General Stress Response Regulator RpoS in Adaptive Mutation and Amplification in *Escherichia coli*

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ABSTRACT

Microbial cells under growth-limiting stress can generate mutations by mechanisms distinct from those in rapidly growing cells. These mechanisms might be specific stress responses that increase mutation rates, potentially altering rates of evolution, or might reflect non-stress-specific processes in rare growing cells. In an *Escherichia coli* model system, both frameshift reversion mutations and gene amplifications occur as apparent starvation-induced mutations. Whereas frameshift reversion ("point mutation") requires recombination proteins, the SOS response, and error-prone DNA polymerase IV (DinB), amplification requires neither SOS nor pol IV. We report that both point mutation and amplification require the stationary-phase and general stress response transcription factor RpoS (σ^S). Growth-dependent mutation does not. Alternative interpretations are excluded. The results imply, first, that point mutation and amplification are stress responses that occur in differentiated stationary-phase (not rare growing) cells and, second, that transient genetic instability, producing both point mutation and genome rearrangement, may be a previously unrecognized component of the RpoS-dependent general stress response.

M ICROBIAL cells exposed to growth-limiting environments can, in some cases, acquire a beneficial or "adaptive" mutation that allows rapid growth. In bacteria and yeast, there are multiple mechanisms of mutation in growth-limiting environments, some demonstrably different from spontaneous mutation in rapidly growing cells (FOSTER 1999b; ROSENBERG 2001). Also called "stationary-phase" mutation mechanisms, these generally occur in populations of cells that are in the stationary phase, defined as displaying no change in population size (HUISMAN *et al.* 1996). The mechanisms and regulation of stationary-phase mutation in these systems are of considerable interest because they represent model systems for understanding interactions between the environment and the genome, particularly whether and how stressful conditions influence genetic change. Induction of genetic change in response to stress has broad relevance to mechanisms of evolution and, thus, to the treatment of bacterial infections; the evolution of bacterial pathogens (for example, the generation of antibiotic resistance); the host immune response; and the genetic instability underlying oncogenesis, tumor progression, and resistance to chemotherapeutic drugs

(*e.g.*, LOEB 1991; STRAUSS 1992; MARTINEZ and BAQUERO 2000).

Since the inception of current interest in stationary-phase mutation mechanisms (SHAPIRO 1984; CAIRNS *et al.* 1988), three broad classes of model have been considered to explain apparent increases in adaptive mutants under some selective conditions (reviewed by FOSTER 1999b; ROSENBERG 2001). In "directed mutation" models (*e.g.*, CAIRNS *et al.* 1988), environmental stress was postulated to stimulate formation or retention of mutations specifically in genes whose altered functions were selected. In "cryptic growth" models, mutation rates are specified to be constant (unaffected by environmental conditions) and the emergence of mutants during stress to result from normal growth-dependent mutation mechanisms operating in rare growing cells (*e.g.*, PARTRIDGE and MORGAN 1988; LENSKI *et al.* 1989; LENSKI and MITTLER 1993; GALITSKI and ROTH 1995; ANDERSSON *et al.* 1998; HENDRICKSON *et al.* 2002; and see DISCUSSION). In "hypermutation" models, mutation rates are suggested to increase globally in response to stress, generating mostly deleterious, but some useful, mutations (*e.g.*, HALL 1990; NINIO 1991; TORKELSON *et al.* 1997; ROSENBERG 2001). The ability to increase mutation could itself be selected (for example, as an advantageous by-product of error-prone DNA repair) or not; either way, increased mutation is a stress response. These models can be viewed as representing different views of evolution (and so have been debated vigorously; see CHICUREL 2001): the first, somewhat Lamarckian; the second, neoDarwinist in its adherence to constant mutation rates and gradual evolutionary change (*e.g.*,

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Mayr 1982); and the third, compatible with Darwinism (which allows for changing rates of generation of heritable variations; Darwin 1859). In this article we examine stationary-phase mutation in a well-studied *Escherichia coli* model system in which directed mutation models were ruled out previously (and hypermutation models supported) by the frequent occurrence of neutral and deleterious mutations along with useful ones (Foster 1997; Torkelson *et al.* 1997; Rosenberg *et al.* 1998; Rosche and Foster 1999; Bull *et al.* 2000, 2001; Godoy *et al.* 2000). Whereas we have suggested that stationary-phase mutation in this system results from transient genetic instability induced in response to stress, a hypermutation model (*e.g.*, Rosenberg 2001), others favor a cryptic growth model (*e.g.*, Hendrickson *et al.* 2002). Here, we examine the regulation of mutation in this system and address whether it is part of a stress response, *i.e.*, a response to environmental conditions.

In the Lac system (Cairns and Foster 1991), *E. coli* cells unable to catabolize lactose (because of a +1 frameshift mutation in *lac* genes carried on an F′ conjugative plasmid) are plated on lactose minimal medium, selecting for Lac$^+$ mutants. Lac$^+$ colonies appear after 2 days and continue appearing for >8 days. Most Lac$^+$ colonies present before day 5 carry a compensatory *lac* frameshift mutation ("Lac$^+$ point mutants"; Foster and Trimarchi 1994; Rosenberg *et al.* 1994). The remainder (5–10%) are Lac$^+$ because they carry amplified tandem arrays of the leaky *lac* allele (20–50 copies) that yield sufficient β-galactosidase for growth ("Lac$^+$ amplified clones"; Hastings *et al.* 2000). The occurrence of amplification in this Lac system (and others previously, see Horiuchi *et al.* 1963; Tlsty *et al.* 1984; Whoriskey *et al.* 1987) was noted previously, but not examined closely (Foster 1994; Foster *et al.* 1995). Recent work demonstrates that amplified clones make up 40–50% of the Lac$^+$ colonies that appear after day 7 (Hastings *et al.* 2000). Thus, both permanent genetic changes (point mutation) and reversible genome rearrangements (amplification) occur during starvation, making this system a useful model for both types of apparent environmentally induced genetic instability.

The mechanism of Lac$^+$ stationary-phase point mutation differs from mechanisms of reversion of the same allele in rapidly growing cells in its requirements for homologous recombination and double-strand-break (DSB) repair proteins (Harris *et al.* 1994, 1996; Foster *et al.* 1996), the SOS response to DNA damage (Cairns and Foster 1991; McKenzie *et al.* 2000), the SOS-regulated error-prone DNA polymerase IV (DinB; McKenzie *et al.* 2001), F′ transfer functions (Foster and Trimarchi 1995a,b; Galitski and Roth 1995), and transiently limiting mismatch repair activity (Harris *et al.* 1997, 1999; Foster 1999a). We have suggested that stationary-phase mutations are formed during error-prone DNA synthesis (involving pol IV) primed from recombination intermediates created as part of repair of double-strand

(DS) ends (Harris *et al.* 1994; Rosenberg 2001; and see Foster *et al.* 1996; Rodriguez *et al.* 2002). Adaptive amplification requires neither SOS nor pol IV (McKenzie *et al.* 2001).

