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The transcription elongation factor NusA is required for stress-induced mutagenesis in *Escherichia coli*

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SUMMARY

Stress-induced mutagenesis describes the accumulation of mutations that occur in non-growing cells, in contrast to mutagenesis that occurs in actively dividing populations, and has been referred to as stationary phase or adaptive mutagenesis. The most widely studied system for stress-induced mutagenesis involves monitoring the appearance of Lac^+ revertants of the strain FC40 under starvation conditions in *Escherichia coli* [1]. The SOS inducible translesion DNA polymerase DinB [2–4], plays a particularly important role in this phenomenon. Loss of DinB (DNA pol IV) function results in a severe reduction of Lac^+ revertants [5,6]. We previously reported that NusA, an essential component of elongating RNA polymerases, interacts with DinB [7]. Here we report our unexpected observation that wild-type NusA function is required for stress-induced mutagenesis. We present evidence that this effect is unlikely to be due to defects in transcription of *lac* genes, but rather due to an inability to adapt and mutate in response to environmental stress. Furthermore, we extended our analysis to the formation of stress-induced mutants in response to antibiotic treatment, observing the same striking abolition of mutagenesis under entirely different conditions. Our results are the first to implicate NusA as a crucial participant in the phenomenon of stress-induced mutagenesis, in addition to its roles in transcription elongation and termination.

RESULTS AND DISCUSSION

nusA$^+$ function is indispensible for the viability of bacteria and its roles in transcription elongation and termination have been appreciated for many years, however our recent data suggest that NusA may also be involved in the process of DNA damage repair/tolerance. We previously reported that the temperature sensitivity of the *nusA11*(ts) allele [8] can be suppressed by overexpression of the TLS (translesion synthesis) polymerase DinB, in a manner that requires the catalytic, and specifically the translesion DNA synthesis, capabilities of DinB [7]. Intrigued by these observations, we looked to see if the DinB dependent phenomenon of stress-induced mutagenesis was altered in a *nusA11* background.

The widely used system devised by Cairns and Foster, utilizes a strain (FC40) which has *lac* deleted on the chromosome and carries an F'128 episome containing a *lacI33-lacZ* fusion with a +1 frameshift in a run of G:C base pairs [1]. When *E. coli* are starved for a carbon source and plated onto minimal lactose medium, Lac^+ mutants occur at a rate of approximately 10^{-7} per cell per day for about 7 days [1]. Furthermore, it has been found that stress-induced

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Lac$^+$ mutations mainly result from a −1 frameshift mutation in a run of consecutive bases [9, 10]. DinB in both prokaryotes and eukaryotes is proficient for the formation of −1 frameshift mutations [11,12]. The analysis of *dinB*'s role in stress-induced mutagenesis is complicated by the fact that *dinB*$^+$ is present on the F' episome as well as on the chromosome. However, depending on the *dinB* alleles used loss of DinB function results in a 2–4 fold reduction in the appearance of Lac$^+$ revertants [5,6]. Additionally, it has recently been found that *dinB*$^+$ is the only SOS regulated gene required at induced levels for stress-induced mutagenesis [13].

Using the *lacI33-lacZ* fusion system described above, we found that FC40 cells carrying the *nusA11* mutation display an approximately 470-fold reduction of the rate of mutation to Lac$^+$ at the permissive temperature (30°C), with reversion to Lac$^+$ occurring at a rate of 0.14 ± 0.03/ 10^8 cells/day compared to the wild-type (67 ± 4.5/10^8 cells/day) (Figure 1A–B). The reduction in stress-induced mutations observed in a *nusA11* background is even more extreme than the reduction that is seen in cells which are deleted for both the chromosomal and episomal copies of *dinB* (1 ± 0.05 Lac$^+$ revertants/10^8 cells/day) (Figure 1A–B). These results suggest that the role of *nusA*$^+$ in stress-induced mutagenesis may extend beyond an interaction with DinB and the formation of −1 frameshift mutations. Additionally we observe that the effect of *nusA11* on stress-induced mutagenesis is independent of the cells' *dinB* status, as FC40 *nusA11* derivatives lacking the episomal copy of *dinB*, the chromosomal copy, or both show the same low level of stress-induced mutagenesis as the *nusA11 dinB*$^+$ parent strain (data not shown). Daily measurements of viable Lac$^-$ cells on the plates show that no significant net growth or death during the course of the experiments and indicate that the *nusA11* cells were not dying upon incubation on minimal lactose plates, but rather were incapable of mutating (Figure 1C).

