A Novel Role for the Transcriptional Modulator NusA in DNA Repair/Damage Tolerance Pathways in *Escherichia coli*

by

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ABSTRACT

All organisms must contend with the consequences of DNA damage, induced by a variety of both endogenous and exogenous sources. Mechanisms of DNA repair and DNA damage tolerance are crucial for cellular survival after DNA damage. Translesion DNA synthesis (TLS) is one such mechanism of DNA damage tolerance which utilizes a specialized translesion DNA polymerase capable of catalyzing DNA synthesis on imperfect templates. There are two TLS polymerases present in *Escherichia coli* encoded by the *dinB* (Pol IV) and *umuDC* (Pol V) gene products. While TLS polymerases provide a variety of benefits to the cell, it is important that they are properly regulated as they have reduced fidelity on undamaged DNA compared to replicative DNA polymerases.

Here I present evidence that the essential transcriptional modulator NusA associates with TLS polymerases in *E. coli* both physically, as noted for DinB, and genetically, with DinB and the *umuDC* gene products. Mutation of *nusA* renders cells sensitive to DNA damaging agents and produces phenotypes reminiscent of mutants with altered DNA processing. Moreover, I report that the *nusA11* mutation completely eliminates the formation of adaptive mutants, revealing that $nusA^+$ function is required for cells to adapt and mutate in response to stress. Though the phenomenon of adaptive mutagenesis also requires $dinB^+$, my data suggest that the role for *nusA* in adaptive mutagenesis extends beyond an interaction with DinB.

Furthermore, I report that NusA in addition to having a role in transcription elongation is also important for promoting survival after DNA damage. Phenotypes of *nusA* mutants are more exaggerated than those of TLS polymerase mutants. Genetic interactions of $nusA^+$ with the nucleotide excision repair pathway suggest that $nusA^+$ may play a role in a new class of NusA-dependent transcription coupled repair. Moreover, I have isolated RNA polymerase mutants with altered ability to survive after DNA damage, and this altered ability is absolutely dependent on $nusA^+$ and $uvrA^+$.

The completion of translesion DNA synthesis requires both the insertion of a nucleotide opposite the adducted template base and extension from that position by several subsequent nucleotide additions. We present evidence that DinB is specialized to perform strikingly proficient extension after insertion opposite an N^2-dG lesion. Our data indicate that cellular survival is coupled to completion of TLS and regulation of these precise steps *in vivo* is genetically complex and involves the toxin-antitoxin module MazEF and the iron import protein TonB.

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Chapter 1

Introduction

Portions of this introduction were published in Y-family DNA polymerases in *Escherichia coli*. Daniel F. Jarosz, Penny J. Beuning, Susan E. Cohen, and Graham C. Walker. *Trends in Microbiology* 2007; 15(2):70 and Lyle A. Simmons, James J. Foti, Susan E. Cohen, and Graham C. Walker. Chapter 5.4.3, The SOS Regulatory Network. *Escherichia coli and Salmonella: cellular and molecular biology.*

Overview

Cellular DNA can be damaged by a wide variety of both endogenous and exogenous sources (23). It is estimated that that nearly 10,000 abasic sites are spontaneously generated in a single eukaryotic cell each day (69), and this statistic is for only one type of DNA damage. If ensuing lesions are left unrepaired, they may lead not only to mutagenesis but also to replication fork collapse, chromosomal breakage and potentially result in cell death. Given the huge spectrum of genomic insults cells must deal with, it is no surprise that all organisms have evolved a variety of mechanisms to deal with DNA damage. Mechanisms of DNA repair, which restore the DNA to its original undamaged state, and DNA damage tolerance, involving the replication of damaged DNA, are of great importance to human health as mutations in these pathways result in a variety of cancers and diseases. Here I will provide an introduction on the many ways cells deal with the problem of DNA damage. My graduate work has primarily focused on the DNA damage tolerance pathway of translesion DNA synthesis (TLS) in *Escherichia coli*.

The SOS Response

SOS, an acronym used as the international distress signal, also describes the induced cellular response after genotoxic stress in *E. coli*. Control of the SOS response is dependent upon the *lexA*$^+$ and *recA*$^+$ gene products. LexA, the transcriptional repressor of the SOS response, binds as a dimer to imperfect palindromic operator sequences present in the promoters of SOS-regulated genes, also referred to as SOS boxes, inhibiting transcription of downstream genes (23). The *recA*$^+$ gene product is important

for not only the induction of the SOS response but also for many other aspects of the cell's ability to deal with DNA damage, including homologous recombination and translesion DNA synthesis. After DNA damage has occurred, continued activity of the helicase ahead of the blocked replication fork leads to accumulation of ssDNA. RecA binds to this single-stranded DNA (ssDNA), forming a nucleoprotein filament in the presence of nucleoside triphosphates. This filament stimulates a latent autoproteolytic activity of LexA, causing LexA to cleave itself into two halves of roughly equal size (38). This cleavage inactivates LexA as a transcriptional repressor and enabling the expression greater than 50 genes, many of which are involved in DNA repair or DNA damage tolerance (117) (Figure 1, sections i and ii). Induction of genes in the SOS regulon ranges from 4-5 fold to 100 fold, due in part to the strength in which LexA binds to particular SOS boxes and the position of the SOS box relative to the promoter (23). *lexA*$^+$ itself is induced as part of the SOS regulon, ensuring that after the DNA damage has been dealt with, LexA levels are restored and SOS returned to an uninduced state.

Identification of genes regulated as part of the SOS network

Genes regulated as part of the SOS response have been identified by the use of gene-fusions, computational searches for consensus SOS boxes and through microarray analysis examining gene expression after exposure to DNA damaging agents (15, 46, 67, 134). In the first approach, the bacteriophage Mu d1 was used to generate random promoterless fusions carrying the structural genes for the *lac* operon. Using this technique, a collection of *E. coli* strains bearing these fusions was used to identify genes that displayed a damage inducible (*din*) phenotype even before the details of the SOS

response had been determined. The inducible expression of β-galactosidase displayed by some of these fusions after treatment with ultraviolet radiation (UV) or mitomycin C (MMC) was dependent on *recA*$^+$ and *lexA*$^+$ (46). Many of the *din* genes and their gene products have still not been characterized in detail. Since then a set of SOS inducible genes have been identified by the identification of LexA binding sites (SOS boxes) within the *E. coli* genome (19, 67, 134), bringing the number of SOS regulated genes to 31 (23). Recent microarray analysis of *E. coli* strains irradiated with UV has identified several more genes that are regulated as part of the SOS network in a *lexA*$^+$ dependent fashion (15). Curiously, *lexA* independent induction of gene expression as well as gene expression that decreased after UV irradiation were also observed (15). Intriguingly, agents that do not damage DNA, such as β–lactam antibiotics can also induce the SOS response through the two-component signal transduction system *dpiBA* (83). This observation raises the possibility that crosstalk between the SOS response and other cellular signaling pathways could be more extensive than previously realized. Finally, the SOS response is one component of a broader cellular response to DNA damage. Exposure of *E. coli* to the DNA-damaging agent MMC results in expression changes of >1000 genes (47). These results indicate that a simplistic view of the SOS response is far from complete, and suggests that other transcriptional networks may be involved in regulating gene expression in response to DNA damage as well as to various other stress responses.

Chronic partial SOS induction

Even without exposure to exogenous DNA damage, the SOS response can become induced by mutations in genes that affect DNA metabolism. Several mutations have been identified that display chronic partial SOS induction, which presumably results in difficulty repairing endogenous DNA damage. Some of these genes include *uvrD* (3'→5' DNA helicase II) (96), *dam* (GATC-specific DNA methylase) (73, 99), *polA* (DNA pol I) (3), *dnaQ* (3'→5' exonuclease epsilon subunit of DNA pol III) (68, 118), and *priA* (primosome component/3'→5' DNA helicase) (90, 107). A missense mutation in the β clamp, an essential component of the replicative DNA pol III, also elicits chronic partial SOS induction (119). Additionally, genetic screens have isolated 42 transposon insertion mutants that display chronic partial SOS induction (91). As expected the majority of mutants isolated in this screen have known functions in the synthesis and repair of DNA. However also isolated were several genes involved in membrane structure and function, comprising a new class of constitutive SOS mutants. Is this chronic partial SOS induction manifested through low levels of SOS induction throughout the entire population of cells or by high levels of SOS induction in only a sub-population of cells? Using a GFP reporter placed under the control of an SOS regulated promoter (P_{sulA}) it was found that for many of these mutations chronic partial SOS induction is due to a high degree of SOS induction in a sub-population of cells (79). These data suggest that even under normal growth conditions; these mutants with altered ability to maintain chromosome integrity induce the SOS response in only a sub-population of cells. This is in contrast to specific mutations in *lexA* or *recA* in which all cells are induced for the SOS response.

DNA damage repair

Repair of damaged DNA is defined as the restoration of DNA to an undamaged state and can occur through mechanisms of direct reversal or excision repair (Figure 2). Direct reversal of DNA damage, used for only a few types of lesions, usually involves a single protein which catalyzes a single step required to restore the DNA to its native state. For example, the Ada protein of *E. coli* irreversibly catalyzes the transfer of alkyl groups from the damaged DNA onto itself (78) and the universal enzyme photolyase found from bacteria to humans, reverses the covalent linkage of two adjacent pyrmidines resulting in the original pyrimidine monomers (23). Excision of the damaged base and synthesis of new DNA can occur through repair mechanisms of mismatch repair (MMR), base excision repair (BER) or nucleotide excision repair (NER). MMR recognizes DNA mismatches produced during DNA replication or by deamination events (23, 56, 110). BER, which primarily acts on endogenously generated oxidative and hydrolytic damage, utilizes a glycosylase to remove the damaged base, leaving behind an abasic site. In *E. coli* this abasic site is then removed by an endonuclease and deoxyribophosphodiesterase, with the ensuing gap filled in by DNA pol I and remaining nick sealed by DNA ligase. NER, on the other hand, acts on all unnatural base modifications and is the only mechanism for repair of bulky adducts. The process of NER in *E. coli* is mediated through the concerted action of the *uvrA*, *uvrB* and *uvrC* gene products resulting in excision of a patch of DNA that includes the damaged nucleotide. This step-wise process is initiated by the $(UvrA)_2UvrB$ complex, required for the recognition and binding of damaged DNA, after which UvrA dissociates from the complex (94, 106). The UvrB-DNA complex is recognized by UvrC, binding of UvrC induces a conformational change

in UvrB, resulting in the incision of the damaged strand on either side of the damaged base. Following nicking UvrD, also called DNA helicase II, mediates release of the oligonucleotide fragment (12-13 nucleotides in length) (95). Completion of NER requires that the gap be filled in by DNA pol I and sealed by DNA ligase.

Transcription coupled repair

Nucleotide excision repair can occur through two distinctive pathways, global NER (described above) and transcription-coupled NER, also known as transcription coupled repair (TCR). It has been appreciated for many years that lesions that block replication by replicative DNA polymerase can also stall RNA polymerase (RNAP), leading to inhibition of transcription. Transcription coupled repair targets nucleotide excision repair processes to actively transcribed genes, resulting in preferential repair of the transcribed strand relative to the untranscribed strand (5, 82). In bacterial cells the *mfd*$^+$ gene product couples the process of NER to transcription and is also known as the transcription coupling repair factor (TCRF) (111, 112). The phenomenon of mutation frequency decline, noted by Evelyn Witkin in the 1950's, describes the loss of UV induced mutations when the *E. coli* culture is held under conditions that inhibit protein synthesis (139, 140). Mfd recognizes RNA polymerases stalled at sites of DNA damage and is capable of translocating or backtracking the RNAP, exposing the DNA lesion. Then through a domain homologous to UvrB, Mfd recruits UvrA to this site of damage, initiating the process of NER. Further investigation has shown that Mfd, highly conserved among bacteria, stimulates the nucleotide excision activity of UvrABC *in vitro* (112). Eukaryotes also perform transcription-coupled repair, in humans, defects in genes

involved in TCR result in hereditary disorders such as xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothio-dystrophy (TTD) (35). The human transcription repair coupling factor (TRCF) gene has been identified as ERCC6 also known as CSB (129). Despite the fact that ERCC6/CSB and *mfd* share no significant sequence homology, they are structurally and functionally homologous.

DNA damage tolerance

Mechanisms of DNA damage tolerance, in which the lesion remains in the DNA through the replication of the damaged template, also play an important role in the cell. This is in contrast to repair mechanisms in which DNA lesions are removed, restoring DNA to an undamaged state. Although not generally considered the first line of defense after DNA damage, tolerance mechanisms are crucial for cellular survival after DNA damage (23). There are two main branches of DNA damage tolerance: recombinational or template switching strategies that avoid using the lesion as a template and translesion synthesis in which specialized DNA polymerases can replicate over lesions, often in a mutagenic manner. Replication fork regression, also referred to as template switching, is one mechanism used to rescue stalled replication forks and allow for bypass of lesion in an error free manner. Upon encountering a replication blocking lesion, the replication fork may migrate backwards, resulting in re-annealing of the two original templates and annealing of the newly synthesized strands. DNA synthesis past the site of the lesion by using one of the newly synthesized strands as a template, results in bypass of the lesion. Fork regression reversal then allows for continuation of DNA replication in an error-free manner, meaning that the newly synthesized DNA doesn't carry any mutations, as an

undamaged strand was used as the template rather than the damaged template strand (Figure 2). This is in contrast to the process of translesion DNA synthesis, which involves direct replication of the damaged template and is potentially error-prone. The process of translesion DNA synthesis (TLS) will be discussed in greater detail (see below).

