Stress-Induced Mutation Rates Show a Sigmoidal and Saturable Increase Due to the RpoS Sigma Factor in *Escherichia coli*

Ram Maharjan and Thomas Ferenci[1]
School of Molecular Bioscience, University of Sydney, New South Wales 2006, Australia

ABSTRACT Stress-induced mutagenesis was investigated in the absence of selection for growth fitness by using synthetic biology to control perceived environmental stress in *Escherichia coli*. We find that controlled intracellular RpoS dosage is central to a sigmoidal, saturable three- to fourfold increase in mutation rates and associated changes in DNA repair proteins.

MUTATIONS provide a source of evolutionary innovation (Radman 2001) but also contribute to the development of antibiotic resistance (Gutierrez *et al.* 2013) and to diseases like cancer (Alexandrov and Stratton 2014). The rate at which mutations occur is not constant, even within a species (Bjedov *et al.* 2003). There is a tradeoff between balancing genetic integrity in good times with increased evolvability in suboptimal environments. A way of shifting this balance is through stress-induced mutagenesis (SIM) (Radman 2001). In the laboratory, mutation rate in bacteria like *Escherichia coli* is increased by suboptimal conditions and regulated by adverse environments (Bjedov *et al.* 2003; Foster 2007; Galhardo *et al.* 2007; MacLean *et al.* 2013). Mutation rates indicative of SIM have been studied (Foster 2007; Galhardo *et al.* 2007; Matic 2013) but aspects of the evidence for SIM have been disputed (Roth *et al.* 2006). Particularly, the role of selection in increasing the frequency of drug-resistant mutants is contentious in studies like Bjedov *et al.* (2003), which relied on aged, stressed colonies. This is because some mutants supposedly have a growth advantage in old colonies on agar plates (Katz and Hershberg 2013).

These uncertainties are best resolved if the impact of stress on genomes could be assessed in the absence of fitness selection in aging colonies over extended periods. A problem for assaying SIM is that the level of stress in cultures and thus selection is difficult to control in colonies. The stress levels, fitness, and mutation rate can change over time in stationary-phase liquid culture as well (Yeiser *et al.* 2002; Loewe *et al.* 2003). An approach to circumventing the problem of selection and varying stress level is to use identical growth conditions, but with bacteria in which the level of SIM is under genetic control. A way of doing this is to control a major input into SIM regulation; RpoS has been implicated as a controller of SIM in several laboratories (Galhardo *et al.* 2007; Storvik and Foster 2010; Gutierrez *et al.* 2013). RpoS (also known as σ^S) is an alternative RNA polymerase sigma factor and the master stress regulator in *E. coli*, which is highly variable and dependent on both environmental signals and growth phase (Battesti *et al.* 2011; Hengge 2011). We use here a surrogate means of fixing stress by artificially setting levels of RpoS in a collection of strains to allow highly replicated measurements of mutation rates.

We recently constructed a set of synthetic strains to achieve fixed, environment-independent RpoS levels (Maharjan *et al.* 2013). The strains in the set do not have transcriptional, translational, and post-translational control over RpoS levels; instead these strains contain distinct synthetic promoters to express fixed levels of *rpoS*. All the strains along with their RpoS level are listed in Table 1 and were standardized to the content of RpoD, which is constant in different environments (Ishihama 2000; Gutierrez *et al.* 2013; Maharjan *et al.* 2013). The strain set covered a 200-fold range of sigma factor ratios and expressed the full range of environmental stress resistance phenotypes controlled by the general stress response (Maharjan *et al.* 2013). The RpoS/RpoD ratios in

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[1]Corresponding author: University of Sydney, School of Molecular Bioscience, G08, University of Sydney, Sydney, NSW 2006, Australia.
E-mail: tom.ferenci@sydney.edu.au

