Effect of translesion DNA polymerases, endonucleases and RpoS on mutation rates in Salmonella typhimurium

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Abstract

It has been suggested that bacteria have evolved mechanisms to increase their mutation rate in response to various stresses and that the translesion DNA polymerase Pol IV under control of the LexA regulon and the alternative sigma factor RpoS are involved in regulating this mutagenesis. Here we examined in *Salmonella enterica* serovar *Typhimurium* LT2 the rates for four different types of mutations (rifampicin-, nalidixic acid- and chlorate-resistance and Lac+ reversion) during various growth conditions and with different levels of four translesion DNA polymerases (Pol II, Pol IV, Pol V and SamAB) and RpoS. Constitutive de-repression of the LexA regulon by a *lexA*(def) mutation had no effect on Lac+ reversion rates but increased the other three mutation rates up to 11-fold, and the contribution of the translesion DNA polymerases to this mutagenesis varied with the type of mutation examined. The increase in mutation rates in the *lexA*(def) mutant required the presence of the LexA-controlled UvrB protein and endonucleases UvrC and Cho. With regard to the potential involvement of RpoS in mutagenesis, neither an increase in RpoS levels conferred by artificial over-expression from a plasmid nor long-term stationary phase incubation or slow growth caused an increase in any of the four mutation rates measured, alone or in combination with over-expression of the translesion DNA polymerases. In conclusion, mutation rates are remarkably robust and no combination of growth conditions, induction of translesion DNA polymerases by inactivation of LexA or increased RpoS expression could confer an increase in mutation rates higher than the moderate increase caused by de-repression of the LexA regulon alone.
Introduction

The neo-Darwinian view of evolution postulates that mutations occur randomly and at a rate that is essentially independent of selection. The fact that at least some spontaneous mutations arise before and independently of selection was demonstrated early by the classic experiments of Luria and Delbruck (30) and Lederberg (27). However, as pointed out by several researchers (7, 16, 17, 45), the lethal selections they used could not have detected potential mutations induced by selective stress conditions and subsequently several genetic systems have been described in which stress seems to increase the yield of mutations (2, 3, 6, 47). The Cairns system has been analyzed in most detail and in this system Lac^- mutant cells (Lac^- due to a +1 frameshift mutation in the lacI part of the fused lacIZ gene) incubated on lactose-containing medium accumulated mutations allowing them to grow on lactose (6, 7). From the observations of Cairns and Foster several models have been proposed, including the gene amplification model (1, 18, 19, 44) and stress-induced mutagenesis (17, 42). One specific model proposes that stress induces a subpopulation of cells (approximately 1/1000) (49) into a hypermutable state, where the genome-wide mutation frequency is increased until a beneficial mutation occurs (i.e. a Lac^+ mutation) and relieves the stress (reviewed in (15)). According to this model mutagenesis is proposed to occur in non-growing cells during local DNA replication initiated by recombination events (reviewed in (13)) and mutagenesis has to be increased about 10^5-fold to account for the number of observed Lac^+ revertants (43). It is notable that such intense general mutagenesis has to our knowledge never been demonstrated under any experimental condition and if realized genome-wide, this mutagenesis would produce approximately 5 lethal mutations per cell under the
course of a Lac\textsuperscript{+} selection experiment (43). Induction of this intense mutagenesis has been suggested to require two factors: induction of the error-prone DNA polymerase IV under control of the DNA damage-inducible LexA regulon (SOS response) and the simultaneous increase in levels of the starvation-induced sigma-factor RpoS (reviewed in (15)).

Bacterial cells frequently encounter various stresses, for example, when cells are exposed to DNA damage, the SOS-response is activated. Damaged DNA is rapidly processed and upon RecA filamentation on ssDNA, filamented RecA mediates proteolytic cleavage of the LexA repressor protein of the SOS-response (34). Induction of the LexA regulon induces expression of \~40 genes, among these the translesion DNA polymerases. (11, 40). These enzymes are responsible for bypassing several different types of DNA lesions in a mutagenic translesion process (35). In \textit{S. typhimurium} four translesion DNA polymerases are known: Pol II (\textit{polB}), Pol IV (\textit{dinB}), Pol V (\textit{umuDC}) and the Pol V homologue SamAB (\textit{samAB}). Under non-induced conditions these polymerases are present at low levels, and during the SOS-response a 10- to 20-fold increase in levels is observed depending on the particular polymerase (10). Because of their mutagenic effect, these polymerases have been suggested to function as inducible global mutators (24, 39).