Daughter strand gaps

While there exist a variety of mechanisms to either repair or tolerate DNA damage, DNA synthesis can re-initiate downstream of a blocked replication fork, leaving behind a lesion containing ssDNA gap. In 1968, Rupp and Howard-Flanders noticed that when *E. coli* lacking *uvrA* (NER) activity were irradiated with UV, they accumulated fragments of newly synthesized DNA that were initially smaller than in un-irradiated controls (104). These smaller DNA fragments eventually, with time, became converted to higher molecular weight DNA, approaching that of undamaged DNA. DNA synthesis required to fill in gaps, corresponding to the low molecular weight fragments, is also referred to as post-replicative repair and implies that DNA synthesis can occur in a discontinuous manner. It was thought for many years that daughter strand gaps could only generated on the lagging strand, with replication resuming at the site of the next Okazaki fragment. However, there is now evidence that gaps can be formed on the leading strand as well. *In vitro* studies in *E. coli* have found that origin independent replication restart can occur downstream of blocked replication fork on the leading strand, resulting in the formation of a gap on the leading strand (37). Furthermore in *S. cerevisiae*, daughter strand gaps have been visualized *in vivo* on both the leading and

lagging strands (74). These daughter strand gaps have been estimated to be approximately 1000 nucleotides in length (37, 39, 53, 104), and can be repaired by recombinational exchange. Using the homologous sequence from a homologous chromosome, which has presumably completed replication, these gaps can be filled in without the loss of any genetic material or introduction of new mutations (105). However, newly emerging models have suggested that some of these lesion containing gaps are filled in by TLS polymerases (14, 37, 65, 74, 138).

Translesion DNA Synthesis

One important mechanism of DNA damage tolerance is translesion DNA synthesis (TLS), a process in which a specialized DNA polymerase copies past DNA lesions that block the highly accurate, stringent replicative DNA polymerases. TLS polymerases are conserved throughout all domains of life, with the majority being members of the Y-family of DNA polymerases (92). Whereas TLS polymerases share no clear sequence homology with replicative polymerases, their tertiary structures reveal a similar right-hand fold consisting of a thumb, palm and fingers domain (Figure 3). Y-family polymerases have an additional little-finger domain that seems to play an important part in both substrate specificity and processivity (6). Unlike the tight grip seen in active sites of canonical DNA polymerases (42), some Y-family polymerases may have open active sites that are relatively solvent accessible. Moreover, an α-helix responsible for several orders of magnitude of fidelity by sterically checking the correct geometry of the newly-forming base pair in canonical DNA polymerases (the O helix) is entirely absent in Y-family polymerases (Figure 3). These structural features provide an

explanation for the comparatively low fidelity of Y-family polymerases relative to replicative polymerases when replicating undamaged DNA. For example, *E. coli* DinB has an error rate of 10^{-4}-10^{-5} compared to10^{-7} for the replicative polymerases. In addition, it appears that, each of the Y-family polymerases is specialized to insert the appropriate nucleotide opposite a cognate lesion (discussed below).

Y-family of DNA polymerases

While Y-family DNA polymerases are present in all domains of life, they are not evenly distributed between them (92). The UmuC family of DNA polymerases is only present in bacteria, while family members Rev1, Pol η (Rad30A), and Pol ι (Rad30B) are specific to eukaryotes. DinB is the most ubiquitous Y-family DNA polymerase, having homologs in bacteria, eukaryotes and archaea. Of the eukaryotic members, Pol η has received the most attention, as loss of Pol η activity in humans results in the cancer-prone syndrome xeroderma pigmentosum variant (XPV), characterized by an increased incidence of skin cancers and sensitivity to sunlight. XPV is similar to the other forms of xeroderma pigmentosum, which result from mutations in nucleotide excision repair and transcription coupled repair. However the other eukaryotic TLS polymerases are also important for damaged induced mutagenesis and thought to be important for somatic hymermutation and immunoglobulin diversification (11).

Escherichia coli has two TLS polymerases, DinB (pol IV) and UmuD'$_2$C (pol V), both of which are transcriptionally induced as part of the SOS network. Mutants of *umuD* and *umuC* display modest sensitivity to UV (23) and abolish mutagenesis induced by UV irradiation and thus have been termed un-mutable (*umu*) (45). In contrast phenotypes of

dinB mutants, originally isolated as a damaged inducible gene using Mu d1 fusions described above (46), have been more enigmatic (25), and will be discussed in greater detail below.

Substrate specificity of TLS polymerases

DinB is capable of bypassing several N^2-dG adducts including N^2-furfuryl-dG (40), DNA-peptide crosslinks (84), N^2-N^2 interstrand cross-link intermediates (55), and N^2-1-carboxyethyl-dG (143). In contrast, UmuD'$_2$C has a much broader substrate specificity. *In vitro* Pol V has been found to bypass abasic sites (97, 124), the two major UV lesions cyclobutane pyrimidine dimmers and 6-4 (T-T) photoproducts (123), as well as several covalent adducts on the C8 position of a template G (88, 114). These lesions were bypassed with varying efficiencies and fidelities. The eukaryotic TLS polymerases pol κ and pol η, have similar substrate specificities to that of DinB and UmuD'$_2$C respectively. pol κ has been implicated in the bypass of mismatched primer template ends (52, 136), thymine glycols (20), and can proficiently and accurately bypass bulky covalent modifications at the N^2 position of guanine (2, 40, 122, 142). In contrast, pol η acts primarily in preferential and accurate bypass of cyclobutane pyrimidine dimers (41, 80, 135), and also participates in modest bypass of thymine glycols (57) and intrastrand crosslinks induced by the chemotheraputic agent cisplatin (130).

The TLS polymerase Rev1, isolated by virtue of its *rev*ersionless phenotype after UV irradiation (66), is perhaps the most uniquely adapted TLS polymerase. Rev1 has a limited catalytic activity restricted to inserting dCMP across from template G's and is important for the bypass of abasic sites and certain covalently adducted guanine residues

(60, 89, 137). Rev1 is not capable of bypassing UV induced lesions *in vitro*, however *rev1* is required for ~95% of all UV induced base pair substitutions (61). Moreover the catalytic activities of Rev1 are dispensable for cellular survival after most DNA damaging agents, leading to the idea that the major role for Rev1 is as a protein-protein interaction scaffold, required to recruit and coordinate other DNA damage tolerance factors at the sites of DNA lesions (33, 36, 125).

Complete TLS involves insertion of a nucleotide opposite an adducted base (i.e. lesion bypass) and extension from that position by several subsequent nucleotide additions. Replication by the TLS polymerase must continue for several nucleotides beyond the site of DNA damage, greater than 5 nucleotides in the case of pol V in *E. coli*, in order to avoid reversal by the proofreading activity of the replicative DNA polymerase (26). In eukaryotes, these steps are often catalyzed by at least two distinct DNA polymerases (102). The increased number of TLS polymerases in a eukaryotic cell may allow for the separation of the insertion and extension phases. Specifically DNA polymerase ζ, a TLS polymerase belonging to the B-family of DNA polymerases in eukaryotes, seems to be particularly specialized for the extension phases of TLS (60, 102). In prokaryotes, the insertion and extension steps of TLS seem to be performed by a single TLS polymerase. In particular, DinB catalyzes strikingly proficient extension after insertion opposite a N^2-furfuryl-dG lesion, and will be discussed in greater detail in Chapter 5.

umuDC

The *umuD* and *umuC* genes, present in an operon, encode for the components of translesion DNA polymerase, DNA pol V. Despite the fact that there are three DNA polymerases induced as part of the SOS response, DNA pol V is thought to be the major polymerase responsible for damaged-induced mutagenesis in *E. coli.* UmuD shares significant homology to the SOS transcriptional repressor LexA, both in sequence and structure (23). Similar to LexA, UmuD forms a homodimer in solution and undergoes a RecA-nucleoprotein activated auto-digestion, removing its N-terminal 24 amino acids to form the truncated product $UmuD'_2$. It is upon binding this form ($UmuD'_2$) that UmuC, the catalytic component of DNA pol V, becomes active as TLS polymerase. This additional layer of regulation, where UmuD is cleaved to UmuD' over the time frame of 20-30 minutes (120) is important for the timing of TLS relative to other forms of DNA repair induced by the SOS response, allowing for TLS activity only when needed. Overexpression of the *umuDC* gene products leads to inhibition of growth at 30°C, a phenomenon referred to as *umuDC*-mediated cold sensitivity. Cold sensitivity seems to result from an exaggeration of a primitive DNA-damage induced checkpoint in which $UmuD_2C$ delays the resumption of DNA synthesis after DNA damage to enable error-free repair processes to occur (93, 121). Phenotypes of $UmuD'_2C$ are reminiscent to those of pol η, in that they are both required for UV induced mutagenesis, capable of bypassing UV lesions *in vitro*, and both are thought to be the major polymerases required for the bypass of these lesions *in vivo* (41, 80, 123, 135). Computational modeling studies have bolstered the notion that the eukaryotic TLS polymerase DNA pol η is a functional homolog of $UmuD'_2C$ (64).

DinB

Isolated in 1980 as a damage inducible (*din*) gene (46), the *dinB* gene product was not found to be a DNA polymerase (pol IV) for two more decades (133), and even for several years after that the physiological role of *dinB in vivo* remained unclear. While deletion of the $dinB^+$ gene has almost no discernable effect on spontaneous mutagenesis (54), $dinB^+$ has been found to be required for untargeted mutagenesis of λ phage. In this phenomenon *E. coli* are UV-irradiated and transfected with unirradiated λ phage, but surprisingly UV-induced mutagenesis is seen in the λ DNA (7). The mutation spectrum observed is distributed between base substitution mutations and -1 frameshift events with a strong preference for mutation at G:C base pairs. DinB is the most conserved TLS polymerase, present in all domains of life, and in fact is the most abundant DNA polymerase after DNA damage in *E.coli*. However, it was difficult to believe that these subtle *in vivo* phenotypes associated with *dinB* accounted for its striking conservation, and implied that there was still much to be discovered.

Several lines of investigation have pointed that one of DinB's specialized functions is in -1 frameshift mutagenesis. Overproduction of $dinB^+$ leads to an increase in spontaneous and 4-nitroquinoline-1-oxide (4-NQO) induced -1 frameshifts and, to a lesser extent, spontaneous base substitutions (49, 50). Specifically, overexpression of DinB resulted in an approximately 800 fold increase in spontaneous – 1 frameshifts occurring in a run of six G:C base pairs (49, 103).

The $dinB^+$ gene is also important for the phenomenon of adaptive mutagenesis in *E. coli*. Adaptive mutagenesis describes the accumulation of mutations that occur in non-growing cells, and is also referred to as stress-induced or stationary phase mutagenesis.

Adaptive mutagenesis is monitored by the reversion of a *lac* allele, allowing for cells that accumulate compensatory mutations to now grow on lactose minimal medium. 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 within a *lacI-lacZ* fusion with a +1 frameshift (fs) in a run of G:C base pairs on a conjugal plasmid (9). Adaptive mutations nearly always almost result from a -1 fs in a run of consecutive bases (22, 103), and mutations of *dinB* result in loss of 50 to 80 percent of adaptive mutants (21, 81).

Another mutant that is deficient for adaptive mutagenesis is the stationary phase sigma factor, encoded by the *rpoS* gene (62). It was found that this is due in part to the fact that *rpoS* regulates *dinB* transcript levels, in addition to the regulation of *dinB* as part of the SOS response. Additionally it has been reported that *dinB* expression can become induced in the presence of β-lactam antibiotics, in an SOS independent manner (98). These observations underscore the notion that the regulation of DinB is genetically complex.

Recent studies have shed light onto native DinB function *in vivo* and suggest that DinB maybe conserved to replicate a class of ubiquitously occurring N^2-dG DNA adducts. Δ*dinB* strains of *E. coli* have been found to display increased sensitivity to the DNA damaging agents nitrofurazone (NFZ) and 4-NQO, which cause a significant fraction of N^2-dG lesions (40). Despite this marked sensitivity to both NFZ and 4-NQO, deletion of the $dinB^+$ gene does not reduce mutagenesis induced by either agent (40). These data suggest that the $dinB^+$ gene product is able to contend with DNA damage produced by at least some DNA damaging agents with comparable fidelity to other repair

processes available to the *E. coli* cell. Strikingly, it has been discovered that purified DinB is 15-fold more proficient at adding dC opposite N^2-furfuryl-dG, a structural analog of a lesion generated by nitrofurazone, than it is opposite undamaged DNA (40). This preference is conserved evolutionarily as DinB orthologue pol κ from *M. musculus* displays similar specificity for replicating N^2-furfuryl-dG containing templates (40). DinB bypass of an N^2-furfuryl-dG lesion is not only proficient but is also relatively accurate, with misinsertion frequencies between 10^{-3} and 10^{-5} (40). Furthermore, this preferential activity on certain damaged templates is governed by DinB's 'steric gate' residue (F13). Mutation of this residue to valine (F13V) completely eliminates DinB's ability to bypass an N^2-furfuryl-dG lesion (40). These data suggest that at least one reason for the DinB conservation throughout evolution is to catalyze TLS across from ubiquitously occurring N^2-dG adducts.

