Chapter 2
Mutagenesis Associated with Repair of DNA Double-Strand Breaks Under Stress

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Abstract Evolutionary theory predicted that mutations occur randomly both in time and in genomic space. This expectation has been revised by the discoveries of stress-induced mutation mechanisms, which activate mutagenesis pathways under the control of stress responses. Stress-induced mutation mechanisms produce mutations preferentially when cells or organisms are maladapted to their environment, i.e., when they are stressed, potentially accelerating evolution. We review stress-induced mutagenesis associated with repair of double-strand breaks in *Escherichia coli*. In this mechanism, the process of DNA break repair by homologous recombination is high-fidelity in unstressed cells, but is switched to a mutagenic mode using the error-prone DNA polymerase DinB, and other error-prone DNA polymerases, under the control of the RpoS general stress response. The switch to mutagenic repair occurs during starvation or if RpoS is upregulated artificially in unstressed cells, and presumably during the many different stresses that activate the RpoS response. Recent work shows that this mechanism accounts for most spontaneous base-substitution and frameshift mutagenesis during starvation in *E. coli*, acts not only in plasmid DNA but also in the chromosomes of plasmid-free cells, illustrates the generality of this mechanism in many organisms and circumstances, and resolves some other old tensions in the field. Stress-induced mutation mechanisms studied in the laboratory are likely to provide supe-

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rior models for mutagenesis underlying pathogen-host adaptation, antibiotic resistance, and cancer progression and resistance mechanisms, all problems of evolution under stress driven by mutations.

Stress-Induced Mutation Is Changing Ideas About Evolution

Before the acceptance of Darwin's theory of natural selection, the impact of environmental stress on induction of natural variation was a popular idea (Mayr 1982). However, lack of strong experimental evidence of environmental influence on induction of genetic variation diminished the idea's traction and it was replaced by the neo-Darwinian modern synthesis of the 1930s. In the neo-Darwinian view (Mayr 1982), mutations were imagined to be random, and to occur randomly in time and in genomic space, with natural selection being the sole driver of evolution. The rise of the neutralists added genetic drift (random survival and proliferation of individuals with mutations) as an additional driver of evolution (Kimura 1991), but did not change the general view of the randomness of mutations.

However, recent discoveries of special mechanisms of mutation induced in bacteria, yeast, plant, and human cells under growth-limiting stress is changing this view (Galhardo et al. 2007; Hastings 2007; Heidenreich 2007; Saint-Ruf et al. 2007; Cirz and Romesberg 2007; Bindra et al. 2007; Fonville et al. 2011; Yao and Kovalchuk 2011; Rosenberg et al. 2012). These mechanisms increase genetic diversity and, potentially, the ability to evolve, specifically when cells are maladapted to their environment, i.e., when they are stressed. The kinds of mutations generated include base substitutions, small deletions and insertions, gross chromosomal rearrangements including copy-number variations (CNVs, amplifications, and large deletions), and transpositions. Although the mechanisms that produce stress-induced mutations vary, the overriding common theme in these pathways is their activation by cellular stress responses, resulting in increased, and sometimes different kinds of, mutations under stress. The kinds of stressors that can provoke stress-induced mutation mechanisms include starvation, hypoxia, oxidative stress, antibiotics, and, presumably, many others that activate the stress responses that promote stress-induced mutations. Understanding of molecular mechanisms of stress-induced mutagenesis is changing how we think about evolution. Stress-induced mutation mechanisms may provide superior models for genetic changes that drive pathogen–host adaptation, antibiotic resistance, aging, cancer progression, and therapy-resistance mechanisms, and, possibly much of evolution, generally. In this chapter, we focus on recent advances in understanding a molecular mechanism of stress-induced mutation, dissected initially in starving *Escherichia coli* cells: double-strand-break-dependent stress-induced mutation. Other stress-induced mutation mechanisms are reviewed by (Galhardo et al. 2007; Hastings 2007; Heidenreich 2007; Saint-Ruf et al. 2007; Cirz and Romesberg 2007; Bindra et al. 2007; Fonville et al. 2011; Yao and Kovalchuk 2011).