Other conditions such as nutrient starvation, oxidative stress or low pH, will induce another cellular stress response that is regulated by the alternative transcription sigma factor RpoS. RpoS induction increases expression of more than 300 genes to varying extent (38, 52). Among these RpoS-induced genes the \textit{dinB} gene encoding the translesion DNA polymerase Pol IV can be found (25). Most studies on stress-induced mutagenesis requiring Pol IV and RpoS induction have focused on the Lac\textsuperscript{+} reversion assay (6, 14, 28, 39) but if, as claimed, these pathways of mutagenesis are
general and not particular idiosyncrasies of the lac system, it is expected that it should be possible to generate a pronounced and continuous mutagenic state by simultaneously inducing these functions by genetic manipulation. Here we studied in *S. typhimurium* the impact of the translesion DNA polymerases and RpoS on four different types of mutations: resistance to the antibiotics nalidixic acid and rifampicin caused by amino acid changes, resistance to chlorate caused by any type of inactivating mutation in genes involved in the synthesis or uptake of molybdate or nitrate reductase and the classic Cairns and Foster reversion to Lac⁺ caused by a reversion of a +1 frameshift mutation. Our results show that constitutive de-repression of the LexA-regulon conferred by a *lexA* (def) mutation causes a moderate increase in mutation rate which for two out of three mutant types was independent of the presence of functional translesion DNA polymerases but for all three mutation types completely dependent on UvrB under LexA control and the endonucleases UvrC and Cho. No further increase in mutation rate was seen during slow growth, long-term stationary phase or when over-expressing RpoS from an arabinose inducible plasmid, in the presence or absence of translesion DNA polymerases or a de-repressed LexA-regulon.
Results

De-repression of the LexA regulon increases mutation rates in a partly translesion DNA polymerase dependent manner for certain types of mutations

Various stresses have been proposed to increase chromosomal mutation rates in bacteria in a translesion DNA polymerase-dependent manner (reviewed in (15)). In this study we investigated how constitutive expression of the DNA-damage inducible LexA-regulon would affect the mutation rate for four different types of mutations. These experiments were performed in a S. typhimurium strain lacking the prophages Gifsy-1, Gifsy-2 and Fels-2 to prevent cellular killing caused by LexA inactivation and resulting phage induction (5). Depending on the mutation types examined, constitutive de-repression of the LexA-regulon (due to a lexA(def) mutation) caused 3.5-fold (chlorate resistance), 8-fold (rifampicin resistance) and 11-fold (nalidixic acid resistance) increases in mutation rates whereas Lac⁰ reversion rates remained unaltered (Fig. 1). Since it has been suggested that the error-prone translesion DNA polymerases II (polB), IV (dinB) and V (umuDC) contribute to this mutagenesis in other systems (14, 24, 36) we determined if in a lexA(def) mutant, inactivation of all four translesion DNA polymerases (including the Pol V homologue SamAB found on the virulence plasmid pSLT of S. typhimurium) caused a reduction in mutation rates. Depending on the mutation type examined, mutation rates remained increased or were reduced to wild type levels when inactivating the translesion DNA polymerases. Thus, for rifampicin resistance mutations the rate increased 8-fold in a lexA(def) mutant compared to the wild type but no decrease in mutation rate could be seen in a lexA(def) mutant lacking all four translesion DNA polymerases (Fig. 1). Likewise, for nalidixic acid resistance mutations, the rate increased 11-fold in the lexA(def) mutant
and in the \textit{lexA}(def) mutant lacking the translesion DNA polymerases no decrease in mutation rate was observed (Fig. 1). For \textit{Lac}^+ reversion no significant increase/decrease in mutation rate was seen in the \textit{lexA}(def) mutant with or without the translesion DNA polymerases present (Fig. 1), making it difficult to evaluate the role of the translesion DNA polymerases for \textit{Lac}^+ reversion in this system. However, chlorate resistance mutations increased 3.5-fold in a \textit{lexA}(def) mutant, an increase that was dependent on the translesion DNA polymerases (Fig. 1). These results show that constitutive de-repression of the LexA- regulon causes a moderate increase in mutation rates for three of the four different types of mutations examined, but that the translesion DNA polymerases contribute to this increase for only one out of these three mutational types. To examine if lack of all four translesion DNA polymerases effects expression of other LexA-controlled functions, we used real-time PCR to measure \textit{uvrB} expression in a \textit{lexA}(def) mutant and a \textit{lexA}(def) mutant lacking all four translesion DNA polymerases. The levels of \textit{uvrB} mRNA were increased 3.5- to 6-fold in both strains as compared to a \textit{lexA}^+ strain (Fig. S1a), implying that LexA-regulated genes were expressed as normal in the multiple polymerase mutant.

\textbf{All four translesion DNA polymerases are required for mutagenesis causing chlorate resistance.} As removal of all four translesion DNA polymerases decreased the mutation rate to wild type levels for chlorate resistance mutations, we determined how the individual translesion DNA polymerases contributed to the increased mutation rate in the \textit{lexA}(def) mutant by inactivating the four polymerases alone and in combinations in the de-repressed mutant. Single inactivation of any one of the translesion DNA polymerases decreased mutation rates from a 3.5-fold increase in a \textit{lexA}(def) mutant to a 1- to 2-fold increase, not significantly different from the wild
type level (Fig. 2). In mutants with combinations of two, three or all four polymerases inactivated, mutation rates were also decreased to wild type levels (Fig. 2).

Inactivation of all four translesion DNA polymerases in wild type background did not decrease mutation rate any further (data not shown). These results indicate that chlorate mutagenesis requires the activity of all four translesion DNA polymerases.