A key question concerns whether adaptive point mutation and/or amplification are stress responses, as predicted specifically by hypermutation models. In this work we examine the role of RpoS, a "general stress response" regulator, in mutation and amplification in the Lac system. RpoS is a sigma (transcription) factor of RNA polymerase that promotes the increased transcription of >50 genes in response to a variety of environmental stresses (Hengge-Aronis 1996, 2000; Loewen *et al.* 1998). Inducers of the RpoS regulon include nutrient deprivation (leading to stationary phase), acidic pH, high temperature, oxidative stress, and osmotic stress (Hengge-Aronis 1996). Cross-protection is an important feature of the general stress response (Hengge-Aronis 1993, 2000). For example, when cells enter stationary phase due to carbon starvation, they become protected against oxidative stress and heat shock (Jenkins *et al.* 1988). RpoS-deficient cells are less viable in long-term culture and are not protected against oxidative, heat, or osmotic stress in stationary phase (Mulvey *et al.* 1990; Lange and Hengge-Aronis 1991; McCann *et al.* 1991). Thus, induction of the RpoS regulon leads to the development of a differentiated, protected state.

We present evidence that RpoS is required for stationary-phase mutation and also amplification in the Lac system. This is the first documented genetic requirement for adaptive amplification and the first shared with adaptive mutation, suggesting a possible common early step(s) in both pathways. The results imply that stationary-phase mutation and amplification are stress responses and that both occur in differentiated stationary-phase cells, as hypermutation, but not cryptic-growth, models predict. Further, the data imply that transient genetic instability is a previously unrecognized component of the RpoS-dependent general stress response, at least in response to stationary phase and, perhaps, in response to other stressors as well.

MATERIALS AND METHODS

Bacterial strains and growth: *E. coli* strains (Table 1) were constructed using standard techniques (Miller 1992). Bacterial growth was at 37°. M9 minimal medium (Miller 1992) had carbon sources added at 0.1% and thiamine (vitamin B1, or B1) at 10 μg/ml, and cultures were incubated for ~40–48 hr to assure saturation. *rpoS* M9 glycerol cultures often saturated at cell densities 3- to 5-fold lower than those of *rpoS$^+$* and were sometimes concentrated 5-fold before plating. Rarely, all *rpoS* cultures of an experiment had normal turbidity but viable cell numbers 10- to 100-fold lower than expected; these experiments were unusable and the variable responsible is unknown. Antibiotics and other additives were used at the following concentrations (μg/ml): chloramphenicol, 25; kanamycin, 50; tetracycline, 10; rifampicin, 100; X-gal, 40; threonine, 50; and tryptophan, 50.

TABLE 1

Escherichia coli **K-12 strains**

Strain	Relevant genotype	Reference or source
CAG12182	MG1655 *cysI3152*::Tn*10kan*	*E. coli* Genetic Stock Center (Yale); SINGER *et al.* (1989)
CAG18425	MG1655 *thrA3092*::Tn*10kan*	*E. coli* Genetic Stock Center (Yale); SINGER *et al.* (1989)
CAG18561	MG1655 *eda-3126*::Tn*10kan*	*E. coli* Genetic Stock Center (Yale); SINGER *et al.* (1989)
CAG18579	MG1655 *trpC3117*::Tn*10kan*	*E. coli* Genetic Stock Center (Yale); SINGER *et al.* (1989)
CAG18604	MG1655 *zgf-3156*::Tn*10kan*	*E. coli* Genetic Stock Center (Yale); SINGER *et al.* (1989)
FC29	Δ(*lac-proB*)$_{XIII}$ *ara thi*[F′ Δ(*lacI-lacZ*)]	CAIRNS and FOSTER (1991)
FC36	Δ(*lac-proB*)$_{XIII}$ *ara thi* Rif^R	CAIRNS and FOSTER (1991)
FC40	Δ(*lac-proB*)$_{XIII}$ *ara thi* Rif^R[F′ *lacI33-lacZ proAB*$^+$]	CAIRNS and FOSTER (1991)
MG1655	*rpoS*$^+$	*E. coli* Genetic Stock Center (Yale)
SMR533	FC40 *malB*::Tn*9*	FC40 × P1(SMR305)
SMR820	FC40 *lexA3 malB*::Tn*9*	MCKENZIE *et al.* (2001)
SMR868	FC40 *lexA3*	MCKENZIE *et al.* (2000)
SMR3462	Δ(*lac-proB*)$_{XIII}$ *ara thi* Rif^R *malB*::Tn*9*	FC36 × P1(SMR533)
SMR3804–3810, SMR3812–3814	FC40 Lac$^+$ (growth-dependent)	Independent spontaneous growth-dependent mutants; MCKENZIE *et al.* (1998)
SMR3855–3856, SMR3858–3865	FC40 Lac$^+$ (day 5)	Independent isolates; MCKENZIE *et al.* (1998)
SMR4562	Δ(*lac-proB*)$_{XIII}$ *ara thi* Rif^R[F′ *lacI33-lacZ proAB*$^+$]	Independent construction of FC40; MCKENZIE *et al.* (2000)
SMR6413, -6416, -6418, -6429, -6451, -6459, -6464, -6474, -6483	SMR4562 Lac$^+$ amplified	Independent isolates; HASTINGS *et al.* (2000)
SMR6499	SMR4562 *cysI3152*::Tn*10kan*	SMR4562 × P1(CAG12182)
SMR6541	SMR4562 *rpoS*::Tn*10*	SMR4562 × P1(ZK1268)
SMR6544–6552	SMR6541 Lac$^+$	Growth-dependent Lac$^+$ isolates of SMR6541
SMR6553–6560	SMR4562 *rpoS*::Tn*10* Lac$^+$	SMR3804–3810, -3812 × P1(ZK1268)
SMR6561–6570	SMR6541 Lac$^+$	Independent day 5 Lac$^+$ isolates of SMR6541
SMR6577	SMR4562 *rpoS*$^+$ (*rpoS*$^+$ from MG1655)	SMR6499 × P1(MG1655)
SMR6578	SMR6541 *lexA3 malB*::Tn*9*	SMR6541 × P1(SMR820)
SMR6579	SMR6541 *lexA3*	Mal$^+$ revertant of SMR6578
SMR6689–6692, SMR6694–6698	SMR4562 *rpoS*::Tn*10* Lac$^+$ amplified	SMR6413, -6416, -6418, -6429, -6451, -6459, -6464, -6474, -6483 × P1(ZK1268)
SMR6993	SMR4562 *thrA3092*::Tn*10kan*	SMR4562 × P1(CAG18425)
SMR6994	SMR6541 *thrA3092*::Tn*10kan*	SMR6541 × P1(CAG18425)
SMR7037	SMR4562 *trpC3117*::Tn*10kan*	SMR4562 × P1(CAG18579)
SMR7038	SMR6541 *trpC3117*::Tn*10kan*	SMR6541 × P1(CAG18579)
ZK1268	*rpoS*::Tn*10* (allele originally named *katF13*::Tn*10*)	LOEWEN and TRIGGS (1984)

The presence of *rpoS*::Tn*10* in SMR6541 was confirmed by PCR with primers 5′-TCAGCAACCGTAGCAATACGTACA-3′ and 5′-AGGCAATTATCGCGACCGCAG-3′ and also by PCR with those primers (separately) in combination with primer 5′-GACAAGATGTGTATCCACCTTAAC-3′, specific to a sequence present at both ends of Tn*10*. The presence of *rpoS*::Tn*10* was confirmed similarly in a sampling of 10 day 2 and 10 day 5 *rpoS* Lac$^+$ colonies, to exclude the possibility that they had lost the Tn*10* and were *rpoS*$^+$. Routinely, *rpoS* cultures were confirmed to be deficient in catalase activity (poor bubbling when hydrogen peroxide is applied to colonies; BOHANNON *et al.* 1991).