Cellular and Environmental Stresses and Stress Responses

Microbes are challenged continuously by environmental stressors that result from a constantly changing environment. At the cellular level, stress can disturb development, affecting structure, function, stability, growth, and survival (Tiligada 2006). Environmental factors that act as potential stressors include starvation, radiation, hypoxia, reactive oxygen species, hyper- or hypothermia, hyper- or hypo-osmotic conditions, factors underlying metabolic deficiencies and other metabolic conditions, heavy metals, toxic agents, and drugs (Tiligada et al. 2002). In nature, microbes constantly face challenges from their environment, from nutrient starvation to variations in temperature, pH, and osmolarity. Antimicrobial agents also act as stressors for microorganisms, because they interfere with important pathways and threaten survival. To overcome these various stresses, all cells have evolved specific adaptive stress responses. Many of these stress-response pathways are quite specific, involving sets of regulated genes that help cells survive specific stress conditions. In addition, all or most cells employ global stress responses that are activated by multiple stressors and provide resistance to many stresses when activated.

The RpoS General Stress Response

In *E. coli*, the general or starvation stress response controlled by the RpoS (σ^S) transcriptional activator is such a general stress response (Weber et al. 2005; Battesti et al. 2011). RpoS (σ^S) is a sigma factor: an interchangeable subunit of the bacterial RNA polymerase. When present, σ^S directs transcription to a large set of stress-responsive promoters and away from "housekeeping" promoters recognized by the RNA polymerase carrying the housekeeping sigma factor. The RpoS response is activated, and σ^S synthesized and stabilized in cells, in response to starvation, stationary phase, cold shock, osmotic shock, and oxidative and pH stress. RpoS directly or indirectly upregulates and downregulates the transcription of approximately 500 genes—about 10% of *E. coli* genes (Weber et al. 2005; Battesti et al. 2011). This program is a general stress response, in that cells challenged with one stressor then display greater resistance to multiple stressors when the RpoS response is activated. In this chapter, the RpoS response is important because, in addition to its immediate stress-tolerating properties, we see that it throws the critical switch that activates stress-induced mutation during repair of DNA breaks.

DNA Damage and Its Repair Are Important to Mutagenesis

DNA damage and repair are important to mutagenesis because repair pathways can create mutations. We explore a mutation mechanism in which the mutagenicity of DNA break repair is upregulated by a stress response, causing stress-induced mutations.

DNA is frequently exposed to DNA-damaging agents such as ultraviolet light, mutagenic chemicals, reactive oxygen species generated by cellular metabolism, ionizing radiation, and radio-mimetic drugs (Friedberg et al. 2005; Hoeijmakers 2001). Frequent types of DNA damage include single- and double-strand breaks (SSBs and DSBs), base lesions, sugar modifications, apurinic/apyrimidinic sites (AP sites), and DNA-DNA and DNA-protein cross-links (Friedberg et al. 2005; Hoeijmakers 2001). If left unrepaired, DSBs can be lethal (Meulle et al. 2008). Both single-strand gaps and DSBs induce the SOS DNA-damage response. The SOS response upregulates transcription of about 40 *E. coli* genes that function in DNA repair, DNA-damage tolerance, mutagenesis, and cell-cycle checkpoint control (Friedberg et al. 2005; Courcelle et al. 2001; Kenyon and Walker 1980). *E. coli* possesses five DNA polymerases. Along with DNA polymerases II and V, the error-prone DNA polymerase Pol IV, encoded by *dinB,* is among the genes upregulated by the SOS response in *E. coli* (Kenyon and Walker 1980; Kim et al. 1997; Wagner et al. 1999). DNA polymerase Pol II is a fairly high-fidelity polymerase (Banach-Orlowska et al. 2005), whereas Pol IV and Pol V are Y-family error-prone DNA polymerases (Ohmori et al. 2001). All three SOS DNA polymerases allow replication forks that are blocked at sites of damaged bases to insert a few bases and move on, a process called translesion synthesis (TLS) (Nohmi 2006). In addition, we see that, although they are not required for efficient repair of DNA breaks, Pol IV, Pol II, and Pol V are permitted to participate in break repair specifically during stress, and make mutations when they do.

Homologous recombination (HR) is the predominant mechanism for DSB repair in *E. coli*, and in some eukaryotes, including baker's yeast (Haber 1999). Mammalian cells use two main mechanisms for the repair of DSBs: HR and non-homologous repair mechanisms, including non-homologous end joining (NHEJ) (Hefferin and Tomkinson 2005; Weterings and van Gent 2004; Wyman and Kanaar 2004) and microhomology-mediated break-induced replication (MMBIR) (Hastings et al. 2009a, b).