The increased mutation rate observed during de-repression of the LexA-regulon is completely dependent on the presence of functional endonucleases (Cho and UvrC) and the UvrB protein. Previously we showed that constitutive de-repression of the LexA-regulon increases the number of DNA breaks in the cell (23). This increase in DNA breaks in the lexA(def) strain is mediated by two endonucleases, UvrC and Cho, and the LexA-controlled UvrB protein (23). UvrC has previously been shown to attack undamaged DNA when present at high concentrations in the absence of DNA damage (4). As increased DNA breaks are associated with increased mutation rates (29, 39), we determined if these endonucleases were required for the increased mutation rate seen during de-repression of the LexA-regulon. Inactivation of the uvrC gene alone in a lexA(def) background did not decrease mutation rates for any type of mutation examined whereas inactivation of the uvrC homologue cho in the same background, decreased mutation rates approximately 3-fold for rifampicin and nalidixic acid resistance mutations (Fig. 3). For chlorate resistance mutations no decrease in mutation rates could be observed. Inactivation of the uvrB gene alone decreased mutation rate almost down to wild type levels for all three types of mutations examined (Fig. 3). Finally, simultaneous inactivation of the uvrB, uvrC and cho genes in a lexA(def) genetic background decreased mutation rates to wild type levels or below for all types of mutations (Fig. 3). This indicates that the increased
mutagenesis associated with de-repression of the SOS-regulon (this work and (12, 31)) is completely dependent on these endonucleases, especially UvrB, and by inference the increase in DNA breaks. To control that other genes (significant for mutagenesis) are expressed normally in the triple endonucelase (uvrB, uvrC, cho) mutant strain, expression of dinB was examined using real-time PCR in both the wild type and lexA(def) backgrounds with or without functional endonucleases. In both the endonuclease defective and proficient strains the expression of dinB increased approximately 15-fold in the lexA(def) background as compared to wild type (Fig. S1b).

**Artificial overproduction of RpoS from an arabinose-inducible plasmid does not increase mutation rates.** The global stress-response regulator RpoS has been suggested to play a role as an activator in the putative stress-induced mutagenesis in the lac system (28). To study the role of RpoS in mutagenesis, we used an arabinose-inducible plasmid with the rpoS gene from Salmonella typhimurium ATCC14028, cloned under an arabinose-inducible promoter. In neither the wild type nor lexA(def) mutant at any concentration of L-arabinose (0.01% to 0.2%) did over-expression of RpoS have any increasing effect on the mutation rates examined (Fig. 4a), except for a small (2.5-fold) increase in the rate of Lac+ reversion at the highest concentration of L-arabinose. Thus, in the wild type mutation rates were largely unaffected by RpoS over-production whereas in the lexA(def) mutant over-production caused a moderate reduction in mutation rates. Western blotting with RpoS antibodies confirmed that RpoS expression increased approximately 5- to 40-fold with increasing concentration of L-arabinose (Fig. 4a). When RpoS was over-expressed from a plasmid (0.1% L-arabinose), real-time PCR experiments showed a corresponding 4- to 10-fold increase
in expression of the RpoS regulated genes *katE* and *otsA* (Fig. 4b), indicating that the over-expressed RpoS protein is functional as a positive regulator. From these results we conclude that increased RpoS expression either alone or in combination with constitutive de-repression of the LexA-regulon is insufficient to cause an increase in mutation rate for any of the four types of mutation examined.

**Slow growth does not increase mutation rates.** During growth on poor carbon sources, RpoS levels and expression of genes under RpoS control are up-regulated, among them Pol IV (20, 25). To test how slow growth affects mutation rates, we compared mutation rates for three different media, LB and minimal M9 media supplemented with 0.2% glucose or 0.2% glycerol (33). As shown previously for the wild type (39), no increase in mutation rate was seen during growth in poorer media for any type of mutations (Fig. 5a). Likewise, for neither the *lexA*(def) mutant nor the *lexA*(def) mutant lacking the four translesion DNA polymerases did growth in poor media increase mutation rates (Fig. 5a). Also, removal of functional RpoS, alone or in combination with de-repression of the LexA operon (*lexA*(def)), had no consistent effect on mutation rates (Fig. 5a), except for a small decrease in mutation rate observed in M9, glycerol for nalidixic acid and rifampicin resistance mutations. These results indicate that slow growth *per se* does not increase mutation rate for any type of mutations. Finally, to examine how expression of the *dinB* gene (encoding PolIV) was regulated in response to altered growth media quality, we showed that expression of a plasmid-borne *dinB::lacZ* protein fusion (β-galactosidase activity) increased with poorer media (Fig. 5b). Interestingly, this increase in *dinB* expression was seen irrespective of whether the *rpoS* gene was functional or not. Furthermore, in *lexA*(def) background the increased expression of *dinB* was strongly dependent on functional
RpoS. These results are different from those observed in *E. coli* (25), where inactivation of RpoS reduced Pol IV protein levels 3- to 5-fold in the wild type background, indicating that regulation of *dinB* expression is different in these two species.