Sequencing: The *rpoS* gene of SMR4562 including its promoter region (LANGE *et al.* 1995) was sequenced using PCR-generated template DNA (primers flanking *rpoS*, above) and

additional *rpoS*-specific primers. One strand was sequenced completely. The difference at codon 33 was confirmed by sequencing across that site in independent PCR products. The *rpoS* allele in SMR6577 was confirmed by sequencing across codon 33, also in PCR-generated DNA. Sequencing was performed by Lone Star Labs (Houston).

Reconstruction experiments: Reconstruction experiments were performed as described (HASTINGS *et al.* 2000). Amplified Lac$^+$ isolates were grown in M9 lactose to maintain selection for the amplified array of 20–50 copies of the *lac* region (HASTINGS *et al.* 2000). The number of amplified cells was determined by plating nonselectively to LBH (1% tryptone, 0.5% NaCl, 0.5% yeast extract, 2 μg/ml thymine, pH 7, plate medium solidified with 1.5% agar) with X-gal to allow scoring of amplified cells (which form sectored blue-and-white colo-

nies) or Lac⁻ cells (which form white colonies, having lost the amplified array; Hastings *et al.* 2000 and references therein).

Mutation, conjugation, and transduction assays: Growth-dependent mutation rates to Lac⁺ were determined using fluctuation tests, as described (Harris *et al.* 1997, 1999) and rates were estimated using a modified method of the median (Lea and Coulson 1949; von Borstel 1978). A total of 29 or 30 independent cultures per strain were plated on M9B1 lactose and Lac⁺ colonies were scored. Rather than scoring only at 48 hr, we controlled for possible differences between genotypes in the time of colony formation and avoided including adaptive mutants (see Harris *et al.* 1999) by plating four to eight independent Lac⁺ derivatives of each strain in parallel with the cultures being scored for growth-dependent mutants. Scoring of all plates at ~1- to 2-hr intervals allows the determination of the median mutant frequency at a time when only 50% of the control Lac⁺ cells of that genotype have formed visible colonies (t_{50}). The t_{100} is set at ~48 hr when ≥95% of the Lac⁺ controls are visible for all genotypes. The mutation rates were determined from the mutant frequencies at the calculated t_{50} and multiplied by two (to give the rates at t_{100}).

Stationary-phase mutation assays were performed as described (Harris *et al.* 1996). All experiments presented had less than twofold net population change during days 1–3 after plating (see results for discussion of the importance of monitoring the Lac⁻ population).

For quantitative conjugation assays, donors were grown to saturation in M9B1 glycerol and then diluted 1:50 into M9 glucose and grown 3–4 hr. Donor and recipient cells (SMR3462, grown to saturation in LBH) were added to pre-warmed M9B1 glucose proline and incubated for 30 min at 37°, during which time dilutions of each were plated on LBH to determine viable cells per milliliter. Mating mixes were vortexed and plated on M9B1 glycerol chloramphenicol to select for Pro⁺ Camᴿ transconjugants. Donor cells were limiting by ≥10-fold to allow calculation of the frequency of conjugation as transconjugants per donor cell.

Transductions were performed as described (Miller 1992) with some modification. Recipient strains were grown to saturation in M9B1 glycerol with threonine or tryptophan added and cells were mixed with phage to give a multiplicity of infection (MOI) of ≤0.01. After 20 min of incubation, transduction mixes were washed once in M9 sodium citrate (20 mM) and plated on M9B1 glycerol 20 mM sodium citrate to select prototrophic transductants. Phage plating efficiencies were similar for both genotypes, indicating that *rpoS* cells are unimpaired for phage P1 infection and production. The phage titer on the *rpoS*⁺ recipient was used in determining the transductant frequency (transductants per phage) for both genotypes.

RESULTS

An *rpoS* mutation decreases stationary-phase mutation and amplification: An *rpoS* null allele (Loewen and Triggs 1984; Ivanova *et al.* 1992) decreases Lac⁺ colony yield substantially (Figure 1A, "total" values). Multiple experiments give similar results (Figure 1D), with some variability. The *rpoS* mutation decreases both point mutation and amplification (Figure 1, A and B; a second experiment gives similar results, data not shown). Lac⁺ point mutant and amplified colonies can be distinguished by streaking to nonselective indicator medium (see materials and methods). The *rpoS* phenotype in amplification is first detectable on day 5 as a threefold

reduction, with greater reductions on later days (five-, six-, and sixfold on days 6, 7, and 8, respectively). RpoS appears to be required for both point mutation and amplification; however, this conclusion depends on control experiments (below) that address other possible explanations for the *rpoS* phenotype.

***rpoS* cells are viable during the stationary-phase mutation assay:** Mutations in *rpoS* can decrease survival in stationary-phase cultures, at least in liquid culture (Mulvey *et al.* 1990; Lange and Hengge-Aronis 1991; McCann *et al.* 1991), suggesting that death of the *rpoS* cells might explain the decreases in observed Lac⁺ point mutants and amplified clones. We monitor viability by counting viable cells in plugs of agar removed from the selective plates each day, beginning the day after plating (day 1). Because Lac⁺ point mutant cells (the majority of Lac⁺ before day 7) take 2 days to form a colony (Table 2; McKenzie *et al.* 1998), any detected growth or death of the population is expected to be reflected in increased or decreased numbers of Lac⁺ colonies 2 days later. The viability data for the experiment in Figure 1, A and B, are presented in Figure 1C. The data are presented offset by 2 days because the Lac⁺ point mutant cells that give newly visible colonies on any given day were present in the population 2 days earlier (for example, the day 1 viability is plotted on day 3 because Lac⁺ cells in the day 1 population grew into the Lac⁺ colonies observed on day 3). The data demonstrate that the *rpoS* mutant strain is not poorly viable under these conditions. It is as viable as *rpoS*⁺ until 5 days after plating (plotted as day 7 in Figure 1C). This is consistent with previous reports of viability of *rpoS* strains on solid medium (Bridges and Timms 1998; Bridges *et al.* 2001). We conclude that net population death does not occur and cannot account for the lack of Lac⁺ point mutant colonies in the *rpoS* strain.

