenotype, stress-inducible mutability is selected.

Fourth, stress-inducible mutagenesis phenotypes are better correlated with ecological niche than with phylogenetic relatedness of strains, suggesting a recent and active role of the environment in modulating the magnitude of this stress response. This implies that, either directly or indirectly, stress-induced mutagenesis is selected (suggested also by Radman, 1974; Echols, 1981).

Fifth, computer modeling shows that stress-inducible-mutagenesis ability can be selected through the beneficial mutations that are generated, and the mutator pathway or genes hitchhike along with them. This second-order selection for beneficial mutations and the hitchhiking of the mutator genes is as reviewed above for constitutive mutators, and is also shown by the negative correlation between constitutive and stress-inducible mutability in the study of Bjedov *et al.* (2003).

The mechanism of mutagenesis in one of the natural isolates from this study is discussed below (Mutagenesis in Aging Colonies) after many of the DNA repair and synthesis components are introduced in the following sections. Table 1 also introduces components discussed.

MECHANISMS OF STRESS-INDUCED MUTAGENESIS BEGINNING WITH CLASSICAL SOS MUTAGENESIS

Most of the examples discussed in this review concern mutagenesis induced during growth-limiting stress conditions to which cells are maladapted: selective environments such as starvation, hypoxia, antibiotic or other stresses. Some of the mutations produced can adapt the cell to the stress condition. These mechanisms have also been called “adaptive mutagenesis” and historically have been discussed separately from classical SOS mutagenesis, which is induced when DNA is damaged (but see Cirz and Romesberg, this volume). This historical separation might reflect a bias that DNA damaging stress might not reflect selective environments *per se*. Both Radman (1974) and Echols (1981) suggested that DNA-damage stress could reflect selective environments, and that SOS mutagenesis might speed evolution specifically during stress producing better adapted variants then. In agreement with their ideas, and because of the similarity between classical SOS mutagenesis and some of the previously-named “adaptive” mutagenesis mechanisms discussed below, we group them together here. The mechanism of stress-induced mutagenesis understood in greatest detail is classical SOS mutagenesis. Two excellent recent reviews cover the SOS DNA-damage response (Friedberg *et al.*, 2006) and present the history and mechanisms of SOS mutagenesis (Schlacher and Goodman, 2007), current understanding of which is outlined in simplified form here.

SOS, the prototypic bacterial DNA-damage stress response, is induced when damage to DNA and/or stalled replication forks cause exposure of single-strand (ss)DNA, the SOS inducing signal. ssDNA becomes coated with RecA protein which together form a nucleoprotein filament. This filament of “activated” RecA can facilitate the autoproteolytic cleavage of LexA, a transcriptional repressor, leading to upregulation of roughly 40 genes involved in DNA repair, DNA synthesis past damaged bases, cell-division arrest, and other functions.

SOS mutagenesis is a consequence of the action of specialized, low-fidelity DNA polymerases induced by the SOS response. “Targeted” SOS mutagenesis occurs when an otherwise replication-blocking lesion in DNA is traversed by a special translesion-synthesis (TLS) DNA polymerase which may insert an incorrect base(s). It is targeted to the damaged DNA. In *E. coli*, DNA polymerase (Pol)V of the Y family of specialized, error-prone DNA polymerases (Table 1), encoded by the *umuC* and *umuD* genes, carries out TLS across from pyrimidine dimers and causes targeted SOS mutagenesis in response to UV damage. TLS allows the DNA to be replicated, and so become double-stranded, which is a prerequisite to repair and so is an important survival mechanism.

Targeted mutagenesis is sometimes regarded as an unavoidable consequence of this necessary damage-tolerance pathway. However, this view might be a simplification of the biology underlying SOS mutagenesis, as first appreciated by Radman (1974) and Echols (1981) (before TLS mechanisms were understood). We now know that TLS events can occur in a high-fidelity manner, as exemplified by *E. coli* DinB (Pol IV) action (Table 1) on guanine adducts, but causes SOS “untargeted” mutagenesis when operating on undamaged template DNA (reviewed by Cirz and Romesberg, 2007; Schlacher and Goodman, 2007). That is, it appears to be possible for TLS to occur without high-frequency mutagenesis, and these TLS polymerases cause mutations when not engaged in TLS, suggesting that mutagenesis might, as Radman and Echols suggested, itself be a selected feature of the SOS response. Regardless of whether or not this is so, both targeted and untargeted mutations are an important biological consequence of the induction of the SOS response.

The SOS response features prominently in many of the experimental systems showing an increase in mutagenesis in response to a variety of stresses, collectively called stress-induced mutagenesis in this essay (see below). Importantly, SOS-controlled inducible mutagenesis is a general phenomenon in bacterial physiology. Several bacterial species have been demonstrated to undergo SOS mutagenesis, and the vast majority of bacterial genomes encode one or more members of the Y-family of specialized DNA polymerases, which includes Pol V and DinB/PoII V discussed here.

THE *E. COLI*/LAC SYSTEM: REGULATION BY TWO STRESS RESPONSES AND THE COUPLING TO DNA BREAK REPAIR

After classical SOS mutagenesis, the most detailed picture of a mechanism of stress-induced mutagenesis has come from studies of the *E. coli* Lac assay, in which current evidence indicates that stress-induced mutagenesis results from a stress-response-controlled switch in the fidelity of DNA double-strand break repair (DSBR) *via* homologous recombination (HR), from a high-fidelity mechanism in growing cells, to an error-prone, mutagenic version of that mechanism during starvation, controlled by two stress responses (Ponder *et al.*, 2005).

In the Lac assay, an *E. coli* strain carrying a *lacI-lacZ* fusion gene with a +1 frameshift mutation in *lacI* in an F' conjugative plasmid, and a deletion of the chromosomal *lac* operon, is grown in a non-lactose carbon source then spread onto lactose plates on which only cells that become Lac⁺ form colonies (Figure 2) (Cairns and Foster, 1991). Generation-dependent Lac⁺ mutants that arose prior to starvation on lactose form visible colonies after ~2 days of incubation. Stress-induced Lac⁺ mutant colonies accumulate continuously from day 3 onward (Figure 2) from a population of stressed starving cells that show no net proliferation (Cairns and Foster, 1991).

The stress-induced mutants include two types (Figure 2): Lac⁺ “point mutants” carry a compensatory frameshift mutation in *lac* (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994), whereas *lac*-amplified cells, carry a tandem array of 20–50 or more repeats of a region of 7–134 kb including the *lac* gene (Hastings *et al.*, 2000; Kugelberg *et al.*, 2006; Powell and Wartell, 2001; Slack *et al.*, 2006). The *lac* frameshift allele is leaky, producing ~1% of the beta-galactosidase activity of wild-type (Foster, 1994) such that multiple copies provide sufficient beta-galactosidase for growth on the lactose medium. *lac*-amplified clones are a minority in earlier days, but constitute up to 40% of colonies appearing on days 8 and 9 (Figure 2). Both the point mutants (Cairns and Foster, 1991; McKenzie *et al.*, 1998) and *lac*-amplified clones (Hastings *et al.*, 2000) result from genetic changes that occur after exposure to starvation (not slow-growing pre-existing mutants). Similarly, formation of both requires the RpoS-controlled general- or starvation-stress response, which is expressed in stationary-phase (Layton and Foster, 2003; Lombardo *et al.*, 2004) (Table 1).

Because RpoS is required specifically for formation of these mutants (not merely for survival or colony formation under the conditions of the experiment, or for generation-dependent reversion) (Lombardo *et al.*, 2004), we feel that these must now legitimately be called “stress-induced” point mutations and amplifications. Previous names for stress-induced mutagenesis (such as adaptive mutagenesis, and others reviewed by Rosenberg, 2001) suggest an artificial distinction from classical stress-induced SOS mutagenesis, which we think are more usefully grouped together.

The point mutagenesis mechanism will be reviewed here and the mechanism of stress-induced amplification of *lac* in a separate section (and in detail by Hastings, 2007).

Double-Strand-Break Repair Proteins, Two Stress Responses, the DinB Error-Prone DNA Polymerase and Limiting Mismatch Repair Function

Stress-induced Lac⁺ point mutations are different from generation-dependent Lac⁺ reversions and form *via* a different mechanism controlled by two stress responses. The following are aspects of the mechanism of stress-induced Lac⁺ point mutagenesis and will be part of common themes in many stress-induced mutagenesis mechanisms reviewed below:

Recombination and DSBR—The *E. coli* RecA (Cairns and Foster, 1991; Harris *et al.*, 1994), RecBCD (Harris *et al.*, 1994), and RuvABC (Foster *et al.*, 1996; Harris *et al.*, 1996) proteins (Table 1), used in DNA DSBR *via* HR, are required. When these genes are inactivated, generation-dependent (day-2) Lac⁺ colonies are present as usual, but late-arising (stress-induced) mutants are reduced ≥ 10 -fold. RecF HR protein is also required for most point mutation (McKenzie *et al.*, 2000). Some of these proteins play roles in induction of the SOS response, in addition to their roles in HR, but all are required in stress-induced point mutation for roles other than or in addition to SOS induction, as seen by the down phenotypes of these mutants in cells with SOS constitutively induced (He *et al.*, 2006).

DinB Error-Prone DNA Polymerase—Formation of most (85%) of Lac⁺ stress-induced point mutants requires the DinB error-prone DNA polymerase (McKenzie *et al.*, 2001), whereas generation-dependent mutagenesis does not (McKenzie *et al.*, 2001, 2003; Wolff *et al.*, 2004). DinB, a Y-family DNA polymerase (Table 1), has homologues in all three domains of life (Ohmori *et al.*, 2001). Most, Y-family DNA polymerases, including DinB (Jarosz *et al.*, 2006), are poorly processive TLS polymerases that allow the replisome to pass over otherwise replication-blocking lesions, promoting cell survival. Most, including DinB, are relatively high-fidelity (non-mutagenic) and insert the correct base(s) opposite their cognate lesions, but are highly error-prone and mutagenic on undamaged template DNA (Bjedov *et al.*, 2007; Jarosz *et al.*, 2006; Nohmi, 2006). Humans have four DinB homologues including one orthologue, DINB1 (Pol kappa), which is upregulated and promotes genome instability in small-cell lung cancers (Bavoux *et al.*, 2005; Wang *et al.*, 2001). The other three (Pol eta, Pol iota and REV1) all play some role in developmentally programmed somatic hypermutation of immunoglobulin genes (reviewed by Diaz and Lawrence, 2005). *E. coli* DinB is normally present in growing cells, but is transcriptionally upregulated about 10-fold by the SOS response (Kenyon and Walker, 1980; Kim *et al.*, 1997) and about twofold by the RpoS stress response (Layton and Foster, 2003). DinB is also upregulated independently of the SOS response in response to beta-lactam antibiotics, which target synthesis of cell wall components (Perez-Capilla *et al.*, 2005), though beta-lactam antibiotics induce the SOS response as well (Miller *et al.*, 2004). Thus, DinB is upregulated by a variety of stressors. Cells lacking the GroE chaperone show reduced levels of DinB protein, indicating that it might also be regulated by its rate of degradation (Layton and Foster, 2005). DinB is very likely to be the DNA polymerase whose errors persist to become stress-induced Lac⁺ point mutations, because the errors spectrum of DinB *in vitro* (Tang *et al.*, 2000; Wagner *et al.*, 1999) and *in vivo* (Wagner and Nohmi, 2000) is predominantly -1 deletions in small mononucleotide repeats, which constitute nearly all stress-induced Lac⁺ point mutations (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994), and also some base substitutions, predominantly G:C to T:A transversions (which would not be observed when selecting reversion of a frameshift allele).

SOS—the DNA Damage Stress Response—Induction of one or more genes of the SOS/LexA DNA damage response regulon (Table 1) is required for most point mutagenesis, roughly the same fraction as requires DinB (McKenzie *et al.*, 2000,2001). DinB is a probable candidate for the SOS-induced component required for stress-induced point

mutagenesis, but this has not been demonstrated. Whereas point mutagenesis requires SOS and DinB, *lac* amplification requires neither (McKenzie *et al.*, 2000,2001).

The RpoS General/Starvation-Stress Response—This response is required for virtually all stress-induced Lac reversion (Lombardo *et al.*, 2004). It is not known which component(s) of this stress response are required, but as we will see below, RpoS in some way licenses the use of DinB in DSB_R during stress, making DSB_R become mutagenic specifically at that time (Ponder *et al.*, 2005). This might work directly *via* the observed ~two-fold upregulation of DinB by RpoS (Layton and Foster, 2003), or it might be *via* some other mechanism. DinB does not contribute to amplification (McKenzie *et al.*, 2001), which requires RpoS (Lombardo *et al.*, 2004), and so clearly RpoS controls at least one other factor that promotes genome instability under stress. At bare minimum, the important role of RpoS in mutagenesis in this system indicates that mutagenesis is a stress response, regulated temporally by RpoS (and SOS).

Mismatch Repair Becomes Limiting—Mismatch repair (MMR) is a highly and widely conserved DNA repair pathway that repairs mispaired bases and 1-few nucleotide insertion/deletion heteroduplexes arising from replication errors or other sources, increasing the fidelity of DNA replication 100 to 1000-fold (Kunkel and Erie, 2005) (Table 1). To begin repair, *E. coli* MutS and MutL proteins recognize the mispaired bases, and their eukaryotic orthologues (called MSH1-MSH6 for MutS homologues, and MLH1-MLH3 and PMS1 and PMS2, the MutL homologues) do similarly. Cells lacking MMR proteins are constitutive mutators (discussed above). The function of the MMR system becomes limiting transiently for mutation avoidance during stress-induced Lac⁺ mutagenesis *via* a limitation in functional MutL, as shown in experiments in which overproduction of MutL specifically reduces stress-induced and not generation-dependent Lac⁺ point mutagenesis (Harris *et al.*, 1997, 1999) (or homologous recombination, Harris *et al.*, 1999). The mechanism of MutL functional limitation is not understood. MutL protein levels do not decrease in most starving cells (Feng *et al.*, 1996; Harris *et al.*, 1997), though they might possibly do so in a cell subpopulation giving rise to stress-induced point mutants (discussed below). One possibility is that excess errors made by DinB polymerase titrate/saturate MutL, transiently exhausting MMR capacity (Harris *et al.*, 1997). Overproduction of DinB has such a titrating effect on MutL (Wagner and Nohmi, 2000), as do other situations in which cells experience a heavy burden of DNA polymerase errors, including in an error-prone mutant of the replicative DNA Pol III (Schaaper and Radman, 1989). Stationary-phase conditions decrease the levels of MutS and MutH MMR proteins (Feng *et al.*, 1996; Tsui *et al.*, 1997), but MutS is not functionally limiting for mutation avoidance in the Lac system (Harris *et al.*, 1997) and MutH has not been tested. MutS does become limiting during other stress situations discussed below.

A Hypermutable Cell Subpopulation—Cells that have experienced a stress-induced Lac⁺ reversion show ~50-fold higher frequencies of unselected secondary mutations affecting many genes throughout their genomes (Godoy *et al.*, 2000; Rosche and Foster, 1999; Torkelson *et al.*, 1997), compared with either unstressed cells or their stressed Lac⁻ neighbor cells from the same selection plates. This implies first, that mutagenesis is not specifically targeted to the *lac* gene or surrounding DNA (as had been hypothesized in early “directed mutation” models, e.g., Cairns *et al.*, 1988). Mutagenesis of unselected non-*lac* genes was also shown in the Lac⁻ stressed cells by direct observation of DSB_R-protein-dependent reversions of a *tetA* frameshift allele next to *lac* in the F' (Foster, 1997), and of the same allele in the chromosome (Bull *et al.*, 2001). The latter was also shown to be DinB-dependent. Second, the 50-fold higher incidence of chromosomal mutations among Lac⁺ point mutants compared with the whole population of Lac⁻ stressed cells on the plate

demonstrates that a subpopulation of cells experiences increased mutagenesis relative to the main Lac⁻ population. This subpopulation is transiently mutable (not composed of constitutive mutator mutants) as described in the following section. The possible origin of the hypermutable cell subpopulation (HMS) and the importance of its contribution to most Lac⁺ stress-induced mutagenesis will be considered below. We will suggest a model in which the HMS is differentiated based on coincident induction of the SOS and RpoS stress responses.