**Long-term stationary phase incubation of cells does not increase mutation rates.**

To test if stationary phase incubation increased mutation rates for any of the four types of mutations, we incubated overnight cultures (plated on selective media at day 1 to determine mutation rates) for an additional 5 days with shaking in the same liquid culture before plating again on selective media at day 6. For wild-type bacteria, the mutation rate remained unchanged in minimal glucose medium when comparing day 1 and 6 (Fig. 6a), except for a 2.5-fold increase seen for nalidixic acid resistance mutations. These results were not affected by whether cells were grown in LB (Fig. S2a-b) or minimal medium supplemented with 0.2% glycerol (Fig. S3a-b), except for a small, 2- to 5-fold, increase in mutation rate seen in minimal glycerol medium for rifampicin and nalidixic acid resistance mutations and a 2-fold increase seen for Lac⁺ reversion in LB. Similarly, for both the *lexA* (def) mutant and the *lexA* (def) mutant lacking all four translesion DNA polymerases the mutation rate remained unchanged in minimal glucose medium when comparing day 1 and 6 (Fig. 6a), with the exception for a small decrease in Lac⁺ reversion rate at day 6 in the mutant lacking all four translesion DNA polymerases. Furthermore, cells lacking *rpoS* alone or in combination with a non-inducible LexA regulon (*lexA*<sup>IND</sup>), did not show a consistently altered mutation rate in minimal glucose medium when comparing day 1 and 6 (Fig. 6b). From these results we conclude that several days of incubation in
stationary phase in combination with either increased expression of the LexA regulon and/or loss of RpoS cause no systematic change in the examined mutation rates.
Discussion

Here we show that de-repression of the LexA regulon either had no effect or increased mutation rate up to 11-fold, depending on the mutation type investigated. Unexpectedly, for the two of the three types of mutations where de-repression of the LexA regulon caused an increased mutation rate, this increase was independent of the presence of functional translesion DNA polymerases. Earlier studies have demonstrated the importance of the translesion DNA polymerases and especially Pol IV, in certain types of mutagenesis (14, 24, 36). For example, for lac adaptive mutations it has been reported that 85% of the adaptive Lac+ reversion mutations require Pol IV for their formation (reviewed in (15)), i.e. inactivation of Pol IV causes a 6-fold reduction in mutant yield. Additional studies highlight the dependence of Pol IV (24) and Pol V (51) in LexA regulated SOS-mutagenesis. In contrast, other studies have shown that cells lacking all three translesion DNA polymerases show no decrease in mutation rates in wild type background for ciprofloxacin resistance mutations (bp substitutions), tonB mutations (bp substitutions, frameshifts or deletions) or Arg+ reversion mutations (bp substitutions) (8, 36, 48). Thus, (i) the contribution of the translesion DNA polymerases to mutagenesis is rather weak and (ii) appears to depend on the particular mutation type and experimental condition.

To study how the individual translesion DNA polymerases contributed to the increase in chlorate resistance mutations, we inactivated the four translesion DNA polymerases alone and in combinations in a lexA(def) mutant background. Interestingly, inactivation of any one translesion DNA polymerase was sufficient to reduce the increased mutation rate close to the wild type levels and no further decrease was seen when inactivating multiple polymerases. One possible explanation
for this is that the polymerases act together as a complex. However, this idea has to our knowledge at present no experimental support.

The translesion DNA polymerases contributed to chlorate resistance mutations whereas no effect of removal of all four translesion DNA polymerases in a \textit{lexA}(def) background could be seen for rifampicin and nalidixic acid resistance mutations (Fig. 1). A possible explanation for this is provided by the mutational spectra of the different polymerases. Chlorate resistance is caused by any mutation that inactivates genes involved in the synthesis or uptake of molybdate or nitrate reductase (base pair substitutions, frameshifts, insertions, deletions etc.). The polymerases Pol II, Pol IV and Pol V generate frameshift mutations and small deletions at a high rate (41, 50, 51), suggesting that all three polymerases are proficient in creating mutations that will cause chlorate resistance (it is presently unknown what the mutational spectrum of SamAB is). In contrast to chlorate resistance mutations nalidixic acid resistance and rifampicin resistance mutations are conferred by a limited number of specific base pair substitutions in the \textit{gyrA} and \textit{rpoB} gene respectively. Thus, we suggest that the translesion DNA polymerases, when expressed at sufficiently high levels contribute to chlorate resistance mutations by generating frameshifts or small deletions. In contrast, for other types of mutations, such as base pair substitutions, the translesion DNA polymerases do not contribute significantly to mutagenesis, at least not at the polymerase levels conferred when the LexA regulon is de-repressed by a \textit{lexA}(def) mutation.