The effect of *rpoS* on formation of Lac⁺ amplified colonies is also not attributable to altered viability relative to *rpoS*⁺. It appears in Figure 1 that lack of growth of the *rpoS* strain when the *rpoS*⁺ begins to grow (late in the experiment) may correlate with the lack of amplified Lac⁺ colonies in the *rpoS* strain. However, amplified Lac⁺ cells take 3–5 days to form a colony (Table 2; Hastings *et al.* 2000). Thus, changes in the population size 5 days after plating (day 7 in Figure 1, at which point *rpoS*⁺ shows growth and the *rpoS* mutant does not) do not affect amplified colony yield until at least day 8, whereas the difference in amplified colony yield in *rpoS* is clear even by day 5. If the *rpoS* Lac⁺ amplified values are adjusted to reflect the viability 3, 4, or 5 days earlier, the effect of *rpoS* on amplification is even greater (data not shown). Also, we observe no loss in viability of the *rpoS* cells, relative to *rpoS*⁺, between plating and day 1 (data not shown). Therefore, poor viability of the population does not cause the *rpoS* phenotype in mutation or amplification.

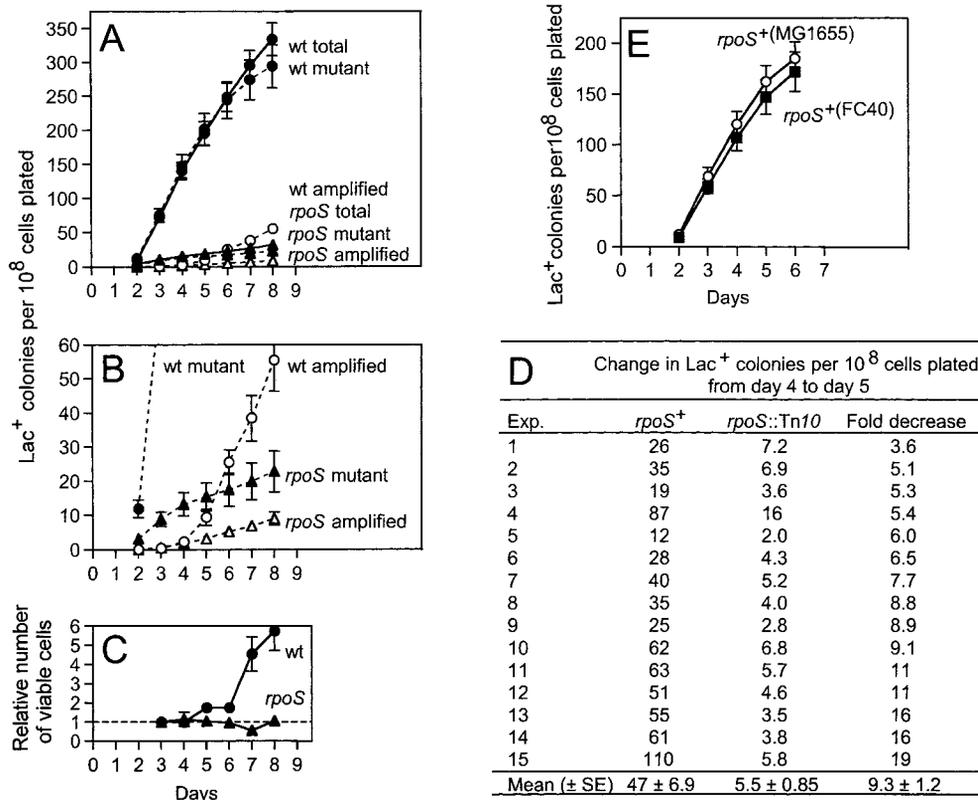Previous data imply that a hypermutable cell subpopu-

FIGURE 1.—RpoS in stationary-phase point mutation and amplification. (A–D) RpoS is required for point mutation and amplification. Strains are: *rpoS*+, SMR4562; *rpoS*, SMR6541. (A) Values are means ±1 SEM for 10 independent cultures per strain from one experiment. "Total" values are all Lac+ colonies scored. Point mutant and amplified Lac+ values are calculated on the basis of sampling of Lac+ colonies (a total of 100–120 colonies/strain/day, 20–30 from each of six cultures) as described (HASTINGS *et al.* 2000): amplified clones form sectored colonies when streaked on LBH rifampicin X-gal, whereas point mutants form solid blue colonies (MATERIALS AND METHODS). (B) Data from A, but with the *y*-axis expanded. (C) Relative viability of the Lac− population on the selection plates was determined as described (HARRIS *et al.* 1994), beginning the day after plating (day 1). The relative viability is offset by 2 days (the day 1 viability value is presented on day 3) because the day 1 population contains the Lac+ point mutant cells that will yield the observed day 3 Lac+ colonies. [Lac+ point mutant cells take 2 days to form a colony and thus are present in the population 2 days before they are scored as colonies (MCKENZIE *et al.* 1998). Amplified Lac+ cells take longer to form colonies (see text; Table 2; HASTINGS *et al.* 2000).] This allows one to track the relationship between the populations that yielded Lac+ on a particular day by looking at the same day on the axis in A–C. Values are means ±1 SEM from five cultures per genotype. Where not visible, error bars are smaller than the symbol. (D) Values are the change in Lac+ colonies per 10^8 cells plated from day 4 to day 5 (the slope of the line where *y* is new Lac+ per 10^8 cells plated and *x* is days) ±1 SEM. Each experiment included 5–10 independent cultures per strain and displayed no net population change before day 4. Experiment 13 is presented in Figure 1, A–C. (E) The variant *rpoS* allele in the *lac* frameshift-bearing strain is not required for stationary-phase mutation (means ±1 SEM, six independent cultures per strain; strains SMR4562 and SMR6577). Relative cell viability of both strains changed less than twofold from day 1 to day 4. A second experiment gave a similar result (data not shown).

