Accumulation of mutants in "aging" bacterial colonies is due to growth under selection, not stress-induced mutagenesis

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Several bacterial systems show behavior interpreted as evidence for stress-induced mutagenesis (adaptive mutation), a postulated process by which nongrowing cells temporarily increase their general mutation rate. Theoretical considerations suggest that periodic stress-induced general mutagenesis would not be advantageous in the long term, due to the high cost of deleterious mutations. Alternative explanations have been tested for very few of the systems used as evidence for stress-induced mutation. In one prominent system, mutants resistant to rifampicin (Rif^R; *rpoB*; RNA polymerase) accumulate in cell populations that "age" on solid medium with little net growth. Mutant accumulation was initially attributed to stress-induced general mutagenesis in nongrowing cells. Evidence is presented that these Rif^R mutants accumulate because they grow faster than parent cells during the aging period. Direct tests revealed no increase in the frequency of other mutant types during the aging period.

Classic experiments have led to the conclusion that mutations arise as errors in replication or repair and show a frequency and site specificity that is random with respect to phenotypic consequences or need (1, 2). Once formed, mutants may change in frequency, depending on their growth rate relative to that of the parent strain. Mutations can also be induced by external conditions: chemical mutagens or DNA-damaging agents (3).

The possibility of a third source of mutations has been entertained for over 150 years but never demonstrated conclusively. That is, do cells possess mechanisms to generate mutations in their own genome in response to environmental stresses that are not directly mutagenic? Stress-induced mutation lost favor after classic demonstrations that bacterial mutants form before exposure to the selective conditions used to detect them (1, 2). Interest was rekindled when it was pointed out that these experiments used lethal selections that could detect only mutants formed before selection, leaving open the possibility of some stress-induced mutations (4–6).

Several bacterial systems show behavior consistent with a mechanism that up-regulates the general mutation rate during stress, thereby contributing new beneficial mutations that circumvent growth limitation. In opposition to this interpretation, the vast excess of deleterious mutations has been cited as evidence that such a mechanism is unlikely to be of long-term benefit (7).

In the Cairns system (8), an *Escherichia coli* mutant defective in lactose catabolism regains the ability to use lactose during exposure to selective conditions, a behavior initially attributed to stress-induced mutagenesis (adaptive mutation). The behavior of this system can now be fully explained by selection alone (9), although the original interpretation retains enthusiastic support (10, 11). Resolution of the question of stress-induced mutagenesis requires that each system be tested for the possibility that its behavior reflects cryptic growth and selection (rather than mutagenesis).

Prominent support for stress-induced mutagenesis is the increase in the frequency of rifampicin-resistant mutants (Rif^R, *rpoB*, RNA polymerase) seen in aging, nongrowing colonies of *E. coli* (12–14). A colony forms during 1 day of growth on rich solid medium. During the following 6 days, the colony population increases very little (1.2-fold), but its frequency of Rif^R mutant cells increases substantially (10–100-fold). The increase in number of Rif^R cells was attributed to stress-induced mutagenesis in nongrowing cells (12). This observation is re-examined here in both *E. coli* and *Salmonella enterica*. The original results are confirmed: Rif^R mutant frequency increases with time and depends on RpoS (a sigma factor of RNA polymerase active in stationary phase). Evidence is presented that the accumulation of Rif^R mutants is due to selection; Rif^R mutants arising before growth limitation grow faster than parent cells during the aging period. No general mutagenesis occurs, as direct tests revealed no accumulation of auxotrophic mutations during aging.

Results

Increases in the Frequency of Rif^R Mutants in Aging Colonies of *S. enterica* and *E. coli*. The accumulation of Rif^R mutants in aging colonies was first described for *E. coli* (13, 14). The extent to which Rif^R mutants accumulated varied widely from one *E. coli* isolate to the next (12) and depended on a functional *rpoS* gene (encoding a stationary phase sigma factor of RNA polymerase). Results below confirm the original results for *E. coli* and demonstrate the same accumulation in *S. enterica*.

Colonies of two well characterized strains of *S. enterica* serovar Typhimurium, LT2 and the more virulent strain 14028s, were initiated by spotting 100–1,000 cells on nitrocellulose filters resting on rich agar plates (LA; *Materials and Methods*). During the first day, the colony population grew to 10^9 viable cells, defined as colony forming units (cfu). The population increased an additional 10-fold (to 10^{10} cells) by Day 2, but showed no further increase or decrease during the next 5 days, as reported earlier for *E. coli* (14).