Discovery of Proteins Required for DNA Break-Dependent Stress-Induced Mutation in *E. coli*

Much of the molecular mechanism of double-strand break-dependent stress-induced mutation has been revealed from studies of the *E. coli* Lac assay for detecting mutation of a plasmid-borne gene, and then generalized using other assays for chromosomal mutations in plasmid-free cells.

The Lac Assay

In the Lac assay, *E. coli* carrying a *lac* +1 bp frameshift allele in an F′ conjugative plasmid are grown to stationary phase in liquid medium with a non-lactose carbon source (Cairns and Foster 1991). During this growth, spontaneous "generation-dependent"

mutations, like those of Luria and Delbruck (Luria and Delbrück 1943), occur. The cells are then spread on solid medium with lactose as the sole carbon source, on which only Lac+ reversion mutants can form colonies. Generation-dependent Lac+ revertant colonies appear after 2 days of incubation (Cairns and Foster 1991). Over subsequent days starving on lactose medium, additional Lac+ colonies arise continuously (Cairns and Foster 1991), and these have been shown to be stress-induced mutants: cells with mutations formed dependently on the activation of three stress responses: the RpoS, SOS and RpoE responses (below).

Most Lac+ revertant colonies visible by day five carry compensatory frameshift mutations ("point mutations") (Rosenberg et al. 1994; Foster and Trimarchi 1994). These are mostly −1 bp deletions in simple repeat sequences, a sequence spectrum more homogeneous than that of generation-dependent reversions (Rosenberg et al. 1994; Foster and Trimarchi 1994). Arising later, and ultimately becoming 40% of Lac+ colonies by day eight, are *lac*-amplified clones (Hastings et al. 2000). These carry 20–100 copies of 7–40 kb tandem repeats containing the leaky *lac* allele in the F′ plasmid, and produce sufficient β-galactosidase activity to allow growth. Both point mutations and amplifications are formed after exposure to the stress condition (Hastings et al. 2000; McKenzie et al. 1998), and both form dependently on activation of the RpoS response, indicating that they are stress-induced (Lombardo et al. 2004), and suggesting that the particular stress was starvation. In new work, we show the specific starvation signaling pathway that activates RpoS during stress-induced mutagenesis (Al Mamun et al. 2012).

The molecular mechanism of stress-induced point mutagenesis is an RpoS-controlled switch from high-fidelity to error-prone repair of DNA double-strand breaks or ends (DSBs/DSEs) (Ponder et al. 2005; Shee et al. 2011a, 2012). This was deduced first by identification of the proteins required for stress-induced point mutagenesis in the Lac assay, and, second, using specific molecular/biochemical demonstrations in living cells to show that mutagenesis is part of DSB/DSE repair.

Proteins

Stress-induced point mutation in the Lac assay requires the proteins used in repair of DSBs/DSEs by homologous recombination (Harris et al. 1994; Foster et al. 1996; Harris et al. 1996), DNA polymerases DinB/Pol IV (McKenzie et al. 2001) and Pol II (Frisch et al. 2010), and the activators of three stress responses: the RpoS transcriptional activator of the general/starvation stress response (Lombardo et al. 2004; Layton and Foster 2003), the SOS DNA-damage response (Cairns and Foster 1991; McKenzie et al. 2000), and the RpoE (σ^E) envelope protein stress responses (Gibson et al. 2010). In addition, DSBs/DSEs are required (Ponder et al. 2005; Harris et al. 1994).

The RpoE envelope protein stress response somehow contributes to the formation of spontaneous DSBs/DSEs (Gibson et al. 2010), at least at some genomic locations. Because DSBs are required for point mutation (Harris et al. 1994) and

amplification (Slack et al. 2006), RpoE is also required for both (Gibson et al. 2010). That is, RpoE is required for both point mutation and amplification; the requirement is for formation of mutations, not for growth of the colony after a mutation is formed, and RpoE is no longer required if a DSB is provided in the same molecule as *lac* using a restriction enzyme expressed in the cell to make a site-specific DSB in that molecule (Gibson et al. 2010).

The SOS response is required solely for upregulation of DinB/Pol IV (Galhardo et al. 2009). Pol IV is present at about 250 molecules per cell and is upregulated about tenfold when the SOS response is induced. This tenfold upregulation of is necessary (Galhardo et al. 2009), but not sufficient for mutation during DSB repair (Ponder et al. 2005; Shee et al. 2011a); the RpoS response must also be activated for Pol IV-dependent mutations to arise (Ponder et al. 2005; Shee et al. 2011a). We will see below that the molecular mechanism of point mutagenesis is that during RpoS-inducing stress, Pol IV, and other error-prone DNA pols are allowed to participate in DNA break repair and make mutations.