Table 1 Properties of strains used in this study

Strain	Relevant genotype (primer set used for construction)	RpoS/RpoD ratio ($m \pm$ SD)[a] Exponential bacteria[b]	Stationary phase bacteria[b]	Source
MC4100	F⁻ *araD*139 Δ(*argF-lac*)U169 *rspL*150 *deoCl relA1 thiA ptsF*25 *flb*5301 *rbsR*	0.00 ± 0.00	0.40 ± 0.06	Ferenci *et al.* (2009)
BW2952	*malG*::λ*plac*Mu55 φ(*malG*::*lacZ*)*rssB*::IS*1*	0.76 ± 0.04	1.00 ± 0.00	Ferenci *et al.* (2009)
BW3709	BW2952 *rpoS*::Tn*10*	0.00 ± 0.00	0.00 ± 0.00	Notley-Mcrobb *et al.* (2002)
BW5205	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R1)	0.30 ± 0.07	0.31 ± 0.02	Maharjan *et al.* (2013)
BW5206	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R2)	0.01 ± 0.01	0.00 ± 0.01	Maharjan *et al.* (2013)
BW5207	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R3)	0.13 ± 0.09	0.16 ± 0.10	Maharjan *et al.* (2013)
BW5208	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R4)	0.86 ± 0.04	0.72 ± 0.13	Maharjan *et al.* (2013)
BW5213	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R5)	0.28 ± 0.03	0.22 ± 0.05	Maharjan *et al.* (2013)
BW5214	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R6)	0.28 ± 0.01	0.27 ± 0.06	Maharjan *et al.* (2013)
BW5219	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R7)	0.26 ± 0.03	0.22 ± 0.01	Maharjan *et al.* (2013)
BW5220	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R8)	0.02 ± 0.00	0.03 ± 0.01	Maharjan *et al.* (2013)
BW5222	BW2952 *rpoS*leader::*bla* (*rpoS*F and *rpoS*R10)	0.31 ± 0.00	0.38 ± 0.03	Maharjan *et al.* (2013)

[a] RpoS/RpoD ratio data for the two culture conditions are from graphs in Maharjan *et al.* (2013) based on Western blot analysis of RpoS and RpoD detected using anti-RpoS and anti-RpoD antibodies. The mean (m) and standard deviation (SD) were from at least three independent experiments.
[b] The growth medium used was Luria-Bertani broth.

the synthetic strains (BW5205–BW5222) remain relatively constant in different growth phases, unlike the wild-type MC4100, as shown in Table 1. To check whether different cultures of strains maintained similar RpoS levels, we tested iodine staining levels of cultures using the method that we used in our previous study; iodine staining is linearly dependent on the RpoS content of cells (Maharjan *et al.* 2013). There was no change in staining level in cultures suggesting that storage and repeat culture did not affect the RpoS levels.

The RpoS strain set was used to investigate SIM by measuring mutation rates (Figure 1, A and B) in fluctuation test-based, >20-fold replicated assays of rifampicin resistance (Rif^R) (Bjedov *et al.* 2003) and cycloserine resistance (Cyc^R) (Feher *et al.* 2006) in strains with 11 different set levels of RpoS but under identical growth conditions. The Rif^R change is limited to a number of point mutations in *rpoB* (Wolff *et al.* 2004), while Cyc^R does not change growth fitness and is due to a wide spectrum of loss-of-function mutations including large indels and transpositions at many points in the *cycA* gene (Feher *et al.* 2006). The strain set was further used to provide a quantitative link between RpoS and the regulation of DNA repair systems involving *dinB* (error-prone polymerase) and *mutS* (mismatch repair) implicated in SIM (Al Mamun *et al.* 2012), as shown in Figure 1C.

The dose-dependent increase in mutation rates with RpoS shown in Figure 1, A and B, strongly implicates the importance of RpoS level in SIM. The mutation rate in the strain with the highest RpoS content was 6.6×10^{-8} per locus per generation for Rif^R and 2.08×10^{-7} per locus per generation for Cyc^R. These were three- and fourfold higher than the mutation rates found in strain with the lowest RpoS (2.4×10^{-8} per locus per generation for Rif^R and 0.53×10^{-7} per locus per generation for Cyc^R per locus per generation respectively (two-tailed $P < 0.05$ in both cases). The RpoS-dependent elevation of mutation rates for Rif^R was slightly lower than the 5.5-fold increase in Rif^R mutants stress-induced in aged colonies (Bjedov *et al.* 2003). Nevertheless, the RpoS dosage is responsible for much of the SIM under stress although stress inputs not sensed through RpoS may contribute to the overall mutation rate (Al Mamun *et al.* 2012).