Because so few cells were used to initiate the colony, no Rif^R mutants were present in the initial inoculum. Thus any Rif^R mutants found later within the colony must have arisen during the time on solid medium. Table 1 describes the time-dependent increase in the frequency of Rif^R mutants within a colony of *S. enterica*. Between Day 1 and Day 7, this frequency increased 21-fold for strain 14028s, but only 4-fold for LT2 (the standard genetic wild-type strain). Because accumulation of Rif^R mutants in *E. coli* was originally shown to depend on RpoS (12), it seemed that differences in RpoS levels might explain the different accumulation rates in the two *S. enterica* strains. The *rpoS* gene of strain LT2 is known to be partially defective due to its atypical start codon, UUG (15), whereas the *rpoS* gene of strain 14028s has a standard AUG start codon.

To test dependence on RpoS, we introduced an *rpoS* mutation

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Table 1. Frequency ($\times 10^9$) of Rif^R mutants per colony

	Rif^R/Day 1 colony*	Dev†	N‡	Rif^R/Day 7 colony*	Dev.†	N‡	Day 7/Day 1§	P¶
S. enterica								
14028s *rpoS*$^+$	6.8	9.4	36	150	58	25	21	0.0001
LT2 *rpoS*	4.9	7.7	18	21	3.5	21	4	0.0015
14028s *rpoS*	3.1	7.1	49	17	2.2	27	5	<0.0001
LT2 *rpoS*$^+$	7.0	3.8	12	240	2.4	12	34	0.0006
E. coli								
MG1655	3.7	2.5	10	34	2.0	10	9	0.0058
CFT073	3.3	19	5	11	4.5	8	3	0.3421
Nu14	3.2	7.2	5	8.7	11	8	3	0.093
C1181	2.7	3.2	5	45	4.3	8	16	0.0067
C1197	2.3	12	5	530	4.7	8	230	0.0044

*Median frequency ($\times$ 10^9) of Rif^R cells/colony on day 1 and day 7.
†Dev. is the absolute average deviation from the median (see *Materials and Methods*).
‡*N* is the number of independent colonies assayed for Rif^R mutants.
§Day 7/Day 1 is the ratio of median frequency of Rif^R mutants in day 7 colonies to frequency in day 1 colonies.
¶Two-tailed *P* values. (The Day 7/Day 1 ratio is significantly different from 1 as measured by the Mann–Whitney nonparametric test).

into strain 14028s, and found that it reduced the Day 7 level of Rif^R mutants to that seen in LT2 (Table 1). Conversely, introduction of a wild-type *rpoS* locus from 14028s into LT2 increased the Day 7 Rif^R mutant frequency to at least the level observed in 14028s. Thus the time-dependent increase in Rif^R mutant frequency is strongly dependent on RpoS in *S. enterica*, as shown previously for *E. coli*.

The original demonstration that Rif^R mutants accumulate in *E. coli* was confirmed for *E. coli* strains MG1655 and CFT073, whose genomes have been sequenced, and for 51 clinical isolates previously characterized for their antibiotic resistance phenotypes (16, 17). See Table 1 for two examples. A ratio of the Rif^R mutant frequency on Day 7 to the frequency on Day 1 ranged from 0.08 to 400, with a median value of 8 (and an absolute average deviation from median of 5). This is similar to the ratio of 7 found previously for 787 natural isolates of *E. coli* (12).

Logarithmic Accumulation of Rif^R Mutants Suggested Growth Under Selection. The time course of Rif^R mutant accumulation was measured in both wild-type (14028s) and *rpoS* mutant cells over 28 days, well beyond the period of the original experiment (7 days) (See Fig. 1.) For both wild-type and the *rpoS* parent strains, the Rif^R mutant frequency increased logarithmically and fit well to exponential curves (R^2 values of 0.93 and 0.91, respectively) with exponents of 0.41 and 0.21 respectively. The increase in Rif^R mutant frequency depended heavily on RpoS.

The logarithmic frequency increase suggested that Rif^R mutants accumulate during aging because they grow faster than the parental strain (or they grow logarithmically while the parent fails to grow) and that this growth advantage depends on RpoS. Assuming that the parent population shows no growth, these data are consistent with Rif^R cells growing with a doubling time of 1.7 days in the presence of RpoS and 3.2 days with the *rpoS* mutation. Thus very little growth is needed to explain the observed increase the frequency of Rif^R cells.