The mechanism of DSB/DSE-dependent stress-induced point mutation was suggested to be peculiar to F′ conjugative plasmids (e.g., Foster and Trimarchi 1995; Radicella et al. 1995; Galitski and Roth 1995; Roth et al. 2006). However, recently we have demonstrated that DSB-dependent stress-induced mutation occurs in starved plasmid-free cells (Shee et al. 2011a) (discussed below). Thus, the same DSB repair proteins, error-prone DNA polymerases SOS, and RpoS stress responses are required for DSB-dependent stress-induced mutation of chromosomal genes in plasmid-free cells (Shee et al. 2011a, 2012; Al Mamun et al. 2012) (discussed below).

DSBs Are Repaired by Homologous Recombination in *E. coli*

DSB-repair in *E. coli* occurs via RecA/RecBCD-mediated homologous recombination. The RecBCD enzyme loads onto DNA at double-strand ends and degrades both strands as exonuclease (Exo) V. RecBCD ceases double-strand degradation and creates single-strand DNA most often at Chi sites (5′ GCTGGTGG 3′) (Dillingham and Kowalczykowski 2008). Single-stranded ends created at Chi are then coated with RecA strand-exchange protein in preparation for recombination. The 3′ end of a RecA nucleoprotein filament invades a homologous DNA sequence (usually in a sister chromosome) to produce a heteroduplex recombination intermediate (Camerini-Otero and Hsieh 1993; Kowalczykowski and Eggleston 1994). Any DNA lost or degraded from the broken molecule is copied from the intact sister chromosome. After repair synthesis, the inter-molecular recombination intermediate is resolved to yield two intact DNA molecules, often by the Holiday-junction resolution proteins RuvABC (West 2003). RecA, RecBC, and RuvABC are all required for stress-induced point mutation (Harris et al. 1994; Foster et al. 1996; Harris et al. 1996). The high-fidelity major replicative DNA polymerase, DNA Pol III, is required for replicative repair of DSBs in unstressed *E. coli* cells (Motamedi et al. 1999), and, in unstressed cells, repair synthesis is non-mutagenic (Ponder et al. 2005; Shee et al. 2011a).

In addition to promoting recombination, the RecA-coated single-stranded DNA filament also activates the SOS DNA-damage response in about 25% of successful DSB repair events (Pennington and Rosenberg 2007). The RecA/ssDNA filament promotes auto-proteolysis of the LexA transcriptional repressor, which upregulates the transcription of about 40 SOS genes that function in DNA repair, DNA-damage tolerance, mutagenesis, and cell-cycle checkpoint control (Courcelle et al. 2001; Sutton et al. 2000).

RpoS Throws a Switch to Mutagenic Repair of Double-Strand Breaks

We showed that stress-induced point mutations are formed in acts of DNA break repair by creating *E. coli* cells that produce site-specific DNA breaks. We cloned the I-*Sce*I double-strand endonuclease under a bacterial regulatable promoter, and placed it into the *E. coli* chromosome (Gumbiner-Russo et al. 2001). Using this tool, we engineered Lac-assay cells to have a single DSB in their F′ near *lac* (Ponder et al. 2005), which could be repaired by homologous recombination with an uncleaved sister DNA molecule. We found that DSBs in the same molecule as *lac* produced 6,000-fold more Lac^+ point mutation; DSBs made in a different plasmid in the cell produced only threefold more Lac reversions. However, if we engineered the DNA near *lac* to carry a sequence identical to one end of the cleaved other plasmid, then DSBs in that other molecule also greatly stimulated Lac reversion during starvation (Ponder et al. 2005). These data show that the mutations occur during acts of DSB repair by homologous recombination. These I-*Sce*I-instigated mutations require DSB-repair proteins, Pol IV, and the SOS and RpoS responses to occur (Ponder et al. 2005), just as standard DSB-dependent stress-induced mutations do. They do not require RpoE, which indicated that the role of RpoE was in spontaneous DSB/DSE formation (Gibson et al. 2010).