The increased mutation rate seen in the \textit{lexA}(def) background for three mutation types was fully dependent on functional UvrC and Cho endonucleases and on UvrB (Fig. 3). There is some precedence for this finding as lack of UvrB has been shown to decrease mutagenesis in aging colonies (47). Our results also indicated that
UvrB, UvrC and Cho do not contribute to mutagenesis equally, as removal of UvrB alone in a *lexA*(def) background decreased mutation rates almost down to wild type levels for rifampicin, nalidixic acid and chlorate resistances whereas removal of UvrC had no effect on these mutation rates. A possible explanation for this is given by the nature of these proteins. UvrC and Cho both possess endonuclease activity that can nick the DNA. In contrast UvrB functions as a matchmaker, recruiting UvrC and possibly Cho to the DNA. We suggest that removal of UvrB abolishes the contact between UvrC and DNA, hence decreasing mutation rates almost to the same levels as inactivation of both genes simultaneously. Inactivation of Cho alone in a *lexA*(def) background decreased mutation rates approximately 2- to 3-fold for rifampicin and nalidixic acid resistance mutations, indicating that Cho contributes to more than half of the increase in mutation rates seen in a *lexA*(def) mutant. As removal of UvrC did not decrease mutation rates for any type of mutation, we suggest that Cho in the absence of UvrC contributes more to mutation formation, buffering for the absence of UvrC, whereas the opposite (UvrC buffering for the loss of Cho) is not occurring.

Taken together, these results indicate that even though a moderate 3.5- to 11-fold increase in mutation rates can be seen for three mutation types when the LexA regulon is constitutively de-repressed, this increase appears, for at least two types of mutation, to be mainly a consequence of the constitutive over-expression of endonucleases rather than translesion synthesis-induced mutagenesis. To rationalize these results we suggest that DNA breaks (caused by UvrB, UvrC and Cho overproduction in the *lexA*(def) mutant) when repaired by either the translesion DNA polymerases or Pol I and III, can result in increased mutagenesis. Even though both Pol I and III are not commonly considered error-prone, some data supports the involvement of these enzymes in mutagenesis. Firstly, inactivation of all three
translesion DNA polymerases in wild type background has in several systems been shown to not cause any reduction in mutation rates (bp substitutions, frameshifts, deletions) (8, 36, 48). Secondly, Pol I and Pol III have been associated with increased mutation rates conferred by DNA breaks (47, 48). Lastly, Pol III has been shown to be involved in the mutagenic bypass DNA damage induced by class 1, 2 and 3 chemicals (22). One possible explanation for these findings could be that replication restart at DNA breaks is a mutagenic process irrespective of which type of polymerase restarts replication.

The global sigma factor RpoS has been proposed to function as one of the main regulators of stress-induced mutagenesis (28). Expression of RpoS in cells with dsDNA breaks has been shown to increase mutation rates in a Pol IV dependent manner (39). In earlier studies, removal of rpoS has been shown to decrease starvation-induced Lac⁺ mutagenesis on lactose-containing medium (39) and conversely over-expression of RpoS has been shown to increase Lac⁺ reversion rates. However, this increase was only seen in combination with over-expression of a restriction enzyme resulting in a large increase in DNA breaks (28). In this study, we over-expressed RpoS (from S. typhimurium ATCC14028) 5- to 40-fold from an arabinose-inducible plasmid (Fig. 4), without observing an increase in mutation rate for any type of mutations, except for a 2.5-fold increase in Lac⁺ reversion at the highest RpoS level. Furthermore, over-expression of RpoS in a lexA(def) mutant conferred no further increase in mutation rate as compared to a lexA(def) mutant with normal RpoS levels. Thus, even though DNA breaks and translesion DNA polymerase levels are increased in the lexA(def) mutant, RpoS has no inducing effect on any of the mutation rates examined. If as suggested, RpoS and the translesion DNA polymerase Pol IV (dinB) in particular (14) act together to increase mutation
rates (15), it is unexpected that no increase in mutation rate can be detected for any type of mutation when inducing RpoS expression in a lexA(def) mutant.

In the classical lac-system of Cairns and Foster (6) lac mutant cells unable to grow on lactose, are starved on lactose-containing plates. As colonies keep arising several days after plating, the starvation has been assumed to induce mutagenesis (6, 32, 42). Here, wild type and lexA(def) mutant cells were grown in poor growth media or incubated in stationary phase before being plated on selective media. With this experimental set-up, we tested the hypothesis if whether any particular combination of slow growth or stationary phase combined with de-repression of the LexA regulon (causing an increase in translesion DNA polymerases and DNA breaks) and RpoS induction could induce pronounced mutagenesis. However, no such induction of mutagenesis could be seen for any one the four different mutation rates measured (except for the moderate increase caused by LexA inactivation alone). In conclusion, our findings are not reconcilable with the stress-induced model generated from studies of Lac⁺ reversion under selection (reviewed in (15)) or aging colonies (47), implying that other explanations have to be sought for the behaviour of these systems. As has been shown by our previous work and other studies, both of them can be explained by growth dependent mutation in a subpopulation of cells able to grow in that specific growth-limiting environment rather than induction of a stress-induced mutagenic mechanism (1, 53). In the lac-system the increased amount of mutations found during prolonged stationary phase can be explained by amplification of lac. Rare cells carrying a pre-existing duplication of the leaky lac gene, can initiate clones within which further amplification of the lac allele increases the growth rate of the cells (reviewed in (44)). In aging colonies rifampicin-resistant mutants have been shown to accumulate in non-growing colonies during 7-days incubation. However, recent
evidence shows that the rifampicin-resistant cells in fact grew faster than the parental strain during the aging process, resulting in an accumulation of rifampicin-resistant cells in the 7-day old colonies (53). In conclusion, for both the lac system and the aging colonies system, selection of pre-existing mutants (duplications and rpoB mutants, respectively) can better explain the results than alterations in mutation rates due to stress-induced mutagenesis. Also, we have been unable to induce a hypermutable state, indicating that if this mutagenic state exists it is not sufficient to increase Pol IV and RpoS levels alone as has been proposed (39).
Materials and methods