If Rif^R mutant accumulation were due to mutagenesis, as originally proposed, the mutant frequency would be expected to increase linearly with time (assuming a nongrowing population and a constant mutation rate). To explain the observed logarithmic increase by mutagenesis of nongrowing cells requires the mutation rate to increase logarithmically, which seems unlikely. To explain the 10^4-fold increase in Rif^R mutant frequency over 28 days by mutagenesis requires an intensity higher than any demonstrated experimentally for a nongrowing population. Standard laboratory mutagenesis of nongrowing cells can increase mutant frequency 100–1,000 fold; larger increases are prevented by loss of viability due to lethal mutations. Larger increases in mutant frequency can be obtained only by mutagenizing a growing population so as to continuously counterselect deleterious mutations. Heavy mutagenesis would predict accumulation of dead cells which was not seen (see *Materials and Methods*), and a large increase in secondary nonlethal mutations in Rif^R mutants, also not observed (see next paragraph).

Rif^R Mutants Show No Evidence of Intensive Mutagenesis. Colonies of wild-type 14028s were aged on rich medium for 1, 7, or 21 days. Day 1 colonies were suspended and spread on LA-Rif selective media. Day 7 and Day 21 colonies were replica-plated directly onto LA-Rif. Rif^R colonies were picked and purified, then patched onto minimal medium and LA. The frequency of auxotrophs among Rif^R mutants (Table 2) were in the range of 1/1,000 as expected for loss-of-function mutations in the roughly 200-gene target used to detect auxotrophs. (Null mutations in a single gene are typically present at 10^{-5} in an unselected overnight culture.) The small increase in the auxotroph frequency over time is similar to that reported initially for resistance to a variety of antimicrobial agents (12). In contrast to this very small increase in auxotroph frequency, the frequency of Rif^R mutants increased 21-fold by Day 7, and 6,800-fold by Day 21 (Fig. 1). The rarity of Rif^R mutants with an associated auxotrophy makes it unlikely that the Rif^R mutants owed their appearance to intense mutagenesis.

Evidence for Clonal Growth of Rif^R Cells in Aging Colonies. If Rif^R mutants accumulate because the few mutants cells that arise during growth (Day 1–2) grow faster than the parent population during the aging period of growth-limiting selection (Days 2–7), then the mutant cells that accumulate under selection should be found as localized clones (papillae) at a few sites within the aging colony. In

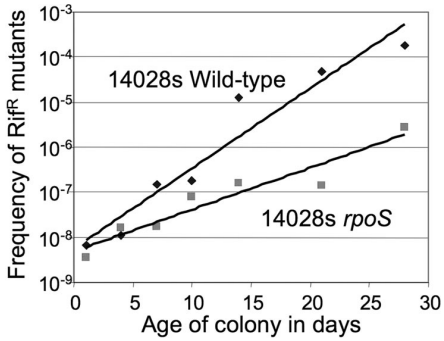

Fig. 1. Increase in the frequency of Rif^R mutants as a function of colony age. Colonies aging on LA plates were assayed for their frequency of Rif^R mutant cells. The two strains tested differ only by substitution of an Amp^R determinant for *rpoS* (*rpoS*::AmpR).

Table 2. Autotroph frequency in aging wild type (14028s)

Age of population	Rif^R mutants tested	No. of auxotrophs	Auxotroph frequency	Fold increase
Day 1	2169	14	3.8×10^{-3}	1
Day 7	794	3	6.5×10^{-3}	1.7
Day 21	459	4	8.7×10^{-3}	2.3

contrast, if growth limitation (aging) induces mutagenesis in a nongrowing cell population as suggested previously (12), then individual Rif^R cells should arise independently and be broadly distributed throughout the aged colony.

To test these predictions, we divided aged colonies into 16 sectors (4×4) and assayed each sector individually for its frequency of Rif^R mutants. Sectors were tested from 30 colonies of strain 14028s on Day 7 and from 5 colonies on Day 14. In each case, $\leq$95% of the Rif^R mutants were contained within 1 or a few of the 16 sectors (see two examples in Fig. 2 and additional data in Fig. 3). The median number of Rif^R mutants per sector on Day 7 was 4. Sectors with many Rif^R mutants (50–10,000) are called "jackpot" sectors. This uneven distribution of accumulated Rif^R mutant cells is expected if preexisting Rif^R mutants (arising by Day 1) form clones that expand during the aging period (Day 1 to Day 7).

Relationship Between Spatial Distribution and Mutant Number. The uneven spatial distribution of Rif^R mutants is a general feature of this system. Two colonies are described in Fig. 2 and additional colonies in Fig. 3. In each colony, the bulk of the mutant cells (>95%) were found in a few of the 16 sectors tested.