To compare our Rif^R mutation rates with the previously estimated mutation rate per genome by Drake (2012) for *E. coli*, we converted our per-locus mutation rates into per genome per generation (Table 2). Given that 79-point mutations in *rpoB* can confer Rif^R (Garibyan *et al.* 2003) and a genome size of 4.6 Mb, we estimated our genomic mutation rate to be 0.0038 per genome per generation for the high RpoS strain and 0.0013 per genome per generation for the low RpoS strain. The low-RpoS mutation rates are similar to the 0.001 per genome per generation estimated by Lee *et al.* (2012) in mutation accumulation (MA) experiments with *E. coli*. On the other hand, the high perceived stress, high-RpoS mutation rate is within the range 0.0022–0.0043 per genome per generation in Drake's studies (Drake *et al.* 1998; Drake 2012). This raises the interesting possibility that the MA data are characteristic of a low-stress environment, which is consistent with the frequent subculture in fresh media in MA experiments (Lee *et al.* 2012).

For Cyc^R, we were unable to convert mutation rates into per genome per generation due to the lack of information on the number of sites in the *cycA* gene that can give rise to the Cyc^R phenotype, which includes point mutations, small and large indels, and transpositions in *cycA* (Feher *et al.* 2006). Nevertheless, the Cyc^R per-locus mutation rate in the strain with low RpoS is very close to the previously estimated mutation rate using the identical method (Table 2;Feher *et al.* 2006; Posfai *et al.* 2006). So for both the Cyc^R and Rif^R assays, the low-RpoS strain reflects previously obtained lab data, but the high-RpoS strain is closer to the SIM state.