Regulation of TLS polymerases by protein-protein interactions

While TLS polymerases provide a variety of benefits to the cell, it is extremely important that their activity is properly regulated as they display reduced fidelity on undamaged DNA relative to replicative polymerases. In addition to transcriptional regulation through the SOS response, TLS polymerases are regulated by their interactions with other proteins. Genetic characterization over the past 30 years has underscored the importance of the *recA* and *umuD* gene products in regulation of *umuC*-dependent mutagenesis (23). Initial reports of UmuD'$_2$C polymerase activity invoked a requirement for UmuD'$_2$, RecA, ssDNA binding protein (SSB) and, in one case, various components of the polymerase III holoenzyme for UmuC activity (27). The demonstration of

polymerase activity of UmuD'$_2$C established UmuD'$_2$ as a subunit of DNA pol V (124). The RecA nucleoprotein filament has also been found to be important for efficient UmuD'$_2$C mediated lesion bypass. It was originally assumed that the RecA bound to the ssDNA template in this activating role, but it has now been proposed that stimulation of UmuD'$_2$C activity by the RecA-nucleoprotein filament occurs *in trans* (108). This has important implications for models of UmuD'$_2$C action given that the most proficient transactivating RecA nucleoprotein filament is one formed on gapped DNA. Recent studies have discovered that regulation by RecA and the *umuD* gene products extends to DinB as well as UmuC (see below) (29).

The mutagenic functions of both UmuD'$_2$C and DinB are also affected by interactions with the molecular chaperone GroEL and the β processivity clamp (16, 29, 76, 132). Mutations in *groE* are defective for *umuDC*-dependent UV induced mutagenesis (17) and also display reduced adaptive mutagenesis, only expressing approx. 1/10th the normal levels of DinB (63). Furthermore, it has been reported that overexpression of GroEL helps to increase the fitness of cells which have been accumulating mutations due to increased expression of DinB in *Salmonella* (75).

Interactions with replicative processivity clamps are crucial for regulating Y-family polymerase activity and dictating their access to DNA. Although they are characterized by low processivity on undamaged DNA, Y-family polymerases exhibit an increased processivity in the presence of the β clamp. Indeed, DinB processivity is enhanced 300 fold by the β clamp (132), whereas that of UmuC is stimulated between 5- and 100- fold (27, 76). In either case, the processivity enhancement as a result of β is far less than that of polymerase III (~10^5-fold) (53). Mutation or deletion of the β interaction

motif in either UmuC or DinB causes a loss of translesion synthesis *in vivo* (4). In eukaryotes, polymerase management is even more complex. The processivity clamp PCNA is subject to several different post-translational modifications that dictate its roles in replication, DNA repair and DNA damage tolerance mediated by Y-family DNA polymerases (44, 100). Specifically, the mono-ubiquinated form of PCNA is important for the recruitment of TLS polymerases to sites of damaged DNA (85). Moreover, the alternative processivity clamp in eukaryotes (Rad9-Rad1-Hus1) is important for modulating the activity of Y-family polymerases (100).

A recent study employing a DinB affinity column was used to search for potential DinB-interacting proteins within lysates of cells that constitutively express the SOS response. This study found that surprisingly, RecA, UmuD, and UmuD' physically interact with and regulate DinB function, in addition to their previously known roles in regulating UmuC. Moreover, these proteins are important for regulating DinB's -1 frameshift activity (29). Characterization of this complex has led to a model where RecA and UmuD may bind and enclose DinB's otherwise open active site. Extension of this study has identified the essential transcriptional modulator NusA as an additional interaction partner and will be discussed in greater detail in Chapter 2. The major focus of my work has been to characterize the interaction between DinB and NusA.

NusA is a general transcription elongation factor

nusA$^+$ function is indispensable for the viability of bacteria and was originally discovered for its requirement in λ phage development. Mutants of *nusA* (*nusA1*) inhibit λ phage development (24). This inhibition arises from the inability of mutant NusA

proteins to bind the phage λ protein N and effect antitermination of the λ genome, thereby allowing RNA polymerase (RNAP) to read through transcription terminators and express the late genes of λ phage. *nusA* was thus named for N utilization substance A. It has also recently been discovered that NusA forms a shield with λQ protein to protect the emerging transcript from termination mechanisms (113).

In *E. coli,* the *nusA* gene encodes an essential transcription factor associated with both termination and antitermination of transcription (12, 18, 30, 58, 70, 72, 109), furthermore *nusA* has been characterized as one of 206 genes making up the minimal bacterial gene set (28), and has orthologs present in archaea as well. Transcription by RNA polymerase proceeds in a series of steps, notably initiation, elongation and termination. In contrast to eukaryotes which possess three forms of RNAP, in bacterial systems, there is only one RNAP comprised of the β and β' catalytic subunits, two α and a ω subunit. Transcription is initiated with the help of a sigma (σ) subunit that recognizes and binds promoter elements in a sequence specific manner, of which there several in *E. coli*. NusA is a key modulator of RNAP that induces pausing of RNAP during the elongation and termination steps of transcription. Effects of NusA on transcription elongation have seemed contradictory and the molecular mechanism of RNAP pausing induced by NusA remains unclear and complicated. *In vitro*, NusA has been shown to slow down the rate of transcription elongation (59, 109, 115), and preferentially enhances pausing associated with nascent RNA hairpins (1, 18, 32, 141). NusA is required for the termination of the λt_{R2} transcript (32), attenuated leader regions of the *rrnB* and *his* operons (13, 51), and at the *trp* operon (18). In contrast or counterintuitively, roles for NusA also extend to the antitermination of transcription. Specifically, NusA is an

essential component of antitermination complexes that form on ribosomal RNA (*rrn*) and phage λ operons (77, 113, 126). NusA, then known as the L factor, was shown to be required for β-galactosidase expression *in vitro* and necessary for antitermination at a Rho sensitive terminator within the *lacZ* coding region (31). Furthermore, *in vivo* synthesis of β-galatosidase is reduced in the *nusA11* background (31), where the *nusA11* mutation is a conditional lethal allele that permits growth at 30°C but not at 42°C (87). Transcription termination occurs through either Rho-dependent or Rho-independent mechanisms. NusA has been shown to both inhibit (8, 59, 115) and stimulate (43) Rho-dependent termination.

NusA is an essential gene in *E. coli* and is conserved in even the smallest of bacterial genomes, however *nusA* function has been found to be dispensable in specific situations. Specifically, it has been reported that under altered Rho function, deletion of *nusA* results in viable cells (144). Though deletion of *nusA* in this background resulted in slow growth (120 minute doubling time) compared to the *rho* isogenic parent (40 minute doubling time), it was the first report of a viable *nusA* deletion strain. These results led to the hypothesis that the essential function of NusA was to enhance RNAP pausing, allowing for the coupling of transcription and translation (144). However, this theory does not explain why NusA is also essential in organisms that lack Rho. More recently it has been reported that *nusA* can be deleted in reduced genome background (10), in which 14 percent of the *E. coli* genome has been removed, by the targeted deletion of horizontally acquired DNA (101). Analysis of this mutant revealed that *nusA* function was required for the suppression of cryptic prophage, specifically the *rac* prophage, expression. This mutant also displays a slow growth phenotype. Together, these results

have specified conditions under which *nusA* function is no longer essential and identified additional alleles for the study of *nusA* and transcription elongation.

Association of NusA with RNA polymerase

The NusA protein has three separate domains: an N-terminal domain required for binding to RNA polymerase, an internal region with RNA binding motifs, and a C-terminal autoinhibitory domain. Details of the NusA-RNAP interaction such as where specifically NusA associates with RNAP and how many molecules of NusA are associated with elongating RNAP's have remained elusive. NusA has been found to compete with sigma factor (σ) for binding to RNAP (131), and NusA has been found to associate with the core RNAP, but not holoenzyme containing σ^{70} *in vitro* (32). Consistent with this finding, NusA has been found by ChIP-chip experiments to be uniformly associated with elongation complexes and was only found bound to RNAP after σ^{70} (the housekeeping sigma factor) dissociation, which occurs after the RNAP escapes from the promoter (86). Additionally crosslinking experiments have found NusA bound to the α (71), β (34, 71, 113, 127) and β' (128)subunits of RNAP, and it has been hypothesized that NusA binds near the RNA exit channel. While the role for $nusA^+$ in transcription has been appreciated for many years, my work has focused on uncovering a role for $nusA^+$ in the DNA repair/damage tolerance.

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Figure 1

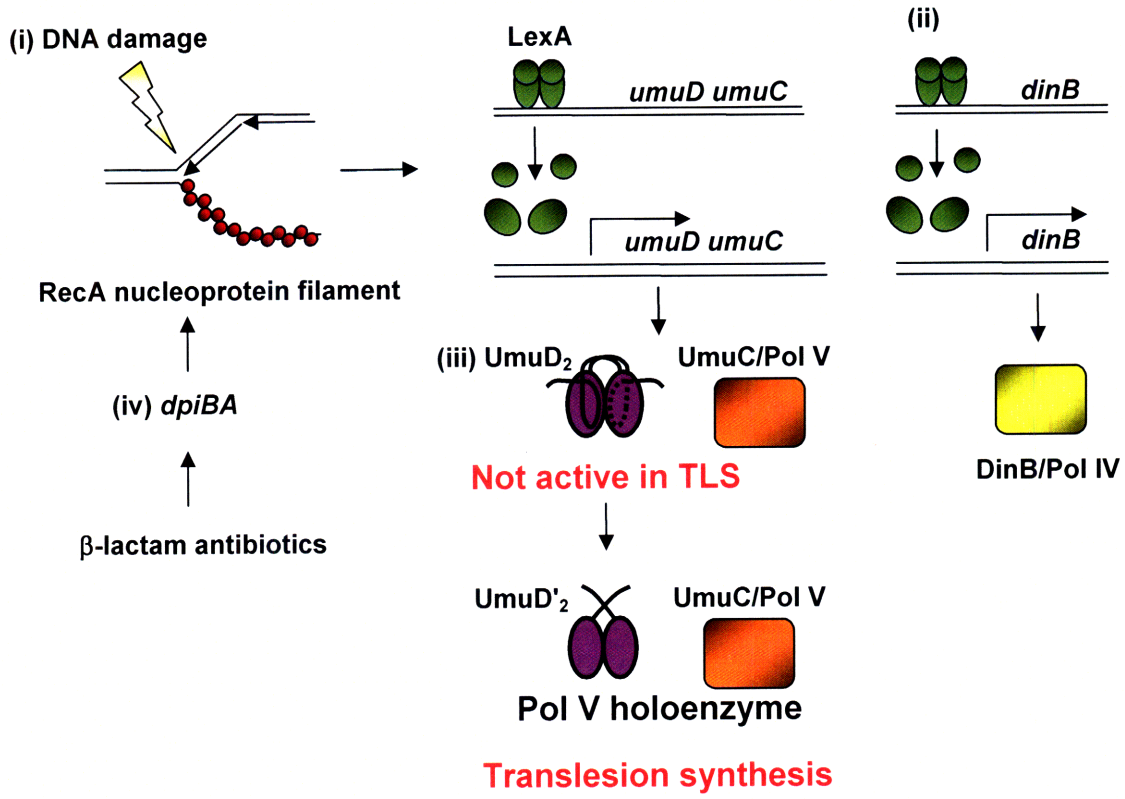

Figure 1. The SOS response to DNA damage. (i) The inducing signal for the SOS response forms when RecA polymerizes on a region of ssDNA, which is formed as a result of the failure to replicate damaged DNA. (ii) Binding to RecA-ssDNA nucleoprotein filament induces LexA to undergo autoproteolytic cleavage, which inactivates it as a transcriptional repressor and leads to induction of at least 50 genes, among which are those that encode the Y-family DNA polymerases UmuD'$_2$C and DinB. (iii) The cleavage of UmuD to UmuD' is also facilitated by the binding of UmuD$_2$ to the RecA-ssDNA nucleoprotein filament, which provides temporal regulation of the potentially mutagenic translesion synthesis activity of UmuC. (iv) Activation of the SOS response is also regulated by *dpiBA* and β-lactam antibiotics.