If the accumulation of Rif^R mutants is due primarily to the growth of clones, then variation in the number of mutants from one colony to the next is expected to depend most heavily on differences in mutant clone size (which increases logarithmically) and less on

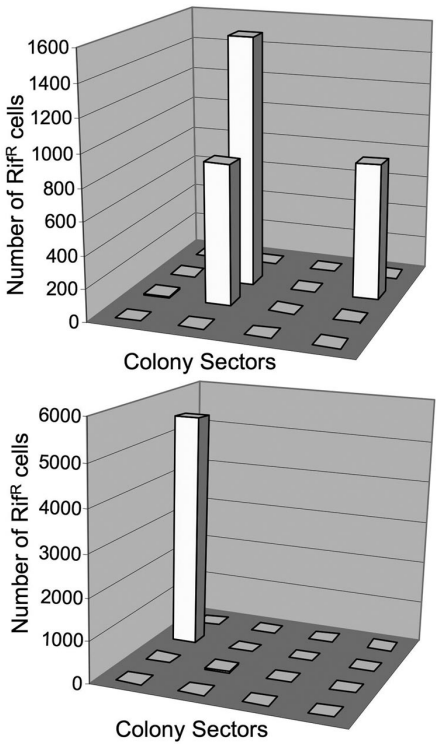

Fig. 2. Distribution of Rif^R cells in two Day 7 colonies of strain 14028s. Each colony was cut into 16 equal sectors, which were assayed for Rif^R cells. More data are in Fig. 3.

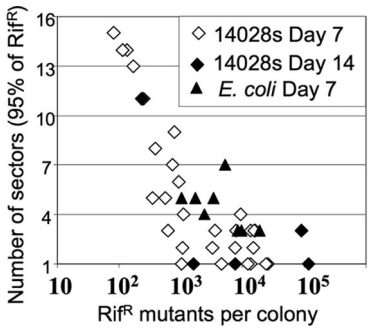

Fig. 3. Colonies with more Rif^R mutants have fewer, but larger, clones. Each point in the graph represents one aged colony. The total Rif^R mutants per colony (sum of all sectors) is plotted against the number of sectors that together include 95% of the mutants. Strain 14028s is an *S. enterica* isolate. The *E. coli* natural isolates were described previously (16).

variability in the number of clones present. Colonies with more mutants are predicted to have larger clones, allowing fewer sectors to contain the bulk of the mutants (>95%). That is, the more mutants a colony contains, the more uneven is the spatial distribution of mutant cells. Conversely, if mutagenesis of nongrowing cells were responsible for the accumulation of Rif^R mutants, one would expect the spatial distribution of mutants to become more even as the total number of mutant cells increases. Data in Fig. 3 support the idea that an increasing number of mutants is associated with more uneven distribution, as predicted by growth under selection.

In *E. coli*, as in *S. enterica*, the accumulated Rif^R mutants were found in a few separated local sectors within the colony. Colonies of the clinical isolates C1181 and C1197 were divided into 16 sectors, and the frequency of Rif^R mutants was found to vary widely from one sector to the next. These results are included in Fig. 3, which portrays the small number of highly populated sectors and the reduced number of sectors required to account for 95% of the Rif^R mutants (as seen in *S. enterica*).

Evidence That Rif^R Cells from a Single Sector Are Clonally Related. The DNA base sequence of the *rpoB* gene (where Rif^R mutations occur) was determined for at least five Rif^R mutants from each of several colony sectors. In almost all cases (see below), the five sequenced mutants from an individual sector carried the same *rpoB* sequence change. Mutants from different sectors seldom shared the same sequence change. This argues strongly that the many Rif^R cells in a single sector (above) belong to one clone that expanded during the selection period. These sequence changes are described in more detail below.

The Nature of Rif^R (*rpoB*) Mutations That Provide a Growth Advantage in Aging Colonies. Although the physiological basis of the growth advantage of Rif^R mutants is not known, it seemed possible that only a particular subset of Rif^R mutations might enhance growth during aging. Mutations of *rpoB* that confer a Rif^R phenotype affect a large number of sites in the *rpoB* gene (18) and can have a wide variety of effects on other aspects of cell physiology (19, 20). Thus one might expect that certain Rif^R (*rpoB*) mutations are more likely than others to be found in the jackpot sectors of Day 7 colonies.