Importantly, the repair of I-*Sce*I-induced DSBs is mutagenic only if the RpoS response is induced; that is, if cells sense another (non-DSB) stress. When the I-*Sce*I cuts were made and repaired in log-phase unstressed cells growing in liquid, mutations were not stimulated (this time using reversion of a *tet* frameshift allele as the mutation reporter) (Ponder et al. 2005; Shee et al. 2011a). If the cells were either allowed to go stationary, or if RpoS was upregulated artificially in the log-phase unstressed cells, then Pol IV-dependent mutagenesis occurred during repair of the I-*Sce*I-induced DSBs (Ponder et al. 2005; Shee et al. 2011a). Thus, the RpoS response throws a switch that causes the otherwise high-fidelity (non-mutagenic) process of DSB repair by homologous recombination to become mutagenic, using Pol IV. This means that even though Pol IV is present at ~2,500–5,000 molecules per cell after a DSB is made and induces the SOS response, it appears not to participate in DSB repair or cause DSB-dependent mutations unless the RpoS response is also activated (Ponder et al. 2005; Shee et al. 2011a).

Thus, put simply, the mutation mechanism requires three simultaneous events: (1) a DSB and its repair by homologous recombination; (2) induction of the SOS response, which DSBs induce, and which upregulates Pol IV; and (3) a second stress that activates the RpoS response, which licenses use of Pol IV and other error-prone DNA pols in DSB repair, causing mutations.

RpoS-Controlled Switch to Mutagenic Break Repair in Chromosomes of Plasmid-Free Cells

These findings hold true in the F′-based Lac assay (Ponder et al. 2005), and also when chromosomal mutations in plasmid-free cells were assayed (Shee et al. 2011a). We showed that chromosomal reversion of a *tet* +1 bp frameshift allele was stimulated 50- to 100-fold by nearby DSBs made by I-*Sce*I endonuclease. These mutations occur by the same DSB-dependent mutation pathway as in the F′, requiring DSB-repair proteins RecA, RecBCD and RuvABC, Pol IV error-prone DNA polymerase, the SOS response (which upregulates Pol IV), and the RpoS stress response (Shee et al. 2011a). This mutagenesis required either a prolonged stationary phase, or an artificial upregulation of RpoS in log-phase growing cells, again demonstrating the RpoS-controlled switch to mutagenic break repair (Shee et al. 2011a). These data put to rest previous concerns that DSB-dependent stress-induced mutation might be peculiar to plasmids (e.g., Foster and Trimarchi 1995; Radicella et al. 1995; Galitski and Roth 1995; Roth et al. 2006).

DNA Polymerases Used

Under stress, the RpoS response causes DSB repair to switch to a mutagenic mode using error-prone DNA polymerases, principally Pol IV (*dinB*), but also Pol II (*polB*) and Pol V (*umuDC*) (Ponder et al. 2005; Shee et al. 2011a). In the *E. coli* Lac assay, ~85% of stress-induced point mutation requires Pol IV (McKenzie et al. 2001) and the remaining ~15% requires Pol II (Frisch et al. 2010), either when I-*Sce*I-promoted (Ponder et al. 2005; Frisch et al. 2010), or when spontaneous (Frisch et al. 2010) stress-induced mutations are assayed. All DSB-dependent chromosomal *tet* reversion requires Pol IV (Shee et al. 2011a). Pol V is partially required for the Pol IV-dependent chromosomal *tet* frameshift reversions (Shee et al. 2011a), and for DSB-repair-protein-, SOS-, and RpoS-dependent forward mutations in a chromosomal *ampD* gene during starvation (Petrosino et al. 2009). Interestingly, DSB repair in unstressed cells requires high-fidelity DNA Pol III (Motamedi et al. 1999) and is non-mutagenic (Ponder et al. 2005; Shee et al. 2011a). We have suggested that the mechanism of the RpoS-controlled switch to mutagenic DSB repair

might be downregulation of Pol III, which might then let the other error-prone DNA polymerases compete more effectively for a spot at the DSB repair replisome (Frisch et al. 2010; Rosenberg et al. 2012). Though, the other DNA polymerases compete with Pol IV during DSB repair under stress (Hastings et al. 2010), the hypothesis that RpoS promotes mutation by downregulation Pol III remains to be tested.

DSB-Dependent Stress-Induced Mutagenesis Produces Spontaneous Mutations

The RpoS-controlled switch to mutagenic DSB repair was demonstrated with artificially created DSBs made by I-*Sce*I (Ponder et al. 2005; Shee et al. 2011a). Importantly, we showed that half of spontaneous frameshift reversions and base-substitution mutations in the chromosomes of starved plasmid-free *E. coli* occur by the DSB-dependent stress-induced mutation pathway when no I-*Sce*I is present: the process requires DSB-repair proteins, RpoS, SOS, and Pol IV (Shee et al. 2011a). Thus, this mechanism is important to evolution. Apparently, without I-*Sce*I, the mutations occur during acts of repair of spontaneous DSBs/DSEs.