Transience of Stress-Induced Mutability—Once cells have acquired a Lac⁺ mutation that restores their ability to grow, they display normal mutation rates in various mutagenesis assays (Longerich *et al.*, 1995), and this is also true for those with evidence of secondary mutations, demonstrating that the HMS is transient (Godoy *et al.*, 2000; Rosche and Foster, 1999; Torkelson *et al.*, 1997). Moreover, Rosenberg *et al.* (1998) reintroduced the *lac* frameshift allele into cells that had become Lac⁺ via stress-induced mutagenesis and showed that, when recycled through a second round of stress-induced-mutagenesis, these were no better than the original Lac⁻ population at generating stress-induced Lac reversions. Thus, as expected for a process controlled by regulated gene expression of two transient stress responses (RpoS and SOS), stress-induced point mutability is a transient state.

A Stress-Response-Controlled Switch from High-Fidelity to Error-Prone Double-Strand Break Repair

The basic model that stress-induced point mutagenesis results from error-prone-DSBR via HR (Harris *et al.*, 1994) (a version shown in Figure 3) has received strong direct support (Ponder *et al.*, 2005). Additionally, the same study made the important discovery that the RpoS stress response controls a switch from a high-fidelity DSBR mechanism (HR) to an error-prone version of that mechanism leading to mutagenesis specifically under stress (Ponder *et al.*, 2005). In this study, the authors introduced a single genomic DSB with *I-SceI* endonuclease during stress-induced mutagenesis and found the following (Ponder *et al.*, 2005):

1. A DSB made near *lac* more than substitutes for the genetic requirement of stress-induced mutagenesis for *TraI*. *TraI* is an F-encoded single-strand endonuclease that nicks constantly at the F origin of transfer. Previous studies showed that some function(s) of the conjugative transfer operon were required for efficient stress-induced mutagenesis of genes in the F' (Foster and Trimarchi, 1995; Galitski and Roth, 1995) and that these could be substituted by a phage-encoded single-strand endonuclease acting in the F, implying that nicking, not transfer, was the relevant component (Rodriguez *et al.*, 2002). Single-strand nicks were proposed to be precursors to DSBs that promoted stress-induced *lac* reversion by error-prone DSBR (Kuzminov, 1995; Rosenberg *et al.*, 1995). The results of Ponder *et al.* (2005) demonstrate that the requirement for *TraI* can be explained by the need to form DSBs. Lower-frequency spontaneous DSBs are thought to be the basis of the roughly 20-fold lower frequencies of DSBR-protein and *DinB*-dependent stress-induced mutagenesis in the *E. coli* chromosome as compared with the F' (Bull *et al.*, 2001).
2. Single DSBs made near *lac* stimulated the rate of mutation by ~6000 fold in a *traI* mutant strain. Both point mutants and *lac*-amplified clones were stimulated. Thus, DSBs promote both mechanisms. The *I-SceI*-promoted point mutations require RpoS, *DinB*, and the same HR-DSBR proteins as normal stress-induced point mutagenesis, and have similar sequences, all indicating that a similar or the same mutation mechanism as usually operates has been elevated 6000-fold (rather than a new mutation pathway activated).

3. I-*SceI*-induced DSBs promoted mutation ~6000-fold when made locally near *lac*, but only ~threefold when induced in different molecule in the cell (a plasmid). This shows that their main stimulatory effect is direct and local, suggesting that it occurs *via* generation of breaks that undergo error-prone repair. The small global stimulation probably occurs *via* enhanced SOS induction.

4. Compellingly, when DNA near *lac* carried DNA sequences identical to one end of the linearized plasmid that previously stimulated mutagenesis only weakly, then linearization of that plasmid promoted efficient mutation in the F' at *lac*. This shows that homologous interaction between a DSE in one molecule and DNA in another, near *lac*, promoted mutation. Figure 3 illustrates how this is thought to occur during repair synthesis primed in acts of HR-DSBR.

5. Moreover, delivering DSBs near to a chromosomal *lac* allele in a different strain background can stimulate mutation, showing that availability of DSBs is the limiting event for chromosomal mutations (Ponder, 2006).

These data provide strong direct support for models in which stress-induced point mutations result from errors made during homologous recombinational DSBR. Additionally, they led to the discovery that a switch in the fidelity of HR-DSBR promotes mutation specifically under stress.

The RpoS-Controlled Switch to Mutagenic DSBR

6. Ponder *et al.* (2005) showed that I-*SceI*-generated DSBs promote local DinB-dependent mutagenesis only in stationary phase, not during exponential growth (Figure 4A). Liquid cultures were grown and allowed to go stationary with continuous low-level I-*SceI* induction, and reversions of a *tetA* gene (near *lac* and the cutsite in the F') were assayed at time points throughout. Only after stationary phase were the DSBs mutagenic (Figure 4A), though their formation and repair were measurable throughout. Again, the mutation was wholly DinB-dependent (Figure 4A).

7. However, when RpoS was expressed weakly from a plasmid, then, even during mid-exponential phase, the induced DSBs promoted mutagenesis (Figure 4B), which was both DinB- and enzyme-and-cut-site-dependent. RpoS did not alter the efficiency of repair (Ponder *et al.*, 2005), merely its fidelity. This result demonstrates that RpoS controls a switch that makes the process of DSBR *via* HR mutagenic during stress, and implies that DSBR is mutagenic in stationary phase because RpoS is expressed then.

REGULATION OF MUTAGENESIS AND EVOLVABILITY

Stress Responses Limit Mutagenesis in Time: A Strategy that Enhances Evolvability

Though long appreciated to promote adaptation to stress by protection of cellular hardware, results from the Lac system and many others now indicate that stress responses in addition to SOS also promote changes to genomic software. Most random mutations are neutral or deleterious. Control of stress-induced point mutagenesis by stress responses (SOS and RpoS, and others discussed below) limits the dangerous experiment of random mutagenesis specifically to times when cells are poorly adapted to their environments, by definition, when they are stressed (Lombardo *et al.*, 2004; Ponder *et al.*, 2005). Computer modeling indicates that as for constitutive mutators (reviewed above), the strategy of transient stress-inducible mutator ability should also enhance evolvability and adaptive evolution in competitive environments (Bjedov *et al.*, 2003). In this regard, it is noteworthy that the most widely conserved (or independently evolved) aspect of various stress-induced mutagenesis programs is the requirement for one or more cellular stress responses, in bacteria, RpoS being the most frequent, and SOS less so but also frequent (Table 2, and reviewed below).

Evolvability and the Regulation of Mutagenesis in Genomic Space via Its Coupling with DNA Repair

The coupling of mutagenesis to DSBR reported by Ponder *et al.* (2005) may have profound implications for the evolvability of organisms that do this, because of its potential to limit stress-induced mutagenesis in genomic space. Unlike housekeeping DNA replication, the replication primed by DSBR is localized to regions near DSBs or between a DSE and the terminus of replication. Ponder *et al.* (2005) saw that DSBs provoke stress-induced mutations well in the same molecule (*in cis*, ~6000X) but poorly *in trans*, in a different molecule (3X), and they suggested that the coupling of mutagenesis to DSBR might also localize mutagenesis within the bacterial chromosome. Whether DSBs do localize mutagenesis *within* molecules has not yet been tested, but if so, such localization could greatly enhance the adaptive value of the mutagenesis mechanism in two ways. Ponder *et al.* (2005) suggested that the DSBs that fuel chromosomal mutagenesis might be random spontaneous DSBs that potentially occur anywhere in the genome. In a large population of cells, therefore, the whole genome would be mutagenized, but in any given cell, only localized regions near one or more DSBs in the cell would be mutagenized. Thus, in rare cells that acquire an adaptive mutation, the chance of having acquired deleterious mutations in distant regions in the genome is much reduced. This principle is seen in somatic hypermutation of immunoglobulin genes and several other examples reviewed below.

Potential for Clustering of Mutations—Second, if localized mutated zones are heavily mutagenized, these might acquire multiple mutations, which could potentially promote concerted evolution within genes or gene clusters, such as operons. Multiple mutations are usually required for evolution of new protein functions (e.g. Camps *et al.*, 2003). Ninio (1996, 2000) has discussed the potential evolutionary benefit of clustering mutations in genomic space for concerted evolution within genes, particularly for simultaneous acquisition of compensatory mutations that can ameliorate negative effects of otherwise beneficial function-altering mutations. He suggests that localized clustering of mutations associated with DNA repair processes is a possible selective advantage of constitutively “error-prone” DNA repair mechanisms and meiotic recombination. We suggest that mechanisms that promote clustered mutagenesis could be yet another potential reason for selection of clustering of functionally related genes in genomes, including in operons. In fact, during the course of the stress-induced mutagenesis experiments in the *Lac* system, mutations accumulating in the *codAB* genes next to *lac* were shown not to behave as independent events from *Lac*⁺ reversion, whereas mutations at an unlinked site did, suggesting that mutations do indeed occur in clusters (Bull *et al.*, 2000). This phenomenon is echoed in several other mutagenesis mechanisms and circumstances discussed below and in Table 2. Moreover, local clustering of mutations has been argued to be more widespread than expected, and likely to arise from states of transient hypermutation (Drake *et al.*, 2005; Drake 2007). In a study of the sequences of mutations and DNA polymerase errors made *in vitro*, Drake and colleagues show that the frequencies of multiple events are higher than predicted from the frequencies of singles, demonstrating clustering of mutations in diverse species and circumstances. More recently, evidence for mutational clustering in “showers” of local simultaneous mutations has been obtained in mice (Wang *et al.*, 2007). The *Lac* system suggests a possible mechanism (coupling of mutagenesis to DNA repair) for mutational clustering.

Hypermutable Cell Subpopulation: Significance and Model for Its Origin

A central question is, how is the HMS differentiated? The coupling of stress-induced point mutagenesis to (at least) two different stress responses, SOS and RpoS, means that cells will not experience increased mutation rates until (at least) two different stress inputs are sensed simultaneously.

Model—We suggest that the overlay of the SOS and RpoS stress responses leads to differentiation of the HMS from the main population, and that this occurs as follows (Figure 5). Our laboratory showed recently that the SOS response is induced from spontaneous DNA damage in about 1% (steady-state levels) of cells in growing cultures, some from spontaneous DNA DSBs/DSEs and others from other spontaneous lesions (Pennington and Rosenberg, 2007). Some of the SOS-induced cells will, we suggest, both carry one or more DSB/DSEs and be induced at the right level of this graded response (Friedberg *et al.*, 2006) to differentiate them into cells able to become HMS cells. However, we suggest that they will not *become* HMS cells unless the RpoS response is activated. This occurs when the growing population enters stationary phase (illustrated Figure 5) but should also occur in response to other RpoS-inducing stresses (reviewed above and Table 1). As discussed above, the SOS-regulated contribution to hypermutability is likely to be its 10-fold upregulation of DinB. The RpoS contribution licenses the use of DinB during DSBR (Ponder *et al.*, 2005, reviewed above), though whether this occurs merely by the RpoS twofold upregulation of DinB levels (Layton and Foster, 2003), or by some other means, remains to be determined.

SOS Induction Properties Can Help Explain HMS Properties—If correct, our model can potentially explain previous perplexing data concerning the HMS. A modern view of the SOS response shows that it is not induced as an all-or-none phenomenon; instead it is heterogeneous in the population, as seen in two studies using SOS promoters fused to *gfp*. Friedman *et al.* (2005) saw that SOS-gene expression occurs in waves of induction and repression, and that the cells in a single culture vary, those with greater induction having more cycles of the waves—staying SOS-induced for longer. Pennington and Rosenberg (2007) sorted spontaneously SOS-induced (green) cells by fluorescence-activated cell sorting, and found vastly different times to resumption of proliferation after the response, some forming colonies normally and others in a senescence-like state of viability without proliferation for many hours or days. Also, they found heterogeneity in the SOS response in cells induced due to a single, repairable chromosomal I-*SceI*-generated DSB: ~27% induced SOS detectably and the rest did not. These results indicate that not all DSB carriers may necessarily induce SOS (relevant to amplification, below), and suggest that those that do may also have a heterogeneous response. All of these results imply that the lengths of time that individual HMS cells spend with SOS induced (and thus in the HMS) is likely to vary considerably.

We suggest that this variability in the length of time that individual cells are induced underlies the observed heterogeneity in accumulation of secondary mutations among Lac⁺ cells: some of the point mutants arising during the experiments have a much higher frequency of unselected mutations than others (Rosche and Foster, 1999; Torkelson *et al.*, 1997). We suggest (per Bull *et al.*, 2001) that this results from individual cells remaining in the HMS for varying times, as dictated by their variable times remaining SOS induced (illustrated in Figure 5), in addition to their variable time before a Lac⁺ mutation is encountered, which would end the HMS state when cells grow and turn off RpoS. Also, as predicted by DSBR models, the extent of genome-wide mutagenesis is expected to vary due to uneven distribution of DSBs in cells in the population. In this way, a single differentiated subpopulation (undergoing the SOS and RpoS stress responses) may include cells with widely varying frequencies of secondary mutations, as a consequence of both different lengths of time spent in the transient mutable state and the limiting availability of DSBR events in the genome.

An alternative explanation is that only some stress-induced Lac⁺ point mutants arise from the HMS (Rosche and Foster, 1999). Those authors suggest that ~90% of Lac⁺ colonies (including those with no detectable secondary mutation) do not and ~10%, including those

with visible secondary mutations, do. In that model, those in the HMS all remain in it for the same length of time and therefore generate about the same number of secondary mutations per cell, but many stress-induced Lac⁺ clones were never in it and arose *via* some other means. We find the first model to be simpler and more harmonious with current understanding of the SOS response and the role of DSBR in stress-induced mutagenesis; however, more experimental work is needed to distinguish these models definitively. Our recent work provides support for the first hypothesis by showing first, that Lac⁺ mutations from demonstrated HMS descendants (cells with a detected secondary mutation) are indistinguishable from most Lac⁺ mutations implying a common origin; and second, that secondary mutations cannot be uncoupled from most Lac⁺ mutagenesis when the latter is increased dramatically by I-*SceI*-induced DSBs. The two processes appear inseparable (Gonzalez C., Ponder R.G., Price M., Hastings P.J., and Rosenberg S.M., unpublished observations).

Other Models

Roth and colleagues (2006) favor a mathematical model for Lac⁺ point mutagenesis in which there is no transient increase in mutation rates caused by stress responses (Roth *et al.*, 2006). In this model, a small fraction of the cells plated in the experiment have a pre-existing spontaneous duplication of a region containing the leaky *lac* gene. They propose that the cells carrying duplications can grow on lactose, starting a very slow process of colony formation. Under the strong selection for lactose utilization, eventual recombination-mediated expansion (gene amplification) to many *lac* copies is favored, improving growth and increasing the chance of acquisition of generation-dependent mutations in the extra copies of *lac*. This is proposed to be unrelated to stress, and in fact to occur in the least stressed cells on the plate: the ones growing most rapidly (which should not have RpoS induced). They propose that Lac⁺ point revertants then take over the colony due their optimal growth rate, giving rise to colonies containing a vast majority of point mutants and some remaining *lac*-amplified cells.

Many aspects of this model are incompatible with previous data, much of it reviewed previously, and we refer readers to the following papers for summary of the literature prior to mid-2004 (Foster, 2004a, 2004b; Rosenberg and Hastings, 2004a, 2004b; Roth and Andersson, 2004a, 2004b, 2004c). At least four important additions to the literature since those reviews have not been reviewed previously. These modern data show that the mathematical model cannot explain most of point mutagenesis in the Lac system.

Evidence that Mutagenesis is Stress Induced

1. Most obviously, mutagenesis in the Lac system has been demonstrated to be a stress response, controlled by RpoS and SOS. The data of Ponder *et al.* (2005) reviewed above show that DSBR switches to an error-prone mutagenic mode, using DinB, either when cells enter stationary phase or when RpoS is expressed in exponential cells (Figure 4). We know of no argument against these data, or the previous demonstrations that RpoS is required for virtually all stress-induced Lac reversion (Layton and Foster, 2003; Lombardo *et al.*, 2004). Moreover, control by RpoS and other similar stress responses is a widespread bacterial strategy (Table 2 and following sections of this review).
2. Second, DSBR and the stress response or stationary phase are all that is needed to elicit the stress-induced point mutagenesis—amplification is not. In the experiments measuring DSB-stimulation of *tet* gene reversion in stationary phase (Figure 4A) or with RpoS expression in log phase (Figure 4B) (Ponder *et al.*, 2005), there is no lactose (or tetracycline) in the medium and thus no selection for amplification of DNA including the *tet* gene near *lac*. Without selection for multiple copies of some gene,

strains carrying amplification do not maintain amplification (Hastings *et al.*, 2000). Thus amplification of *lac*, *tet*, or even the *dinB* gene (proposed to be required in some versions of the model, Roth *et al.*, 2006) cannot be precursors to these stress-induced mutations.