Two factors are expected to contribute to the likelihood of a particular Rif^R mutation appearing in a jackpot clone. One is the magnitude of its conferred growth advantage over wild type during the aging period. Another factor is the frequency with which a particular mutation arises. This frequency influences not only the number of times a particular mutation appears, but also the time in the history of the colony at which the Rif^R mutation arises. Any Rif^R mutation that arises early during growth is expected to have more

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Table 3. Nature and selective value of mutations arising during aging

rpoB mutation		No. of occurrences			Competitive index (CI)			
No.	Substitution	Day 1	Day 7, nonjackpot	Day 7, jackpot	CI	Dev*	$N^{\dagger}$	*P* value‡
1	D516G	14	8	14	2.7	2.3	11	0.0002
2	S531F	10	3	0	1.0	0.4	12	0.1362
3	H526Y	5	3	8	1.1	7.6	14	0.6312
4	S512P	3	5	3	1.1	0.4	16	0.8103
5	R529H	3	4	0	5.2	3.6	8	<0.0001
6	P564L	3	4	4	27.8	93.2	11	<0.0001
7	S522F	2	3	0	0.2	0.7	12	0.0014
8	I572F	2	1	0	1.7	0.7	5	0.1188
9	K504N	1	1	0	3.0	1.0	11	<0.0001
10	L511P	1	1	0	3.1	1.5	11	<0.0001
11	S512F	1	4	2	0.9	0.3	17	0.2891
12	R529C	1	6	1	17.2	7.2	16	<0.0001
13	S512Y	0	2	0	0.8	0.6	18	0.3628
14	Q513L	0	2	0	5.7	3.7	13	0.0061
15	Q513H	0	1	0	12.9	5.9	15	0.0001
16	D516Y	0	2	0	2.2	0.9	23	<0.0001
17	S522Y	0	0	1	4.6	8.0	13	0.0105
18	H526L	0	2	0	1.4	1.0	12	0.2041
19	H526P	0	1	0	1.3	2.3	25	0.2225
20	R529S	0	1	0	40.6	45	12	<0.0001
21	R529L	0	1	1	8.1	9.4	18	<0.0001

*Dev is the absolute average deviation from the median (see *Materials and Methods*). Note: the CI was positive for all competitions.
†*N* is the number of independent colony competitions for each mutant.
‡Two-tailed *P* values (CI of mutant significantly different from CI of wild-type) measured by Mann–Whitney nonparametric test.

time in which to grow and achieve a large clone size, both before and during the aging period. Frequent mutations are likely to appear early in the rapid growth period (10^6-fold) on Day 1. Thus growth advantage and frequency of occurrence are expected to contribute independently to the likelihood of particular mutations being found in large clones after the aging period.

To test these expectations, we sequenced the *rpoB* gene from 271 different Rif^R mutants from the following sources:

1. From Day 1 populations (that experienced very little selection), one Rif^R mutant was sequenced from each of 46 independent filter colonies. This set included 12 different mutant *rpoB* alleles (see Table 3).
2. From each of 55 nonjackpot sectors of Day 7 colonies (median 4 Rif^R cells/sector), a single randomly chosen Rif^R mutant was tested. This yielded 20 different *rpoB* alleles.
3. From each of 32 jackpot sectors (with 50–10,000 Rif^R cells), five Rif^R mutants were tested. This collection included 8 different sequence changes. For 30 of the 32 sectors, all five mutants tested had the same *rpoB* sequence change, demonstrating clonality of the population. The two other sectors each included two mutant types; both of these sectors had a very small jackpot (<200 mutants).
4. Mutants from different jackpot sectors of the same colony (6/7 colonies with multiple jackpots) showed different *rpoB* sequence changes.

From all sources combined, 21 different mutant *rpoB* sequences were found among the 271 mutant genes sequenced. Thus many mutant types recurred. Results are described below and in Table 3.

The 8 different *rpoB* mutations recovered from 32 distinct jackpot sectors could be either types that conferred the largest growth advantage during aging or types that conferred a smaller advantage but arose early during growth and had the most time to expand their clones both before and during aging. The 12 *rpoB* mutant types that recurred repeatedly among Rif^R mutants identified before selection (Day 1) are inferred to arise commonly (top of Table 3). To test whether there is a correlation between conferred growth advantage and likelihood of being found in a jackpot sector, we tested the 21 different *rpoB* mutations in reconstruction experiments for their ability to provide improved growth within an aging colony.