Strikingly, we observe that *dinB* deletion strains display an approximately 75-fold reduction in Lac$^+$ revertants at 30°C (Figure 1A–B), which is significantly greater than the reported 2–4-fold reduction observed at 37°C [5, 6]. This discrepancy could be due to either the *dinB* deletion allele used or the temperature at which experiments were performed. We therefore performed these experiments at both temperatures utilizing the same doubly deleted Δ*dinB* strain. Compared to wild-type (FC40) the Δ*dinB* strain displays an approximately 6-fold reduction at 37°C and 75-fold reduction at 30°C (Figure 2 A–C). Most notable, is that while the number of Lac$^+$ revertants in the doubly deleted Δ*dinB* strain is similar at the two temperatures, there are more Lac$^+$ revertants produced at 30°C in the FC40 background than at 37°C, indicating that there are more *dinB*-dependent mutants at 30°C. To our knowledge, this is the first report of the temperature dependence of stress-induced mutagenesis in the *lacI33-lacZ* system.

One caveat to our above results is that, *in vitro*, NusA is known to be required for β-galactosidase transcription [14]. *In vivo*, at the permissive temperature, the *nusA11* mutation is reported to cause a 40 percent reduction in β-galactosidase activity [8], due to a Rho-dependent termination signal within the *lacZ* coding region. Could this defect in *lacZ* expression explain the elimination in stress-induced mutagenesis we are seeing? To test this, we isolated stress-induced mutants that arose from the wild type FC40 strain (FC40 Lac$^+$) and *nusA11* strains originally appearing at day 6 of selection and then monitored β-galactosidase activity as well as the ability to re-form colonies on lactose medium at 30°C in reconstruction experiments. We observe that a *nusA11* derivative of a Lac$^+$ strain shows an approximately 40 percent reduction in β-galactosidase activity from the episomal *lacI33-lacZ* reporter compared its *nusA*$^+$ parent (Figure 3A). However a *nusA11* derivative displays an approximately 75 percent reduction in residual β-galactosidase activity from the leaky *lacI33-lacZ* allele in a FC40 Lac$^-$ strain compared to its *nusA*$^+$ parent (Figure 3B). Potential implications of this difference are discussed later.

Reconstruction experiments, taking Lac^+ revertants and re-plating a known number of cells in the presence of ~10^9 scavenger cells to recapitulate lactose selection conditions, have been used to determine if mutations leading to Lac^+ arose during selection or were preexisting in the population [15]. If the Lac^+ colony originally appearing at day six after selection reappears after 6 days, it is presumed to be a slow growing preexisting mutant. If however, the number of days to reform colonies is less than the number of days when the original Lac^+ colony arose, it is presumed to have occurred on the plate in response to stress. We observe that Lac^+ colonies, from both the wild-type (FC40) and *nusA11* strains take much less than 6 days to reform colonies at 30°C. FC40 *nusA*$^+$ Lac^+ colonies begin to appear at day 2, with the remaining colonies becoming visible at day 3. Consistent with their moderately reduced levels of β-galactosidase expression, *nusA11* Lac^+ colonies however, begin to appear on day 3, with remaining colonies developing on day 4, a delay of *ca.* 24 hours compared to the *nusA*$^+$ parent (Figure 3C). These results suggest that, even under the conditions of reduced β-galactosidase activity in a *nusA11* strain, there remains sufficient β-galactosidase function to grow when lactose is the only carbon source, albeit at a slower rate. Thus, the elimination of stress-induced mutagenesis seen in the *nusA11* strain is likely not due to a defect in *lacZ* expression, but rather to an inability to adapt and mutate in response to environmental stress.

Although the above observations suggest that loss of stress-induced mutagenesis in a *nusA11* background is not due to altered expression of the *lacI33-lacZ* fusion, we wanted to see if this observation would repeat in another context. In an effort to accomplish this, we thought we might take advantage of a pre-existing reporter that has been previously used to monitor unselected mutations in stress-induced mutagenesis experiments employing the *lacI33-lacZ* system. The strain FC722, carries an inactivated *tetA* gene due to a +1 frameshift mutation in a run of G:C base pairs within a Tn*10* transposon on the episome (F'128), positioned approximately 5 kb away from the *lac* genes [16,17], and has a reversion rate to tetracycline resistance of 3×10^{-9}/cell/generation during exponential growth [18]. Tetracycline is a bacteriostatic antibiotic which functions through inhibition of the 30S ribosome [19]; resistance imparted by a Tn*10* insertion is achieved by tetracycline efflux [20]. The tetracycline resistance (Tet^R) of a strain carrying a chromosomal Tn*10* insertion was not affected in a *nusA11* background, indirectly showing that *tetA* expression is not altered. In fact, the presence of the *nusA11* allele even modestly improves tetracycline resistance at higher doses (Figure 4A). At the concentration of tetracycline used in the experiments described below (12 μg/mL), both the *nusA*$^+$ and *nusA11* Tet^R strains showed 100 percent survival. Stress-induced mutagenesis is defined as mutations that arise in non-growing bacteria held under non-lethal selection, allowing for growth [21]. Thus we hypothesized that, by plating FC722 cells on tetracycline medium, non-growing cells should accumulate mutations in the defective Tn*10* that would then allow for growth on tetracycline medium, analogously to the Lac^+ mutations that accumulate in starved FC40 cells plated on minimal lactose medium.