3. In work leading to a similar conclusion, Stumpf *et al.* (2007) showed that high-level reversion of a *tetA* allele in the same molecule with *lac* is not higher if that gene is close to *lac* and can be co-amplified with *lac*. This again indicates that amplification is not a precursor to mutagenesis.

4. Some versions of the model of Roth *et al.* (2006) suggest that *dinB*, which is near *lac*, must be located in the F', where it is expressed more highly (Kim *et al.*, 2001), to cause mutagenesis: a presumed special circumstance, not general to most bacteria. This has been shown not to be true for DinB-dependent chromosomal Lac⁺ reversions (Ponder, 2006) and is also not the case for the many examples of DinB-dependent stress-induced mutagenesis in *E. coli* and in other bacterial genera reviewed below (and Table 2).

Finally, previously, the very strongest experimental support for the model of mutations promoted by selection for amplification and growth without stress came from a single experiment reported by (Hendrickson *et al.*, 2002). These authors tested the idea that amplification of *lac* was a requirement for point mutagenesis by asking whether counter-selection of increased copy number of a *tet* gene next to *lac*, which would co-amplify with *lac*, inhibited stress-induced point mutagenesis, and they reported that it did and that counter-selection of a distant copy of that gene did not.

5. That strongest result has proven to be unrepeatable by another laboratory. Stumpf *et al.* (2007) find that counter-selection of the gene inhibits *lac* reversion regardless of its proximity to *lac* (and ability to affect *lac* amplification). Thus, this experiment can no longer provide clear support (see also Rosenberg and Hastings, 2004a).

6. Finally, the specific predictions of colony composition and the relationship of amplification to point mutagenesis have not held up to experimental testing (Hastings *et al.*, 2004). In the mathematical model, a microcolony of 10⁵ cells with 100 copies of *lac* (10⁷ total copies) was suggested to mutate at a nearly "normal" generation-dependent rate near 10⁻⁷. That means that colonies should contain, on average, only *lac*-amplified cells until they grow to be ~10⁵ cells big. Hastings *et al.* (2004) showed that very young colonies are either purely point mutant or amplified, not all purely amplified, as the model demands. Even colonies at the two-cell stage were purely point mutant. This is impossible if 10⁷ *lac*⁻ copies must precede a Lac⁺ point mutation. They also separated amplification and point mutagenesis genetically, showing that amplification can be eliminated by *polA* mutation, inactivating DNA Pol I (Hastings *et al.*, 2004; Slack *et al.*, 2006), without altering point mutagenesis. This is not possible if amplification is a precursor to point mutations. Hastings *et al.* (2004) report several other critical specific tests, results of which were incompatible with the model.

Thus, we feel that there can be no real controversy regarding whether mutation rates are increased in response to stress in the Lac system. Moreover, the generality of this strategy in many other systems (Table 2 and reviewed below) makes the conclusion widespread.

STRESS-INDUCED AMPLIFICATION: GENOME REARRANGEMENT INDUCED BY DSBS AND CONTROLLED BY A STRESS RESPONSE

Gene amplification is a phenomenon widely observed both in prokaryotic and eukaryotic cells, where it may play important roles in antibiotic resistance and tumorigenesis. For example, amplification of oncogenes is associated with several types of cancer (reviewed by

Savelyeva and Schwab, 2001; Vogelstein and Kinzler, 1998); amplification of the *DHFR* gene induced by the chemotherapeutic agent methotrexate confers tumor resistance to that drug (Huennekens, 1994), and amplification of the beta-lactamase-encoding *ampC* gene of *E. coli* promotes resistance to beta-lactam antibiotics such as ampicillin (Edlund and Normark, 1981). As reviewed above, in the *E. coli* Lac system, amplification of DNA spanning the *lac* frameshift allele is an adaptive outcome, alternative to point mutation (Hastings *et al.*, 2000, 2004), in which cells acquire 20 to 50 or more tandem repeats of a region containing 7–134 Kb, of the *lac*-containing DNA, allowing growth on lactose medium (Hastings *et al.*, 2000; Kugelberg *et al.*, 2006; Slack *et al.*, 2006). The *lac*-amplified colonies appear later than the point mutants (Figure 2) and can be distinguished from point-mutant colonies based on their unstable Lac⁺ phenotype; upon re-plating on rich medium containing a color indicator for beta-galactosidase activity, such as X-gal, *lac*-amplified cells give rise to both white (Lac⁻) and sectored colonies (blue colonies with white sectors), which indicates deamplification of the repeat array by recombination during growth of the colony (discovered and understood by Horiuchi *et al.*, 1963; see also Tlsty *et al.*, 1984). These contrast with the solid blue colonies formed by true *lac* revertants allowing discrimination between the two classes of event. This has allowed determination of the genetic requirements specific to each (reviewed by Hastings, 2007).

Current understanding of the possible mechanism of stress-induced amplification is reviewed in detail (Hastings, 2007), and here we review only three aspects relevant to the comparison of many mechanisms, though the genetic requirements are summarized in Table 2. Please see that paper for discussion of possible alternative models to the one described here.

The RpoS Response, Coupling to DSBR, and Long-Distance Template-Switching During Repair Replication

Stress-induced *lac* amplification is a stress response and requires the RpoS general-stress-response master regulator (Lombardo *et al.*, 2004) (Table 1). Amplification requires homologous recombination and DSBR proteins RecA, RecBCD, RuvABC (Slack *et al.*, 2006), and is strongly stimulated by an I-SceI-introduced DSB (Ponder *et al.*, 2005), indicating that DSBs provoke amplification and that HR-DSBR is part of some stage of the stress-induced amplification mechanism. However, the junction sequences for the tandemly repeated DNA segments containing *lac* indicate that the event that creates a tandem duplication is not homologous recombination, but rather is a “non-homologous” or microhomologous event between 3- and 6-base G-rich imperfectly identical sequences (Kugelberg *et al.*, 2006; Slack *et al.*, 2006). The process also specifically requires the 5'-to-3' flap endonuclease activity of DNA polymerase I (Pol I, Table 1) (Slack *et al.*, 2006) which is used for removal of RNA primers during lagging-strand replication, and also in some DNA repair reactions, which are not part of amplification (Slack *et al.*, 2006). Those authors propose and support a model in which repeats are formed specifically during stress, by an act of template-switching of the nascent lagging strand from one replication fork to another where it base pairs to a region of microhomology. The switch requires that the initial DNA replication fork should stall. Slack *et al.* (2006) suggested that the role of DSBs in this processes is that the DNA replication that stalls, allowing the template switch, is replication initiated in acts of DSBR (Figure 6) and thus not under the multiple layers of control of a replication origin, which disallow replication initiation in starving cells. Replication from origins, they suggest, would not pause as frequently. The specific model for how the duplication is generated, and its subsequent amplification by HR between sister chromosomes, is reviewed in detail in another review in this series (Hastings, 2007). Here we wish to highlight three points.

No Hypermutation, SOS or DinB

Unlike Lac⁺ point mutants, *lac*-amplified cells show no evidence of genome-wide hypermutation (Hastings *et al.*, 2000), and amplification does not involve the DinB polymerase or the SOS response (McKenzie *et al.*, 2001). The lack of hypermutation in *lac*-amplified clones probably reflects the lack of requirement for SOS, which we proposed is required, along with RpoS induction, for cells to enter the HMS (Figure 4). Pennington and Rosenberg (2007) find that only ~27% of cells with a reparable chromosomal DSB generated by I-SceI induce SOS, such that only 27% of the amplified clones are predicted to have been hypermutated, a figure which is not ruled out by current data on (lack of) hypermutation among *lac*-amplified clones (Hastings *et al.*, 2000). Thus, amplification is a process of genome rearrangement controlled by RpoS and not SOS.

Stress-Response Regulation, Genomic Localization of Genome Rearrangements and Evolvability

First, Like Lac⁺ point mutagenesis, stress-induced amplification is regulated temporally by the RpoS response (Lombardo *et al.*, 2004), such that, in this case, genome rearrangement will occur specifically when cells are stressed and induce the RpoS response. A general model for the switch to rearrangement-provoking replication under stress is shown in Figure 6. Second, the coupling of stress-induced amplification to acts of DSBR (Ponder *et al.*, 2005, reviewed above) should limit amplifications, and other non-homologous rearrangement events, in genomic space to presumably rare sites of spontaneous double-strand breakage in the bacterial chromosome. Could the coupling of genome rearrangement to DSBR be evolutionarily advantageous, *e.g.*, for limiting deleterious rearrangements in the genomes of rare adaptive mutants? Amplifications are transient and unstable, such that those not selected are likely to be lost. However, other genome rearrangements might also be induced *via* the proposed template-switch model, such as deletions and/or inversions. Limitation of the number of sites per genome that experience rearrangement *via* its coupling to DSBR could provide an evolutionary advantage similar to that suggested for point mutagenesis: the limitation of deleterious rearrangements in cells that acquire a rare beneficial rearrangement.

ANTIBIOTIC-INDUCED RESISTANCE MUTATIONS

Ciprofloxacin Resistance in *E. coli*: SOS Response and Error-Prone Double-Strand Break Repair

Stress-induced mutagenesis was predicted to be important for evolution of drug resistance by pathogens (Martinez and Baquero, 2000), and recent work with the antibiotic ciprofloxacin shows a drug-induced mutation pathway nearly identical to stress-induced point mutagenesis in the Lac system described above. Ciprofloxacin is a widely used broad-spectrum antimicrobial agent of the fluoroquinolone family. Quinolones are inhibitors of type II topoisomerases, and known inducers of the SOS response (reviewed by Drlica and Zhao, 1997). In *E. coli*, point mutations in the gyrase genes *gyrA* and *gyrB*, and in the topoisomerase IV genes *parC* and *parE* are the main source of resistance to this drug (Drlica and Zhao, 1997). After exposure to this bacteriocidal drug, the ~1% of cells that survive experience no net growth while they generate new resistant mutants for days, mutants that demonstrably did not pre-exist in the cultures before treatment (Riesenfeld *et al.*, 1997). No stepwise improvement of the resistance was detected in the cells on the plate, as determined by measuring the minimum inhibitory concentrations (MICs) of sensitive cells surviving in the plate.

SOS DNA Pols—A recent breakthrough study has provided insight into the mechanism of stress-induced mutagenesis giving rise to ciprofloxacin resistance. Using both laboratory

culture experiments and a murine infection model, Romesberg and colleagues showed that *E. coli* cells are unable to develop resistance either in culture or during infection if they carry a special uncleavable LexA transcriptional repressor, which prevents induction of SOS genes (Cirz *et al.*, 2005; and reviewed in detail by Cirz and Romesberg, this volume). The requirement for the SOS genes in producing ciprofloxacin-resistant mutants probably reflects a need for the induction of the SOS-controlled DNA polymerases Pol II, DinB/Pol IV and Pol V (Tables 1, 2), which they showed are required for mutation to resistance. Mutants defective for one or more of these polymerases were defective in post-exposure mutagenesis, but not in survival during the experiment. Of these DNA polymerases both DinB and Pol V are Y-family error-prone DNA polymerases, and all three are specialized DNA polymerases implicated in translesion DNA synthesis (Table 1). The results reveal a mechanism of SOS-dependent increase in mutation rates after antibiotic stress, that, like point mutagenesis in the Lac system, requires DinB, in addition to the two other SOS and translesion DNA polymerases. They suggest that the Lac system appears to require only DinB because only frameshift reversions are selected (not other alterations of sequence achieved by the other polymerases), *i.e.*, that the two mechanisms are the same in this respect (Cirz and Romesberg, 2007). Moreover, the ciprofloxacin-induced mutagenesis pathway is different from spontaneous mutagenesis leading to ciprofloxacin-resistance in that the latter requires neither SOS nor the SOS DNA polymerases (Cirz *et al.*, 2005; Cirz and Romesberg, 2006). Thus, the mutations arising before and after the antibiotic stress are formed by fundamentally different molecular mechanisms controlled by the SOS response.

DSBR Mechanism—Also like stress-induced Lac point mutagenesis, the ciprofloxacin-induced resistance mutagenesis requires HR-DSBR proteins RecA, RecBC and RuvABC (Cirz *et al.*, 2005). The authors propose a mechanism for the mutagenesis that begins when the antibiotic traps a type II topoisomerase (one that cleaves both strands of DNA prior to strand passage) on DNA that it has cloven but not religated, in a DSB intermediate (see Cirz and Romesberg, this volume). They propose that these DSBs are converted into SOS inducing signals (single-strand DNA), the SOS DNA polymerases are upregulated, and that HR-DSBR then provides an opportunity for error-prone synthesis catalyzed by the SOS DNA polymerases. Their model is similar to the error-prone DSBR model now well supported in the Lac system (*e.g.*, Figure 3). Their data imply a mutagenesis mechanism of error-prone HR-DSBR induced by stress, this time with the stress being caused by the antibiotic rather than starvation. As in the Lac example reviewed above, and the stress is communicated *via* the SOS stress response.

Antibiotic Therapy—An important implication of this work of interest to the authors is that new drugs that inhibit the mutagenic process itself should be included with the antibiotics to prevent evolution of resistance mutations induced by antibiotics (Cirz *et al.*, 2005, 2006a). In this case, inhibitors of the SOS response, RecA, RecBCD, Ruv proteins or the SOS DNA polymerases might all be appropriate candidates.

Rifampicin Resistance in *Mycobacterium tuberculosis*

A very similar involvement of the SOS response in the generation of antibiotic resistance during infections was shown in *Mycobacterium tuberculosis*. In this bacterium, the *dnaE2* gene, encoding a second copy of the catalytic subunit of Pol III (Table 1), is regulated by the SOS response (Davis *et al.*, 2002), and is responsible for mutations resulting in rifampicin resistance during infection (Boshoff *et al.*, 2003). Rifampicin inhibits bacterial RNA polymerase. *dnaE2* is always accompanied in bacterial genomes by a putative Y-family DNA polymerase (Table 1) encoded by the *imuB* gene (Galhardo *et al.*, 2005), and these genes seem to be highly conserved components of the SOS response in bacteria (Abella *et al.*, 2004), as recently demonstrated by their LexA-dependent upregulation by ciprofloxacin

in *Pseudomonas aeruginosa* (Cirz *et al.*, 2006b). Thus, upregulation of specialized polymerases by the SOS response is a widely conserved response in bacteria, likely to be relevant in antibiotic-resistance acquisition. The importance and generality of the upregulation of SOS by antibiotics is also indicated by the positive regulation of both the SOS response and error-prone polymerases by various antibiotics (Miller *et al.*, 2004; Perez-Capilla *et al.*, 2005), including, in the latter two studies, beta-lactam antibiotics, which act on cell wall components (not DNA).

MUTAGENESIS IN AGING COLONIES (MAC)

A Natural Isolate: Two Stress Responses, RecA, DNA Pol II, Limiting MMR

The assay system with which 787 *E. coli* world-wide natural isolates were screened for stress-inducible mutability, described above and in Figure 1, uses *E. coli* colonies that are allowed to grow old on solid medium, from which the frequencies of unselected rifampicin-resistant (Rif^r) mutants are determined after one and 7 days of incubation. The stress-inducible mutagenesis mechanism operating in one natural isolate requires two stress responses: the RpoS-controlled general- or starvation-stress response (reviewed Table 1), and release from catabolite-repression, a carbon-starvation response (Table 1) (Bjedov *et al.*, 2003). Clearly, genesis of the mutants is stress induced. The mechanism also requires oxygen, and DNA Pol II, a relatively high-fidelity DNA polymerase, RecA recombinational DNA repair protein, and a transient limitation/down-regulation of MMR protein MutS, decreasing MMR activity, as shown by the specific inhibition of stress-induced mutagenesis by overproduction of MutS, restoring MMR function (Table 1). The authors suggest a possible mutagenesis mechanism of error-prone repair of oxidative damage to DNA, a known function of Pol II. Components of this mechanism that are common among other stress-induced mutation mechanisms include—regulation by stress responses, particularly RpoS (most common, Table 2); involvement of RecA; use of a specialized (though not an error-prone) DNA polymerase; and transient limitation of MMR, which becomes limiting *via* limiting MutS (Table 2), not MutL as in the Lac system (discussed above). The proposed mechanism also fits with a theme of mechanisms that potentially localize mutations in genomic space *via* coupling to a localized process (DNA repair).