Demonstrating the Selective Advantage of Rif^R Mutants in Reconstruction Experiments. Strains carrying each of the 21 different *rpoB* mutations (described above) were marked with a chromosomal insertion of the transposition-defective *Tn*10-Tet^R. Cells of wild-type 14028s were grown as a colony for 1 day, after which a mixture of Tet^R-marked Rif^R mutants and Cam^R-marked (wild-type) cells were spotted onto the colony (see *Materials and Methods*). These constructed colonies were incubated for a further 7 days, then assayed for the relative frequency of Tet^R (marking the Rif^R mutant being tested) and Cam^R (marking the control wild-type cells). After 7 days, the ratio of Tet^R to Cam^R cells was determined and compared to the ratio seen on Day 1. The factor by which the ratio changes over the course of the 7-day aging period is defined as the competitive index (CI), which expresses the magnitude of the growth advantage of Rif^R cells over parent cells. Results (Table 3) suggest that most of the Rif^R mutant types (12/21) have a significant growth advantage over the wild type (Fig. 4). A small intrinsic effect of the Tet^R versus Cam^R markers on competitive advantage was measured (1.8-fold over 7 days) and was taken into account to normalize the Rif^R/wild-type competition data (see *Materials and Methods*).

Four of the 21 mutant types showed a very large growth advantage, with a CI > 10. The magnitude of the growth advantage of a mutant type in the competition assay does not correlate well with the frequency of these *rpoB* mutations in Day 7 jackpot sectors. This supports the hypothesis that the particular Rif^R alleles found in Day 7 jackpots are also biased in favor of those that arose early and had the longest time to expand their clones during both the growth and aging periods rather than solely in favor of those with the largest growth advantage. Both of the two mutations (numbers 17 and 21) found in Day 7 jackpots, but not among mutants recovered on Day 1, have a large selective advantage (4- and 8-fold respectively). Conversely the 4 jackpot mutant types with the lowest

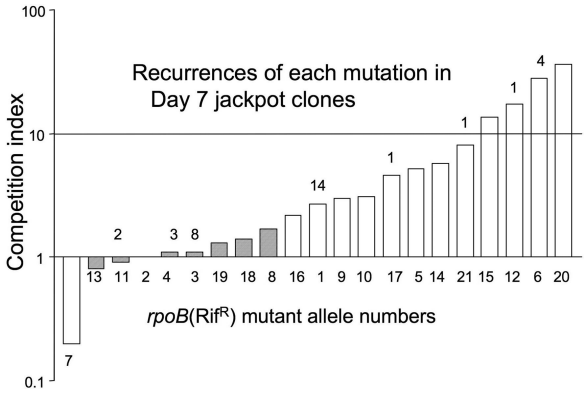

Fig. 4. The competitive advantage of 21 selected Rif^R mutants over the parent strain. Above each column is the number of independent occurrences in Day 7 jackpots. Below the columns are the *rpoB* mutation numbers (Table 3). Open columns indicate CI values statistically significant from wild type; hatched columns indicate values not statistically significant from wild type.

selective advantage (mutations 1, 3, 4, and 11) were frequent enough to appear (some repeatedly) among the Day 1 mutants.

Demonstrating that the Characterized *rpoB* (Rif^R) Mutation Is Sufficient to Provide a Growth Advantage During Aging. The data in Fig. 4 show that most Rif^R mutants recovered from aged colonies have a growth advantage in colony competition against the wild type. The growth advantage during aging is actually conferred by the same mutation (*rpoB*) that confers the Rif^R phenotype. This was shown by moving three Rif^R *rpoB* mutations (mutants 6, 15, and 20) into a wild-type strain (14028s) that had never experienced selection. Each of these three constructed Rif^R strains was then used in a competition experiment with wild-type cells. In each case, the constructed Rif^R mutant strain showed a clear growth advantage over the isogenic wild-type strain in the aging colony (Table 4). We conclude that each of these three *rpoB* mutations is sufficient to confer a growth advantage over wild-type cells in an aging colony.

Discussion

An increase in the frequency of Rif^R cells within aging colonies was initially attributed to stress-induced general mutagenesis (14) and has been cited as support for the idea that cells mutagenize their own genome when growth is prevented (10, 11). Evidence is presented here that the accumulation of Rif^R mutants is due to growth, not mutagenesis; Rif^R cells grow during the aging period, whereas the parent population does not. In this system, as in the Cairns *lac* system (9), accumulation of mutants in stressed cells can be explained by growth under selection and requires no increase in mutation rate.