When independent cultures were plated onto minimal glucose plates supplemented with tetracycline, we observed Tet^R colonies appearing over a period of several days (25 ± 4.6/10^9cells/day), an observation reminiscent of the appearance of Lac^+ colonies over several days in standard *lacI33-lacZ* stress-induced mutagenesis (Figure 4B). Compared to the Lac system, the total number of mutants that are produced is lower when compared to FC40 strains plated on lactose minimal medium. Additionally, we were not able to isolate tetracycline sensitive (Tet^S) cells from the plates, by removing agar plugs from spaces in between colonies. However, if the plates were then overlaid with top agar containing glucose, growth of the lawn population was then visible (data not shown). These data indicate that although we were unable to quantify the Tet^S population over the course of the experiment, many (if not all) the cells remain viable upon continued exposure to tetracycline.

We find that the genetic requirements for stress-induced mutations that occur after tetracycline treatment are similar to those that occur in response to starvation in that they require *dinB*$^+$ and the recombination functions of *ruvC*$^+$ [22,23] (Figure 4B), with reversion to Tet^R occurring at $3 \pm 0.5/10^9$cells/day and $1 \pm 0.2/10^9$cells/day in the doubly deleted Δ*dinB* mutant and Δ*ruvC* mutant respectively. Furthermore, stress-induced hypermutability after tetracycline treatment can be achieved through mutation of *recG* (125 ± 34 Tet^R revertants/10^9cells/day) (Figure 4C), as occurs in the Lac system [22,23]. *nusA*$^+$ function is also required for tetracycline adaptive mutagenesis, as the *nusA11* mutation virtually eliminates the formation of stress-induced mutants and has a reversion rate to Tet^R of $0.1 \pm 0.07/10^9$cells/day (Figure 4B). Isolating Tet^R revertants, from both the wild-type and mutant strains, that originally appeared at day 5 or 6 allowed us to perform reconstruction experiments to test if these mutants are truly stress-induced. By plating a known number of cells, in the presence of ~10^9 scavenger cells (FC29) to mimic a lawn population of non-growing Tet^S cells, we observe that colonies appear after two days of incubation on minimal glucose plates supplemented with tetracycline with no notable difference in colony size between wild-type, *nusA11*, Δ*dinB*, and Δ*ruvC* Tet^R strains (Figure 4D). These data imply that the Tet^R mutants that appear after tetracycline exposure are indeed stress-induced and further support the notion that the defect in stress-induced mutagenesis seen in the *nusA11* strain is not due to improper transcription but rather to a disruption of a process that allows the cells to adapt and mutate in response to stress. Interestingly, *E. coli* cells plated on medium containing a bacteriostatic concentration of the gyrase inhibiting antibiotic, ciprofloxacin, acquired resistant mutants in non-growing cells over the course of a week [24], and display a dependence on the SOS response, homologous recombination and DinB as has been shown in the Lac system [25]. These data suggest that stress-induced mutagenesis in response to bacteriostatic antibiotic treatment may be a general phenomenon.

In summary, we have discovered a previously unrecognized role for the essential gene *nusA*$^+$ in the process of stress-induced mutagenesis. Specifically a function of *nusA* altered by the *nusA11*(ts) mutation at the permissive temperature is critical for the formation of stress-induced mutations. The reduction in stress-induced mutagenesis caused by the *nusA11* mutation is 470-fold, which is larger than the reductions caused by mutation of other genes whose products have been implicated in stress-induced mutagenesis, e.g. *rpoS* (10–20 fold) [26,27], *dinB* (4–5 fold) [5,6], *recA* (10–100 fold) [1,28], *groE* (10–20 fold) [29], *ppk* (5 fold) [21], and *ruvC* (10 fold) [22,23]. Furthermore, the *nusA11* mutation does not affect the frequency of UV induced mutagenesis in a standard mutagenesis assay (Supplemental Table 1). Since *nusA11* cells are viable at 30°C, the function of NusA altered by the *nusA11* mutation is genetically separable from the essential roles of NusA in normal RNA polymerase elongation and termination/antitermination. Our finding that the reduction in stress-induced mutants seen in the *nusA11* background is greater than that seen when *dinB* is deleted, leads us to hypothesize that NusA may play a role in stress-induced mutagenesis that extends beyond a potential interaction with DinB. Though future experiments will be required to elucidate what this role may be, we propose that NusA is required to link transcription to stress-induced mutation.