Mutagenesis Is Not an Unavoidable Consequence of DNA Break Repair

The idea that the upregulation of mutagenesis by a stress response would accelerate evolution, and that this might be selected for its evolution-enhancing ability, was suggested first for the SOS DNA-damage response by Radman (Radman 1975) then Echols (McPartland et al. 1980). However, because the SOS response upregulates DNA repair and damage-survival functions, from its original proposal until the present, others have argued that mutagenesis is an unavoidable consequence of repairing DNA. They argue that high-fidelity (non-mutagenic) DNA repair cannot evolve (e.g., Sniegowski et al. 2000; Erill et al. 2007; Andersson et al. 2010; Lynch 2010). This argument cannot be resolved in the context of the SOS response, because SOS *is* required for survival of DNA damage. However, our work in DNA break-dependent stress-induced mutation shows that survival of the break does not require mutagenesis; neither RpoS nor Pol IV, which throw the switch to mutagenic repair, is required for DSB survival, even under RpoS-inducing stress conditions (Ponder et al. 2005; Shee et al. 2011a). Thus, use of the error-prone DNA polymerase during repair synthesis is not an unavoidable consequence of repair. It is a regulated response that boosts mutagenesis, potentially accelerating evolution, and it remains possible that it might have been selected by that property.

Other Old Problems Resolved

Recent results with the chromosomal Tet assay for DSB-dependent stress-induced mutation resolve other old problems.

DSB-dependent stress-induced mutation was studied initially in *E. coli* cells carrying a specific F′ conjugative plasmid that carries an extra copy of *dinB* encoding Pol IV, prompting concerns that the mutation mechanism might be peculiar to the assay system (Foster and Trimarchi 1995; Radicella et al. 1995; Galitski and Roth 1995; Roth et al. 2006; Slechta et al. 2002a), conjugative plasmids, or the specific F′ used (Radicella et al. 1995; Slechta et al. 2003). Our recent work shows that DSB-dependent stress-induced mutagenesis occurs in the chromosome of starved, plasmid-free *E. coli*, and even accounts for half of spontaneous frameshift and base-substitution mutations there, putting this concern to rest (Shee et al. 2011a).

A related concern was that perhaps the stress of starvation selects, rather than induces, the mutations—a possibility in the Lac assay in which Lac$^+$ reversions were selected during starvation on lactose plates. When Lac$^+$ mutants are selected, spontaneous gene amplifications of the *lac* region could be selected, causing multiple *lac* copies, and increasing mutation rate per cell by simply increasing copy number (Roth et al. 2006; Slechta et al. 2003; Roth 2010). A preexisting *lac* gene duplication might undergo amplification, allowing slow growth by production of beta-galactosidase from the weakly functional *lac* gene, and increased *lac* mutation per cell. This concern was addressed by use of the Tet assay in the F′ (Ponder et al. 2005) or chromosome (Shee et al. 2011a), in which cells are starved without selection for function of the defective *tet* gene, then assayed for Tet^R mutations after rescue from starvation. In the Tet assay, mutagenesis occurred by the same DSB-repair-protein-, SOS-, RpoS-, and Pol IV-dependent mechanism (Ponder et al. 2005; Shee et al. 2011a), and the amount of mutagenesis related to the length of time the cells were starved (Shee et al. 2011a). Thus, DSB-dependent stress-induced mutation occurs independently of selection for the function of mutated gene, so that selected amplification of the target gene can be ruled out as a component of the mechanism.

Duplications Are Attractive

Although the specific amplification-selection-mutation model discussed above is not supported, spontaneous gene duplications may nevertheless be important. Duplications are an attractive solution to the problem of which segment of DNA is used for repair of a chromosomal DSB during homologous recombination in starving, haploid *E. coli* (Shee et al. 2011b; Rosenberg et al. 2012). It could be that spontaneously duplicated segments are the source of homology for repair by recombination in chromosomes of starving cells.