Table 4. Mutations (*rpoB*) fully explain growth advantage

	Selected *rpoB* mutant			Constructed *rpoB* mutant		
Mutation	Median CI	(Dev)†	*N*‡	Median CI	(Dev.)†	*N*‡
Q513H	12.9	(5.9)	15	5.5	(10)	16
R529S	40.6	(45)	12	73.9	(100)	11
P564L	27.8	(93.2)	11	42.3	(18.4)	16

*P values (CI of mutant significantly different from CI of wild type by Mann–Whitney nonparametric test) were <0.0001 for all competitions.
†Dev. is the absolute average deviation from the median (see *Materials and Methods*). Note: the CI was positive for all competitions.
‡N is the number of independent colony competitions for each mutant.

The following lines of evidence support selection (and argue against mutagenesis):

1. The frequency of Rif^R mutants increased logarithmically during the aging period. A linear increase is expected if mutants arise by mutagenesis of a nongrowing population.
2. Mutant cells were found in localized sectors within the aging colony, consistent with growth of the mutant population from individual precursor cells. An even spatial distribution is predicted if mutants accumulate due to mutagenesis of a nongrowing population.
3. Mutant cells within a single sector of the colony carried the same sequence alteration, demonstrating their clonal relatedness.
4. In colonies with more mutant cells, mutants (95% of the total) were found in a smaller number of localized subclones, consistent with variation due to differences in clone growth rate rather than differences in the number of clones.
5. The Rif^R mutants that accumulate during aging showed no increase in the frequency of secondary mutations, suggesting that they were not made by induced general mutagenesis.
6. The growth advantage of Rif^R mutants during aging can be demonstrated in reconstruction experiments and is conferred on naïve cells that receive the particular *rpoB* (Rif^R) mutation.

Part of the evidence initially used to support stress-induced general mutagenesis was an increase in the number of cells resistant to other antibiotics during aging. However, the increase in Rif^R was much larger than that of the other resistances. The particular *E. coli* isolate (C4750) showed a 77-fold increase in Rif^R mutant frequency between Days 1 and 7, but no increase in the frequency of resistance to streptomycin or nalidixic acid. In the same experiment, mutants resistant to 5-fluorouracil, mecillinam, or fosfomycin increased only 3.4-, 1.9-, and 4.8-fold, respectively (12). If general mutagenesis were responsible, one might expect a similar magnitude of increase for all resistant mutants. We suggest that the small increases in other drug-resistant mutants seen previously (like the small increases in auxotroph frequency seen here) are likely to reflect the additional rounds of replication enjoyed by a subset of the population during the aging process.

Although it is not clear why many Rif^R mutations have a growth advantage during aging, it seems likely that some mutant forms of RNA polymerase may allow expression of genes that promote growth under the aging conditions. Many Rif^R mutations are known to affect transcription elongation or termination (19–25), including two of the *rpoB* mutations shown here to enhance growth during aging. Mutation 20 (R529S) increases the probability of transcription through Rho-dependent terminators (23). Mutation 6 (P564L) causes constitutive expression of the pyrimidine biosynthetic gene *pyrE* by preventing attenuation (26).

The importance of RpoS for the accumulation of Rif^R mutants was noted initially (12) but was attributed to a role of RpoS in regulating a proposed mechanism for "stress-induced" mutagenesis. We suggest instead that RpoS and the mutant RNA polymerase (RpoB) produced by Rif^R mutants act together to enhance expression of genes that facilitate growth in the aging colony. This fits with evidence that RpoS is up-regulated during growth limitation and serves to enhance expression of many genes during strong growth limitation (27).

The majority of the different Rif^R mutants (12/21) found in jackpot clones after selection showed a statistically significant growth advantage when retested. Some of the other jackpot clones may reflect frequent mutations that arise early and expand during the growth period, requiring little or no advantage over the wild type. Other clones may have Rif^R mutations that confer a growth advantage only when the *rpoB* gene is selectively amplified. The rpoB gene is located between *rrn* repeats and is subject to frequent

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amplifications, which would be subject to loss during unselected growth before retesting.

The phenomenon described here may represent a situation in which a cellular mechanism limits growth under particular conditions. The Rif^R mutants can be viewed as cheaters that ignore or override the growth controls (28) and continue growing within the quiescent parent colony. The behavior of these Rif^R mutants may parallel the loss of growth control in cancer cells that proliferate in a multicellular body, where most cells are quiescent.

Materials and Methods

Bacterial Strains and Growth Conditions. Strains of *S. enterica* serovar Typhimurium were derived from wild-type strains ATCC 14028s and LT2. *E. coli* strains are described in the text. Transduction crosses used phage P22 HT (high transducing). Bacteria were grown at 37°C in Luria-Bertani broth (LB) and on plates of LB medium solidified with 1.5% agar (Oxoid), 0.2% glucose, and 3 mM $CaCl_2$ (LA plates). Rifampicin was in media at 100 μg/ml, tetracycline at 15 μg/ml, and chloramphenicol at 50 μg/ml.