Our previous report suggested that a problem occurring in the *nusA11* mutant, at the restrictive temperature, might be at the DNA level as overexpression of DinB can rescue growth in a manner requiring the catalytic and specifically the translesion DNA synthesis properties of DinB [7]. A possible role for NusA under stressed conditions could arise from a deficiency in DNA repair that results in unrepaired endogenous lesions being present in the transcribed strand. Alternatively, the very limited growth allowed by the leaky *lacI33-lacZ* allele might result in DNA replication intermediates that have more single stranded gaps than in exponentially growing cells. Either of these situations could stall an RNA polymerase if transcription is attempted. If this were the case, the function lost by the *nusA11* mutation might be the ability to recruit DinB and potentially other cellular factors required for stress-induced

mutagenesis to the stalled RNA polymerase. The process of transcription-coupled translesion synthesis we have hypothesized [7] might therefore be one mechanism that could contribute to the generation of stress-induced mutations. Below we suggest how this idea might fit into the proposed models to explain the phenomenon of stress-induced mutagenesis.

There are currently two alternative classes of models to explain how Lac^+ mutations occur during lactose selection: models involving error-prone DNA synthesis during double-strand break repair and the amplification model [30–32]. In the models involving error-prone DNA synthesis during double-strand break repair, it is suggested that amplification results in an unstable Lac^+ colony and is separate from, and does not lead to, mutation. The process through which actual mutation occurs is postulated to be initiated by a double-strand break and controlled by the SOS and RpoS stress responses. The process of repair of a double-strand break, which could be formed by replication encountering a nick generated at the episomal origin, could become error-prone through DNA replication by DinB. This switch to error-prone double-strand break repair by DinB is regulated by the SOS and RpoS stress responses [17, 30,31,33]. The limited DNA replication initiated at the episomal origin or during double-strand break repair could generate DNA substrates that could stall RNA polymerase and result in the recruitment of factors required for stress-induced mutagenesis by NusA.

The amplification model suggests that an amplified array of DNA of up to 100 copies containing the *lac* genes is an intermediate in the formation of Lac^+ mutants. In this model it is suggested that the residual β-galactosidase activity, which is now amplified by up to 100 times, allows cells to grow very slowly on minimal lactose media and reversion to Lac^+ occurs with a higher probability due to the repeated array [16,32,34–36]. Considering this model, it is possible that *nusA11* cells, since they display a 75 percent reduction in residual β-galactosidase activity (Figure 3C), may be further restricting the slow growth of the Lac^- cells thus inhibiting their ability to mutate to Lac^+. Similar effects have been previously observed through the addition of LacZ inhibitors or through inactivation of the *gal* operon [36]. However, it is less clear how this type of explanation could account for the almost complete loss of stress-induced Tet^R mutants in a *nusA11* strain. It is possible, however, that the *nusA11* mutation could interfere with the creation of the illegitimate recombination event that creates the initial duplication or the subsequent events involved in generating the amplified arrays under stressed conditions.

The amplification model also proposes that the number of revertants depends highly on the pre-growth conditions in which the original duplication required for amplification is generated [32]. We observe that when an equivalent number of FC40 *nusA*$^+$ and *nusA11* cells are plated on minimal media with better carbon sources (glucose or glycerol) the *nusA11* mutation does not affect colony formation with respect to either time of appearance or their size whereas, on poor carbon sources (acetate or pyruvate), the *nusA11* mutation delays the appearance of colonies by *ca.* 24 hours, suggesting that *nusA*$^+$ is important for growth under poor carbon sources. The replication involved in the generation of the amplified arrays under stressed conditions could generate the substrates we suggest might stall RNA polymerase and the recruitment of factors involved in stress-induced mutagenesis by NusA.