Hypermutable Cell Subpopulation

We and others found that during DSB-dependent stress-induced mutation, the cells with Lac$^+$ mutations have more mutations in other genes in their genome than Lac$^-$ starved cells taken from the same petri plates (Torkelson et al. 1997; Rosche and Foster 1999; Godoy et al. 2000; Slechta et al. 2002b). This indicates that a cell subpopulation has an increased mutation rate in unselected chromosomal genes: that there is hypermutable cell subpopulation (HMS). Further, those Lac$^+$ mutants with the additional, unselected "secondary" mutations did not have heritably higher mutation rates once the Lac$^+$ colonies were picked. The HMS appears to be a transiently differentiated subpopulation, not rare hypermutator mutants. Secondary mutations are increased proportionately in hyper-recombining DSB repair mutants *recD* and *recG,* which also increase Lac$^+$ mutation, implying that they form by a similar DSB-repair-protein-dependent mechanism (Bull et al. 2000).

These results ended a previous Lamarck versus Darwin debate for stress-induced mutation (Bridges 1997), but are now the focus of a different problem. Those who favor models of constant, gradual evolutionary change dislike the idea of stress-induced increase in mutation rate that potentially accelerates evolution (Chicurel 2001). They, and some others (Rosche and Foster 1999), suggest that although the HMS exists, it is not important: that most Lac$^+$ adaptive mutants arise from cells not in the HMS—specifically that 90% do not, and only 10% of Lac$^+$ mutants do arise from the HMS (Slechta et al. 2003; Slechta et al. 2002b; Roth and Andersson 2004; Hendrickson et al. 2002). In contrast, the following evidence supports the hypothesis that the HMS generates most stress-induced mutants. First, the sequences of Lac reversions from cells demonstrably from the HMS (with a secondary mutation) are identical to the majority of Lac$^+$ stress-induced mutations, indicating that both arise from a similar mechanism (Gonzalez et al. 2008). Second, when the dominant DSB-dependent mutation mechanism is increased by providing I-*Sce*I cuts, the associated secondary mutations increase proportionately (Gonzalez et al. 2008). This shows that the HMS cannot be uncoupled from the major stress-induced mutation pathway. In the future, possible isolation of the HMS during mutagenesis may be possible, and could provide direct evidence concerning this point.

The discovery of a Pol IV-dependent, but DSB-independent, stress-induced mutation mechanism activated by the ComK-controlled competence stress response in *Bacillus subtilis* (Sung and Yasbin 2002) suggests that a different bacterium might also create a transiently differentiated HMS. The competence response is famously induced in only a subpopulation of starving cells, which it makes competent for uptake of DNA from the environment, allowing natural transformation (Dubnau and Losick 2006). Thus this stress-induced mutation mechanism seems likely to occur in a transiently differentiated hypermutating subpopulation.

DSBs and Mutation Hotspots

Mutation hotspots, clusters, and showers are apparent in cases ranging from phage to human (Drake 2007a; Drake 2007b; Caporale 2006; Wang et al. 2007), but the mechanisms that form hotspots are unknown. We and others have suggested that a consequence of linking mutagenesis to DSB repair might be that mutations could be formed in localized hotspots, near the sites of repair (Galhardo et al. 2007; Ponder et al. 2005; Ninio 1996; Yang et al. 2008). Mutational hotspotting is important because it could promote evolution, including evolution of tumors and pathogens: first, by potentially targeting regions in which variability might provide a growth advantage, as occurs with somatic hypermutation of immunoglobulin genes (Di Noia and Neuberger 2007) and pathogen "contingency" genes (Moxon et al. 1994), and, as is seen in the cancer-driving Philadelphia chromosome (Albano et al. 2010). Additionally, hotspot formation could allow high-level mutagenesis that can promote concerted evolution (multiple mutations) within genes or gene clusters, without accumulation of deleterious mutations throughout the genome (Ponder et al. 2005; Ninio 1996; Yang et al. 2008). Recent data showing mutation clusters in human cancers have been interpreted in support of the hypothesis that DSBs cause hotspots (Nik-Zainal et al. 2012; Roberts et al. 2012). However, we, and others (Nik-Zainal et al. 2012), note that this is not demonstrated, and other sources of the hotspots are possible. DSB-dependent mutation was discovered in *E. coli* (Rosenberg et al. 1994; Harris et al. 1994), then demonstrated in yeast (Yang et al. 2008; Strathern et al. 1995; Deem et al. 2011; Hicks et al. 2010), in which it is not known to be stress-inducible, and might be constitutive. Until recently, no experiments in either organism had shown whether or not the strong stimulation of mutagenesis near DSB sites also occurs distantly from the DSB in the same molecule. Hotspots would be produced only if the mutagenesis were localized. In the sole yeast study to address this point, mutations occurred equally well next to and 36 kb away from a DSB, and greater distances were not assayed (Deem et al. 2011). Further, these yeast experiments were done in conditions under which long-distance replication was demanded, which might make them unusual. In *E. coli*, we have recently demonstrated that DSBs provoke both strong local mutation hotspots like in cancers (Nik-Zainal et al. 2012; Roberts et al. 2012) and weak long-distance hotspots (Shee et al. 2012). This work provides a plausible molecular mechanism for hotspotting, an important force in genome evolution.