A Lab Strain: Three Stress Responses, RecA, DNA Pol I, Limiting MMR

The same assay system used to examine the 787 *E. coli* natural isolates was developed and used first with a laboratory strain of *E. coli*. In these experiments (Taddei *et al.*, 1995), cell number increased ~10-fold (only 3 to 4 generations), but Rif^r mutant frequencies increased 50-fold over the 7 days, implying mutagenesis induced during growth-limiting stress. In the laboratory strain used initially (Taddei *et al.*, 1995), the increased mutagenesis in ageing colonies requires at least two stress responses: the SOS DNA damage response and cAMP synthesis, part of the release of catabolite repression, a response to carbon starvation (Table 1), indicating that it is a cellular response to starvation stress (Taddei *et al.*, 1995). The authors showed that mutations that block the induction of these responses block the mutagenesis. RecA and RecBCD were required, perhaps for their roles in SOS induction (Taddei *et al.*, 1995, 1997a). Also in this particular laboratory strain, the DNA nucleotide-excision repair protein UvrB and DNA Pol I were required for the stress-induced mutagenesis. The stress-induced mutagenesis was specific to colonies on solid medium, not occurring in liquid cultures, probably because cAMP was used as a necessary communication molecule between cells in close proximity (Taddei *et al.*, 1997a). The requirements for RecA and cAMP were as observed with the natural isolate strain examined by Bjedov *et al.* (2003) whereas that study found a requirement for Pol II rather than Pol I in the natural isolate. The UvrB requirement suggests possible coupling of mutagenesis to DNA repair, which could cluster mutations, as discussed above.

MutS and MMR Become Limiting

As in stress-induced mutagenesis in the natural isolate of Bjedov *et al.* (2003) and described above for stress-induced Lac⁺ point mutagenesis, and several systems discussed below, MMR function becomes limiting during stress-induced mutagenesis of this laboratory strain (Taddei *et al.*, 1997a); mutation in *mutS*-defective cells did not cause a significant increase in stress-induced mutations, demonstrating that it occurs in cells already experiencing a limitation in MMR activity. Further, overproduction of MutS reduced mutagenesis indicating that MutS was the limiting component.

HOW MMR BECOMES LIMITING

The limitation of MMR activity *via* limited MutS during MAC is unlike several experimental systems in which MutL becomes limiting, but is like two others in which MutS does: the natural isolate of Bjedov *et al.* (2003), and an assay measuring MMR itself (not mutagenesis) *via* repair of heteroduplex DNA molecules introduced into stationary-phase cells (Brégeon *et al.*, 1999). The assays in which MutL becomes limiting are the Lac system (reviewed above, see also Harris *et al.*, 1997, 1999), cells expressing an error-prone mutant DNA polymerase III (Schaaper and Radman, 1989) or overproducing DinB/Pol IV (Wagner and Nohmi, 2000), and cells in which MMR proteins are titrated by overproduction of the interacting repair protein VSR (Doiron *et al.*, 1996). This difference can be understood as follows (Rosenberg and Hastings, 2003): the cases in which MutL becomes limiting appear to be situations in which the number of errors (or protein molecules) potentially titrating MMR are very high, and so might titrate MMR before the significant decline in MutS and MutH levels occurs in stationary phase (Feng *et al.*, 1996; Harris *et al.*, 1997; Tsui *et al.*, 1997). When titrated before the decrease in MutS levels in stationary phase, MutL is proposed to be limiting first. In the studies in which MutS was limiting, the DNA polymerases making the mutations are not “error-prone” DNA polymerases: Pol II (Bjedov *et al.*, 2003) and Pol I (Taddei *et al.*, 1997a), and MMR might not be overwhelmed until later, when MutS levels have dropped significantly in stationary phase. According to this hypothesis, whether MutL or MutS becomes limiting during stress-induced mutagenesis depends on how error-prone the synthesis is and its timing relative to the decline of MutS levels in stationary phase. Regardless of the specific mechanism, work discussed above and below shows that MMR limitation and the resultant increased mutagenesis in response to stress is a general theme in bacteria and human cells.

STARVATION-INDUCED MUTATIONS IN SOME OTHER BACTERIA

***Bacillus subtilis*: Competence Stress Response, DinB, MMR Limitation, and Possible Transcription Coupling of Mutagenesis**

Two other bacterial species are emerging as alternative models for the study of stress-induced mutations, *Bacillus subtilis* and *Pseudomonas putida*. Starvation-associated mutations have been studied in *B. subtilis* using different alleles conferring histidine, methionine or leucine auxotrophy (reviewed in detail by Robledo *et al.*, this volume). All can be reverted by any of several different base substitutions, restoring ability to grow in minimal medium lacking the corresponding amino acid. Using this system, Sung and Yasbin found that stress-induced prototrophic revertants arise after plating of the culture on minimal medium (Sung and Yasbin, 2002). Unlike the *E. coli* Lac system, mutations form independently of RecA (the SOS response or recombination) but like many of the systems discussed here, a starvation-stress response and a Y-family DNA polymerase are required, and MMR becomes limiting. The competence stress-response genes *comA* and *comK*, encoding transcriptional regulators of differentiation and competence development after exponential growth, are required for the generation of stress-induced mutations (Sung and Yasbin, 2002), as is the stringent stress response to aminoacid starvation (Rudner *et al.*,

1999; reviewed Robleto *et al.*, 2007). Competence (for natural transformation with DNA taken up from the environment) is a starvation-stress response, indicating the importance of stationary phase, starvation, and the appropriate stress response to the mutagenesis. The requirement for Com functions is analogous to requirements in other systems discussed above for the RpoS stress response and cAMP (see Table 2). As expected, the competence genes are not involved in mutagenesis in exponentially growing cells. Also paralleling the Lac system, and antibiotic-induced mutations, YqjH, a putative Y-family polymerase homologous to DinB, is responsible for at least half of the adaptive mutations in this system (Sung *et al.*, 2003). Overproduction of MutS and MutL or MutS alone caused a two- to threefold reduction in mutagenesis, indicating that MMR limitation occurs *via* limitation of MutS (Pedraza-Reyes and Yasbin, 2004).

Possible Transcription Coupling and Limitation in Genomic Space—In addition to its temporal regulation by the Com stress response, the *B. subtilis* system also shows a hint that there might be spatial restriction of the mutagenesis within the genome. Mfd is a protein that couples DNA nucleotide excision repair to transcription in bacteria, causing preferential repair of actively transcribed genes. Mfd is required for stress-induced mutagenesis in this organism (Ross *et al.*, 2006). This suggests that mutagenesis might be part of transcription-coupled repair, and thus might be localized to genes actively transcribed during the stress, and perhaps further limited to those undergoing a DNA repair event. Like the coupling of mutagenesis to HR-DSBR in the Lac system, this suggests a mechanism with the potential to localize and cluster mutagenesis in genomic space, which could facilitate concerted evolution (per Ninio, 1996; Ponder *et al.*, 2005, discussed above). An important area for future work in this system will be to determine whether or not these mutations are localized and clustered in genomes, and whether this mutation mechanism is in fact coupled to transcription or transcription-coupled repair.

***Pseudomonas putida*: RpoS Stress Response, DinB, MMR Limitation, and More Than One Mechanism**

The *P. putida* system also involves analysis of mutation arising after imposition of a non-lethal stress. In the first versions of the assay, cells carrying a plasmid containing a promoterless *pheAB* operon were assayed for post-selection mutagenesis (Kasak *et al.*, 1997). The *pheAB* genes mediate the degradation of phenol and its use as a carbon source, but cells carrying the promoter-less plasmid version cannot grow on phenol minimal plates. However, mutations occur after plating, creating functional promoters. These mutations are various, and different from those generated during growth prior to selection. They include base substitutions, deletions and insertion of a transposon containing an outward-facing promoter. Intriguingly, mutations do not form in other starvation conditions, but require phenol in the medium. The point mutation sequences differ in the earlier days of starvation from the later days, suggesting that more than one mutagenesis mechanism contributes (Saumaa *et al.*, 2002). Base substitutions prevailed in the first days, and small insertions in the later days.

These mutations are stress-response regulated by RpoS response (Table 1). RpoS is required for 2- to 3-bp insertions occurring in the later days and positively regulates the transposition of Tn4652 by actively regulating the transposase promoter (Ilves *et al.*, 2001; Saumaa *et al.*, 2002, this mechanism discussed below). In another version of the assay for Phe⁺ mutations, the plasmid-borne *pheA* gene was altered in the coding sequence by introducing three different nonsense mutations or a +1 frameshift mutations. With this set of alleles, DinB was largely responsible for the generation of -1 frameshift mutations from day 8 onward. These mutations form independently of RecA suggesting that the DinB activity did not require the SOS response in this system (Tegova *et al.*, 2004). Removing the *P. putida* MMR system by

mutations in *mutL* and *mutS* caused a marked increase in the base substitutions, but not in the -1 frameshift mutations (Saumaa *et al.*, 2006), suggesting either that the frameshift mutations are generated from a precursor structure not subject to MMR [unlikely given avidity of MMR in other organisms for -1 frameshift mutation intermediates (Kunkel and Erie, 2005)], or that they originate in cells already depleted in MMR activity. The latter possibility would fit with these mutations specifically requiring DinB. Perhaps in this system, as proposed for the *E. coli* Lac assay (Harris *et al.*, 1997; McKenzie *et al.*, 2001), MMR becomes titrated by high frequency errors generated by DinB, which in this system is not the source of the substitution mutants.

Both the *P. putida* and *B. subtilis* examples share many of the key features of *E. coli* Lac point mutagenesis: temporal regulation by stress responses, DinB-like error-prone polymerases, MMR limitation, and, perhaps for *B. subtilis*, the possibility of linkage of mutagenesis to local DNA repair events.

CONTROL OF MUTAGENESIS BY PHOPQ: RPOS AGAIN?

PhoPQ is yet another stress response that controls mutagenesis in *E. coli*. PhoPQ constitute a two-component system of signal transduction that upregulates several genes in response to diminished levels of cellular Mg^{2+} , plays an important role in the expression of virulence genes in pathogenic enterobacteria (Monsieurs *et al.*, 2005), and also stabilizes RpoS (Tu *et al.*, 2006). In a mutagenesis assay used by Hall (1998), the cryptic *ebg* operon is employed in an elegant selection for forward (gene-inactivating) mutations in the *ebgR* repressor gene during starvation. Following a screen for mutants with decreased stress-induced *ebgR* mutagenesis, Hall showed that PhoP and PhoQ are required for the stress-induced mutagenesis specifically, not generation-dependent *ebgR* mutation, and excluded possible effects of PhoPQ on the growth of *ebgR* mutants (Hall, 1998). Many of the mutations in *ebgR* are insertions of mobile elements (IS elements) (Hall, 1999), which left open the possibility that movement of these genetic elements might be regulated by PhoPQ. In light of recent evidence that PhoPQ stabilizes RpoS (Tu *et al.*, 2006), this work now begs the question of whether the RpoS starvation- and general-stress response is required for the *ebgR* mutagenesis during starvation, and whether the need for RpoS is the reason PhoPQ are required. Whatever its mechanistic basis, these results provide another example of the control of mutagenesis by a stress response(s). Other examples involving movement of mobile elements follow.

MOBILE GENETIC ELEMENTS AND STRESS-INDUCED GENOME REARRANGEMENTS

Mu-Mediated Deletions

The idea that transposons might be a source of genetic variability in response to stress was proposed by Barbara McClintock (1984) working in corn, and the observation of stress-induced movement of mobile genetic elements in bacteria was one of the early instances of stress-inducible genetic change reported. Shapiro used an *E. coli* strain carrying the transposing prophage Mu inserted between the *araB* and *lacZ* genes. The insertion renders the strain Lac⁻. However, Mu excisions can produce in-frame fusions of *araB* and *lacZ*, making cells able to grow on lactose, as long as arabinose is also provided to induce the transcription of the fusion gene. The excision events are very rare in growing cells, but increase by several orders of magnitude after prolonged incubation of cells in lactose-arabinose plates (Maenhaut-Michel and Shapiro, 1994; Shapiro, 1984), during long-term incubation of glucose cultures (Mittler and Lenski, 1990), or in response to starvation generally (Foster and Cairns, 1994; Maenhaut-Michel and Shapiro, 1994; Sniegowski, 1995): all starvation stresses. However, the structure of the fusions is different depending on

the environment (*i.e.*, selected on lactose-arabinose plates or in liquid with other carbon sources), indicating that a complex regulatory network operates in response to the environment, influencing the genetic rearrangements (Maenhaut-Michel and Shapiro, 1994).

The occurrence of excisions absolutely requires the RpoS stress response (Gomez-Gomez *et al.*, 1997), and proper controls demonstrated that the lack of excisions in the mutant was not due to decreased viability or growth rate of the *rpoS* strain carrying the *araB::lacZ* fusion. RpoS mediates de-repression of the lytic promoter of Mu in stationary phase (Lamrani *et al.*, 1999). Similarly, CRP, the transcriptional activator, which upregulates promoters during the release from catabolite repression upon carbon starvation, is also required (Lamrani *et al.*, 1999) (Table 2), indicating positive control of these fusions by two starvation stress responses. Conversely, depletion of the histone-like protein H-NS, which promotes repression of some RpoS-regulated promoters, stimulates excision events (Gomez-Gomez *et al.*, 1997), indicating that several host inputs act to balance the formation of fusions in response to stress.

Stress-Induced Transposition in Old Colonies

More recently, Coros *et al.* used a papillation assay to monitor transposon movement in single *E. coli* colonies, and found that transposition of the mobile element IS903 occurs predominantly late in colony development (Coros *et al.*, 2005). Various host factors affect this transposition, including AspA, which implies nutritional regulation of transposition (Twiss *et al.*, 2005). *aspA*⁺, encoding aspartase, represses transposition generally and particularly during early colony growth, and also inhibits transposition of the unrelated elements Tn10 and Tn552. Aspartase converts L-aspartate to fumarate in the TCA cycle, suggesting that its inhibitory effect results from avoidance of nutritional deprivation. Demonstration that nutritional stress indeed induces transposition was obtained by supplying extra fumarate in growth medium, which suppressed the early-transposition phenotype of *aspA* mutants. On the other hand, H-NS, which antagonizes the RpoS stress response, was shown to promote Tn10 transposition directly, by binding to the uncleaved transpososome (Wardle *et al.*, 2005).

Stress-Induced Transposition in *Pseudomonas*

In *P. putida*, a similar induction of transposition events by stress was observed using the promoter-creating assay described above (Kasak *et al.*, 1997). Several of the mutations allowing expression of the promoterless *pheAB* operon were Tn4652 insertions, and these required RpoS, which transcriptionally upregulates the Tn4652 transposase (Ilves *et al.*, 2001). Two other regulatory inputs of Tn4652 transposition specific to stationary phase illustrate a fine-tuned regulation of transposon movement in response to stress: IHF and the ColS-ColR two component system (Horak *et al.*, 2004; Ilves *et al.*, 2004). Upregulation of transposon movement during stress is an interesting observation, because these elements are viewed by some as selfish parasites of the genome. Whatever the possible selective pressure for (or lack of selective pressure against) mechanisms leading to enhanced transposition in times of stress, the result is that transposon movement may be an important source of generation of variability in response to environmental changes.