Figure 3

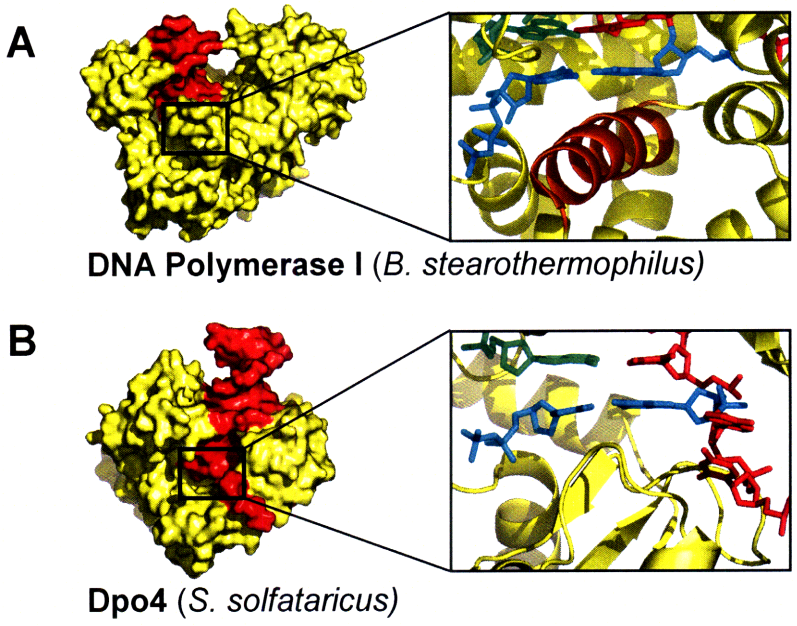

Figure 3. X-ray structures reveal key mechanistic details of TLS. (A) The structure of *Bacillus stearothermophilus* replicative DNA polymerase I in a closed conformation (48) shows numerous close protein (yellow) contacts with DNA (red). An α-helix (orange) performs a geometric check to ensure the fidelity of the incipient base pair (blue). (B) By contrast, the Y-family polymerase Dpo4 from *Sulfolobus solfataricus* (116) shows a loose grip on the DNA, a relatively open active site, and has no α-helix to check the geometry of the incipient base pair.

Figure 2

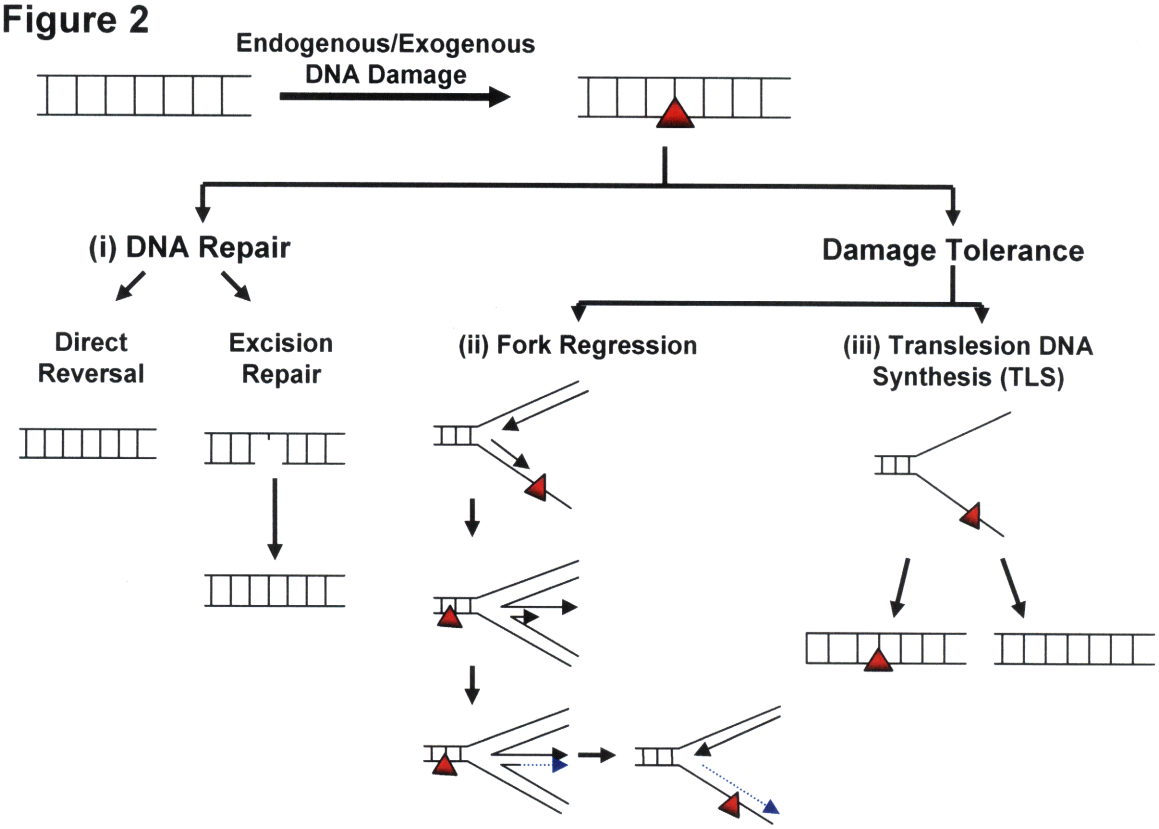

Figure 2. DNA repair vs. DNA damage tolerance. DNA damage can be generated by endogenous or exogenous sources. (i)The resulting DNA lesion, designated by the red triangle, can be removed through DNA repair mechanism of direct reversal or excision repair, resulting in undamaged DNA. (ii) Damage tolerance through fork regression. Replication stalled by a DNA lesion can migrate backward, re-annealing of the original templates allowing for the newly synthesized strands to anneal. DNA synthesis past the site of the lesion using the newly synthesized daughter strand as a template (shown in blue) and reverse regression allow for continuous DNA synthesis. (iii) DNA damage tolerance mechanism of translesion DNA synthesis, utilized a translesion DNA polymerase to replicate the damaged template.

Chapter 2

Transcriptional Modulator NusA Interacts with Translesion DNA Polymerases in *Escherichia coli*

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ABSTRACT

NusA a modulator of RNA polymerase, interacts with DNA polymerase, DinB. Increased expression of *dinB* or *umuDC* suppresses the temperature sensitivity of the *nusA11* strain, requiring the catalytic activities of these proteins. We propose NusA recruits TLS polymerases to RNA polymerases stalled at gaps, coupling translesion DNA synthesis to transcription.

INTRODUCTION

DNA is damaged from a variety of endogenous and exogenous sources, which can result in a variety of cellular problems, including cell death (12). All organisms have mechanisms of DNA repair and DNA damage tolerance to help them to survive DNA damage (12). One important mechanism of DNA damage tolerance is translesion DNA synthesis (TLS), a process in which a specialized DNA polymerase copies past DNA lesions that block the highly accurate, stringent replicative DNA polymerases. Although certain TLS polymerases can catalyze proficient DNA synthesis across from cognate lesions, they have reduced fidelity on undamaged templates (11, 16). TLS polymerases are conserved throughout all domains of life, with the majority being members of the Y-family of DNA polymerases (37). *Escherichia coli* has two Y-family DNA polymerases, DinB (Pol IV) and $UmuD'_2C$ (Pol V).

DinB (termed DNA pol kappa in eukaryotes) is the only Y-family polymerase found in all domains of life, yet despite its striking conservation, the role of DinB *in vivo* is still incompletely understood. *E. coli* DinB is known to be involved in the phenomenon of λ untargeted mutagenesis (4), adaptive mutagenesis (19), and when expressed at increased levels it causes an increase in -1 frameshift mutations (22). It was recently discovered that Δ*dinB* strains are sensitive to DNA damaging agents nitrofurazone and 4-nitroquinolone-1-oxide, and that DinB preferentially and accurately bypasses certain N^2-dG adducts (21, 24, 32, 51). This ability to preferentially bypass these N^2-dG adducts is conserved evolutionarily (21), suggesting a possible reason for the conservation across all domains of life. Additionally, DinB has been shown to incorporate oxidized nucleotides (50), possess lyase activity (42) and is suggested to be

involved in replication-arrest-stimulated recombination (29). Intriguingly mammalian pol kappa has been implicated in nucleotide excision repair (NER) and is proposed to function in the patching or gap filling step (36) . Given that improper access to DNA or misregulation by increased expression of TLS polymerases can be mutagenic under normal conditions, it is extremely important that TLS polymerases are properly regulated. In *E. coli, dinB* and *umuDC* are both transcriptionally induced as part of the SOS response to DNA damage (12). In addition, the activity of UmuC is controlled by an elaborate post-transcriptional regulatory process that includes the RecA mediated cleavage of its partner UmuD to UmuD' and interactions with the β processivity clamp and RecA (12, 31, 46). DinB also interacts with the β clamp and its activity has recently been shown to be controlled by the *umuD* gene products and RecA (14). Both DinB and UmuC also interact with the molecular chaperone GroEL (8, 14, 48).

RESULTS AND DISCUSSION

A DinB affinity column used to search for potential DinB-interacting proteins within lysates of cells that constitutively express the SOS response found that UmuD, UmuD' and RecA physically associate with DinB (14). Extension of this study (14) by binding purified recombinant $(His)_6$-HMK-DinB to a Ni^{2+} charged affinity resin to generate a DinB affinity column identified NusA as a potential interactor as determined by N-terminal sequencing (Figure 1A). However, identification of protein interaction partners by affinity methods can lead to a high frequency of false positive interactions (9, 38). Furthermore, confirmation of an interaction by other methods does not necessarily imply any relevance *in vivo*. Here we report that NusA, long known to be a RNA polymerase associated factor, physically interacts with DinB and that *nusA* genetically interacts with both *dinB* and *umuDC*. These unexpected findings suggest additional biological roles for NusA besides modulating RNA polymerase function.

NusA is an essential protein that functions in both termination and antitermination of transcription, and is thought to be associated with the RNA polymerase throughout the elongation and termination steps of transcription (6, 10, 17, 25, 26, 28, 40). Originally reported in 1974, NusA forms an antitermination complex with λN protein that is required for successful λ phage infection (13), and has been recently found to form a shield with λQ protein to protect the emerging transcript from termination mechanisms (41). NusA is highly conserved throughout bacterial and archaeal domains of life, however to date no eukaryotic sequence or functional homolog has been identified.

To test whether the interaction between DinB and NusA detected by affinity chromatography is direct or indirect, we performed a far-western experiment using cell lysates expressing NusA and NusA derivatives (30) (Figure 1B). As shown in Figure 1C, our observations indicate that DinB and NusA do indeed interact and that they do so directly. Interestingly, the C-terminal 263 amino acids of NusA, which seem to be especially important for the interaction with DinB, are also implicated in binding to RNA polymerase (30), implying that this region is involved in the binding of both RNA polymerase and DinB. We also used cellulose filter peptide arrays to search for peptides of NusA which might potentially interact with DinB. Filter peptide arrays containing 12 mer peptides of NusA each overlapped by two residues were probed with purified recombinant DinB (Figure 2A), or were performed without a DinB incubation step (Figure 2B). Interacting peptides were then mapped to a homology model of NusA based on the *Thermotoga maritima* crystal structure (43) (Figure 2C). Interestingly, we found that one potential DinB binding region of NusA encompasses several surface residues around the site of the temperature sensitive mutation of the *nusA11*(ts) allele (33). While some peptides found to potentially bind to DinB are located within the C-terminal 263 amino acids of NusA, consistent with the far-western results, others are found outside of this region. Further study will be required to define the exact details of how DinB and NusA interact, but it is possible that there are multiple contact sites since neither the far western approach nor the peptide array approach take into account the full tertiary structures of the proteins.

Nevertheless, the peptide array data led us to consider the possibility that elevated levels of DinB might stabilize the NusA11 protein, resulting in *dinB*$^+$ serving as a multicopy suppressor of the temperature sensitivity of a *nusA11* strain. We found that increased expression of DinB, from a low copy number plasmid under the *lac* promoter, indeed suppresses the temperature sensitivity of the *nusA11* strain and does so by approximately three orders of magnitude (Figure 3A).

Although this multicopy suppression could have resulted from DinB stabilization of the NusA11 protein, it could also have resulted from DinB functioning as a TLS DNA polymerase. To distinguish between these possibilities, we tested the ability of various *dinB* derivatives to serve as a multicopy suppressor of *nusA11*. Strikingly, we found that DinB requires its catalytic activity to act as a multicopy suppressor of the *nusA11* temperature sensitivity as shown by the failure a *nusA11* strain to grow at 42°C when harboring p*dinB*003, which encodes a catalytically inactive DinB (D103N). Furthermore p*dinB*Δβ, which encodes for a truncation mutation of DinB eliminating its β processivity motif (7) and p*dinB*(F13V), which encodes for a variant of DinB that can catalyze DNA synthesis but not across N^2-adducted-dG residues-*in vitro* (21), also fail to support growth of the *nusA11* strain at 42°C (Figure 3A). These observations indicate that the suppression of the temperature sensitivity of the *nusA11* strain by DinB, not only requires DinB's ability to catalyze DNA synthesis and to interact with the processivity clamp, but also its ability to perform translesion DNA synthesis. Western blotting experiments show that the various *dinB* mutants are expressed as well as *dinB*$^+$ (Figure 3E) and none of the above mentioned plasmids affect growth of a *nusA*$^+$ strain (Figure 3B). It is possible that

the interaction with DinB is also stabilizing the NusA11 protein but, if so, this stabilization is not sufficient to account for the multicopy suppression we have observed.