Cancer Cells Display a Switch to Mutagenic Break Repair Under Stress

Cancer is an evolutionary process, driven by mutations that fuel oncogenesis, tumor progression, and development of resistance to chemotherapies. Human cancer cells display a stress-induced mutation mechanism somewhat analogous to DSB-dependent stress-induced mutagenesis in *E. coli*. In *E. coli*, the RpoS stress

response throws a switch from high fidelity to mutagenic repair of DSBs, causing mutations preferentially under stress (Ponder et al. 2005; Shee et al. 2011a). In cancer cells, hypoxic stress activates hypoxic stress responses, which have been shown to downregulate the *BRCA1* and *RAD51* genes required for DSB repair by homologous recombination (HR) (Bindra et al. 2005; Bindra et al. 2004). In humans, non-homologous end-joining (NHEJ) pathways take over when HR is not operative. NHEJ can cause genomic rearrangements. Thus, downregulation of *BRCA1* and *RAD51* is expected to cause a switch from genome-stabilizing repair by HR in unstressed cells to genome-rearranging end-joining under hypoxic stress (Bindra et al. 2005; Bindra et al. 2004). Though not the same mechanism as in *E. coli*, it is analogous in using a switch from high-fidelity to mutagenic repair of DNA breaks under stress.

Antibiotic Resistance

Mutation is a major route to resistance to antibiotics, an important and urgent clinical problem (Davies and Davies 2010). Recent work has shown that antibiotics themselves are stressors (Davies and Davies 2010; Kohanski et al. 2007; Miller et al. 2004) that induce mutagenesis, causing mutations that confer resistance to the same antibiotic (Cirz et al. 2005), and to different antibiotics, or in other genes (Perez-Capilla et al. 2005; Kohanski et al. 2010). In two instances, this mutagenesis appears to occur by mechanisms similar to DSB-dependent stress induced mutation. Mutagenesis induced by the fluoroquinolone antibiotic ciprofloxacin (cipro) causes cipro resistance via a mutation pathway that requires DSB-repair proteins, the SOS response, and all three SOS DNA polymerases (including Pol IV) (Cirz et al. 2005). Whether RpoS is also required is not known, but this implicates DSB-dependent mutagenesis. In pathogenic Salmonella, bile-resistance mutations are induced by exposure to bile (an antibacterial agent produced in our bodies), and this mutation pathway also requires DSB-repair proteins, Pol IV, the SOS response, and, additionally, RpoS (Prieto et al. 2006; J Casadesus Pers. commun.). Bile-induced mutagenesis bears all the hallmarks of DSB-dependent stress-induced mutagenesis, and in this case, the stressor appears to be bile, a membrane-disrupting agent. Understanding the molecular mechanisms by which antibiotics provoke mutation is critical to combating resistance. We have suggested that the development of new drugs that would block the induced mutagenesis would block the evolution of resistance (e.g., Rosenberg et al. 2012; Al Mamun et al. 2012).

Conclusion

Stress-induced mutation mechanisms appear to be both ubiquitous and important. Of 787 natural isolates of *E. coli* collected world-wide, about 80% showed induction of mutation under the generic laboratory stress of aging in a colony

(Bjedov et al. 2003). This ability appears to have been selected in that it correlates with ecological niche, as expected for traits under recent selection, implying that stress-induced mutation mechanisms are important to bacterial evolution. Regarding the more general question of the relevance of stress-induced mutation mechanisms as a whole, it seems clear that mutability induced by and allowing growth under stress is an important departure from the classical Luria/Delbrück paradigm (Luria and Delbrück 1943), and that many systems previously modeled on Luria/Delbrück principles may now need to be rethought, in the light of inducible genetic change mechanisms that might provide more appropriate models. Our understanding of evolution needs to move into the molecular age. Understanding stress-induced mutation mechanisms will propel treatment and prevention options for evolution-based diseases such as cancer and infectious disease, and is critical to a realistic picture of the molecular mechanisms of evolution.

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