EXPERIMENTAL PROCEDURES

Mutagenesis Assays

All strains used are listed in Supplemental Table 2 and constructed using standard techniques. Lactose stress-induced mutagenesis assays were performed as previously described, except when noted incubation steps were performed at 30°C [37]. Briefly, ~10^8 cells, of at least five independent cultures of each strain were grown to saturation in M9 minimal media with either (0.1% Glycerol or Glucose), were mixed with ~10^9 cells of FC29 scavenger cells and 2.5mL

minimal top agar and overlaid onto M9 minimal lactose (0.1%) plates. Viable Lac^- cells were monitored as described in [1]. Tetracycline stress-induced mutagenesis assays were performed by growing each strain to saturation in M9 minimal media (0.2% Glucose) and diluted 10^5 fold into fresh medium (0.2% Glucose), divided into several cultures, and allowed to reach saturation, to produce at least five independent cultures. Approximately 10^9 cells from each independent culture is mixed with minimal top agar and overlaid onto M9 minimal glucose (0.2%) plates containing tetracycline (12 μg/mL). Viable cells were determined by dilution in M9 salts and plating cells on to M9 minimal glucose (0.2%) plates without tetracycline. Plates were incubated at 30°C and tetracycline resistant colonies are counted and marked every day. Reversion of *argE3* allele in standard UV induced mutagenesis were performed according to the published method [38], all incubation steps were performed at 30°C.

β-galactosidase activity assays

Strains were grown at 30°C in minimal M9 lactose (0.2%) (Lac^+ strains) or glycerol (0.2%) (Lac^- strains) medium and β-galactosidase activity assays were performed as previously described [39].

Determination of *nusA11* effects on reporter genes or growth on different carbon sources

TetA expression from Tn*10*. Strains harboring chromosomal Tn*10* (SEC527, SEC29) were grown in LB medium, diluted in M9 salts and plated onto LB plates supplemented with tetracycline, CFU/mL were determined after incubation at 30°C. Reconstruction assays were performed by collecting Lac^+ or Tet^R strains and plating a known number of cells with ~10^9 scavenger cells (FC29) onto either minimal lactose or tetracycline media and incubated at 30° C. Colony number and size were scored as a factor of time. When applicable tetracycline was used at 12 μg/mL. Growth on different carbon sources. FC40 *nusA*$^+$ and *nusA11* cells (SEC182) were diluted in M9 salts and plated onto M9 minimal plates containing glycerol, acetate or pyruvate at 0.2% and incubated at 30°C. Colonies number and size were scored as a factor of time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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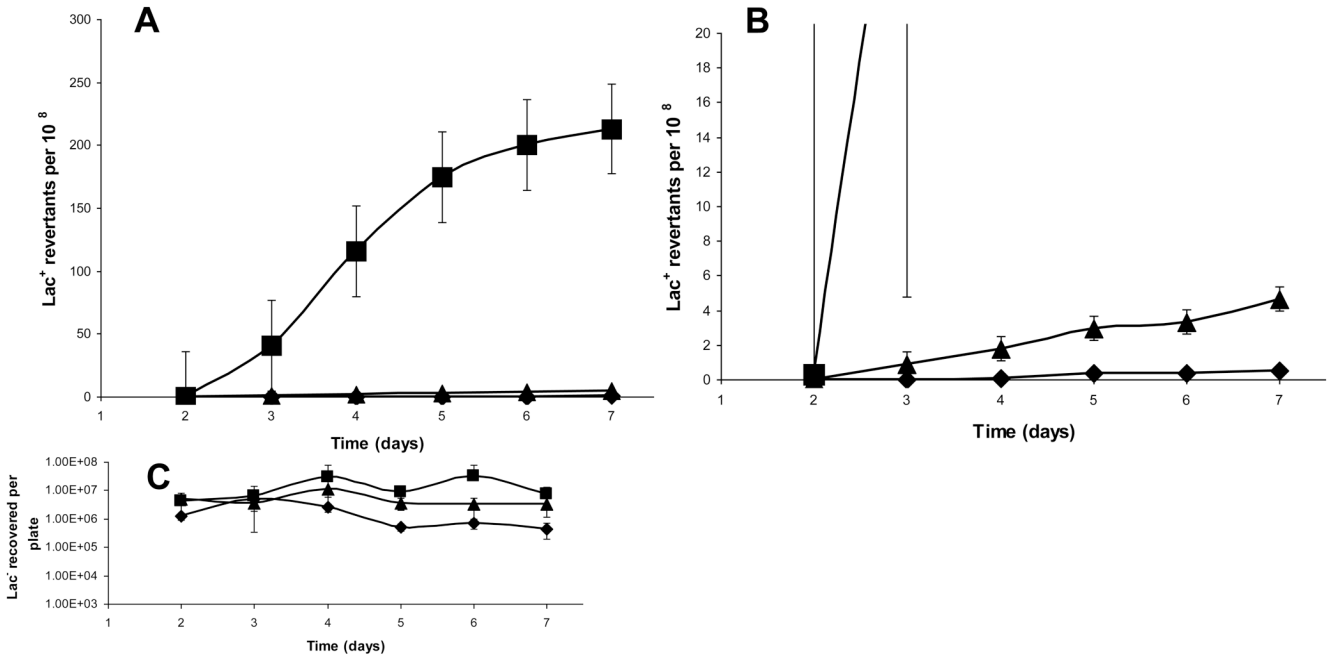


Figure 1.