Our discovery that the TLS function of DinB is necessary for multicopy suppression of the *nusA11* temperature sensitivity prompted us to test whether *umuD*$^{+}$*C*$^{+}$ could also function as a multicopy suppressor. Using a plasmid with *umuD*$^{+}$*C*$^{+}$ under its native promoter with an operator constitutive mutation (o_1^c) on a medium copy number plasmid, pDC, we found that indeed elevated levels of the *umuDC* gene products increases the survival of the *nusA11* strain at 42°C by approximately 4 orders of magnitude (Figure 3C). The difference in suppression between *dinB* and *umuDC*, may reflect a qualitative difference, but it may also be explained at least in part by the differences in the vector and promoter used for expression. Neither of the *umuD* gene products alone, pD or pD', under the operator constitutive promoter (o_1^c), can support growth of the *nusA11* strain at 42°C indicating that UmuC function is required for multicopy suppression of the *nusA11* strain. Similar to the suppression by DinB, suppression of *nusA11* strains at 42°C requires UmuC's catalytic activity. This is shown by the facts that the temperature sensitivity of a *nusA11* strain cannot be suppressed by pDC(101), which encodes a catalytically inactive UmuC (D101N), nor by pDC(Δβ), which encodes for a variant of UmuC that is deficient for binding to the β clamp. It also requires the autoproteolytic activities of UmuD, shown by pD(S60A)C, which encodes for a non-cleavable UmuD variant. However to our surprise, pD'C, which directly expresses UmuD'C from the plasmid, does not support growth of the *nusA11* strain at 42°C, suggesting that both UmuD and UmuD' must be present along with UmuC for the multicopy suppression to occur. Interestingly UmuD and UmuD' form a heterodimer that

is considerably more stable than the $UmuD_2$ or $UmuD'_2$ homodimers (1), raising the possibility that the UmuD·UmuD' heterodimer may be required for the multicopy suppression by *umuDC*. Alternatively, the additional methionine used to initiate the translation of UmuD' in our pD'C construct may alter the ability for multicopy suppression. pDC(122), which encodes for a truncation of the UmuC protein that results in hydroxyurea resistance (15), also does not support growth at 42°C, implying that the requirements for hydroxyurea resistance are not the same for multicopy suppression (Figure 3C). Additionally, western blotting experiments show that the various mutant *umuDC* gene products are expressed at least as well a $umuD^+C^+$ (Figure 3E), and that expression of these variants does not alter growth of $nusA^+$ strains (Figure 3D).

In summary, we find that NusA, an essential *E. coli* protein, physically interacts with DNA polymerase DinB, in addition to its well known RNA polymerase contacts. We have shown that peptides implicated in DinB binding form distinct patches on the NusA surface including residues around the site of mutation of the *nusA11*(ts) allele. Furthermore elevated expression of $dinB^+$ or $umuD^+C^+$, results in multicopy suppression of the temperature sensitivity of the *nusA11*(ts) strain. For both of these translesion DNA polymerases, this multicopy suppression requires their catalytic activities, as well as their ability to bind to the β clamp.

Taken together, our results suggest the existence of a previously unsuspected cellular process involving physical and genetic interactions between an important RNA polymerase modulator and translesion DNA polymerases. Furthermore, the fact that the lethality of a *nusA11* mutant at 42°C can be suppressed by elevating the expression of either of two translesion DNA polymerase implies a hitherto unrecognized role for NusA

in DNA repair and/or DNA damage tolerance. Such a role for NusA might be an additional reason that the *nusA* gene is present in all bacteria and archaea.

What type of molecular mechanism could account for these unanticipated results? We propose that, by binding to DinB or some complex involving UmuC and the *umuD* gene products, NusA can couple the process of transcription to the process of translesion synthesis to enable transcription-coupled translesion synthesis (TC-TLS), in a manner analogous to the coupling of transcription to nucleotide excision repair during transcription-coupled repair (TCR). In principle, a process of TC-TLS could be helpful when transcription becomes stalled by gaps in the transcribed strand that are opposite lesions in the non transcribed strand. Such gaps can be caused by lesions that cannot be bypassed by the replicative DNA polymerase. On the lagging strand, gaps are generated by replication resuming at the site of the next Okazaki fragment, while replication re-start can generate similar gaps on the leading strand; gaps formed in this manner are estimated to average about 1,000 nucleotides in length (18, 20, 23, 39). Alternatively, gaps opposite lesions could also be formed by UvrABC-dependent nucleotide excision repair if two lesions are very close together but on opposing strands or by UvrABC-dependent incisions during repair of an intrastrand crosslink (12). In these latter two cases, the gaps would be smaller, 12-13 nucleotides (12). DinB has recently been shown to be capable of accurately filling the gaps that could be generated during repair of N^2,N^2 guanine intrastrand crosslinks (24), an observation that might help rationalize the involvement of DNA pol Kappa in mammalian nucleotide excision repair (36).

We hypothesize that, if an RNA polymerase encounters one of these gaps in the transcribed strand opposite a lesion, it would stall. In this case NusA, which is associated with the RNA polymerase throughout the elongation phase of transcription, might then recruit a TLS polymerase to fill in the gap in the template strand (Figure 4). Repairing the gap would permit transcription of the gene by subsequent RNA polymerases, possibly even by the original RNA polymerase if it is retained during process as in TCR. TC-TLS would provide a way of prioritizing the use of the cells translesion DNA synthesis resources to benefit maximally transcription, the same way TCR prioritizes nucleotide excision repair resources to maximally benefit transcription.

The mechanism of TC-TLS we are proposing is unrelated to the phenomenon of template strand gap bypass that has been characterized previously (27, 52). It has been shown that two phage RNA polymerases and *E. coli* RNA polymerase are capable of bypassing a small gap in the transcribed strand, thereby generating faithfully transcribed, but internally deleted, mRNAs that would be non functional in most cases. Although T7 RNA polymerase can bypass a template strand gap of up to 24 nucleotides, *E. coli* RNA polymerase only inefficiently bypasses a one nucleotide gap and generates the equivalent of a –1 frameshift mutation in the process (27, 52). Many of the gaps we are considering would be too big to bypass in this fashion and, the mechanism we are proposing would usually result in the production of biologically functional mRNAs.

If a process of transcription-coupled translesion synthesis does exists, and if it is universal as most DNA repair and DNA damage tolerance processes tend to be, it might be of particular importance in mammals where some mRNAs can take many hours to transcribe (47) so that the consequences of encountering a template gap late in the

transcriptional process would be severe. Alternatively NusA may be important in recruitment of DinB to serve as the polymerase in the patching step of NER that takes place during transcription coupled repair, as DNA pol Kappa has been implicated in eukaryotes (36).

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MATERIALS AND METHODS

Affinity Chromatography

IMAC (Immobilized Metal Ion Chromatography) where purified recombinant $(His)_6$-HMK-DinB was bound to a Ni^{2+} charged affinity resin using conditions and reagents as recommended by QIAGEN. Recombinant $(His)_6$-HMK-DinB was purified as previously described (3).

Farwestern assays and peptide array experiments

Farwestern blots were performed as previously described (14, 45). Briefly, BL21 (DE3) cell lysates expressing NusA and NusA derivatives (30) (Table 1) were separated by SDS-PAGE, transferred to a PVDF membrane and probed with ^{32}P labeled $(His)_6$-HMK-DinB. Cellulose filter peptide array was synthesized with overlapping 12-mer peptides scanning the primary sequence of NusA, each peptide offset by 2 residues from the previous (MIT CCR Core Facility) probed with 150nM purified recombinant DinB or without a DinB incubation step and developed with an anti-DinB antibody as described in (34). Recombinant DinB was purified as previously described (3).

Multicopy suppression assays

nusA11 cells (SEC29) or *nusA*$^+$ cells (P90C) harboring plasmids containing *dinB*$^+$ and *dinB* derivates (Table 1) were grown to saturation in LB plus ampicillin (100 μg/mL) at 30°C and diluted (1:100) into LB supplemented with ampicillin and IPTG (1mM) and again allowed to reach saturation. The cultures were then diluted in M9 salts and plated onto LB agar plates, supplemented with ampicillin and IPTG, pre-heated to either 30°C or 42°C and incubated at the respective temperature, CFU/mL were then scored on plates growing at 30°C and 42°C. Survival of *nusA11* strains at 30°C vs. 42°C harboring

plasmids. *umuDC* suppression assays were performed as for the *dinB* suppression assays except IPTG was omitted.

Western Blots

nusA11 cells harboring *dinB* plasmids were grown as they would for multicopy suppression assays except cells were harvested, lysed with lysozyme, and treated with deoxyribonuclease I (Sigma). 15μg total protein of each lysate was loaded onto 4-20% SDS-polyacrylamide gel. After electrophoresis proteins were transferred to a PVDF membrane and probed with an anti-DinB antibody, with western conditions as described in (2) except with the addition of a high salt (0.5M NaCl) wash after incubation with the secondary antibody. Antibodies against UmuD and UmuC can only detect plasmid borne protein levels after SOS induction. Thus expression of *umuDC* under the conditions used for *nusA11* multicopy suppression cannot be detected by western blotting. In order to check that the different mutants used were stably expressed as compared to the wild type, AB1157 cells harboring *umuDC* plasmids were irradiated with UV to induce the SOS response, western blots were then performed as described in (2).

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Figure 1

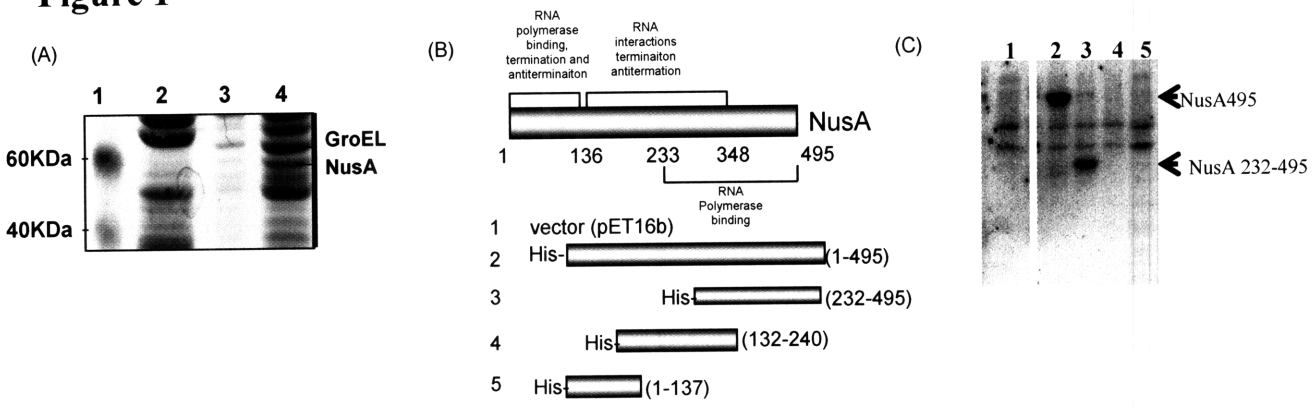

Figure 1. DinB physically interacts with NusA.

(A) Coomassie stained SDS-PAGE gel showing several steps of traditional IMAC (Immobilized Metal Ion Chromatography) where purified recombinant $(His)_6$-HMK-DinB was bound to a Ni^{2+} charged affinity resin using conditions and reagents as recommended by QIAGEN. Recombinant $(His)_6$-HMK-DinB was purified as previously described (3). Lane 1- Molecular weight markers. Lane 2- Non specific binding to resin. Lane 3- Washes with 20mM imidazole. Lane 4- DinB affinity column eluate, eluted with 300mM imidazole. Interacting proteins NusA and GroEL were identified by Edman degradation (MIT CCR Core Facility).

(B) Schematic of NusA constructs used for far western experiment, adapted from (30).

(C) Far Western Blot demonstrates that the interaction between DinB and NusA is direct. BL21 (DE3) cell lysates expressing NusA and NusA derivatives (30) (Table 1) were separated by SDS-PAGE, transferred to a PVDF membrane and probed with ^{32}P labeled $(His)_6$-HMK-DinB as previously described (14, 45). Lane 1- vector (pET16b), Lane 2- pNusA(1-495), Lane 3- pNusA(232-495), Lane - pNusA(132-240), and Lane 5- pNusA(1-137).