Bacterial strains, genetic methods and growth conditions. The bacterial strains used in this study were derived from Salmonella enterica serovar typhimurium LT2 (designated S. typhimurium throughout this paper) and are listed in Table S1. All gene transfers were made by phage P22 transduction (9). The presence of the relevant alleles was confirmed by PCR after construction of each strain. Plasmids were transferred by electroporation, except for F’128, which was transferred by conjugation. Bacteria were grown in standard Luria Bertani-broth (LB) or M9 minimal media supplemented with 0.2% glucose or glycerol (33). When grown overnight, bacteria were incubated at 37°C and liquid cultures were shaken at 200 rpm. Antibiotic concentrations used were as follows: ampicillin (Amp) 100mg/L, kanamycin (Kan) 50mg/L, chloramphenicol (Cam) 20mg/L and rifampicin (Rif) 100mg/L and nalidixic acid (Nal) 50mg/L, in both agar plates and culture media. Nalidixic acid and rifampicin plates were LA based. Chlorate resistance was studied on MacConkey agar plates supplemented with 0.2% Sodium Chlorate and 0.2% Galactose. Lactose utilization was studied on M9 minimal media plates supplemented with 0.2% Lactose.

Mutation rate assay. We studied mutation rates for four different types of mutations. Rif and Nal resistance are formed through a few point mutations in rpoB or gyrA respectively on the S. typhimurium chromosome (21, 54). Cells become chlorate resistant through the acquisition of mutations that inactivate any of the genes involved in the synthesis or uptake of molybdate or nitrate reductase (37). Finally, a well studied genetic construct allows cells to become Lac+ after reversion of a +1
frameshift mutation at the promoter-proximal of the fused lacI\textsuperscript{Z} gene (6). To
determine mutation rates, cells were grown overnight from 10\textsuperscript{5} cfu (for chlorate
resistance and lactose mutations) or 10\textsuperscript{6} cfu (nalidixic acid and rifampicin resistance)
cfu in LB or M9 minimal media supplemented with 0.2\% glucose or glycerol. To
over-express RpoS from pBAD30::rpoS, cells were induced with L-arabinose (sigma)
0.01-0.2 \% final concentration in LB broth. 20 independent cultures from each strain
were plated on Nal, Rif, chlorate or lactose containing plates respectively and viable
counts were made from 5 independent cultures for each strain. Plates were incubated
at 37°C for 24h for Rif and Nal containing plates, 24h anaerobically and 6h
aerobically for chlorate plates and 48h for M9 minimal media plates containing
lactose. Mutation rates were calculated by the method of the median or by the P\textsubscript{0}−
method (26) and are given relative to the mean wild-type mutation rate for each type
of mutation in each medium (Table 1), calculated from at least 2 rounds of plating.
The P\textsubscript{0} method was only used when three or more cultures lacked mutants and in all
other cases the method of the median was used. Rates calculated by the two different
methods were compared with rates calculated with the same method and were not
significantly different. Also, an example of relative mutation rates are shown in
supplementary Table S2, to indicate the extent of variability and what data were used
to calculate the SEMs.

**Western blot.** Cells were grown and induced with L-arabinose as for the mutation
assay with pBAD30::rpoS. 1ml of over night culture was spun down for 2 min at
13000 rpm at 4°C and the pelleted cells were frozen at -80°C until the blot was run.
Samples were thawed on ice and resuspended in 250\textmu l lysis buffer (0.02 M HEPES,
0.3 M KCl, 0.05\% Triton X-100) supplemented with 1\times Complete protease inhibitor
Cells were lysed by sonication for 5 s, repeated three times. The lysis mixture was spun for 1 min at 13000 rpm at 4°C to remove cell debris and the supernatant was transferred to a fresh tube. Total protein concentration was calculated with the BCA protein assay kit as described by the manufacturer (Thermo Scientific). Equal amounts of total protein were loaded onto a 12% SDS acrylamide gel and run for 1 h at 100 V and then for 2 h at 150 V. Proteins were blotted onto a PVDF membrane (BioRad) using a semi-dry transfer system (BioRad), for 35 min at 15 V. The PVDF membrane was blocked in 1× TBS supplemented 0.1% Tween and 5% BSA (In Vitro) at 4°C overnight. After washing twice with 1× TBS supplemented with 0.1% Tween, primary antibody (commercial mouse anti-RpoS, Neoclonne), was diluted 1:1000 in 1× TBS supplemented with 0.1% Tween and 5% BSA and incubated with the membrane for 1.5 h at RT. The secondary antibody (goat IgG-HRP, Amersham ECL Plus™ Western Blotting Reagent Pack from GE Healthcare) was diluted 1:25000 in 1× TBS supplemented with 0.1% Tween and 5% BSA and incubated with the membrane for 1 h at RT. Immunoactivity was detected using Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and photographic film (Amersham Hyperfilm ECL, GE Healthcare).