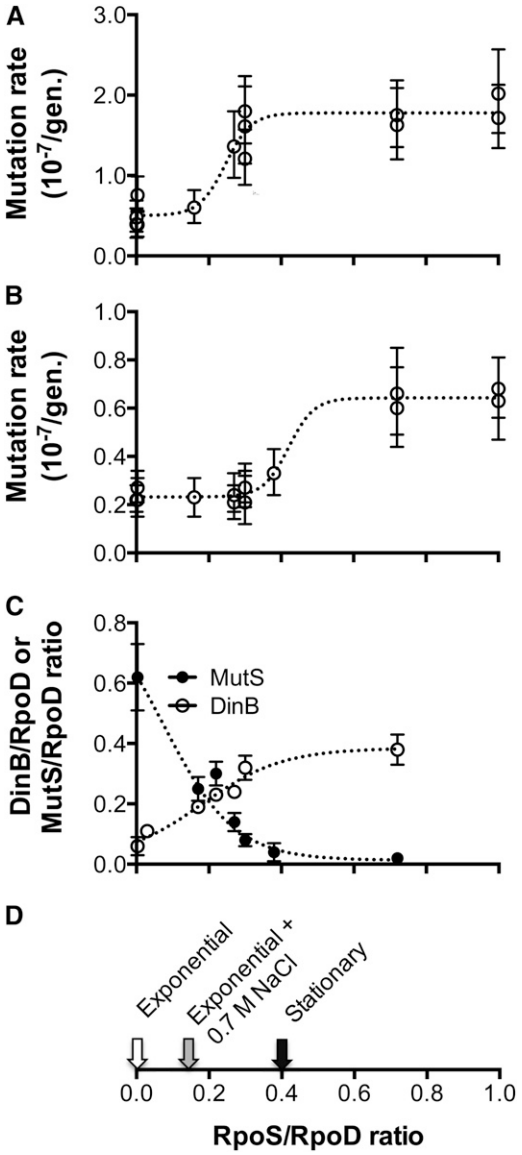

Figure 1 The relationship between intracellular RpoS dosage and mutation rates. (A and B) The mutation rate of *E. coli* with set levels of RpoS was estimated using two different assays, involving resistance to cycloserine (Cyc^R, A) and rifampicin (Rif^R, B). The mutation rates were estimated by fluctuation analyses in engineered strains with fixed ratios of RpoS/RpoD (plotted on the *x*-axis based on the data in Maharjan *et al.* 2013). The engineered strains do not have transcriptional, translational, and post-translational control over RpoS levels and contained synthetic promoters to express a fixed level of *rpoS* (Maharjan *et al.* 2013). A single colony of each strain was inoculated into 5 ml Luria–Bertani broth (LB) and allowed to propagate overnight at 37° with shaking. The overnight culture was diluted in 5 ml fresh LB medium and allowed to grow to optical density of 0.6 at 600 nm. The exponentially growing cultures were further diluted 10,000-fold and 150 μl was distributed into each of 40 wells in 96-well plates and incubated at 37° with shaking at 200 rpm. Aliquots of each well were then plated on rifampicin and cycloserine plates to detect Cyc^R and Rif^R mutant colonies. The mutation rates were estimated from the number of resistant colonies per culture and total cell count by using the fluctuation analysis calculator (FALCOR) web tool (Hall *et al.* 2009). Error bars in A and B are upper and lower limits with 95% CI and are based on at least 20 replicate cultures from fluctuation tests. The mutation rates were then estimated from the number of resistant colonies per culture and total cell count by using the FALCOR web tool (Hall *et al.* 2009). At each RpoS/RpoD ratio, two to three independent fluctuation analyses, each with >20 replicates, were carried out on the same strain. (C) The levels of DinB and MutS proteins in exponentially growing cultures of engineered strains were determined by using anti-DinB and anti-MutS rabbit antibodies. The RpoD, MutS, and DinB proteins were quantified by Western blotting. Error bars represent the standard deviations from two independent experiments. For estimation of DinB and MutS levels in the strain set with fixed level of RpoS/RpoD, a single colony of each strain was grown overnight in 5 ml LB in a MacCartney bottle with shaking (200 rpm) at 37°. The overnight cultures were diluted 500-fold in 5 ml of fresh LB and allowed to grow to optical density 0.4–0.6 at 600 nm. The cultures (1 ml) were harvested by centrifugation (10000 × *g*) for 1 min and 200 μl of 1× SDS PAGE sample buffer was added after removing supernatant. The tubes containing protein samples were then snap frozen using dry ice and stored until use. of Protein samples, 10 μl, were revolved on a 12% polyacrylamide gel (Bio-Rad, BioRad Australia, Sydney, N.S.W., Australia). Proteins were then transferred in Optitran BA-S85 blotting membrane (GE Whatman, GE Healthcare Australia Pty. Ltd, Sydney N.S.W., Australia). Proteins DinB and MutS were detected using anti-DinB (Santa Cruz Biotechnology, Santa Cruz Biotechnology, Dallas, Texas 75220 U.S.A.) and anti-MutS (US Biological, United States Biological, Massachusetts, MA 01907, U.S.A.) rabbit antibodies using the same protocol as that used for the detection of RpoS and RpoD and band intensities were also quantified by densitometry as previously described (Maharjan *et al.* 2013). DinB and MutS protein levels in the RpoS strain set were expressed as a ratio to the RpoD band as reference. (D) The physiological levels of RpoS/RpoD in wild-type *E. coli* K12 bacteria (strain MC4100; Maharjan *et al.* 2013) are shown in different stress situations. Unstressed bacteria were grown to exponential phase in LB medium (open arrow) or grown to exponential phase in LB with 0.7M NaCl added to initiate osmotic stress (shaded arrow) while the ratio found in 16-hr culture to stationary phase in LB medium is shown by the solid arrow.