D	Change in Lac+ colonies per 10^8 cells plated from day 4 to day 5		
Exp.	*rpoS*+	*rpoS*::Tn*10*	Fold decrease
1	26	7.2	3.6
2	35	6.9	5.1
3	19	3.6	5.3
4	87	16	5.4
5	12	2.0	6.0
6	28	4.3	6.5
7	40	5.2	7.7
8	35	4.0	8.8
9	25	2.8	8.9
10	62	6.8	9.1
11	63	5.7	11
12	51	4.6	11
13	55	3.5	16
14	61	3.8	16
15	110	5.8	19
Mean (± SE)	47 ± 6.9	5.5 ± 0.85	9.3 ± 1.2

lation gives rise to point mutants (TORKELSON *et al.* 1997; ROSCHE and FOSTER 1999; GODOY *et al.* 2000), but not amplified Lac+ clones (HASTINGS *et al.* 2000). We cannot assess the viability of any subpopulation directly. However, it is unlikely that *rpoS* reduces point mutation by causing inviability of the hypermutable subpopulation because *rpoS* also decreases amplified clones, which are not derived from it (HASTINGS *et al.* 2000).

rpoS phenotype cannot be attributed to poor colony formation or loss of amplification: Perhaps Lac+ mutations and amplified arrays form normally in *rpoS* cells, but cells carrying them form colonies slowly, giving the appearance of decreased mutation and amplification. We performed reconstruction experiments (as previously, MCKENZIE *et al.* 1998; HASTINGS *et al.* 2000) with multiple day 2 (presumed growth-dependent) and day 5 (stationary-phase) Lac+ isolates to determine how efficiently and rapidly *rpoS* Lac+ cells form colonies under the exact conditions of the stationary-phase mutation assay. Both day 2 and day 5 *rpoS* Lac+ point mutant

cells form Lac+ colonies with unit efficiency (∼100% of viable cells plated yield Lac+ colonies) and as rapidly as the *rpoS*+ controls (Table 2A). To control for the possibility that *rpoS* Lac+ cells had acquired mutations that facilitate colony formation under selective conditions, we performed similar experiments with *rpoS*+ day 2 Lac+ point mutants that were transduced with *rpoS*::Tn*10*. These gave similar results (data not shown).

To determine whether *rpoS* slows colony formation of Lac+ amplified cells, we introduced *rpoS*::Tn*10* into 9 independent amplified Lac+ strains with different amplified arrays that confer varying growth rates on lactose medium (HASTINGS *et al.* 2000). The *rpoS* derivatives retain the sectoring phenotype (Figure 1 legend) that is the hallmark of *lac* amplification, and the time required for colony formation is similar (Table 2B). Thus, *rpoS* does not decrease numbers of mutant and amplified colonies by slowing colony formation. Rather, *rpoS* cells appear to be impaired in forming mutations and amplified arrays.

TABLE 2

rpoS::Tn10 does not affect Lac⁺ colony formation

A. Point mutant Lac⁺	Average days to Lac⁺ colony formation $\pm$ SD[a]	
	$rpoS^+$	$rpoS$::Tn10
Day 2	2.1 $\pm$ 0.2	2.1 $\pm$ 0.03
Day 5	2.1 $\pm$ 0.04	2.1 $\pm$ 0.05

B. Amplified Lac⁺	Average days to Lac⁺ colony formation (single experiment)[b]		Average of $rpoS^+/rpoS \pm$ SD (three experiments)[c]
	$rpoS^+$	$rpoS$::Tn10	
Isolate 1	3.5	3.3	1.0 $\pm$ 0.12
Isolate 2	4.5	4.7	0.92 $\pm$ 0.06
Isolate 3	4.0	3.8	1.2 $\pm$ 0.15
Isolate 4	5.0	3.8	1.2 $\pm$ 0.10
Isolate 5	5.0	4.2	1.2 $\pm$ 0.04
Isolate 6	4.6	4.7	0.93 $\pm$ 0.07
Isolate 7	4.5	3.7	1.2 $\pm$ 0.03
Isolate 8	5.8	4.6	1.0, 1.3[c]
Isolate 9	3.3	2.6	1.4 $\pm$ 0.19

[a] Values are the averaged "average days to colony formation" (determined in reconstruction experiments as described; Hastings *et al.* 2000) of 10 independent Lac⁺ isolates for $rpoS^+$ and 9 for $rpoS$::Tn10. Lac⁺ colonies were scored each day for 5 days. Point mutant isolates are: day 2 $rpoS^+$, SMR3804–3810, SMR3812–3814; day 5 $rpoS^+$, SMR3855–3856, SMR3858–3865; day 2 $rpoS$, SMR6544–6552; day 5 $rpoS$, SMR6561–6570. Two additional experiments gave similar results.

[b] Values are for a single culture of each genotype from one experiment. Lac⁺ colonies were scored each day for 9 days. Amplified isolates (in order 1–9) are: $rpoS^+$, SMR6413, SMR6416, SMR6418, SMR6429, SMR6451, SMR6459, SMR6464, SMR6474, SMR6483; $rpoS$, SMR6689–6692, SMR6694–6698.

[c] Average of the ratio ($rpoS^+/rpoS$) of the "average days to colony formation" from three experiments with one culture of each genotype, except for isolate 8 (ratios for two experiments are given).

The retention of the amplified (sectored colony) phenotype when $rpoS$::Tn10 is introduced by transduction also indicates that $rpoS$ does not cause loss of amplified arrays, which presumably occurs by homologous recombination and segregation. This contrasts with *recA* mutations, which stabilize preformed *lac* amplification in *E. coli* (Tlsty *et al.* 1984) and Salmonella (Andersson *et al.* 1998). Apparently $rpoS$ mutation does not promote loss of amplified arrays, but instead inhibits their formation.

$rpoS$ and growth-dependent Lac⁺ mutation: Several genes required for Lac⁺ stationary-phase mutation (*e.g.*, *recA*) are not required for reversion of the same allele in rapidly growing cells (growth-dependent mutation), providing clear distinctions between the stationary-phase and growth-dependent mutation mechanisms (Cairns and Foster 1991; Harris *et al.* 1994, 1996; Foster *et al.* 1996; McKenzie *et al.* 2001). We find that growth-dependent Lac⁺ mutation is higher in $rpoS$ cells than in $rpoS^+$ (Table 3; paired Student's *t*-test on the three sets of data, $t = -4.33$, d.f. $= 2$, $P = 0.050$), which is the opposite of its effect on stationary-phase mutation (Figure 1). This is the first direct evidence we are aware of that RpoS can affect mutation in growing cells. We conclude that RpoS is required specifically for Lac⁺

mutation and amplification in stationary phase and that it may suppress mutation in growing cells.

The $rpoS$ genotype of the scavenger cells does not affect Lac⁺ colony yield: In adaptive mutation experiments, the *lac* frameshift-bearing cells are plated with an excess of nonrevertible (Δlac) "scavenger" cells to remove nonlactose carbon sources (Cairns and Foster 1991). $rpoS$ cells might appear deficient in mutation because the $rpoS^+$ scavenger cells might, for example, prevent growth of $rpoS$ Lac⁺ mutant colonies. If so, $rpoS$::Tn10 scavengers might have given a different result. However, we find that the yield of Lac⁺ colonies in $rpoS^+$ and $rpoS$::Tn10 strains is unaffected by the $rpoS$ allele of the scavengers (data not shown). This excludes a key role for the scavengers in the $rpoS$ mutation/amplification phenotype.

DNA recombination and F transfer are not defective in $rpoS$ cells: The results presented above indicate that RpoS is required specifically for formation of stationary-phase mutations and amplified arrays and exclude several trivial reasons for the $rpoS$ phenotype. The experiments in this and the following section address several possible mechanisms by which RpoS (and the genes it controls) might promote stationary-phase mutation and amplification.