β-galactosidase assay. Pol IV-expression was measured as β-galactosidase activity using a fusion of the dinB promoter to lacZ on the pRS552 plasmid (46). Strains were grown overnight in LB or M9 media supplemented with 0.2% glucose or glycerol as described for the mutation assay. Cells from 1 ml of overnight culture were used to measure β-galactosidase activity as described by Miller (33) but adapted for readings in a BioscreenC reader (Labsystems). In Fig. 5b all values are represented as relative values of the β-galactosidase activity of the mutant as compared to the wild type.
**Real-time PCR.** Cells were grown overnight and diluted 1:500 in 5ml fresh LB or M9-media supplemented with 0.2% glucose and grown to OD$_{600} = 0.5$. For cells grown in M9 glucose, 1ml of the culture was removed at OD$_{600} = 0.5$ and RNA was prepared with the SV Total RNA Isolation System (Promega) according to the manufacturer. For arabinose induction, cells were grown in LB and induced by adding 0.1% L-arabinose (for controls no arabinose was added) and subsequent incubation for 1h at 37°C after which RNA was prepared as described above. RNA concentrations were measured with a Nanodrop 1000 (Thermo scientific) and 0.1- to 0.3 µg RNA was used for cDNA synthesis. mRNA was converted to cDNA using the cDNA reverse transcription kit from Applied Biosystems according to the manufacturer. Quantitative real-time PCR technique based on the high affinity of SYBR Green dye for double-stranded DNA was used to measure relative mRNA levels according to the manufacturer (Bio-Rad). The fluorescence signal was monitored on-line, using the MiniOpticon real-time PCR system (Bio-Rad). The mRNA levels were calculated relative to *dnaE* mRNA in each individual RNA-sample, and normalized to expression of the same gene in wild type cells (DA10212). Primers for the real-time PCR are found in Table S3.

**Construction of plasmid.** The gene encoding RpoS was amplified from *Salmonella enterica* serovar typhimurium, ATCC14028 chromosomal DNA with rpoS_F and rpoS_R primers (Table S3) and cloned into the pBAD30 vector with SacI and SmaI. Primers were designed to contain restriction sites for SacI and SmaI restriction enzymes and the gene was amplified with its own ribosome binding sites and start codon. The gene was inserted in the correct orientation for expression from the
arabinose inducible araBAD promoter. PCR products were amplified with GeneAmp 9700 (Applied Biosystems) and run on a gel to verify the correct size. PCR reactions containing bands corresponding to the size of the gene were purified using GFX illustra PCR DNA and Gel purification kit (Amersham). Purified PCR products and purified pBAD30 plasmid were restricted with FastDigest SacI and SmaI enzymes (Fermentas) for 2h at 37°C and heat inactivated for 20min at 65°C. Ligation of the fragments was performed at 16°C overnight. The ligation mixture was transformed into New England Biolabs (NEB) high efficiency chemically competent cells as described by the manufacturer (In Vitro). Transformants were selected on LA-plates containing 100mg/L ampicillin at 37°C overnight. Random colonies were selected and purified on LA-plates containing 100mg/L ampicillin and screened for inserts by colony PCR with pBAD30 primers (Table S3). Plasmids with the correct size of insertion were prepared with E.Z.N.A plasmid purification kit (Omega-biotek) and inserts were verified by sequencing with pBAD30 primers.

**Acknowledgements**

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References


17. Hall, B. G. 1990. Spontaneous point mutations that occur more often when advantageous than when neutral. Genetics 126:5-16.


Table 1. Mutation rates in different growth media for the wild-type strain DA10212.

Rif= rifampicin, Nal= nalidixic acid, and Chl= chlorate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Mutation type</th>
<th>Mutation rate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(mutation /cell /generation +/- SEM)</td>
<td></td>
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<tr>
<td>DA10212</td>
<td>LB</td>
<td>Rif resistance</td>
<td>1.7 x 10^{-8} ± 0.2</td>
</tr>
<tr>
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<td>LB</td>
<td>Nal resistance</td>
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</tr>
<tr>
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<td>LB</td>
<td>Chl resistance</td>
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<td>minimal M9 glucose</td>
<td>Rif resistance</td>
<td>3.5 x 10^{-9} ± 0.5</td>
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<tr>
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<td>DA10212</td>
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<tr>
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Figure legends

**Fig 1.** Relative mutation rates for four different types of mutations, rifampicin resistance, nalidixic acid resistance, chlorate resistance and Lac\(^+\) reversion. Rates are given relative to the mean mutation rate of the wild-type for each type of mutation in minimal M9 glucose medium (Table 1). Error-bars are SEM.