Telomere Shortening- and DNA Damage-Response-Activated Retrotransposition and Point Mutagenesis in Yeast

Yeast telomeres are stably maintained by the addition of simple DNA repeats at chromosome ends by telomerase. Telomerase uses the reverse transcriptase activity of the Est2 enzyme to synthesize the DNA using an RNA template. In *est2* mutants, telomeres shorten progressively, triggering a cell-cycle checkpoint and ultimately provoking cell senescence (Lundblad and Blackburn, 1993). Scholes and colleagues demonstrated that

mobility of the yeast LTR-retrotransposon Ty1 is greatly stimulated in response to telomere erosion stress in *est2* mutants (Scholes *et al.*, 2003). This enhanced retrotransposon mobility requires the Rad9, Rad17 and Rad24 signal-transduction proteins of a DNA-damage checkpoint pathway, analogous to the SOS DNA-damage response in bacteria.

Additionally, telomere shortening in *est2* mutants is associated with increased point mutagenesis in telomere-proximal regions, mediated by the error-prone polymerases Rev1 and Rev3/Rev7 (Meyer and Bailis, 2007), the first of which is a Y-family DNA polymerase homologous with DinB, and all of which are specialized, error-prone translesion polymerases. These authors did not measure mutation of the same gene close to and distant from the telomere, so the differences in mutagenesis upon telomere shortening might be due to either the mutation-target sequences or to telomere proximity *per se*, in either case, activated by stress. Although the signal transduction pathway(s) of this later phenomenon have not been determined, enhanced movement of the Ty1 retrotransposon and increased mutagenesis might be part of the same global response to telomere shortening. The regulation of retrotransposition by a DNA damage response, and both it and point mutagenesis by stress, make these events analogous to those discussed for bacteria in which the SOS response upregulates genetic instability.

TRANSCRIPTION-ASSOCIATED MUTATIONS IN YEAST AND BACTERIA

A dramatic example of circumscription of mutations in genomic space is the association of mutation and transcription. In *Saccharomyces cerevisiae*, in experiments in which transcription of a *lys2* gene with a frameshift mutation was controlled with an inducible promoter, increased transcription rates were correlated with increased reversion rates (Datta and Jinks-Robertson, 1995). In *E. coli*, a transcription association with mutagenesis was suggested by genomic analysis (Francino *et al.*, 1996). More directly, auxotrophic mutants carrying mutations in amino acid biosynthetic genes show increased rates of reversion in conditions that stimulate the expression of those specific biosynthetic genes (Wright *et al.*, 1999).

In a particular *E. coli* example, reversion rates of a *leuB* allele were perfectly correlated with the expression levels of the gene in several different conditions (Wright *et al.*, 1999). Synthesis of the small starvation-stress signal molecular, ppGpp, and starvation for leucine are necessary for the high levels of expression of *leuB*, and consequently, elevated reversion rates. Modulation of expression of the biosynthetic gene by a non-native, artificially inducible promoter also demonstrated that increased transcription rates correlate with increased reversion. A similar requirement for ppGpp was observed for the mutation rates in *B. subtilis* cultures (Rudner *et al.*, 1999). In this assay, reversion rates of two amino acid auxotrophy markers were positively correlated with the production of ppGpp by cells, unlike mutations giving rise to streptomycin resistance, which were unaltered in ppGpp-synthesis-defective mutant strains.

In one model for a possible mechanism for transcription associated mutagenesis, Wright notes that transcription induces local modifications to supercoiling, which she proposes facilitate the formation of stem-loop structures with unpaired bases, which would be particularly prone to mutation (Wright, 2004). The author observes a correlation between predicted ability of DNA sequences to form stem-loop structures and their acquisition of mutations. This locally facilitated mutagenesis might be an unavoidable consequence of other DNA transactions or a specifically selected mechanism. Either way, it is expected to have broad evolutionary impact, because a link between the need for a gene product in a given situation and mutagenesis could facilitate the evolution of new and better adapted gene functions. The impact of transcription rates on mutagenesis of various genes assayed in

stress-induced mutagenesis assays is a very interesting question that still remains to be addressed in most experimental models for stress-induced mutagenesis. Transcription-associated mutagenesis is also well documented in somatic hypermutation of immunoglobulin genes (discussed below).

OTHER BACTERIAL EXAMPLES

What other examples of increased mutation rates in response to stress are available? In an interesting approach, Lowe *et al.* demonstrated that *E. coli* cultures held in stationary phase for long periods show a high rate of mutations, as shown by the loss of fitness caused by deleterious mutations (Loewe *et al.*, 2003). Some concerns were raised regarding this study about the possible effects of GASP mutations on the results (de Visser and Rozen, 2004), but the GASP phenomenon itself (see below) might be an example of stress-induced mutagenesis.

GASP: the SOS/Translesion DNA Polymerases and RpoS

GASP (growth advantage in stationary phase) mutants arise in long term stationary-phase populations as a result of new mutations conferring advantageous phenotypes that allow growth in the limiting resources. The cells that acquire these mutations out-compete previous genotypes and overtake the population (Finkel, 2006). While the overall population size stays constant (the definition of stationary phase), the fitter mutant types rise in number, outcompeting their siblings, then later fall as they are overtaken by fitter mutants in successive clonal sweeps.

Early GASP mutations that confer an advantage are diminished function (but not null) alleles of RpoS (Table 2) (Zambrano *et al.*, 1993) in apparent contrast with the several examples given in which RpoS function is required for mutagenesis (Table 2 and above). It appears that in GASP conditions, less activity of the RpoS regulon and more of the housekeeping genes is advantageous, but still some RpoS activity appears to be required in that null alleles are not selected. Ferenci has discussed the inherent problem of the tradeoff between housekeeping genes and the RpoS stress genes in limiting resources (Ferenci, 2003), which this appears to illustrate.

Little is known about the generation of the GASP mutations, including whether they are spontaneous mutations present in cultures before stationary-phase stress, or are stress-induced during starvation. However, the following results imply that they are stress-induced *via* the SOS response. Intriguingly, all three of the SOS-inducible DNA polymerases (Tables 1, 2) are required for the production of GASP mutants (Yeiser *et al.*, 2002). Strains that lack any of the SOS polymerases are consistently overtaken by wild-type strains, despite the fact that they show no significant alteration in viability in stationary phase. This implies that the three SOS DNA polymerases are required for production of the GASP mutations themselves. These results echo the stories of ciprofloxacin-induced resistant mutagenesis and Lac⁺ point mutagenesis discussed above, and suggest that the SOS-controlled DNA polymerases may underlie the generation of genetic diversity in stationary phase in this system as they do in others. This is particularly likely, given the very small contribution of these polymerases to spontaneous mutagenesis in growing cells in the absence of DNA damage (reviewed by Nohmi, 2006).

Similarly, in *Pseudomonas putida* cultures, the presence of a plasmid encoding a homologue of DinB/Pol V facilitates the acquisition of the GASP phenotype (*i.e.*, population takeover in stationary phase) (Tark *et al.*, 2005). GASP seems to be an important principle in microbial evolution that is wide open for exploration of the mechanisms and regulation of the mutagenesis that drives it.

STRESS-INDUCED MUTATIONS IN YEAST

Yeast cells exposed to starvation also accumulate adaptive mutations that relieve the growth impairment (reviewed in detail by Heidenreich, this volume). Histidine auxotrophic mutants containing a nonsense mutation in the *HIS4* gene, show accumulation of HIS^+ adaptive mutants detected as papillae in colonies grown in medium with limiting amounts of histidine (Hall, 1992). Cells from the papillae were shown to have formed during starvation, and were not slowly growing mutants present before plating.

A similar observation was made in strains containing a *lys2* frameshift mutation, which shows reversion to lysine prototrophy after plating in medium lacking this amino acid, in the absence of detectable cell growth (Steele and Jinks-Robertson, 1992). This allele has been used for studies of stress-induced mutation in two laboratories using different yeast strains. In one strain background housing this allele, lack of functional mitochondria does not affect starvation-stress-induced mutations, strongly suggesting that oxidative DNA lesions are not precursors in the mutagenic process (Heidenreich and Wintersberger, 1998). In a remarkable similarity with stress-induced mutations in the *lac* system, adaptive reversion of the +4 *lys2* allele shows a strong bias for -1 deletions in mononucleotide repeats, in contrast to a variety of different mutations occurring in the same locus in growing cells (Heidenreich and Wintersberger, 2001), although, when assayed in the other strain, both the stress-induced and generation-dependent mutations were mostly deletions in simple repeats (Greene and Jinks-Robertson, 1999). Yeast strains can be quite different genetically, and we will confine the rest of this discussion to the strain used by Heidenreich and colleagues (2003, 2004), only because more information exists about the mechanism of mutagenesis in it. In that strain, the mutation sequences are suggestive of diminished MMR function, but this has not been tested directly.

A Possible Switch To Mutagenic DSBR *via* NHEJ Under Stress

In the Heidenreich yeast system, two proteins that function in DSBR by non-Homologous End Joining (NHEJ), Ku70 and DNA ligase IV, are required for at least half of the mutations arising specifically under stress (Heidenreich *et al.*, 2003) (Tables 1 and 2).

NHEJ (Table 1) is a mechanism of DSBR that is completely different from homologous recombination (HR), discussed above for the Lac system, and which is inherently mutagenic. In bakers yeast, NHEJ is the less used DSBR pathway and is specific to situations in which there is no homologous DNA available for repair *via* HR-DSBR (reviewed by Dudasova *et al.*, 2004). In NHEJ, two DSEs are united without any need for homology, involving only recognition of the DNA ends, recruiting of proteins involved in resection, and ligation (Bowater and Doherty, 2006).

In the yeast stress-induced *lys2* reversion assay, enhancement of DNA breakage by gamma irradiation increased the rate of adaptive mutagenesis in a NHEJ dependent manner, showing that NHEJ can indeed lead to mutations in starved cells (Heidenreich *et al.*, 2004). The process by which NHEJ produces the frameshift mutations in starved cells is not understood. Although it has not been demonstrated in this yeast system (but see below for human), a plausible mechanism for the stress-induced mutagenesis is that, as in the *E. coli* Lac system, mutagenesis might result from a switch from high-fidelity DSBR to mutagenic DSBR under stress. This could occur by a switch from HR-DSBR to DSBR *via* NHEJ. If this hypothesis is correct, the mechanism emerging would appear to be analogous to that in *E. coli* but evolutionarily unrelated: a common strategy (possible coupling of stress-induced mutagenesis to DSBR) evolved independently and differently, but, we suggest, potentially with similar selective advantages for localization of mutagenesis in genomic space. Below we will see evidence that in human cells, such a switch from high-fidelity HR-DSBR to

mutagenic NHEJ is induced by stress, *via* stress-induced transcriptional down-regulation of key HR-DSBR genes.

Mutagenic HR-DSBR in Yeast

Mutagenesis coupled with HR-DSBR events, just as now appreciated in the Lac system, are also well documented in yeast. Strathern and colleagues showed that the induction of DSBs by an endonuclease specifically provokes both frameshift and base substitution mutations near the break, in the region that has undergone HR-DSBR (Strathern *et al.*, 1995). The error-prone, translesion DNA polymerase REV3 is required for the generation of the base substitutions mutations (Holbeck and Strathern, 1997). Whether these mutations have any relationship to cellular stress or stress responses (including DNA-damage response) has not been investigated, so it is unknown whether there is any temporal regulation of this process in yeast, as there is in *E. coli* (Ponder *et al.*, 2005). However, the coupling of mutagenesis to HR-DSBR events using an error-prone DNA polymerase is just as observed in the Lac system, and may confer similar potential advantages for evolvability, discussed above.

PROGRAMMED ERROR-PRONE DNA BREAK REPAIR IN THE IMMUNE RESPONSE

An interesting parallel with point mutagenesis in the Lac system, antibiotic-induced mutagenesis, and yeast mutagenesis coupled to HR-DSBR is seen in the diversification of the immunoglobulin genes. Somatic hypermutation of immunoglobulin genes involves Y-family polymerases to generate the mutations giving rise to the antibody repertoire, and apparently concentrates those mutations near to DNA breaks, in this case, programmed DNA breaks in the immunoglobulin genes, also associated with transcription (Diaz and Lawrence, 2005; Neuberger *et al.*, 2003).

STRESS-INDUCED MUTAGENESIS IN HUMAN CELLS AND CANCER

Transcriptional Down-regulation of MMR and High-Fidelity DSBR

Tumor development is a multi-stage process, during which cells progressively acquire a series of characteristics that lead to unrestricted growth. The cascade of events bestowing cancer development requires mutations in multiple genes, and tumor cell lines often display a mutator phenotype (Bielas *et al.*, 2006). The parallels between cancer and evolution, and the role of mutagenesis in promoting both, are discussed in detail in the following review and hypothesis articles (Cairns, 1975; Loeb *et al.*, 1974; Nowell, 1976; Echols, 1981; S.M. Rosenberg, E.C. Cox, R.C. von Borstel and L.A. Loeb, manuscript in preparation). In early tumor microenvironments, cells often experience states of hypoxia, which is counteracted by angiogenesis in later stages of tumor development. Hypoxia plays a very important role in tumor progression, because this condition itself increases genetic instability, as detected in wide variety of cell lines and assay systems (reviewed by Huang *et al.*, 2007).

Although hypoxic conditions can cause DNA damage directly, an interesting set of findings reveals that genetic instability itself is induced by hypoxia, triggered by down-regulation of at least five DNA repair genes by stress responses. This regulated process bears two striking parallels to stress-induced mutagenesis in the Lac system and other microbes.

Down-Regulated MMR—First, mismatch repair is down-regulated by decreased transcription of three genes: *MLH1*, *MSH2*, and *MSH6*, homologues of *E. coli* MutL and MutS (Table 1). Repression of *MLH1* transcription involves histone deacetylation and the Max regulatory network, and is accompanied by an increase in (CA) dinucleotide-repeat instability and increased mutation in a chromosomal marker, demonstrating that *MLH1*

becomes functionally limiting (Bindra and Glazer, 2007a; Mihaylova *et al.*, 2003). MLH1 down-regulation is p53-independent, and thus likely to fuel mutagenesis and tumor progression in the large numbers of cancers in which the *p53* tumor-suppressor gene is mutant. Additionally, *MSH2* and *MSH6* are transcriptionally down-regulated by the HIF-1alpha repressor protein, which is removed normally by the ubiquitin-proteasome but becomes stable during hypoxia (Koshiji *et al.*, 2005). This decreases MSH2 and MSH6 levels and is also accompanied by genetic instability in a mononucleotide repeat marker. The MSH2/MSH6 down-regulation is p53-dependent and thus potentially relevant to fewer tumors, those with functional p53. Finally, the Max network may also regulate *MSH2* in a HIF1-alpha-independent manner (Bindra and Glazer, 2007a). These findings parallel the microbial examples of down-regulation of MutS and functional limitation of MutL and MutS in response to stress. The transcriptional regulatory networks are different, and are understood in greater detail, but the outcomes for MMR function are similar, and the same components (homologues of MutS and MutL) become limiting (Table 2).

Altered DSBR—Second, HR-DSBR is down-regulated *via* transcriptional repression of *BRCA1* (breast cancer tumor suppressor protein) and *RAD51* (RecA homologue, see Table 1 for both). *RAD51* is transcriptionally down-regulated in response to hypoxia, and this down-regulation is correlated with a substantial decrease in HR in hypoxic conditions (Bindra *et al.*, 2004). The repression is mediated by the formation of repressive E2F4/p130 complexes, triggered by p130 dephosphorylation in response to hypoxia (Bindra and Glazer, 2007b). Similarly, the *BRCA1* gene, which functions in HR-DSBR and suppresses use of the more error-prone NHEJ pathway of DSBR (reviewed by Gudmundsdottir and Ashworth, 2006), is also transcriptionally down-regulated by repressive E2F complexes (Bindra *et al.*, 2005). *BRCA1* depletion leads to decreased HR, while the NHEJ pathway remains unaffected, potentially leading to increasing genomic instability *via* a switch to reliance on the inherently mutagenic NHEJ-DSBR pathway during hypoxia. This is highly analogous to, though a different mechanism from, the switch to mutagenic HR-DSBR induced by stress and controlled by RpoS in the Lac system. This resembles the yeast system in which stress-induced mutations require NHEJ proteins (reviewed above), suggesting a switch to error-prone NHEJ from HR-DSBR under stress.

Whatever the selective pressure (or lack of it) that allowed evolution of these responses in multicellular animals, both are expected to drive cancer evolution by promoting mutagenesis under stress. As suggested elsewhere (SM Rosenberg, EC Cox, RC von Borstel and LA Loeb, manuscript in preparation), anti-evolution drugs designed to interfere with the mutator mechanisms themselves would provide powerful accompaniments to standard chemotherapies, most of which block cell growth, induce stress, and have the potential to induce stress-induced resistance mutations.