Stress-induced mutation in response to starvation A) *nusA11* strains are defective for stress-induced mutagenesis. Wild-type FC40 (squares), Δ*dinB* (SEC1414) (triangles) deleted on both chromosome and episome, and *nusA11* (SEC182) (diamonds). B) Magnification of A). C) Recovery of viable Lac^{-} cells from agar plugs reveals that no significant growth or death occurring during the duration of the experiment. Error bars represent the standard deviation from five independent experiments.

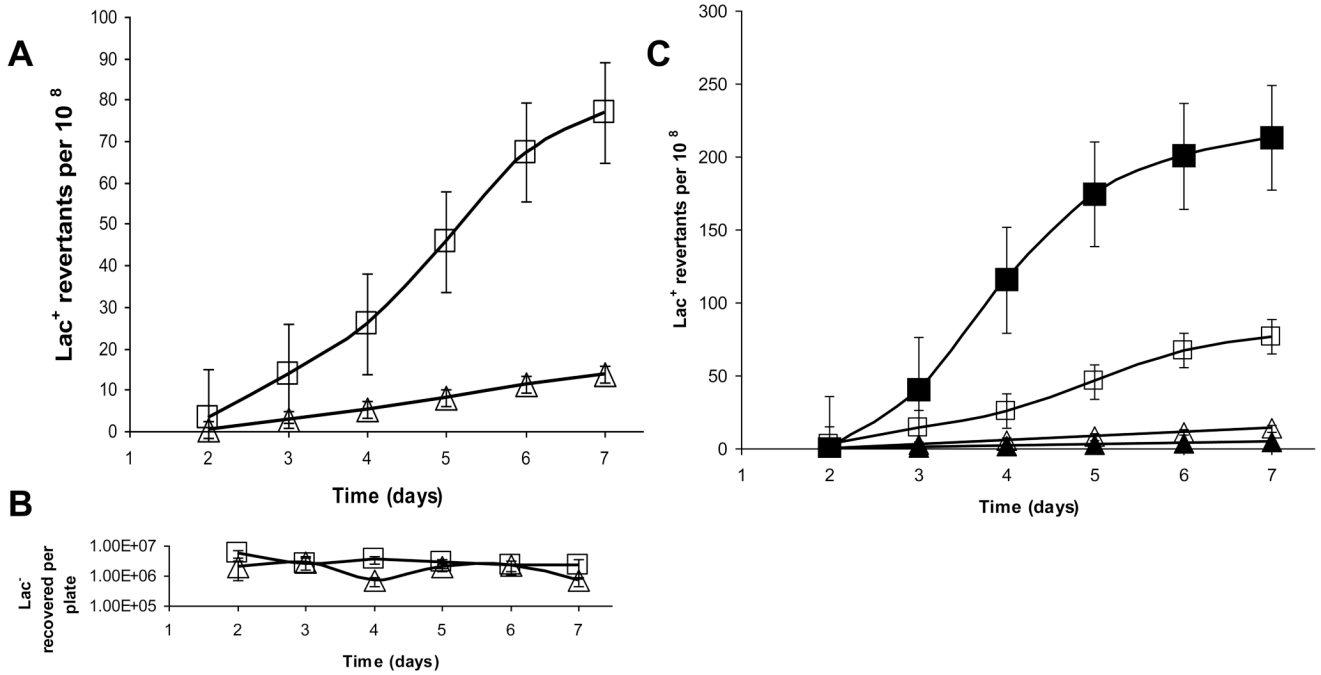
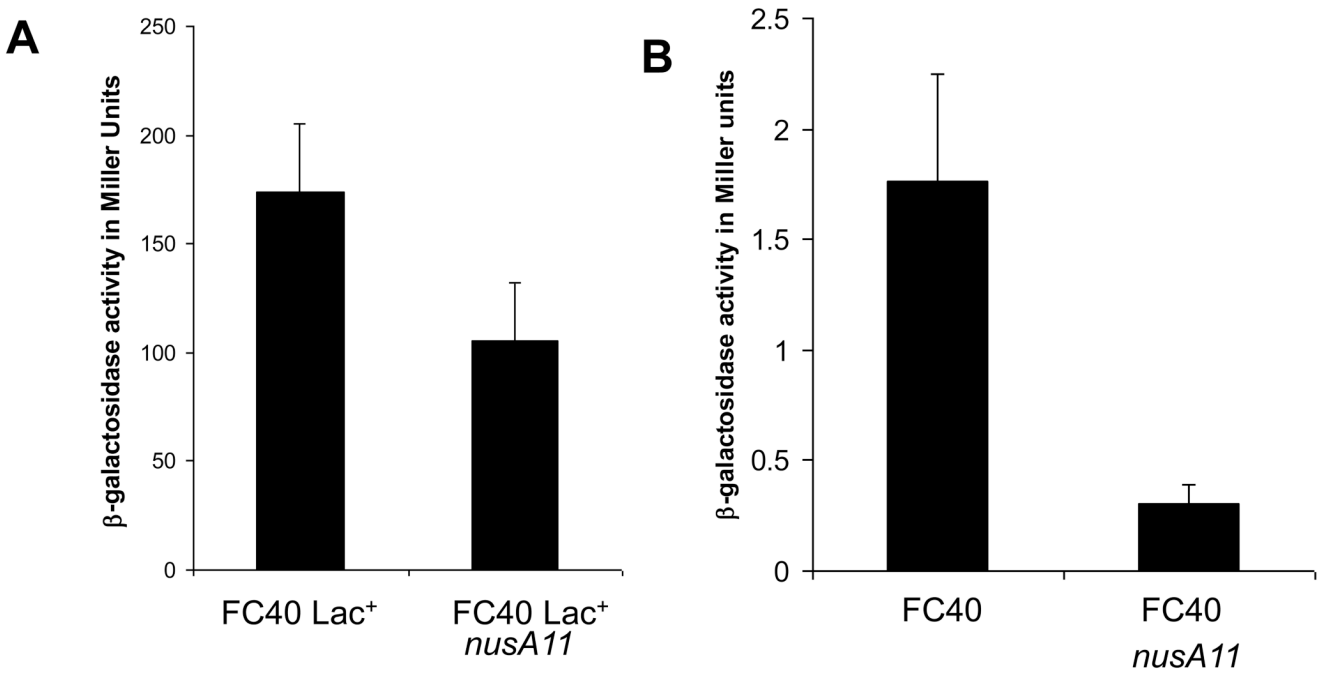