Figure 2

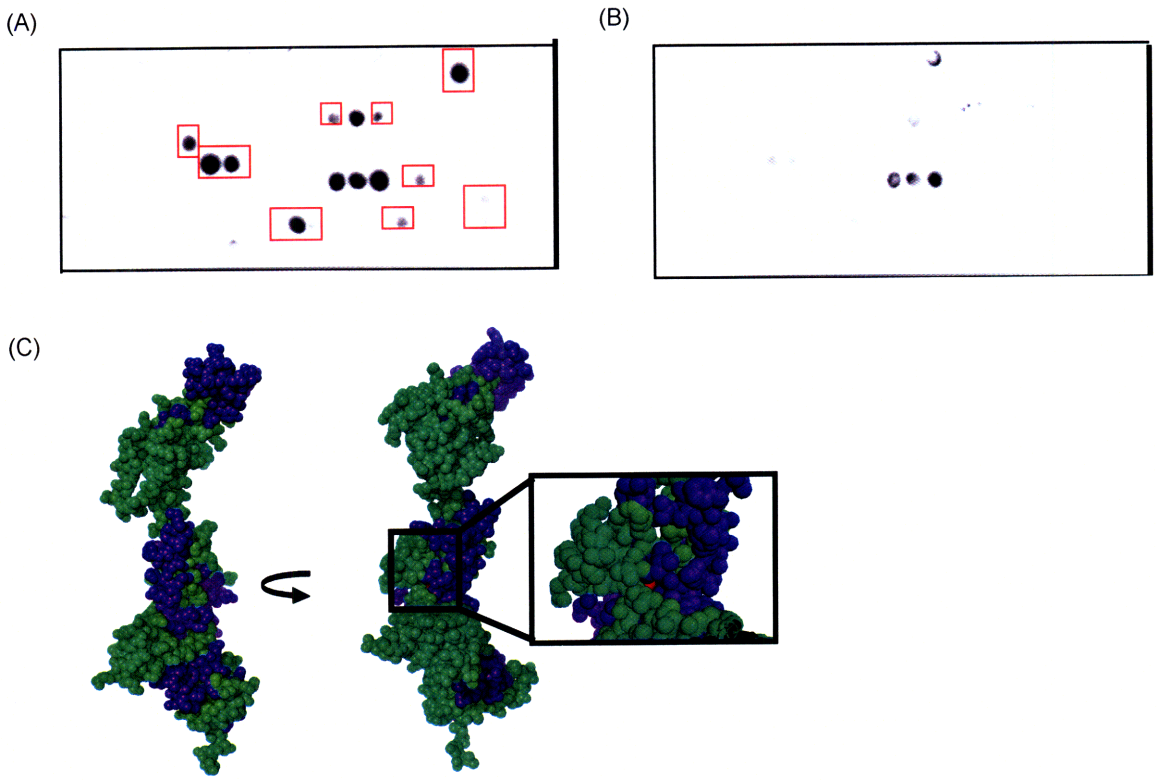

Figure 2. Peptides of NusA which bind to DinB encompass the site of *nusA11* temperature sensitive mutation.

(A) Depicts a 1 hour exposure of a cellulose filter peptide array consisting of 12-mer peptides scanning the primary sequence of NusA, each peptide offset by 2 residues from the previous (MIT CCR Core Facility) probed with 150nM purified recombinant DinB and developed with an anti-DinB antibody as described in (34). Recombinant DinB was purified as previously described (3). Red boxes highlight peptides that interact with DinB and were mapped onto homology model in (C).

(B) Depicts a 1 hour and 15 minute exposure of a control peptide array which was performed as in (A) except without a DinB incubation step.

(C) Mapping of interacting peptides onto a homology model of NusA. NusA residues shown in green, interacting peptides are shown in blue and temperature sensitive mutation of the *nusA11* allele, shown in red. NusA homology model, constructed with SWISS-MODEL, based on crystal structure of full length NusA from *Thermotoga maritime*, with the N terminus of NusA at the top.

Figure 3

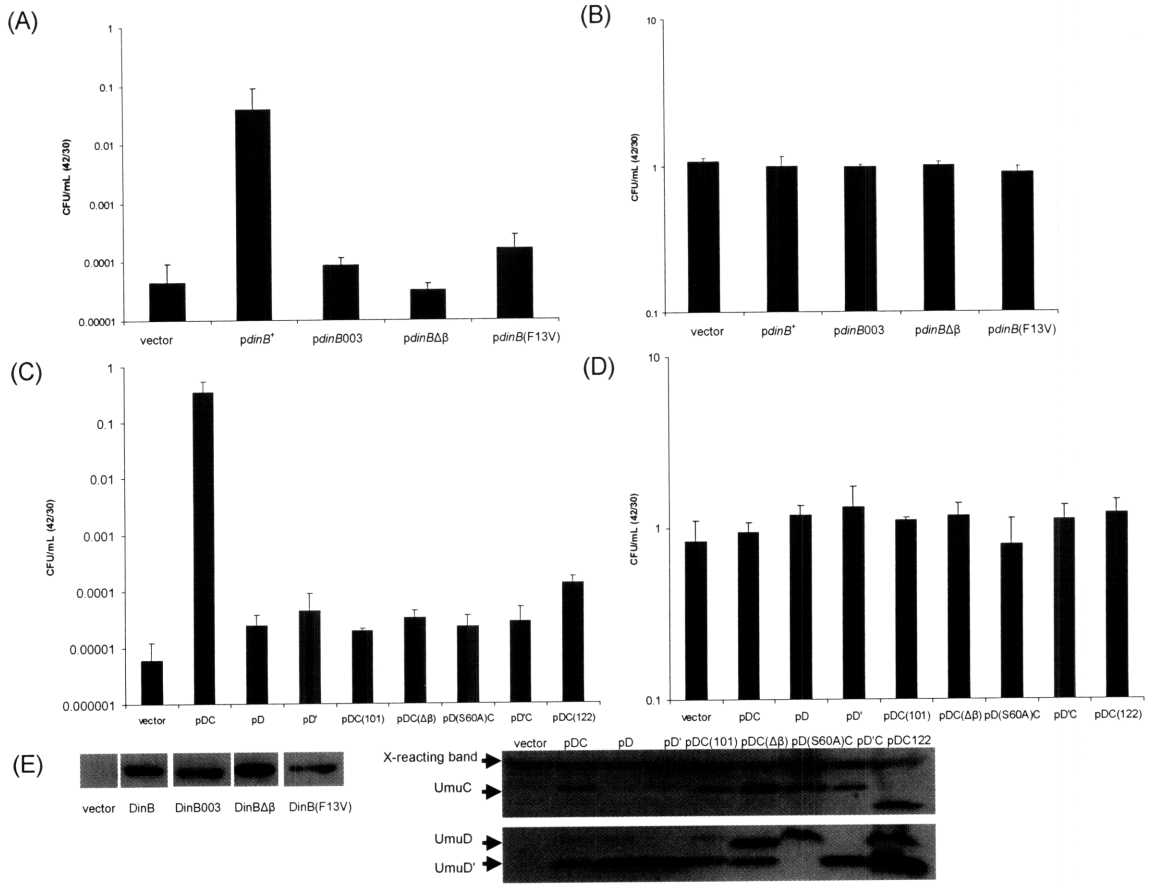

Figure 3. DinB or *umuDC* act as multicopy suppressors of the *nusA11* temperature sensitivity.

(A) Survival of *nusA11* strains at 30°C vs. 42°C harboring plasmids. pWSK29 the empty vector, p*dinB*$^+$, p*dinB*003, which encodes a catalytically inactive DinB, p*dinB*Δβ, encodes for a truncation mutation of DinB eliminating its β processivity motif, and p*dinB*(F13V), which encodes for a variant of DinB which can catalyze DNA synthesis but not across N_2-adducted-dG residues (*in vitro*).

(B) Survival of *nusA*$^+$ strains (P90C) at 30°C vs. 42°C harboring the same set of plasmids as in (A), show that these plasmids do not confer any growth phenotypes in a wild type background.

(C) Survival of *nusA11* strains at 30°C vs. 42°C harboring plasmids. pBR322 (empty vector), pDC_{oc} (p*umuD*$^+$*C*$^+$, operator constitutive promoter), pD, expresses only *umuD*, and pD', expresses only UmuD', pDC(101), which encodes a catalytically inactive UmuC, pDC(Δβ), encodes for a variant of UmuC with the β binding motif mutated to alanines, pD(S60A)C, which encodes for a non-cleavable UmuD variant, $pD'C_{oc}$, which contains UmuD'C under the operator constitutive promoter, and pDC(122), which encodes for a truncated variant of UmuC.

(D) Survival of *nusA*$^+$ strains at 30°C vs. 42°C harboring the same set of plasmids as in (C), show that these plasmids do not confer any growth phenotypes in a wild type background.

(E) Western blots demonstrate that *dinB* and *umuDC* derivatives are stably expressed as well as *dinB*$^+$ or *umuD*$^+$*C*$^+$.

Figure 4

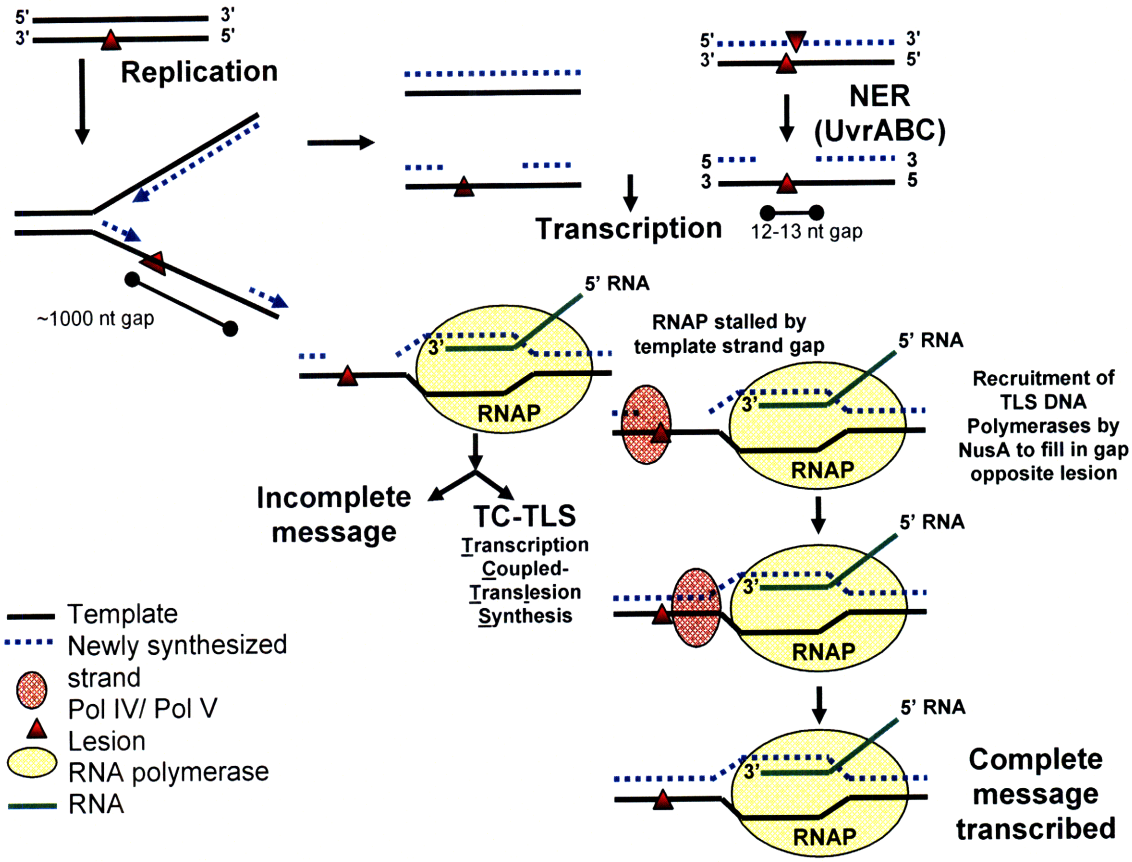

Figure 4. Proposed model of transcription coupled translesion synthesis (TC-TLS). Model of transcription coupled translesion synthesis (TC-TLS) is as described in text. Briefly we propose that an RNA polymerase stalled by a gap in the template strand opposite a DNA lesion on the non transcribed strand, could recruit TLS polymerases through NusA to fill in the gap opposite the lesion to allow for the continuation of transcription.

Table 1. Strains and Plasmids.

Strains	*Genotype*	*Reference*
P90C	Δ(*lac-pro*)$_{XII}$ *ara gal*	(5)
IQ419	*zha-132*::Tn10 *arg rpsL257*	CGSC
YN2351	*metB trpE9829*(am) *tyr*(am) *sup*-126 *nusA11*	(33)
SEC26	Same as YN2351 except *zha-132*::Tn10, constructed by P1*vir* transduction (IQ419 x YN2351)	This work
SEC29	Same as P90C except *nusA11 zha0132*::Tn10	This work
BL21 (DE3)		Novagen
AB1157	*thr-1 leuB6 proA2 hisG4 thi1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*	Lab Stock

Plasmids		
pWSK29	Vector, pSC101-like replicon, Amp^R	(21)
pYG782 (p*dinB*$^+$)	*dinB* gene cloned in the pWSK30 plasmid (Amp^R) which is the same as pWSK29 but with the MCS cloned in the opposite orientation under the lac promoter	(22)
pd*inB*003	Same as pYG782, except *dinB* gene encoding D103N mutation	(49)
p*dinB*Δβ	Same as pYG782, except *dinB* gene encoding truncation mutant to delete C terminal residues, deleting the β binding motif, premature stop codon inserted using site directed mutagenesis kit by Stratagene.	This work
p*dinB*(F13V)	Same as pYG782, except *dinB* gene encoding F13V mutation	(21)
pBR322	Vector, Amp^R	New England Biolabs
pDCoc	*umuDC* cloned into pBR322 under its operator constitutive promoter o_1^c	(2)
pDC(101)	Same as pDCoc except that it encodes a catalytically inactive UmuC (D101N), constructed using site directed mutagenesis kit by Stratagene.	This work
pDC(Δβ)	Same as pDCoc except UmuCβ[1] motif, residues 357-361, are mutated to alanine	(2)
pD'Coc	UmuD'C cloned into pBR322 under the	This work

	operator constitutive promoter o_1^c. Constructed by subcloning from pGY9738 (44).	
pDC(122)	Same as pDCoc expect stop codons, in all three reading frames using site directed mutagenesis kit from Stratagene, were inserted to correspond to the UmuC122 truncation, lacking the last 102 residues.	This work
pD(S60A)C	Same as pDCoc except *umuD* gene encoding S60A mutation, constructed using the site directed mutagenesis kit from Stratagene.	This work
pGW2020 (pUmuD)	*umuD* cloned into pBR322 under the operator constitutive promoter o_1^c	(35)
pGW2122 (pUmuD')	UmuD' cloned into pBR322 under the operator constitutive promoter o_1^c	(35)
pET16b	Vector, pBR322 origin of replication Amp^R, similar to pET11d except contains His_6	Novagen
pNusA(1-495)	N-terminal His_6-tagged full length NusA cloned into pET11d	(30)
pNusA(232-495)	N-terminal His_6-tagged amino acids 232-495 of NusA cloned into pET11d	(30)
pNusA(132-240)	N-terminal His_6-tagged amino acids 132-240 of NusA cloned into pET11d	(30)
pNusA(1-137)	N-terminal His_6-tagged amino acids 1-137 of NusA cloned into pET11d	(30)

Chapter 3

The transcriptional modulator NusA is essential for adaptive mutagenesis in *Escherichia coli*

This chapter has been submitted for publication as: Susan E. Cohen and Graham C. Walker. The transcriptional modulator NusA is essential for adaptive mutagenesis in *Escherichia coli*.