TABLE 3

RpoS is not required for Lac⁺ mutation in rapidly growing cells

Relevant genotype[a]	Experiment	Mutation rate ($\times 10^{-10}$; mutations per cell per generation)[b]	Average mutation rate ($\times 10^{-10}$; ±SEM)
$rpoS^+$	1	1.5	3.1 ± 1.0
	2	2.8	
	3	5.0	
$rpoS$::Tn*10*	1	8.4	12 ± 1.9
	2	15	
	3	11	

[a] Strains are: $rpoS^+$, SMR4562; $rpoS$::Tn*10*, SMR6541.

[b] Mutant frequencies were determined in 29 or 30 tube fluctuation tests and mutation rates were calculated using control Lac⁺ strains: $rpoS^+$, SMR3804–3810, SMR3812; $rpoS,$ SMR6544–6551 (see MATERIALS AND METHODS). In experiment 1, four $rpoS^+$ Lac⁺ and eight $rpoS$ Lac⁺ control strains were used, and in experiments 2 and 3, eight Lac⁺ control strains of each genotype were used. Lac⁺ controls were independent isolates confirmed as mutant, not amplified, by colony appearance on LBH rifampicin X-gal plates (Figure 1 legend).

Stationary-phase mutation of the F′-borne *lac* allele requires F transfer functions (FOSTER and TRIMARCHI 1995a,b; GALITSKI and ROTH 1995) and RecBC-dependent homologous recombination proteins (HARRIS *et al.* 1994, 1996; FOSTER *et al.* 1996). We find that neither F transfer nor RecBC-dependent recombination is deficient in *rpoS* cells. $rpoS^+$ and *rpoS* stationary-phase cells are similarly active as donors in F transfer assays (consistent with previous results; FROST and MANCHAK 1998). The average numbers of transconjugants per donor cell are (experiment 1 and experiment 2; values are the means of three cultures): $rpoS^+$ (strain SMR4562), 7.7 × 10⁻³, 4.0 × 10⁻³; *rpoS* (SMR6541), 14 × 10⁻³, 3.8 × 10⁻³. Also, the frequency of transduction (a RecBC-dependent process; LLOYD and LOW 1996) is not different for $rpoS^+$ and *rpoS* minimal B1 glycerol stationary-phase cultures (Table 4; also true for minimal B1 glucose and rich medium cultures, data not shown). These are the only experiments we are aware of that address a

role for RpoS in homologous recombination. Because *rpoS* mutation affects neither F transfer nor homologous recombination, the *rpoS* requirements in stationary-phase mutation and amplification are unlikely to be to due to defects in either of these processes.

rpoS **and the SOS response:** Additional evidence supporting the involvement of RpoS in the SOS-dependent point mutation pathway (CAIRNS and FOSTER 1991; MCKENZIE *et al.* 2000) comes from epistasis analysis of *lexA3* and *rpoS* mutations. *lexA3* encodes a noncleavable LexA repressor; a *lexA3* strain is therefore unable to induce the Lex-dependent genes of the SOS response (WALKER *et al.* 2000). *lexA3* prevents most stationary-phase mutation (CAIRNS and FOSTER 1991; MCKENZIE *et al.* 2000), but has no effect on amplification (MCKENZIE *et al.* 2001). If RpoS and the SOS response act in separate pathways leading to point mutation, then the *rpoS lexA3* double mutant should have a more severe decrease in Lac⁺ yield than either single mutant. We

TABLE 4

***rpoS*::Tn*10* does not reduce transductional recombination**

Selection[a]	Experiment	Transductant frequency ($\times 10^{-4}$)[b]		Fold decrease	Mean fold decrease (±SEM)
		$rpoS^+$	$rpoS$::Tn*10*		
Thr⁺	1	5.8	3.4	1.7	0.94 ± 0.4
	2	1.2	2.0	0.60	
	3	1.2	2.3	0.52	
Trp⁺	1	0.86	2.7	0.32	1.2 ± 0.8
	2	0.28	0.10	2.8	
	3	6.1	13	0.47	

[a] For threonine or tryptophan prototrophy (MATERIALS AND METHODS).

[b] Recipient and donor strains are: $rpoS^+$ *thr*, SMR6993; $rpoS$::Tn*10* *thr*, SMR6994; $rpoS^+$ *trp*, SMR7037; $rpoS$::Tn*10* *trp*, SMR7038; P1 phage donor, SMR4562. Transductant frequencies are means from two to four transductions of one $rpoS^+$ or *rpoS* recipient culture at varying MOIs (all <0.01 phage per cell).
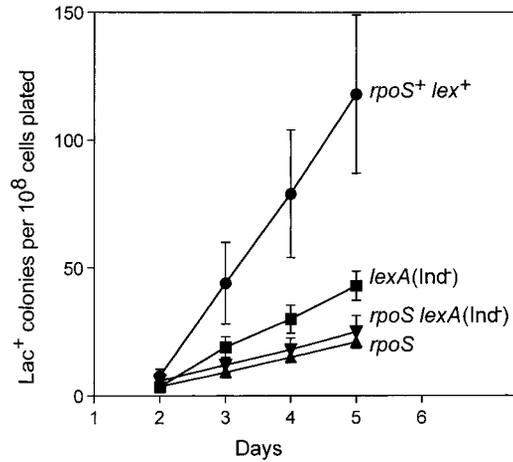
FIGURE 2.—*rpoS*::Tn*10* is epistatic to *lexA3*(Ind$^-$). Values are means ±1 SEM of Lac$^+$ colony counts per 10^8 cells plated from three experiments of six cultures each. Viability in each of the experiments varied less than twofold from day 1 to day 4 for all strains (data not shown).

find, in contrast, that the double mutant is similar to the *rpoS* strain (Figure 2), indicating that the RpoS and the SOS regulons act in the same pathway. That amplification also requires RpoS (Figure 1; Table 2), but not SOS (MCKENZIE *et al.* 2001), suggests that RpoS might act upstream of SOS before a common pathway branches into SOS-dependent point mutation and SOS-independent amplification routes. However, it is not yet clear whether RpoS plays the same role in amplification as it does in point mutation.

Mutation and amplification do not require a special *rpoS* allele: Many *E. coli* laboratory strains carry *rpoS* mutations that attenuate RpoS function (IVANOVA *et al.* 1992; VISICK and CLARKE 1997; ATLUNG *et al.* 2002), and the acquisition of an *rpoS*^{att} allele is a key feature of the *g*rowth *a*dvantage in *s*tationary *p*hase (GASP) phenotype (ZAMBRANO *et al.* 1993; ZAMBRANO and KOLTER 1996). In GASP, cells in long-term culture acquire advantageous mutations, the first being *rpoS*^{att}, and then outcompete their parental genotypes in successive waves, each requiring additional mutations (ZAMBRANO and KOLTER 1996). Might Lac$^+$ stationary-phase mutation require a special allele of *rpoS*?