Determination of Rif^R Frequency in Aging Colonies. Colonies were initiated by spotting 2 μl (100–1,000 cfu) of a diluted fresh overnight culture onto a nitrocellulose filter (82-mm diameter, 0.2-μm pore size, Protran BA83; Whatman, Schleicher & Schuell) on an LA plate. A maximum of four colonies were grown per filter. Plates with filters were incubated in sealed plastic bags at 37°C for 1 or 7 days typically, but up to 28 days in some cases. After incubation, colonies were cut from the filter and suspended in 1 ml 0.9% NaCl. Appropriate dilutions were spread on LA and LA–rifampicin plates and incubated for 24 h.

In the earlier work by Bjedov *et al.*, it was claimed that mutagenesis occurred in a nongrowing population (12). To determine whether the rather constant cell number during aging reflected a balance between more substantial rates of cell growth and death, we assessed the number of dead cells in the colony. This number did not increase between Day 1 and Day 7 colonies based on microscopy using the LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes).

Colony Sectoring. An aged colony (typically 1.5 cm in diameter after 7 or 14 days) was cut into 16 pieces by using a sharp sterile scissors and fine tweezers, and cells on each piece were suspended in 500 μl of 0.9% NaCl solution. Less than 10% of the cells in a colony were lost during the cutting and processing of colonies. Dilutions were plated on LA and LA–rifampicin plates and incubated 24 h at 37°C to determine total cells and number of Rif^R mutant cells.

Colony Competition Assay. Rif^R mutants isolated from colonies were assayed for their growth competitiveness in reconstructed colonies. A filter colony was initiated as described above. After 24 h incubation, 4 μl of a competition mix was spotted onto the Day 1 colony. This mixture contained equal numbers of Rif^R competitor cells (marked with a tetracycline resistance marker, *zhe*-8953::Tn*10*dTet) and wild-type cells (marked with a chloramphenicol resistance marker, *zcd*-3677::Tn*10*dCam). To test the effect of these markers on fitness, we conducted 59 competition experiments obetween Tet-marked and Cam-marked wild-type strains. The *zhe*-8953::Tn*10*dTet marker confers a small advantage over 7 days of competition: median Tet^R/Cam^R = 1.8 (Mann–Whitney: $P = 0.0017$), which was used to normalize the reported competitive index for Tet^R-marked mutant versus Cam^R-marked wild-type strains.

Mixtures with either $\approx 10^2$ or $\approx 10^3$ cfu of each competitor were tested in parallel (Day 0). After 7 days of additional incubation (Day 7) the colony was suspended in 1 ml of 0.9% NaCl and appropriate dilutions plated on LA, LA–tetracycline, and LA–chloramphenicol plates. The CI was calculated for each Rif^R mutant from the change in the RifR/wild-type ratio from Day 0 to Day 7.

Statistical Analysis. Median and average absolute deviations from the median (AADM) were calculated using a descriptive statistics package available online (http://www.physics.csbsju.edu/stats/descriptive2.html). Note that an AADM larger than the median is caused by the non-normal distribution of the CI values (the presence of outliers, colonies in which the positive CI was very large) and does not imply negative CI values in any competitions. The significance of the differences in CI between mutants and wild-type (*P* values, two-tailed 95% confidence value) were calculated using the Mann–Whitney nonparametric test available on the VassarStats Web Site for Statistical Computation (http://faculty.vassar.edu/lowry/VassarStats.html).

PCR Amplification and DNA Sequencing. To prepare DNA, we suspended a single bacterial colony in 100 μl sterile water and boiled it for 5 min. PCR was performed with Ready-2-Go PCR beads (Amersham Bioscience) in a 25-μl volume containing 0.4 μM forward and reverse primers and 1 μl DNA sample. PCR conditions were as follows: denaturation at 95°C for 5 min, then 30 cycles of 95°C (for 15 s), 55°C (20 s), and 72°C (2 min). Primers for amplifying *rpoB* were 1421F: 5′-CGGTGAAAGAGCGTCTGTCT and 2170R: 5′-CAGTACCGCCACGTTTAGCT. Each sequencing reaction (Uppsala Genome Center, Rudbeck Laboratory, Uppsala, Sweden) contained $\approx$10 ng purified PCR product and 1.6 pmol sequencing primer 1453F: 5′-GATACCCTGATGCCTCAG.

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