Interestingly, both the Cyc^R and Rif^R assays in Figure 1, A and B, showed a sigmoidal increase before saturation at high RpoS levels. The RpoS threshold needed to induce Cyc^R is slightly lower compared to Rif^R, possibly because different mutations are involved. Nevertheless, as shown in Figure 1D, mutation rates with either Rif^R or Cyc^R increased at RpoS levels higher than those found in rapidly growing bacteria. Even osmotic stress levels that elicit partial induction of the general stress response are not high enough to induce SIM. This stress threshold for inducing SIM makes ecological sense in balancing genetic integrity, so that low levels of environmental stress do not elevate mutation rates. These results are consistent with a stationary-phase increase in mutation rates, when RpoS levels are elevated to the position of the solid arrow shown in Figure 1D, where mutation rates are elevated. It should be noted though that the cellular levels of RpoS can differ even in closely related *E. coli* K-12 strains (Spira *et al.* 2008), which in turn can affect the mutation rate. The parentage strain used in this study as the wild type (MC4100) exhibits similar RpoS level to MG1655 (Spira *et al.* 2008), the strain used in other mutation rate measurements (Lee *et al.* 2012).

The effect of RpoS on SIM is thought to be through modulating both the mismatch repair system and error-prone polymerase DinB (Godoy *et al.* 2007; Al Mamun *et al.*

Table 2 Mutation rates in *Escherichia coli* K12

Strain	Assay	Mutation rate per locus ($\times 10^8$) [95% CL]	Mutation rate per bp ($\times 10^{10}$)	Mutation rate per genome	References
BW2952 (high RpoS)[a]	Rif^R fluctuation test	6.55 [4.9–7.9]	8.3	0.0039	This study
BW5206 (low RpoS)[a]	Rif^R fluctuation test	2.2 [1.3–2.6]	2.8	0.0013	This study
BW2952 (high RpoS)[a]	Cyc^R fluctuation test	20.8 [15.7–26.7]	NA	NA	This study
BW5206 (low RpoS)[a]	Cyc^R fluctuation test	5.3 [3.5–7.5]	NA	NA	This study
E. coli K-12[b]	Cyc^R fluctuation test	6.5	NA	NA	Feher *et al.* (2006); Posfai *et al.* (2006)
E. coli K-12[b]	Mutation Accumulation	NA	2.2	0.0010	Lee *et al.* (2012)
E. coli K-12[b]	*lacI* (Lac reversion)	NA	4.1–9.3	0.0019–0.0043	Drake *et al.* (1998); Drake (2012)

[a] Rif^R and Cyc^R mutation rates and 95% confidence interval (CL) in the strains with high and low levels of RpoS were calculated as described in Figure 1. The Rif^R mutation rates per base pair (bp) per generation were estimated from mutation rates per locus by assuming Rif^R is conferred by 79 different point mutations in the *rpoB* gene (Garibyan *et al.* 2003).

[b] Mutation rates were based on published studies.

2012; Gutierrez *et al.* 2013). Our strain set allows testing of these notions. We find, as shown in Figure 1C, that RpoS level changes regulation of both repair systems simultaneously, so both together contribute to SIM, even though different networks regulate the two processes (Al Mamun *et al.* 2012; Gutierrez *et al.* 2013). For example, other influences besides RpoS (such as from RpoE;Gibson *et al.* 2010) may additionally affect the overall mutation rate. Nevertheless, the mutation rate threshold in Figure 1 is correlated with the regulation of *mutS* and *dinB* in the strains set.

In conclusion, stress sensed solely through RpoS resulted in a three- to fourfold increase in mutation rates in Figure 1. Thus even in the absence of selection for competitive fitness as may occur in aging colonies, SIM clearly has a component influenced by the general stress response. A central conclusion of this study is that SIM exhibits a nonlinear stress–dose response, with a clear threshold. The sigmoidal dose effects and saturability prevent a simple linear extrapolation between levels of stress, mutation rates, and evolvability. The final conclusion is that the mutation rate we observe for both Cyc^R and Rif^R with high levels of perceived stress, but not low RpoS, is higher than that found in the oft-used MA laboratory experiments (Lee *et al.* 2012). Given the use of an unstressed environment in MA experiments, the threefold discrepancy in mutation rates between MA experiments and other estimates of genomic mutation rates (Drake *et al.* 1998) may well be due to different levels of environmental stress in different laboratory experiments and in nature.

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