We find that the *rpoS* allele of the *lac* frameshift strain SMR4562 [an independent construction of FC40 (CAIRNS and FOSTER 1991) from the same parental strains] has a mutation relative to the sequence in the *E. coli* K12 "wild-type" strain MG1655 (BLATTNER *et al.* 1997). *rpoS* codon 33 in SMR4562 is TTG, encoding leucine, instead of the CAG, encoding glutamine, in MG1655. This codon varies in laboratory strains (glutamine or an amber codon are common; IVANOVA *et al.* 1992; VISICK and CLARKE 1997; ATLUNG *et al.* 2002). However, this variant allele is not important for stationary-phase mutation and amplification because neither is altered in an SMR4562 derivative carrying a nonattenuated *rpoS*$^+$ allele (ATLUNG
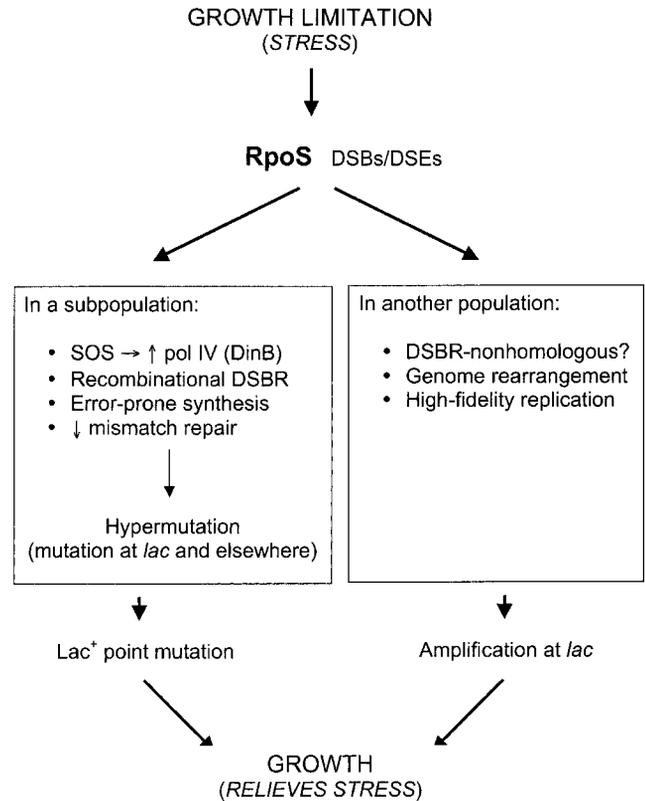

FIGURE 3.—A branched pathway model for adaptive point mutation and amplification as RpoS-dependent stress responses (modified from HASTINGS *et al.* 2000; MCKENZIE *et al.* 2001; ROSENBERG 2001; HASTINGS and ROSENBERG 2002). Amplification and point mutation are proposed to be alternative outcomes of a branched pathway, in which the only common genetic requirement known currently, RpoS, acts early, before the pathways diverge. Both processes are proposed to be stress responses that result in genetic change, some of which can promote growth in the growth-limiting environment. In point mutation, double-strand-break repair (DSBR) becomes error prone in stationary phase via use of pol IV. DSBs, double-strand breaks; DSEs, double-strand ends.

et al. 2002) from wild-type MG1655 (Figure 1E and additional data not shown). Therefore, the FC40 variant *rpoS* allele does not specifically promote mutation and amplification, and further, attenuated *rpoS* function conferred by mutation, which underlies GASP, does not underlie these stationary-phase mutations.

DISCUSSION

Summary: We found that RpoS, which controls a large regulatory network induced in response to various environmental stresses, including the onset of stationary phase (LOEWEN and HENGGE-ARONIS 1994), is required for most stationary-phase point mutation and amplification in the Lac system (Figure 1; Table 2; results reported in the text). RpoS is required specifically for mutation in stationary phase and not in rapidly growing cells (Table 3). RpoS and the SOS regulon act in the

same pathway leading to stationary-phase point mutation (Figure 2), with RpoS possibly acting upstream of SOS at a point before the amplification route branches from the point mutation route (*e.g.*, Figure 3). These results support the idea that transient genetic instability in this system is a stress response, specifically one induced in stationary phase.

These conclusions are possible only because other explanations for the *rpoS* phenotype (poor viability, poor colony formation, and loss of amplified arrays) were excluded experimentally. A previous study reported data indicating that an *rpoS* mutation reduced late Lac⁺ colonies, but the authors dismissed the phenotype because it correlated with altered growth relative to $rpoS^+$ before day 4 (Bridges *et al.* 2001). Their experimental conditions differed sufficiently as to allow growth during the early days of the experiment, which confounds interpretation of the data. For example, if the population grows, some unknown number of Lac⁺ colonies must be attributed to non-stationary-phase (*rec-* and *din-*independent) mutation mechanisms. Here, we controlled carefully for effects on growth and viability and other alternative explanations, including RpoS effects on mutation in growing cells, and conclude that RpoS is required specifically for most stationary-phase mutation and amplification.

Mutation and amplification are stress responses—implications for hypermutation and cryptic growth models: Three broad classes of model, two of which persist, were proposed as possible explanations for adaptive mutation in this and other assay systems (reviewed by Foster 1999b; Rosenberg 2001). One class, postulating mutations directed to genes under selection (directed mutation models, *e.g.*, Cairns *et al.* 1988), was rejected for the Lac system (see Introduction; Rosenberg 2001). The two extant classes of model evoke either a global increase in mutation rates in a stress response to the environment (hypermutation models) or "normal" mutation rates and mechanisms acting on multiple DNA copies in rare growing cells (cryptic growth models, an example is discussed below). In the Lac system, hypermutation models are supported not solely by detection of mutation at non-*lac* sites (see Introduction; reviewed by Rosenberg 2001), but also by requirements for components well known to contribute to increased mutation in other contexts and/or assay systems (Friedberg *et al.* 1995; Kim *et al.* 1997; Wagner and Nohmi 2000; Walker *et al.* 2000): the mutagenic SOS response (Cairns and Foster 1991; McKenzie *et al.* 2000), the low-fidelity DNA pol IV (McKenzie *et al.* 2001), and limiting mismatch repair (Harris *et al.* 1997, 1999).