Figure 2.

Stress-induced mutation in response to starvation at 30° and 37°C. A) Reversion to Lac^+ of wild-type FC40 (open squares), Δ*dinB* (SEC1414) (open triangles) deleted on both chromosome and episome, at 37 °C. B) Overlay of graphs from Fig. 1A and Fig. 2A. Open symbols represent experiments carried out at 37°C, and closed symbols at 30°C. Symbols as in A). C) Recovery of viable Lac^- cells from agar plugs reveals that no significant growth or death occurring during the duration of the experiment. Error bars represent the standard deviation from five independent experiments.

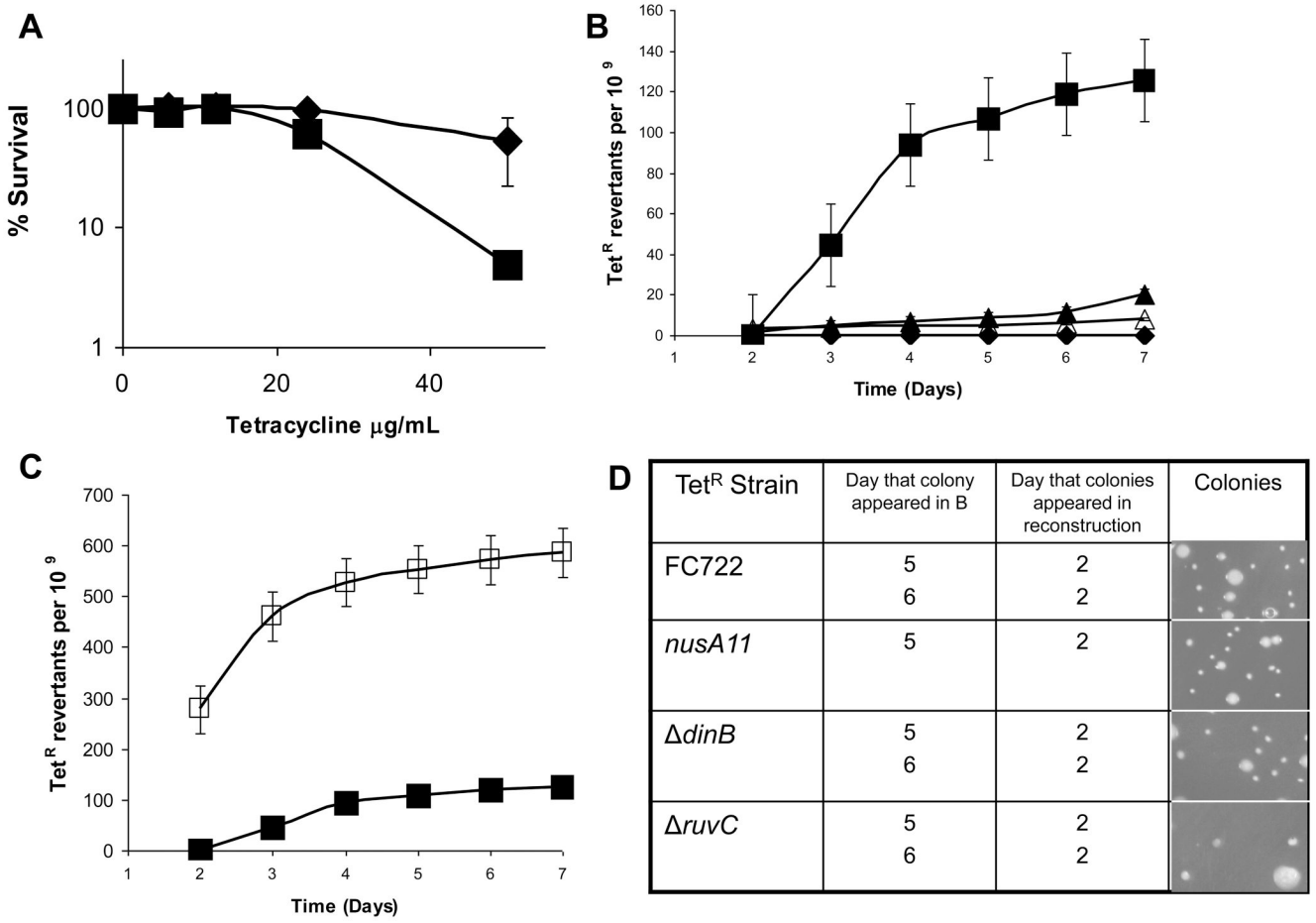


C

Lac+ strain	Day that colony appeared in A	Day that colonies appeared in reconstruction	Colonies
FC40	6	2-3	
nusA11	6	3-4	

Figure 3.

β-galactosidase activity and growth on lactose medium of *nusA11* strains at 30°C. A) FC40 Lac$^+$ *nusA11* (SEC1429) strains show an approximately 40 percent reduction in β-galactosidase activity as compared to FC40 Lac$^+$ *nusA*$^+$ (SEC1419). B) FC40 *nusA11* (SEC182) strains show an approximate 75 percent reduction in β-galactosidase activity as compared to FC40 *nusA*$^+$. Error bars represent the standard deviation determined from three independent experiments. C) Reconstruction experiments showing that Lac$^+$ revertants picked from day 6, take only two-three days for wild-type (91.3% ± 4.5 of colonies formed by day two) and three-four days for *nusA11* strains (71.8% ± 13.8 of colonies formed by day three) to form colonies on lactose minimal media. Pictures of colonies taken on day 4 of reconstruction experiment. Data collected from the examination of 3–5 clones for each reconstruction.