ABSTRACT

Adaptive mutagenesis describes the accumulation of mutations that occur in non-growing cells over time. In *Escherichia coli*, a *lacI33-lacZ* fusion reporter is used to monitor the appearance of adaptive mutants from a Lac^- to a Lac^+ phenotype. While many cellular processes are implicated in the adaptive response, loss of DinB (DNA pol IV) function results in loss of 50 to 85 percent of adaptive mutants. I have previously reported that the essential RNA polymerase modulator NusA, physically and genetically interacts with DinB. Here I report my unexpected observation that a function altered in the *nusA11*(ts) allele, at the permissive temperature, eliminates the formation of adaptive mutants. I present evidence that this reduction in adaptive mutation is not due to a defect in transcribing *lacI33-lacZ*, but rather an inability to adapt and mutate in response to environmental stress. Furthermore, I have extended a previously existing reporter used to monitor the appearance of unselected mutations to be able to monitor the formation of adaptive mutants in response to antibiotic treatment. My results are the first to implicate a specific function of NusA, a key component of elongating RNA polymerases, in adaptive mutagenesis.

INTRODUCTION

Adaptive mutagenesis describes the accumulation of mutations that occur in non-growing cells, and is also referred to as stress-induced or stationary phase mutagenesis. 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 with a *lacI33-lacZ* fusion with a +1 frameshift in a run of G:C base pairs (2). 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 (2). The reversion of this mutant from a Lac^- to Lac^+ phenotype has been found to occur by both adaptive mutation and adaptive amplification (1, 21, 23, 24). Furthermore, it has been found that adaptive Lac^+ mutations nearly almost result from a -1 frameshift mutation in a run of consecutive bases (8, 38). While the mechanisms underlying adaptive mutation and amplification remain unclear, a variety of cellular factors have been implicated, such as DNA recombination functions (9, 19, 20, 22) and induction of the SOS response (2, 31, 32). The *dinB*$^+$ gene product, DNA Pol IV, is also important for -1 frameshift mutations. The analysis of *dinB*'s role 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 loss of 50 to 85 percent of the -1 frameshift mutations in the *lacI33-lacZ* fusion that lead to a Lac^+ phenotype (6, 32).

DinB, also known as Pol IV in *E. coli* (pol Kappa is its ortholog in eukaryotes), is a translesion DNA polymerase which is induced as part of the SOS regulon in response to DNA damage (11, 26, 36). Translesion DNA polymerases are a specialized class of DNA polymerases that are capable of replicating imperfect or damaged templates in a process

termed translesion DNA synthesis (TLS) (11). While TLS polymerases provide a variety of benefits to the cell, it is extremely important that they are properly regulated as their fidelity on undamaged DNA is lower than that of their replicative counterparts (10, 14, 16). DinB in both prokaryotes and eukaryotes is proficient for the formation of -1 frameshift mutations, and has been found to be responsible for both adaptive -1 frameshift mutations as well as for -1 frameshift mutations that occur during exponential growth (27, 32, 35). Furthermore, it has recently been found that *dinB*$^{+}$ is the only SOS regulated gene required at induced levels for adaptive mutagenesis (12).

I have previously reported that DinB physically and genetically interacts with the essential transcriptional modulator NusA (4). NusA associates with RNA polymerase (RNAP) throughout the elongation and termination steps of transcription, where it functions as both a terminator and antiterminator (3, 5, 17, 28-30, 39). Here I report that a specific function of NusA modified by the *nusA11* temperature sensitive mutation at the permissive temperature is essential for the formation of adaptive mutations. The deficiency of a *nusA11*mutant for adaptive mutagenesis was observed both in the starvation conditions using the *lacI33-lacZ* reporter as well, as in a second system based on the reversion of a +1 frameshift *tetA* allele. I propose that NusA, in addition to its well established roles in transcription, plays a previously unrecognized role in the phenomenon of stress induced mutagenesis. My results are the first to implicate a specific function of NusA, a key component of the elongating RNA polymerase, in the phenomenology underlying adaptive mutagenesis.

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 my recent data suggest that NusA may also be involved in the process of DNA damage repair/tolerance. I previously reported that the temperature sensitivity of the *nusA11*(ts) allele (34) can be suppressed by overexpression of the TLS polymerase DinB, in a manner that requires the catalytic capabilities of DinB (4). Intrigued by these observations, I looked to see if the DinB-dependent phenomenon of adaptive mutagenesis was altered in a *nusA11* background.

Using the *lacI33-lacZ* fusion system described above, I found that FC40 cells carrying the *nusA11* mutation are incapable of adaptive mutation to Lac$^+$ at the permissive temperature (30°C) (Figure 1A and B). This reduction in adaptive 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* (Figure 1A and B). These results suggest that the role of *nusA*$^+$ in adaptive mutagenesis may extend beyond an interaction with DinB and the formation of -1 frameshift mutations. Agar plugs taken from plates on day 7 contained roughly equal amounts of Lac$^-$ bacteria for the *nusA*$^+$ and *nusA11* strains. This observation indicates that the *nusA11* cells were not dying upon incubation on minimal lactose plates, but rather were incapable of mutating (Figure 1C).

One caveat to our results above is that NusA is known to be the L factor required for β-galactosidase transcription *in vitro (18)*. At the permissive temperature, the *nusA11* mutation is reported to cause a 40 percent reduction in β-galactosidase activity *in vivo*

(34), due to a Rho-dependent termination signal within the *lacZ* coding region. Could this defect in *lacZ* expression explain the elimination in adaptive mutagenesis we are seeing? To test this I isolated adaptive mutants that arose from the wild type FC40 strain (FC40 Lac^+), introduced the *nusA11* mutation, and then monitored β-galactosidase activity as well as the ability to form colonies on lactose medium at 30°C. Although *nusA11* strains showed an approximately 40 percent reduction in β-galactosidase activity from the episomal *lacI33-lacZ* reporter compared its wild type parent (Figure 2A), colony formation on minimal lactose medium was unaltered, matching colony formation on glucose medium (Figure 2B). These results suggest that, even under the conditions of modestly reduced β-galactosidase activity in a *nusA11* strain, there remains sufficient β-galactosidase function to grow when lactose is the only carbon source. Thus, the elimination of adaptive mutagenesis seen in the *nusA11* strain is not likely due to a defect in *lacZ* expression, but rather in an inability to adapt and mutate in response to environmental stress.

Although the above observations suggest that loss of adaptive mutagenesis in a *nusA11* background is not due to improper expression of the *lacI33-lacZ* fusion, I wanted to see if this observation would repeat in another context. In an effort to accomplish this, I thought I might take advantage of a pre-existing reporter that has been previously used to monitor unselected mutations in adaptive 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) (7). Tetracycline is a bacteriostatic antibiotic which functions through inhibition of the 30S ribosome (15). Tetracycline resistance (Tet^R) as measured from a

chromosomal Tn*10* was not altered 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 3A). 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. Adaptive mutagenesis is defined as mutations that arise in non-growing bacteria held under non-lethal selection, allowing for growth (40). Thus I 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, I observed Tet^R colonies appearing over a period of several days, an observation reminiscent of the appearance of Lac$^+$ colonies over several days in standard *lacI33-lacZ* adaptive mutagenesis (Figure 3B). Although the total number of mutants that are produced is lower when compared FC40 strains plated on lactose minimal medium, mutations occur with similar kinetics. The genetic requirements for adaptive mutations that occur after tetracycline treatment are similar to those in response starvation in that they require *dinB*$^+$ and the recombination functions of *ruvC*$^+$ (9) (Figure 3B). Furthermore, adaptive hypermutability after tetracycline treatment can be achieved through mutation of *recG* (Figure 3C), as occurs in the Lac system (9, 19). *nusA*$^+$ function is also essential for tetracycline adaptive mutagenesis, as the *nusA11* mutation eliminates the formation of adaptive mutants (Figure 3B). These data further support the notion that defect in adaptive 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. The role of adaptive amplification in adaptive mutagenesis experiments is not manifested until later time points, usually day 7, and thus not likely a key factor within the time frame of our experiments. 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 (37), implying that adaptive mutagenesis in response to bacteriostatic antibiotic treatment may be a general phenomenon.

In summary, I have discovered a previously unrecognized role for the essential gene *nusA*$^+$ in the process of adaptive mutagenesis. Specifically a function of *nusA* altered by the *nusA11*(ts) mutation at the permissive temperature is critical for the formation of adaptive mutations. Since the *nusA11* cells are viable at 30°C, the function of NusA altered by the mutation is genetically separable from the essential roles of NusA in normal RNAP elongation and termination/antitermination. Our finding that the reduction in adaptive 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 adaptive mutagenesis that extends beyond an interaction with DinB. Though future experiments will be required to elucidate what this role may be, I propose that NusA is required to link transcription to adaptive mutation. Specifically, I suggest that the process of adaptive mutagenesis is initiated by the stalling of elongating RNA polymerases by an irregularity in the DNA. For example, under the conditions of glucose starvation used in adaptive mutagenesis experiments, 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. Alternatively, a deficiency in DNA repair under stressed conditions would result in lesions being present in the transcribed strand or in the generation of gaps opposite lesions when DNA replication is attempted. If this were the case, the function lost by the *nusA11* mutation might be the ability to recruit the other cellular factors, including DinB, required for adaptive mutagenesis to the stalled RNA polymerase. The process of transcription-coupled translesion synthesis we have hypothesized (4) might therefore contribute to the generation of adaptive mutations.

MATERIALS AND METHODS

Mutagenesis Assays

All strains used are listed in supplemental Table I and constructed using standard techniques. Lactose adaptive mutagenesis assays were performed as previously described, except all incubation steps were performed at 30°C (13). Tetracycline adaptive 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 (12mg/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.

β-galactosidase activity assays

Strains were grown at 30°C in minimal M9 lactose (0.2%) medium and β-galactosidase activity assays were performed as previously described (33).

Determination of *nusA11* effects on reporter genes

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. CFU/mL formation on minimal glucose and minimal lactose was determined by growing cultures in M9 minimal glucose (0.2%) media to saturation, cultures were then diluted in M9 salts and plated onto minimal glucose (0.2%) or minimal lactose (0.2%) plates, incubated at 30° C.

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Figure 1

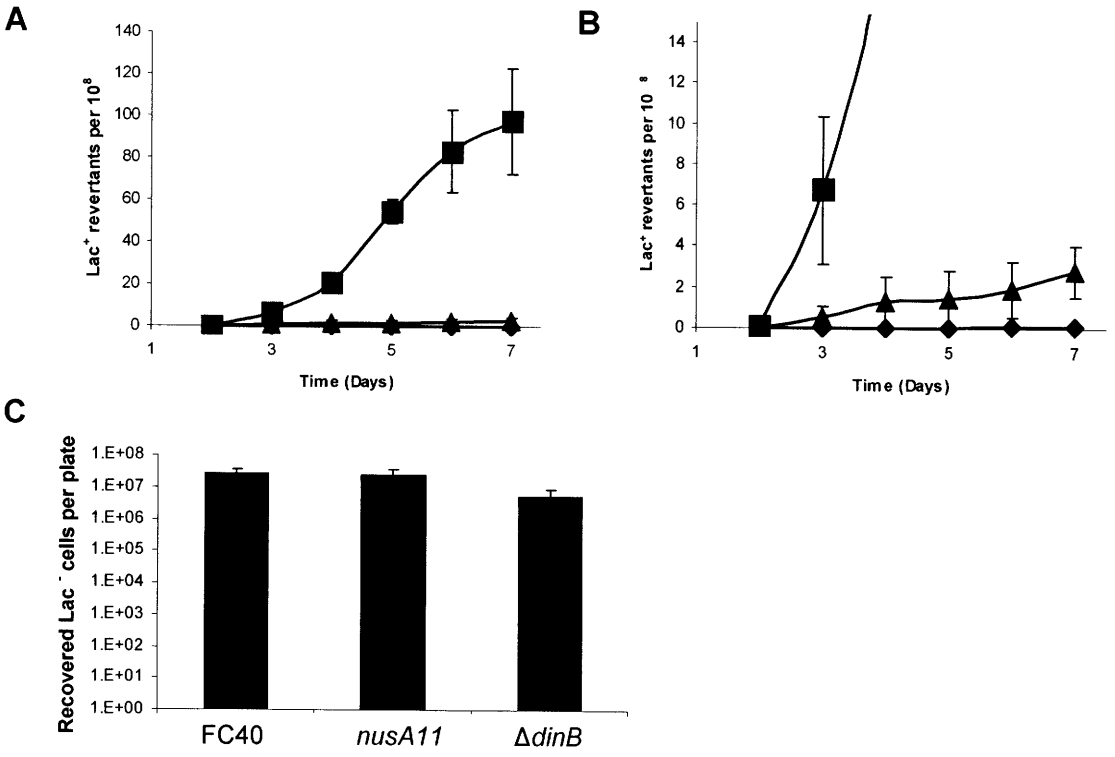

Figure 1. Adaptive mutation in response to starvation A) *nusA11* strains are defective for adaptive mutagenesis. Wild-type FC40 (squares), *ΔdinB* (SEC1414) (triangles) deleted on both chromosome and episome, and *nusA11* (SEC182) (diamonds). B) Magnification of A). C) Recovery of viable Lac$^-$ cells from agar plugs on day 7 reveals that roughly equal number of bacteria were recovered from all strains. Error bars represent the standard deviation from three independent experiments.