A current cryptic growth model (Andersson *et al.* 1998; Hendrickson *et al.* 2002) proposes preexisting spontaneous amplification of the *lac* region as a mechanism to achieve cryptic growth and mutation in some rare cells: multiple *lac* copies (more β-galactosidase) allow growth; mutation rates are normal, and the in-

creased Lac⁺ mutants in stationary phase result mainly from growth and extra *lac* copies. Recent versions of this model account for the extensively documented genome-wide hypermutation in Lac⁺ mutants (Torkelson *et al.* 1997; Rosche and Foster 1999; Bull *et al.* 2000, 2001; Godoy *et al.* 2000) and for the requirement for SOS-inducible DNA pol IV (McKenzie *et al.* 2001) by invoking induction of the SOS response by amplification (Hendrickson *et al.* 2002) or by amplification of the gene encoding pol IV (Kofoid *et al.* 2003). This cryptic growth model thus makes hypermutation a by-product of, rather than central to, formation of Lac⁺ adaptive point mutants. Lac⁺ mutations are proposed to result mainly from growth and increased replication of multiple gene copies, occurring more robustly with increasing growth rate, not as part of a stress response or in differentiated stationary-phase cells. The requirement for an RpoS-dependent stress response (Figure 1; Table 2) and the implication that the mutating cells are in stationary phase are incompatible with such cryptic growth models that allow no special circumstances (other than growth) and instead support hypermutation models, which feature increased mutation rates as part of a stress response in growth-limited cells (*e.g.*, Figure 3).

RpoS and models for transient genetic instability: In Figure 3, we suggest that RpoS acts upstream in a common stress response pathway that branches to yield either point mutation or amplification, rather than downstream in promoting survival of cells carrying mutation or amplification. This order of action is supported by the well-characterized role of RpoS in establishing the differentiated state of stationary phase (Hengge-Aronis 2000) and by the epistasis of *rpoS* to a *lexA*(Ind⁻) mutation (Figure 2). Although it is economical to hypothesize a single role for RpoS in the two genetic instability routes in the common part of a branched pathway, it is also possible that RpoS plays different roles in point mutation and amplification.

The two branches (to point mutation or to amplification; Figure 3) are clearly delineated: the SOS response and DNA pol IV are required only for mutation, not for amplification (McKenzie *et al.* 2001), and amplified isolates do not display hypermutation as point mutants do (Hastings *et al.* 2000). The point mutation pathway is better defined, requiring DSB-repair proteins (Harris *et al.* 1994, 1996; Foster *et al.* 1996), F transfer proteins (Foster and Trimarchi 1995a,b; Galitski and Roth 1995), an SOS response (Cairns and Foster 1991; McKenzie *et al.* 2000), SOS-induced DNA pol IV (DinB; McKenzie *et al.* 2001), and limiting mismatch repair (Harris *et al.* 1997, 1999), and is associated with transient genome-wide hypermutation (Torkelson *et al.* 1997; Rosche and Foster 1999; Bull *et al.* 2000, 2001; Godoy *et al.* 2000). In contrast, amplification requires neither SOS nor pol IV (McKenzie *et al.* 2001) and is not associated with genome-wide hypermutation

(Hastings *et al.* 2000). We suggest that point mutation occurs in a transiently hypermutable cell subpopulation in which the SOS response is induced, leading to increased production of error-prone pol IV. The mutations could result from error-prone synthesis (using pol IV) primed during homologous recombinational DSB repair (Harris *et al.* 1994). Amplification might result when DS ends (possible sources reviewed by Rosenberg 2001) are repaired beginning with a nonhomologous end-joining event, creating either a duplicated segment or a rolling circle intermediate, using high-fidelity DNA synthesis in non-SOS induced cells (Hastings and Rosenberg 2002).

In this model (Figure 3), acquisition of either a Lac$^+$ point mutation or amplified *lac* DNA allows growth, curtails the RpoS-dependent stress response, and restores genetic stability. That amplification is an endpoint, rather than an intermediate en route to point mutation (as in single-pathway models, *e.g.*, Hendrickson *et al.* 2002), is supported by previous findings that amplified clones are neither hypermutated nor particularly mutable and do not lead readily to formation of adaptive point mutants under selection (Hastings *et al.* 2000).

RpoS in other stationary-phase mutation mechanisms: RpoS is required for stress-induced mutation in another assay system in *E. coli* (Bjedov *et al.* 2003). In that system mutation in aged colonies also requires RecA, but in contrast to mutation in the Lac system does not have strong dependence on either RecB or the LexA regulon. Thus this appears to be a different mechanism, but one that also requires the RpoS regulon, supporting a general role for RpoS in multiple stationary-phase mutation mechanisms (although not in all stationary-phase mutation systems; see Bridges and Timms 1998). RpoS is required for mutation-dependent adaptation to long-term culture conditions that can give some cells a growth advantage in stationary phase (see RESULTS; Zambrano and Kolter 1996), as are pol IV and two other SOS-inducible polymerases (Yeiser *et al.* 2002). Also, apparent stationary-phase mutations in Pseudomonas seem to require RpoS (Saumaa *et al.* 2002) as do stationary-phase genome rearrangements associated with transposable elements in *E. coli* (Gomez-Gomez *et al.* 1997; Lamrani *et al.* 1999) and other bacteria (Ilves *et al.* 2001; Saumaa *et al.* 2002).

Candidate genes in the RpoS regulon: RpoS-regulated gene products (Loewen *et al.* 1998) that might be involved at the DNA level in mutation and amplification include proteins involved in base excision repair (Exonuclease III), methylation damage repair (AidB), chromosome segregation/cell division (FtsQ, FtsZ), and prevention of oxidative damage (catalase HPII, Dps). In addition, RpoS downregulates methyl-directed mismatch repair in stationary phase, apparently by decreasing the levels of MutS and MutH (Feng *et al.* 1996; Tsui *et al.* 1997). An apparent insufficiency of functional MutL

facilitates stationary-phase mutation in this system (Harris *et al.* 1997, 1999; and perhaps amplification, because the work was done prior to the discovery of amplification as a major component on later days).

Transient genetic instability and the general stress response: The data presented here imply that transient genetic instability in the Lac system occurs in cells that are differentiated by RpoS-dependent gene expression. The well-characterized role of the RpoS regulon (Hengge-Aronis 2000) in protecting cells (particularly at the DNA level) from environmental stresses may seem at odds with a role in supporting genetic instability. However, the evidence that only a subpopulation of cells experiences genetic instability in this system (reviewed in Rosenberg 2001) means that the population as a whole is not threatened. Only a subpopulation is subject to the potentially damaging effects of increased point mutation rates. Recent work demonstrates that the majority of wild *E. coli* experience stress-inducible mutation, supporting the relevance of increased mutation rates to bacterial survival (Bjedov *et al.* 2003; Rosenberg and Hastings 2003). The role of RpoS as the regulator of the general stress response (Hengge-Aronis 2000) suggests that stationary phase might not be the only condition that stimulates mutation and genetic rearrangements such as amplification. Other inducers of the general stress response might also cause transient genetic instability and so promote bacterial adaptation to stressful environments.

Note: A prepublication report released during the review of this manuscript shows that RpoS positively regulates the DinB error-prone DNA polymerase (pol IV; Layton and Foster 2003). Because DinB is required for adaptive point mutation (McKenzie *et al.* 2001), this could account for some or all of the role of RpoS in adaptive point mutation. Because DinB is not required for adaptive amplification (McKenzie *et al.* 2001), RpoS must play some other role in that process.

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Communicating editor: R. S. Hawley