Tet^R Strain	Day that colony appeared in B	Day that colonies appeared in reconstruction	Colonies
FC722	5 6	2 2	
nusA11	5	2	
Δ*dinB*	5 6	2 2	
Δ*ruvC*	5 6	2 2	

Figure 4.

Stress-induced mutagenesis in response to tetracycline at 30°C. A) Tetracycline resistance from chromosomal Tn*10*. Percent survival of *nusA*$^+$ (SEC527) (squares) and *nusA11* (SEC29) (diamonds) strains to tetracycline (0–50μg/mL) shows that *nusA11* strains are not altered in their ability to express tetracycline resistance. Error bars represent the standard deviation determined from three independent experiments. B) Stress-induced mutagenesis of FC722 upon tetracycline treatment. Wild-type FC722 (SEC361) (closed squares), *nusA11* (SEC369) (closed diamonds), Δ*dinB* on the chromosome and episome (SEC611) (closed triangles), and Δ*ruvC* (SEC1466) (open triangles). C) Stress-induced Tet^R hypermutability. Hypermutation observed in FC722 Δ*recG* mutants in response to tetracycline as is seen during lactose stress-induced mutagenesis of FC40. Wild-type FC722 (SEC361) (closed squares) and Δ*recG* (SEC1464) (open squares). D) Reconstruction experiments showing that Tet^R revertants picked from day 5 or 6, take only two days to reform colonies on tetracycline medium. Data collected from the examination of 3–5 clones for each reconstruction, all colonies formed by day two.