OVERVIEW AND SIGNIFICANCE FOR EVOLVABILITY

There are similarities and differences in the various molecular mechanisms of mutagenesis discussed above, with several repeating examples of use of common components and/or common strategies that limit when and where mutagenesis occurs. These are summarized in Table 2. The many examples support a picture of mutagenesis as a highly regulated process, regulated both in time, and at least sometimes, in genomic space. The restriction of mutagenesis to times of stress by coupling it to stress responses provokes mutagenesis specifically when cells are maladapted to their environments, which is when mutator phenotypes will drive their adaptation. The limitation of mutagenesis to local genomic regions might, as reviewed above, limit deleterious mutations in genomes of rare adaptive mutants, and promote concerted evolution within genes and linked gene clusters (shown compellingly by Drake *et al.*, 2005; Drake, 2007; Wang *et al.*, 2007). Although these

principles have been demonstrated most directly in the Lac system, many other systems share common components—like use of RpoS, the SOS response, RecA, specialized and Y-family and SOS-controlled DNA polymerases, and transiently limiting mismatch repair—and/or common strategies: control of mutagenesis by stress responses, and evidence for localization of mutagenesis in genomic space. We have discussed the probable adaptive value of these strategies in microbial populations. It is less clear why multicellular organisms like humans should share these strategies, but it is clear that they do, and that stress-induced mutagenesis is likely to underlie evolution of tumorigenesis. Elsewhere the case is made that cancer formation, progression and evolution of resistance to treatments are also evolutionary processes driven by mutation and selection, often under stress (SM Rosenberg, EC Cox, RC von Borstel and LA Loeb, manuscript in preparation).

ARE STRESS-INDUCIBLE MUTAGENESIS MECHANISMS SELECTED?

Second-Order Selection

Is stress-inducible mutator character of itself a selectable trait, or is it an incidental byproduct of a stress-inducible function that is selected? There is little evidence to reject or favor either hypothesis (Tenaillon *et al.*, 2004). That some aspect of stress-induced mutagenesis mechanisms is selected is strongly implied by the correlation of stress-inducible mutability with ecological niche rather than phylogeny (Bjedov *et al.*, 2003). As for constitutive mutators, mutator alleles become fixed in populations under strong selective pressure by hitchhiking along with the advantageous mutations that they may induce (Introduction and Tenaillon *et al.*, 1999). This second-order selection could also apply to stress-induced mutation mechanisms, and computer simulations suggest that both constitutive and stress-inducible mutator phenotypes can be selected in this way (Bjedov *et al.*, 2003). The other possibility is that natural selection favors some characteristic(s) that are accompanied by the byproduct of increased mutability during stress: the pleiotropy hypothesis. In this case, evolution favors transient increases in mutation rates *via* other benefits, such as a strategy for saving energy for other cellular functions by shutting down replication fidelity mechanisms, or upregulating error-prone polymerases to achieve increased resistance to DNA damage encountered during the stress period, *etc.* Although, as noted, the “error-prone” DNA polymerases are usually error-prone only when *not* performing translesion synthesis; so random mutagenesis using them would be expected to be avoidable if it were selected against. That is, survival promoted by (high-fidelity) translesion synthesis could occur without concurrent mutagenesis on non-lesion template DNAs. Importantly, stress-inducible mutability will accelerate evolution in competitive environments regardless of why cells possess the ability.

Periodic Selection

We have seen above that there is strong evidence that constitutive mutator alleles are repeatedly lost and regained, revealed by the mosaic phylogeny of the sequence of *mutS* (Denamur *et al.*, 2000). This tells us that a mutator phenotype is advantageous in some environments/circumstances and not in others, *i.e.*, it is subject to periodic (positive then negative) selection. Could this also apply to the possession of a stress-inducible mutator character? The answer to this question bears profoundly the question of whether stress-inducible mutability is itself a selectable trait.

We see evidence, presented above, that it is indeed likely that stress-inducible mutability is a capacity that is lost and regained as needed. First, there is huge variability in the strength of stress-inducible mutators in wild isolates (Bjedov *et al.*, 2003; Saint-Ruf and Matic, 2006) suggesting that different stress-inducible mutator activities have different mechanisms. Second, as reviewed above, there is in fact wide variation in the molecular mechanisms of

stress-inducible mutagenesis among the different systems, as revealed by the differing genetic requirements. The picture appears to be patchwork of mechanisms with common parts and strategies used interchangeably in different stains, species, and stresses, as if stress-inducible mutability has been “invented,” lost and “reinvented” independently many times. Third, the distribution of stress-inducible mutability does not correlate with phylogenetic relationships among natural isolates, but rather, correlates with habitat (Bjedov *et al.*, 2003). This strongly suggests that it is under short-term environment-specific selection. These facts argue strongly that stress-inducible mutability is under periodic selection.

RpoS in Periodic Selection?—Finally, we note that in bacteria, regulation of mutagenesis by RpoS is a common feature even in systems with different mutagenesis mechanisms. We suggest that periodic selection of RpoS could provide a critical master “switch” underlying periodic selection of some and perhaps many bacterial stress-inducible mutagenesis mechanisms. Like *mutS*, the *rpoS* gene itself is highly polymorphic in different *E. coli* populations demonstrating that its function is under periodic selection (Ferenci, 2003). Ferenci has argued that loss of RpoS function is selected due to fitness costs associated with this stress response in decreasing transcription of needed housekeeping genes, as in GASP (Ferenci, 2003). We suggest that that could account for selection against stress-inducible mutagenesis pathways by selecting against their most appropriate master regulator, and that second-order selection for stress-inducible mutability in competitive habitats swings the pendulum back. Stress-inducible mutagenesis mechanisms should be advantageous in competitive environments, and irrelevant in environments to which cells are well adapted, in which the RpoS component is at times demonstrably sometimes costly.

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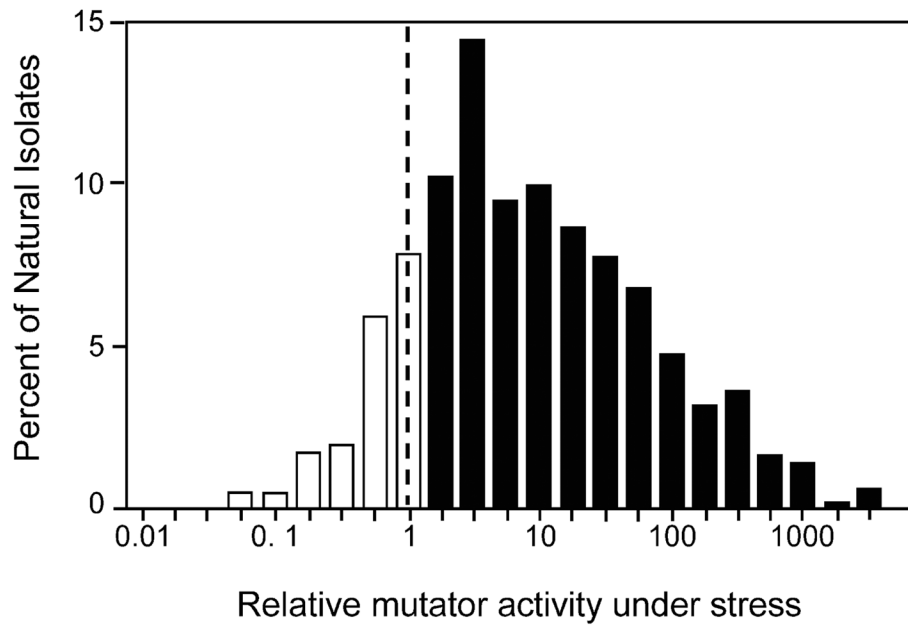


FIGURE 1.

Most *E. coli* natural isolates display stress-inducible mutagenesis. Bjedov *et al.* (2003) examined 787 natural isolates from various habitats for mutagenesis in aging colonies on solid medium, a starvation stress. The frequencies of rifampicin-resistant (base-substitution) mutants per viable cell after seven days in an aging colony relative to that after only one day are shown for all 787 strains. Numbers greater than one indicate induction of mutagenesis. More than 80% showed stress-inducible mutagenesis, indicating a common feature of many natural isolates. Figure re-drawn from Rosenberg and Hastings's (2003) review of the Bjedov *et al.* paper.

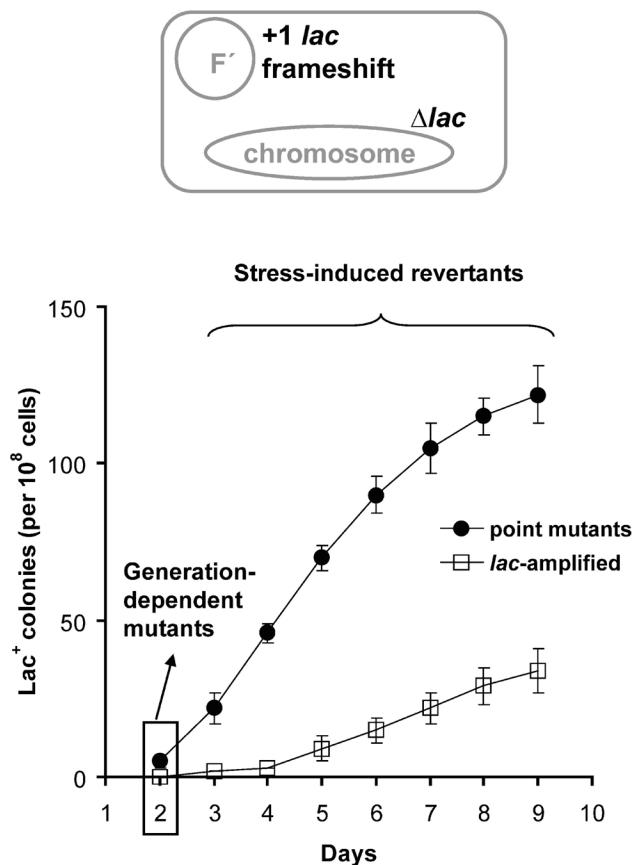


FIGURE 2.

The *E. coli* Lac assay for stress-induced mutagenesis. Above: schematic representation of the strain used in the Lac assay. This strain bears a ~200 kb F' conjugative plasmid carrying the mutant *lacIZ33* allele, a *lacI-lacZ* fusion gene with a +1 frameshift mutation. Below: generation-dependent mutation events occurring during growth of the culture prior to plating on lactose medium are detected as Lac⁺ colonies present on about day 2. During subsequent days of incubation, stress-induced Lac⁺ colonies accumulate, and include both Lac⁺ point mutants with a compensatory frameshift mutation or *lac*-amplified cells, with 20 to 50 copies of DNA spanning the *lac* gene, which provides sufficient beta-galactosidase activity for growth without a frameshift reversion mutation (Hastings *et al.*, 2004).

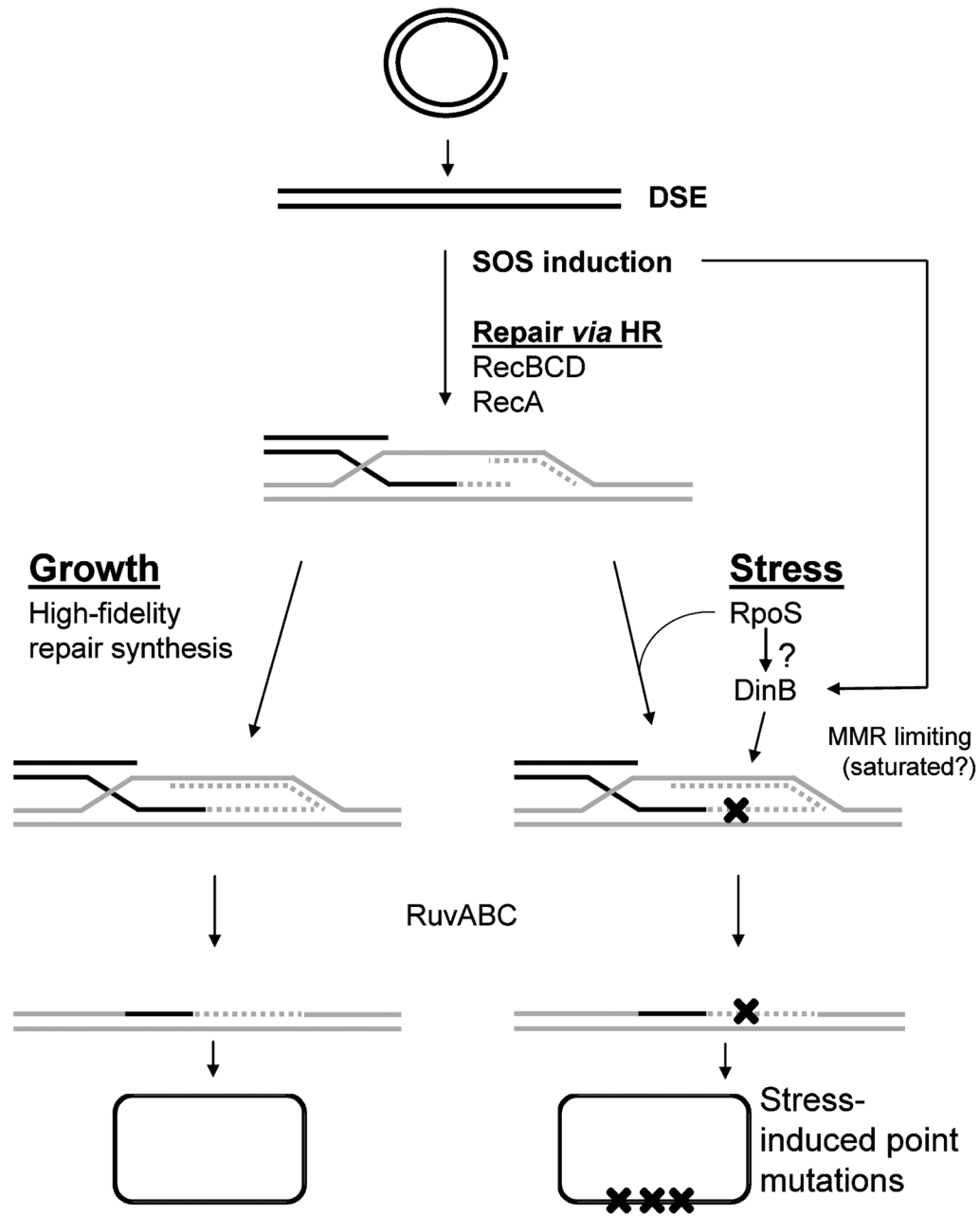
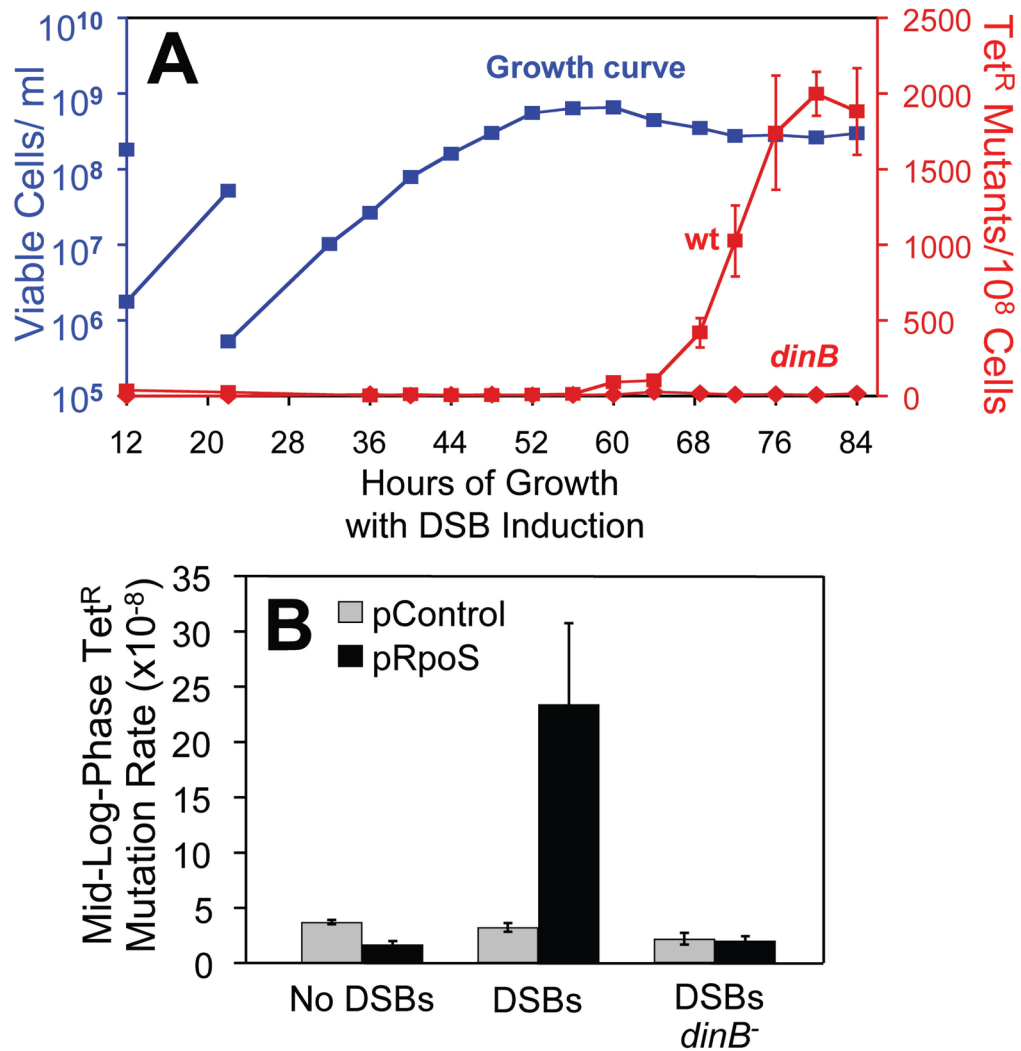


FIGURE 3.