**Fig 2.** Relative mutation rates for chlorate resistance mutations. Rates are given relative to the mean mutation rate of the wild-type in minimal M9 glucose medium (Table 1). Error-bars are SEM.

**Fig 3.** Relative mutation rates for three types of mutations, rifampicin resistance, nalidixic acid resistance and chlorate resistance. Rates are given relative to the mean mutation rate of the wild-type in minimal M9 glucose medium for each type of mutation (Table 1). Error-bars are SEM.

**Fig 4.** a) Relative mutation rates for four different types of mutations, rifampicin resistance, nalidixic acid resistance, chlorate resistance and Lac\(^+\) reversion in the presence or absence of the arabinose inducible plasmid pBAD30::rpoS. Rates are given relative to the mean mutation rate of the wild-type for each type of mutation in LB medium (Table 1). + indicate presence of pBAD30::rpoS in strain DA14346 and lexA(def) mutant DA14400 induced with the indicated concentration of L-arabinose (0.01-0.2%) (top panel). – indicate the mutation rate in wild type DA10212 and lexA(def) DA10598 without the plasmid. The bottom panel shows a Western blot with
commercial mouse anti-RpoS antibodies for the respective strain in the same experiment. Error-bars are SEM.

b) Relative mRNA levels measured by real-time PCR in cells grown with or without induction of the pBAD30::rpoS plasmid by 0.1% L-arabinose. mRNA levels are given relative to the wild type level set to 1. Error-bars are SEM.

**Fig 5.** a) Relative mutation rates for four different types of mutations, rifampicin resistance, nalidixic acid resistance, chlorate resistance and Lac⁺ reversion in different media: LB, minimal M9 glucose and minimal M9 glycerol. Rates are given relative to the mean mutation rate of the wild-type for each type of mutation in minimal M9 glucose medium (Table 1). b) Relative Pol IV (dinB)-expression measured as β-galactosidase activity (dinB promoter-lacZ fusion on pRS552 plasmid, see Materials and Methods). Rates are given relative to the wild-type Pol IV expression in each experiment in LB medium. Error-bars are SEM.

**Fig 6a-b.** Relative mutation rates for four different types of mutations, rifampicin resistance, nalidixic acid resistance, chlorate resistance and Lac⁺ reversion for wild-type and mutant cells grown in minimal M9 glucose medium. Rates are given relative to the mean mutation rate of the wild-type for each type of mutation in minimal M9 glucose medium at day 1 (Table 1). Error-bars are SEM.
Fig 4a

<table>
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<tr>
<th>L-arabinose concentration</th>
<th>pBAD30::rpoS</th>
<th>wild type (DA10212 / DA14346)</th>
<th>lexA- (DA10598 / DA14400)</th>
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<tr>
<td></td>
<td>-</td>
<td>0.01% 0.05% 0.1% 0.2%</td>
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</table>

- Relative mutation rate to nalidixic acid resistance
- Relative mutation rate to rifampicin resistance
- Relative mutation rate to chlorate resistance
- Relative mutation rate to Lac+ reversion

RpoS antibody
Relative mRNA levels

- katE levels
- otsA levels

Fig 4b

- wt DA10212
- lexA⁻ DA10598
- pBAD30::rpoS DA14346
- lexA⁻ pBAD30::rpoS DA14400
Relative mutation rate to rifampicin resistance

Relative mutation rate to nalidixic acid resistance

Relative mutation rate to chlorate resistance

Relative mutation rate to Lac+ reversion

Fig 5a
Fig 5b

Relative $dinB$-$lacZ$ expression measured as $\beta$-galactosidase expression

- LB
- M9 glucose
- M9 glycerol

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>lexA&lt;sup&gt;-&lt;/sup&gt;</th>
<th>rpoS&lt;sup&gt;-&lt;/sup&gt;</th>
<th>lexA&lt;sup&gt;-&lt;/sup&gt;rpoS&lt;sup&gt;-&lt;/sup&gt;</th>
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</tbody>
</table>
Fig 6a

Relative mutation rate to rifampicin resistance in M9 glucose

Day 1 Day 6

Relative mutation rate to nalidixic acid resistance in M9 glucose

Day 1 Day 6

Relative mutation rate to chlorate resistance in M9 glucose

Day 1 Day 6

Relative mutation rate to Lac+ reversion in M9 glucose

Day 1 Day 6
Relative mutation rate to rifampicin resistance in M9 glucose

Day 1  Day 6

Relative mutation rate to nalidixic acid resistance in M9 glucose

Day 1  Day 6

Relative mutation rate to chlorate resistance in M9 glucose

Day 1  Day 6

Relative mutation rate to Lac+ reversion in M9 glucose

Day 1  Day 6