A switch from high-fidelity to error-prone DNA double-strand-break repair underlies stress-induced mutagenesis in the Lac system. Double-strand breaks are repaired *via* high-fidelity HR-DSBR in cells growing in optimal conditions. However, in growth-limited cells expressing the RpoS stress-response regulon, DSBs are repaired mutagenically under the control of RpoS, which somehow licenses the use of the DinB error-prone polymerase, which is upregulated by the SOS and RpoS responses. Mismatch repair (MMR) becomes limiting under this condition, and fails to correct many DNA polymerase errors, we suggest due to saturation/titration of MutL protein by excess DNA polymerase errors (described in the text). Single lines represent strands of DNA except in the two circular molecules at the bottom, where they represent whole bacterial chromosomes composed of double-stranded

DNA. X's represent DNA polymerase errors, and then mutations in the bottom-most molecule.

**FIGURE 4.**

The switch to mutagenic double-strand-break repair in stationary phase, or if RpoS is expressed. Data reprinted with permission from Ponder *et al.* (2005), on stress-induced mutagenesis in the *E. coli* Lac system. Here, reversion not of *lac* but of a *tetA*+1 frameshift allele near to *lac* is assayed in cells grown in medium without lactose (such that no DNA amplification is selected, reviewed in the text). (A) An I-SceI endonuclease-generated DSB made near *tetA* is induced at a low level continuously during exponential growth and then stationary phase (*blue line* represents growth curve), but provokes DinB-dependent *tetA* reversion (*red lines*) only in stationary phase. (B) Mutation rate data from a mid-logarithmic-phase time point from experiments as in A, but this time with the RpoS, general stress-response transcriptional activator protein produced weakly from a plasmid. DinB-dependent *tetA* reversion occurs during mid-log phase if RpoS is expressed and DSBs are induced, showing that stationary phase is not necessary if RpoS is supplied even at low levels.

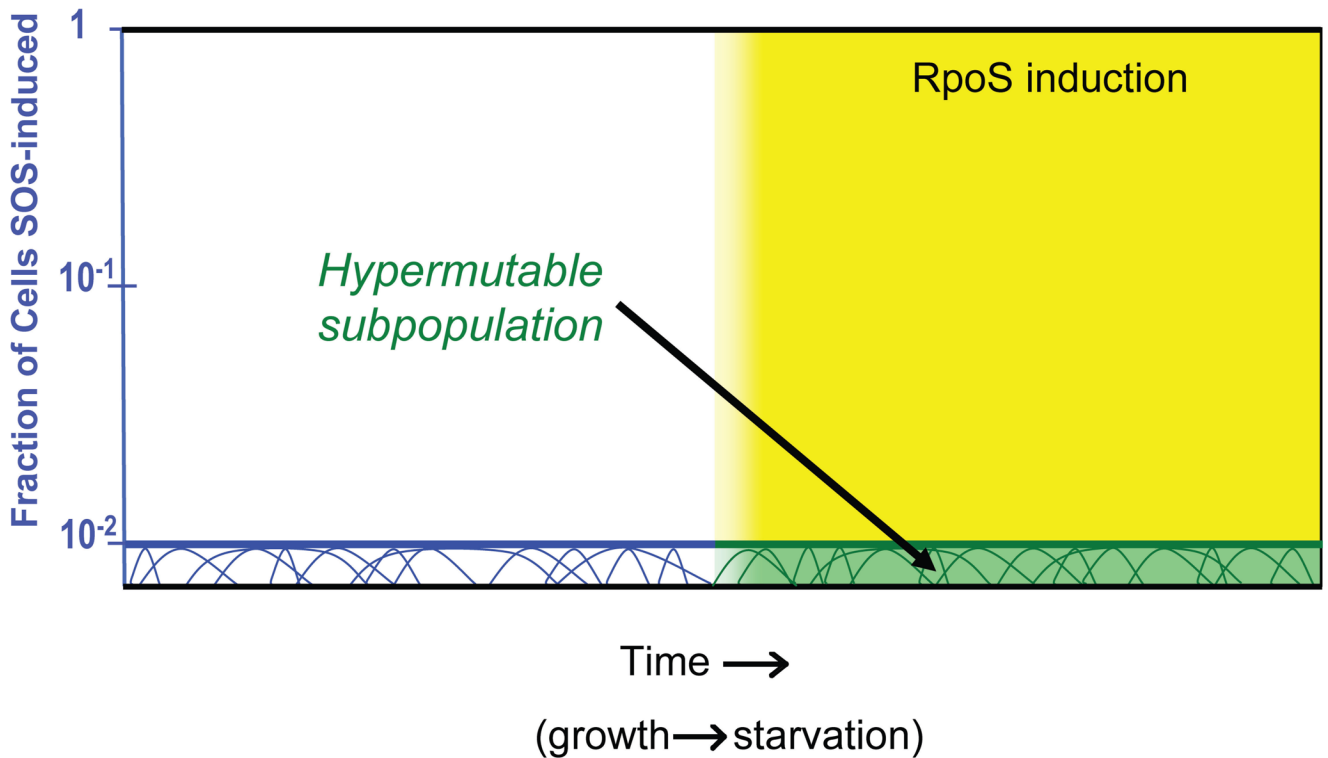


FIGURE 5.

Model for the origin of the hypermutable cell subpopulation in the Lac system: convergence of two stress responses. We suggest that the hypermutable cell subpopulation (HMS) associated with stress-induced point mutagenesis in the *E. coli* Lac assay system results from the convergence of cells experiencing both the SOS response (with a DNA double-strand break) and the RpoS response simultaneously. In this version of the model, spontaneous SOS induction and DNA double-strand breakage are constants at the roughly 0.6% level observed in logarithmically growing *E. coli* cells, with cells cycling into and out of the SOS-induced population at varying rates depicted by curves under the 1% SOS-induced population (Pennington and Rosenberg, 2007). SOS and DSBs are necessary but not sufficient for producing an HMS cell; induction of RpoS, shown as cells enter stationary phase, is also required. This model explains why there is a switch from high-fidelity to error-prone DSBR as cells enter stationary phase or when RpoS is induced (Ponder *et al.*, 2005 and Figure 4).

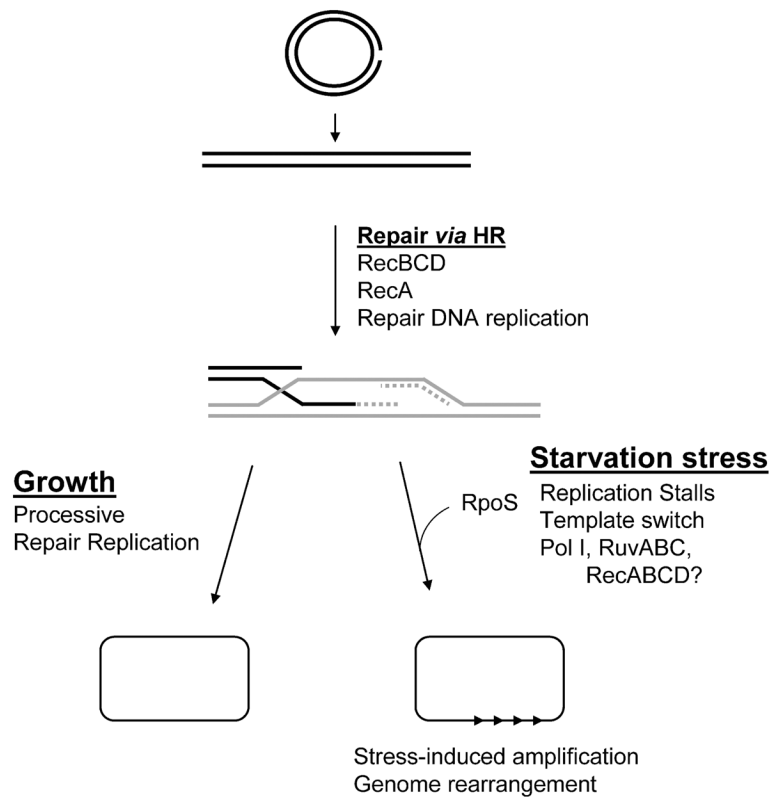


FIGURE 6. Model for amplification induced by rearrangement-provoking replication fork stalling events, and template switching, during acts of DSB repair under stress. Model from Slack *et al.* (2006), and discussed in the text. Single lines represent strands of DNA except in the two circular molecules at the bottom, where they represent whole bacterial chromosomes composed of double-stranded DNA.

TABLE 1

Definitions of some components in stress-induced mutagenesis pathways

RpoS/ σ^S (General Stress Response Activator)	A bacterial transcriptional activator protein (sigma factor) that controls a large general stress response. The RpoS (AKA general-, starvation-, or stationary-phase stress) response is induced, upon entry into the stationary phase and in response to starvation, acid pH, osmotic shock, cold shock and oxidative stresses. RpoS upregulates transcription of ~340 genes (Weber <i>et al.</i> , 2005), many of which play various roles in stress resistance. Reviewed by Hengge-Aronis (2002).
SOS Response	The DNA-damage stress response of bacteria. SOS upregulates ~40 genes that function in DNA repair, cell-cycle check-point control (cell division arrest), DNA damage-tolerance including translesion DNA synthesis <i>via</i> the use of three DNA polymerases Pol II, Pol IV (DinB), and Pol V (UmuD ₂ C). RecA senses DNA damage and then facilitates auto-proteolytic cleavage of LexA transcriptional repressor, thus de-repressing the expression of the SOS regulon. Reviewed by Friedberg <i>et al.</i> (2006).
Competence Response of <i>B. subtilis</i>	Competence (for natural transformation) is a differentiated state in which cells take up and incorporate foreign DNA. Competence development occurs in response to nutrient deprivation and high cell density, controlled by the ComP/ComA two-component regulatory system. The ComA response regulator activates expression of competence genes when phosphorylated by ComP. When cells become competent, auto-regulated transcriptional activator, ComK, mediates DNA synthesis arrest, inhibition of cell division, and expression of DNA-binding, uptake and recombination genes. Reviewed by Claverys <i>et al.</i> (2006).
Stringent Response	Amino-acid-starvation-stress response activated by nutritional deprivation in bacteria that upregulates aminoacid-biosynthesis genes, and is activated by guanosine nucleotides GDP 3'-diphosphate or GTP 3'-diphosphate, commonly referred to as (p)ppGpp. Other effects of ppGpp include facilitation of RpoS-dependent gene expression, and DNA replication arrest. Reviewed by Chatterji and Ojha (2001).
Release from Catabolite Repression	A response to carbon starvation that globally regulates carbon utilization genes. When released from catabolite repression, the repression of genes involved in the utilization of some sugars in the presence of better carbon sources, like glucose is released. The CAP/CRP protein mediates this response by binding cAMP and becoming a transcriptional activator for promoters containing a CAP binding site, like the <i>lac</i> , <i>mal</i> , <i>gal</i> , and <i>ara</i> operons. Synthesis of cAMP by adenylate cyclase (product of the <i>cya</i> gene) is turned on when the levels of glucose in cells are low. Reviewed by Snyder and Champness (2002).
PhoPQ Response	A two-component transcriptional regulatory system, that responds to several stresses such as acidic pH, low intracellular concentrations of divalent cations and antimicrobial peptides. Several genes involved in stress response and virulence are regulated by this system. Reviewed by Groisman (2001).
MadI/Max and Mnt/Max Complexes	Max is a mammalian transcriptional regulator whose activity is modulated by interaction with several members of the helix-loop-helix leucine zipper family, like c-Myc and the Mad subfamily. c-Myc/Max complexes function in transcriptional activation, whereas complexes with Mad proteins such as Mad1, Mnt and Mx1 are repressors. Expression of MMR genes, normally activated by c-Myc/Max complexes, is down-regulated during hypoxia due to the formation of repressive complexes of Max with Mad proteins. See Bindra and Glazer (2007a) and references therein.
p53	Mammalian transcription factor regulating the expression of genes implicated in apoptosis, DNA repair and cell cycle arrest in response to DNA damage. p53 is a very important gate keeper of genome integrity, acting as a tumor suppressor gene. Mutations inactivating p53 are found in at least 50% of all human cancers. Reviewed by Toledo and Wahl (2006).
HIF-1 α	A mammalian transcription factor regulating gene expression in response to hypoxia. Normally degraded by the ubiquitin-proteasome, this protein is stabilized under hypoxia conditions, leading to the transcriptional activation of genes containing HRE (hypoxia responsive element) sequences in their promoters. Reviewed by Ke and Costa (2006).
E2F4/p130	E2Fs compose a family of mammalian transcriptional factors controlling genes involved in cell-cycle regulation, DNA-damage response and apoptosis. Depending on the family member and association with other proteins, E2Fs may function as transcriptional activators or repressors. In particular, E2F4 and p130 associate to form a repressive complex demonstrated to inhibit gene expression under hypoxia conditions. See (Bindra and Glazer, 2007b) and references therein.
BRCA1	Human protein required for DSB repair <i>via</i> homologous recombination. Acts with RAD51. Defects cause breast-cancer predisposition. Reviewed by Gudmundsdottir and Ashworth, 2006).
RAD51	Eukaryotic RecA homologue required for DSB-repair <i>via</i> homologous recombination. Acts with BRCA1 protein in humans. Reviewed by Friedberg <i>et al.</i> (2006).
RecA	Bacterial DNA repair protein that catalyzes homologous genetic recombination (used in single-strand-gap repair and double-strand-break repair) and also induction of the SOS response. RecA forms a nucleoprotein filament on single-stranded DNA that promotes its interaction with homologous sequences in double-stranded DNA, and also activates the SOS response by promoting proteolytic cleavage of the LexA transcriptional repressor of the DNA damage-response genes. The eukaryotic homologue, Rad51, functions similarly in recombinational DNA repair. Reviewed by Cox (2007).

RecBCD	The main double-strand DNA end (DSE)-recognition and double-strand-break-repair (DSBR) enzyme complex of <i>E. coli</i> and related bacteria, composed of RecB, RecC, and RecD subunits. An exonuclease and helicase. RecBCD processes only DSEs at which it promotes DSB repair by exposing single-strand DNA and loading RecA onto the single-strands. Reviewed by Friedberg <i>et al.</i> (2006).
RuvABC	<i>E. coli</i> recombinational DNA repair proteins that function in DSBR and other homologous recombination. This complex catalyzes endonucleolytic cleavage of Holliday-junction intermediates in recombination. Reviewed by Yamada <i>et al.</i> (2004).
DinB and Y-family DNA Polymerases	A DNA polymerase superfamily conserved in all three domains of life, with two representatives (of the five total DNA polymerases) in <i>E. coli</i> (DinB or Pol IV and Pol V or UmuD ₂ C), and four (of nearly 20 total) in humans. These are poorly processive, specialized DNA polymerases that catalyze "translesion" DNA synthesis, which allows replication forks halted at non-instructive DNA lesions to insert one or two bases and continue, allowing cell survival. Most are high-fidelity (not mutagenic) when inserting bases across from their cognate lesions but are highly error-prone/mutagenic when synthesizing on undamaged DNA. The DinB subfamily contains bacterial members required for stress-induced mutagenesis in several different assay systems and organisms, and is the only subfamily conserved in eubacteria, archaea, and eukaryotes (human orthologue of <i>E. coli</i> /DinB: DINB1). Other subfamilies: UmuC (eubacterium-specific) Rad30 and Rev1 (eukaryote-specific). Reviewed by Nohmi (2006).
DNA Polymerase I	<i>E. coli</i> high-fidelity DNA polymerase used in DNA replication (Okazaki-fragment processing), various DNA repair pathways, and stress-induced gene amplification. Expressed constitutively. Reviewed by Friedberg <i>et al.</i> (2006).
DNA Polymerase II	<i>E. coli</i> high-fidelity DNA polymerase used in some DNA repair reactions and some stress-induced point mutagenesis pathways. Expressed constitutively and upregulated by the SOS response. Pol II may also assist the Y-family DNA polymerases IV (DinB) and V in translesion synthesis (Napolitano <i>et al.</i> , 2000) and is also considered a specialized DNA polymerase. Reviewed by Friedberg <i>et al.</i> (2006).
DNA polymerase III	<i>E. coli</i> major replicative DNA polymerase, a high-fidelity DNA polymerase and an essential protein. Reviewed by Friedberg <i>et al.</i> (2006).
DNA polymerase V (UmuD ⁺ C)	<i>E. coli</i> Y-family translesion DNA polymerase responsible for "targeted" ultraviolet-light-induced SOS mutagenesis, and some other stress-induced mutagenesis responses. Reviewed by Friedberg <i>et al.</i> (2006); Schlacher and Goodman (2007).
Rev3/DNA Pol zeta	Eukaryotic translesion DNA polymerase not in the Y family. Also error-prone on non-lesion template DNAs. Causes mutations associated with DSB repair in yeast (Holbeck and Strathern, 1997).
Mismatch Repair (MMR)	Highly and widely conserved DNA repair pathway that corrects errors in DNA synthesis post-synthetically by recognizing mispaired bases in DNA, and excising them from the new DNA strand. Perhaps the most important cellular enforcer of genetic and chromosomal stability, MMR—reduces mutations <i>via</i> post-synthesis DNA-polymerase-error correction; suppresses recombination between partially identical DNA sequences, which can lead to chromosome rearrangements; and inhibits transposon mobility. MMR-defective organisms/cells have high mutation rates ("mutator" phenotype) and cancer-predisposition in humans. Reviewed by Kunkel and Erie (2005). In several bacteria and human cells, stress induces transient dysfunction of MMR promoting transiently increased mutagenesis (reviewed here).
Non-Homologous DNA End Joining (NHJ) and Ku Protein	A low-fidelity DNA DSB repair pathway, alternative to high-fidelity homologous recombination, in which DSEs are processed and then religated, mediated by Ku end-binding proteins. More likely to cause genome rearrangements (<i>e.g.</i> , translocations, deletions) than DSB repair <i>via</i> homologous recombination. Operative in eukaryotes and some bacteria, not <i>E. coli</i> . Reviewed by Bowater and Doherty (2006).

TABLE 2
Common elements between various stress-induced mutagenesis pathways/mechanisms

Components	Organism	Mutation Assay	Reference
Stress responses			
Requirement for σ^5 /RpoS general stress response	<i>E. coli</i>	Stress-induced point mutation, Lac assay	(Layton and Foster, 2003; Lombardo <i>et al.</i> , 2004)
	<i>E. coli</i>	Stress-induced amplification, Lac assay	(Lombardo <i>et al.</i> , 2004)
	<i>E. coli</i>	DSBR-associated <i>tetA</i> -frameshift-reversion mutation ¹	(Ponder <i>et al.</i> , 2005)
	<i>E. coli</i> natural isolate	Mutagenesis in aging colonies (MAC)	(Bjedov <i>et al.</i> , 2003)
	<i>E. coli</i>	Starvation-induced Mu excisions	(Gomez-Gomez <i>et al.</i> , 1997; Lamrani <i>et al.</i> , 1999)
	<i>P. putida</i>	Starvation-induced Phe ⁺ point mutations	(Saumaa <i>et al.</i> , 2002)
	<i>P. putida</i>	Starvation-induced Tn4652 transposition	(Ilves <i>et al.</i> , 2001)
Requirement for decreased function of σ^5 /RpoS general stress response	<i>E. coli</i>	GASP mutations ²	(Zambrano <i>et al.</i> , 1993)
SOS DNA damage response	<i>E. coli</i>	Stress-induced point mutation, Lac assay	(McKenzie <i>et al.</i> , 2000)
	<i>E. coli</i>	Mutagenesis in aging colonies (MAC) in laboratory strain	(Taddei <i>et al.</i> , 1995)
	<i>E. coli</i>	Ciprofloxacin-induced resistance mutations in <i>E. coli</i>	(Cirz <i>et al.</i> , 2005)
Rad9/Rad17/Rad24 DNA-damage checkpoint	yeast	Telomere-shortening-stress-induced retrotransposition of Ty element	(Scholes <i>et al.</i> , 2003)
ComAK competence differentiation/starvation-stress response	<i>B. subtilis</i>	Starvation-induced reversion of amino acid auxotrophy	(Sung and Yasbin, 2002)
Stringent response to amino acid starvation (ppGpp synthesis)	<i>E. coli</i>	Amino acid-starvation-induced, transcription-associated mutagenesis	(Wright <i>et al.</i> , 1999)
Cyclic AMP/release from catabolite repression starvation response	<i>B. subtilis</i>	Amino acid-starvation-induced mutagenesis	(Rudner <i>et al.</i> , 1999)
	<i>E. coli</i>	Mutagenesis in aging colonies (MAC) in laboratory strain	(Taddei <i>et al.</i> , 1995)
PhoPQ regulon ³	<i>E. coli</i>	Starvation-induced Mu excisions	(Lamrani <i>et al.</i> , 1999)
Transcriptional repression by MtdI/Max and Mnt/Max complexes, which down-regulate genes in response to growth arrest	<i>E. coli</i>	Starvation-induced mutations in the <i>ebgR</i> gene	(Hall, 1998)
HIF-1/alpha hypoxia-stress response and p53 DNA-damage response	Human	Hypoxia-induced transcriptional down-regulation of <i>MLH1</i> (mismatch-repair function in human cells causing genetic (dinucleotide-repeat) instability	(Bindra and Glazer, 2007a; Mihaylova <i>et al.</i> , 2003)
E2F4/p130-mediated transcriptional repression (response to radiation and oxidative damage and hypoxia stress)	Human	Hypoxia-induced transcriptional down-regulation of <i>MSH2</i> and <i>MSH6</i> mismatch-repair genes in human cells causing genetic (dinucleotide-repeat) instability Hypoxia-induced down-regulation of RAD51, BRCA1 and DSBR <i>via</i> HR presumably leading to genome instability in human cells <i>via</i> NHEJ substituting in DSBR	(Koshiji <i>et al.</i> , 2005) (Bindra <i>et al.</i> , 2005; Bindra and Glazer, 2007b)

Components	Organism	Mutation Assay	Reference
Specialized DNA polymerases			
DinB/Pol IV and other Y-family polymerases	<i>E. coli</i>	Stress-induced point mutation, Lac assay (DimB)	(Foster, 2000; McKenzie <i>et al.</i> , 2001)
	<i>E. coli</i>	Ciprofloxacin-induced resistance mutations, requiring three SOS-inducible DNA polymerases, two in the Y-family: DinB/Pol IV and UmuD'C/PolV	(Cirz <i>et al.</i> , 2005)
	<i>P. putida</i>	Starvation-induced Phe ⁺ point mutations (DimB homologue)	(Tegova <i>et al.</i> , 2004)
	<i>B. subtilis</i>	Starvation-induced reversion of amino acid auxotrophy (DimB homologue)	(Sung <i>et al.</i> , 2003)
Pol II	<i>E. coli</i> natural isolate	Mutagenesis in aging colonies (MAC)	(Bjedov <i>et al.</i> , 2003)
	<i>E. coli</i>	Ciprofloxacin-induced resistance mutations, requiring SOS-inducible DNA Pols II, IV and V	(Cirz <i>et al.</i> , 2005)
Pol I	<i>E. coli</i>	Stress-induced amplification, Lac assay	(Hastings <i>et al.</i> , 2004)
	<i>E. coli</i>	Mutagenesis in aging colonies (MAC) in laboratory strain	(Taddei <i>et al.</i> , 1997a)
Rev3/DNA Pol zeta	<i>S. cerevisiae</i>	Mutations associated with DNA DSB repair <i>via</i> homologous recombination ⁴	(Holbeck and Strathern, 1997)
Limiting Mismatch Repair Function			
MutL/MLH1 the limiting component	<i>E. coli</i>	Stress-induced point mutation, Lac assay	(Harris <i>et al.</i> , 1997, 1999)
	Human	Mutations induced by hypoxia in human cells, <i>MLH1</i> transcriptionally down-regulated	(Mihaylova <i>et al.</i> , 2003)
MutS/MSH2/MSH6 the limiting component	<i>E. coli</i> natural isolate	Mutagenesis in aging colonies (MAC)	(Bjedov <i>et al.</i> , 2003)
	<i>B. subtilis</i>	Starvation-induced reversion of amino acid auxotrophy	(Pedraza-Reyes and Yasbin, 2004)
	<i>E. coli</i>	Decreased mismatch repair of heteroduplexes in phage DNA in stationary-phase cells	(Brégeon <i>et al.</i> , 1999)
	Human	Dinucleotide instability in human cells, <i>MSH2</i> and <i>MSH6</i> transcriptionally down-regulated	(Koshiji <i>et al.</i> , 2005; To <i>et al.</i> , 2005)
MutH a minor limiting component	<i>E. coli</i>	Decreased mismatch repair of heteroduplexes in phage DNA in stationary-phase cells	(Brégeon <i>et al.</i> , 1999)
DNA Repair and Recombination			
RecA/Rad51	<i>E. coli</i>	Stress-induced point mutation, Lac assay	(Cairns and Foster, 1991; Harris <i>et al.</i> , 1994)
	<i>E. coli</i>	Stress-induced amplification, Lac assay	(Slack <i>et al.</i> , 2006)
	<i>E. coli</i>	Ciprofloxacin-induced resistance mutations	(Cirz <i>et al.</i> , 2005)
	<i>E. coli</i>	Mutagenesis in aging colonies (MAC) in laboratory strain	(Taddei <i>et al.</i> , 1995, 1997a)
	<i>S. cerevisiae</i>	Mutations associated with DNA DSB repair <i>via</i> homologous recombination ⁴	(Strathern <i>et al.</i> , 1995)
Down-regulation of RecA homologue RAD51 required	Human	Hypoxia-induced down-regulation of homologous recombination, presumably leading to genome instability	(Bindra <i>et al.</i> , 2005)

Components	Organism	Mutation Assay	Reference
RecBCD	<i>E. coli</i>	Stress-induced point mutation, Lac assay	(Harris <i>et al.</i> , 1994)
	<i>E. coli</i>	Stress-induced amplification, Lac assay	(Slack <i>et al.</i> , 2006)
	<i>E. coli</i>	Ciprofloxacin-induced resistance mutations	(Cirz <i>et al.</i> , 2005)
	<i>E. coli</i>	Mutagenesis in aging colonies (MAC) in laboratory strain	(Taddei <i>et al.</i> , 1997a)
	<i>E. coli</i>	Stress-induced point mutation, Lac assay	(Foster <i>et al.</i> , 1996; Harris <i>et al.</i> , 1996)
RuvABC	<i>E. coli</i>	Stress-induced amplification, Lac assay	(Slack <i>et al.</i> , 2006)
	<i>E. coli</i>	Ciprofloxacin-induced resistance mutations	(Cirz <i>et al.</i> , 2005)
Ku/NHEJ proteins	<i>S. cerevisiae</i>	Stress-induced amino acid-auxotrophy reversion	(Heidenreich <i>et al.</i> , 2003)
	Human	Presumed for hypoxia-induced genome instability in human cells caused by NHEJ substituting for homologous recombination in DSB repair due to down-regulation of RAD51 and BRCA1	(Bindra <i>et al.</i> , 2005)
Localized Mutagenesis Processes?			
Mutational clustering of unknown mechanisms	Mouse	Temporally and spatially clustered spontaneous mutations in mouse somatic cells ⁵	(Wang <i>et al.</i> , 2007)
	Many organisms	Multiple lines of evidence for clustering of mutations in many diverse organisms and <i>in vitro</i> ⁵	(Drake <i>et al.</i> , 2005; Drake 2007)
Mutagenesis associated with DSB repair	<i>E. coli</i>	Stress-induced point mutation, Lac assay	(Ponder <i>et al.</i> , 2005)
	<i>E. coli</i>	Stress-induced amplification, Lac assay	(Ponder <i>et al.</i> , 2005; Slack <i>et al.</i> , 2006)
	<i>E. coli</i>	Presumed for ciprofloxacin-induced resistance mutations	(Cirz <i>et al.</i> , 2005)
	<i>S. cerevisiae</i>	Mutations associated with DNA double-strand-break repair <i>via</i> homologous recombination ⁴	(Strathern <i>et al.</i> , 1995)
	<i>S. cerevisiae</i>	Presumed for stress-induced amino acid auxotrophy reversion dependent on Ku/NHEJ proteins	(Heidenreich <i>et al.</i> , 2003)
	Human	Presumed for hypoxia-induced genome instability in human cells caused by NHEJ substituting for homologous recombination in DSB repair due to down-regulation of RAD51 and BRCA1	(Bindra <i>et al.</i> , 2005; Bindra <i>et al.</i> , 2004)
Mutagenesis associated with nucleotide-excision repair (NER)	<i>E. coli</i>	Suggested for mutagenesis in aging colonies (MAC) assay based on requirement for UvrB NER protein	(Taddei <i>et al.</i> , 1997a)
Mutagenesis associated with transcription	<i>S. cerevisiae</i>	Increased mutation in highly transcribed genes ⁵	(Datta and Jinks-Robertson, 1995)
	<i>E. coli</i>	Amino acid-starvation-induced, transcription-associated mutagenesis	(Wright <i>et al.</i> , 1999)
	<i>E. coli</i>	Transcription-coupled-repair-associated mutations suggested by genome sequence data	(Francino <i>et al.</i> , 1996)
	<i>B. subtilis</i>	Possible transcription association implied by the requirement for the transcription-coupled-nucleotide-excision-repair factor, Mfd, for these stress-induced mutations.	(Ross <i>et al.</i> , 2006).

- ¹ These mutations associated with repair of a restriction-enzyme-produced DSB *in vivo* can form only if the cells are in stationary phase or if RpoS is expressed inappropriately in the log phase (implying that RpoS expression in stationary phase accounts for the stationary-phase specificity of the mutagenesis) (see Figure 4).
- ² GASP (Growth Advantage in Stationary Phase) mutations are not demonstrated to be stress-induced mutations, but might possibly be. They are mutations that confer increased fitness that allows stationary-phase stressed cells to out-compete neighboring cells. It has not been determined whether the mutations occur before or after the cells enter stationary phase (the stress condition). Mutations that partly diminish function of σ^S arise early in the GASP process and confer a fitness advantage.
- ³ PhoPQ are part of a two-component transcriptional regulatory system involved with scavenging phosphorus and magnesium and are implicated in stress responses partly because they stabilize RpoS (Tu *et al.*, 2006).
- ⁴ These mutations are not known to be starvation- stationary-phase- or stress-associated. Whether stress or stationary phase were involved the activity of this DSBR-associated mutagenesis mechanism was not examined.
- ⁵ Not known to be stress-associated.