Figure 2

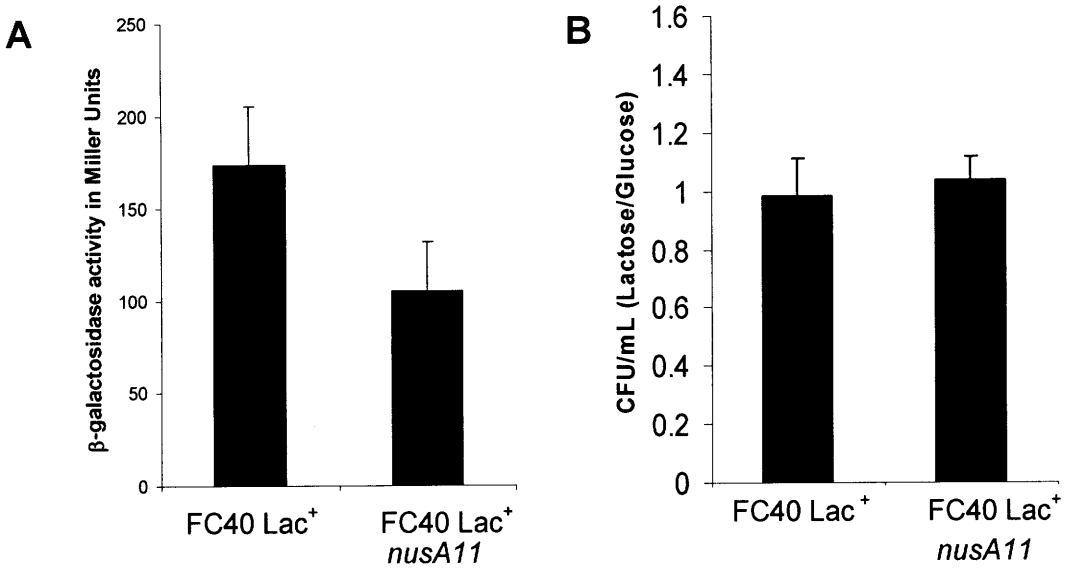

Figure 2. β-galactosidase activity and growth on lactose medium of *nusA11* strains at 30°C. A) FC40 Lac$^+$ *nusA11* (SEC1429) strains show an approximately 40 percent reduction in β-galactosidase activity as compared to FC40 Lac$^+$ *nusA*$^+$ (SEC1419). B) FC40 Lac$^+$ *nusA11* cells do not show altered ability to form colonies on minimal lactose medium compared to FC40 Lac$^+$ *nusA*$^+$ determined by CFU/mL on lactose divided by CFU/mL on glucose. Error bars represent the standard deviation determined from three independent experiments.

Figure 3

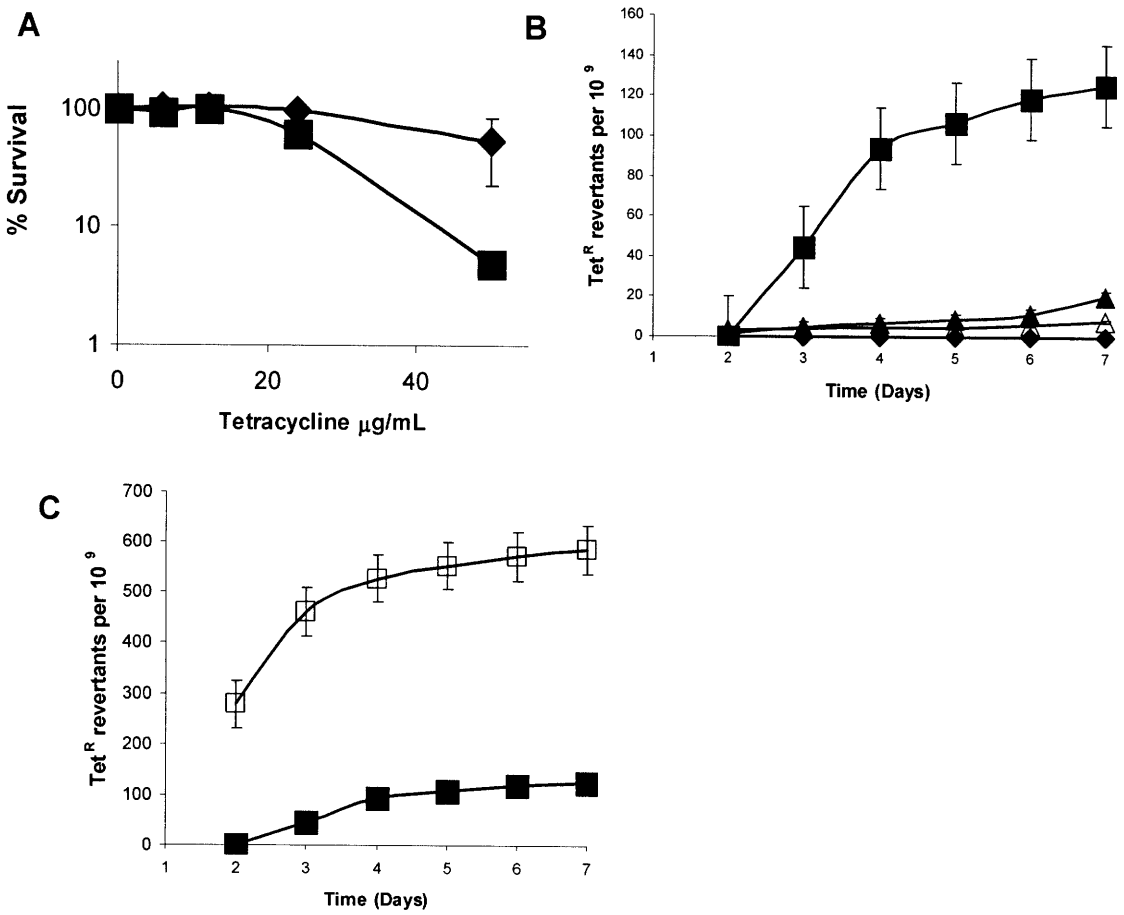

Figure 3. Adaptive mutagenesis in response to tetracycline. A) Tetracycline resistance from chromosomal Tn*10*. Percent survival of *nusA*$^+$ (SEC527) (squares) and *nusA11* (SEC29) (diamonds) strains to tetracycline (0-50μg/mL) shows that *nusA11* strains are not altered in their ability to express tetracycline resistance. Error bars represent the standard deviation determined from three independent experiments. B) Adaptive mutation of FC722 upon tetracycline treatment. Wild-type FC722 (SEC361) (closed squares), *nusA11* (SEC369) (closed diamonds), *ΔdinB* on the chromosome and episome (SEC611) (closed triangles), and *ΔruvC* (SEC1466) (open triangles). C) Tetracycline adaptive hypermutability. Hypermutation observed in FC722 Δ*recG* mutants in response to tetracycline as is seen during lactose adaptive mutagenesis of FC40. Wild-type FC722 (SEC361) (closed squares) and Δ*recG* (SEC1464) (open squares).

Table I. Strains used in this study

Strain	Genotype	Reference or Source
FC40	*ara* Δ(*lac-proB*)$_{XIII}$*thi* Rif^R/ F' Φ(*lacI33-lacZ*)	(2)
SEC182	as FC40 except *nusA11 zha0132::Tn10*	This work
SEC1414	as FC40 execpt Δ*dinB*::frt on chromosome, Δ*dinB*::CAT on episome	This work
SEC1419	as FC40 except Lac$^+$ revertant	This work
SEC 1429	as SEC182 except Lac$^+$ revertant	This work
FC722	as FC40 except with a defective Tn*10* (tetracycline sensitive)	(7)
SEC361	as FC722 except *sfsB3198::Tn10*kan	This work
SEC364	as SEC361 except Δ*dinB*::frt on chromosome	This work
SEC369	as SEC361 except *nusA11*	This work
SEC 611	as SEC364 except Δ*dinB*::CAT on episome	This work
SEC1466	As FC722 except Δ*ruvC*::Kan	This work
SEC1464	As FC722 except Δ*recG*::Kan	This work
SEC527	as P90C except *zha0132::Tn10*	This work
SEC29	as P90C except *nusA11 zha0132::Tn10*	(4)
IQ419	*zha0132::Tn10 arg rpsL257*	CGSC
CAG12127	F$^-$ *LAM sfsB3198::Tn10kan rph-1*	CGSC
P90C	*ara* Δ(*lac-proB*)$_{XIII}$*thi* Rif^R	(2)
JW1852	F$^-$ *Δ(araD-araB)567 ΔlacZ4787*(::rrnB-3) *LAM Δ ruvC789::kan rph-1 Δ(rhaD-rhaB)568 hsdR514*	Keio Collection (Baba et al., 2006)
JW3627	F$^-$ *Δ(araD-araB)567 ΔlacZ4787*(::rrnB-3) *LAM Δ recG756::kan rph-1 Δ(rhaD-rhaB)568 hsdR514*	Keio Collection (Baba et al., 2006)
AB1157 Δ*dinB*::CAT	*thr-1 leuB6 proA2 hisG4 thi1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsl31 supE44* Δ*dinB*::CAT	(25)

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Chapter 4

Unraveling the crosstalk between transcription and DNA repair/damage tolerance in *Escherichia coli*

Authors for this work are Susan E. Cohen, Cindi A. Lewis, and Graham C. Walker. Manuscript in preparation.

ABSTRACT

All organisms must contend with the challenges of DNA damage, which can result in a variety of cellular catastrophes including cell death. Here I report my unexpected observations that the essential transcriptional modulator NusA is also involved in promoting survival after the challenge of DNA damage in *Escherichia coli*. My results are the first to implicate NusA, a protein associated with RNA polymerase throughout elongation and termination phases of transcription, in the processes of DNA repair/damage tolerance. Mutation of *nusA* results in reduced viability after exposure to DNA damaging agents and, even without the addition of exogenous DNA damaging agents, *nusA* mutants display phenotypes reminiscent of mutants with altered DNA processing. Furthermore, I can genetically link transcription/RNA polymerase to the *nusA*-dependent events that help promote survival after DNA damage through the isolation of RNA polymerase mutants with altered ability to deal with the lethal effects of DNA damage. Together, my results suggest that NusA, in addition to its roles in transcription elongation and termination, is important for coordinating the cellular responses to DNA damage by coupling the processes of translesion DNA synthesis and nucleotide excision repair to transcription.

INTRODUCTION

DNA can become damaged from a variety of endogenous and exogenous sources, which can result in a variety of cellular problems including cell death (14). All organisms must contend with the challenges of DNA damage and thus possess a variety of mechanisms for dealing with DNA damage, including mechanisms of DNA repair and DNA damage tolerance to help them to survive DNA damage (14). One mechanism of DNA repair, in which the ensuing lesion is removed from the DNA, occurs through the nucleotide excision repair (NER) pathway. In *Escherichia coli* the NER factors UvrA, UvrB and UvrC can recognize and repair damaged DNA on either a global scale or by being specifically recruited to transcribed DNA in the process of transcription-coupled repair (TCR) (14). The process of translesion DNA synthesis (TLS) is one important mechanism of DNA damage tolerance, in which a specialized DNA polymerase copies past DNA lesions that block the highly accurate, stringent replicative DNA polymerases. While there exist mechanisms to recruit NER to transcription no conceptually similar recruitment mechanism has yet been identified for TLS.

DNA polymerases belonging to the Y-family of DNA polymerases exhibit this specialized ability to perform TLS and are present in all domains of life (46). Y-family DNA polymerases have been implicated in diverse biological phenomena ranging from adaptive mutagenesis in bacteria to several human cancers. Although certain TLS polymerases can catalyze proficient DNA synthesis across from cognate lesions, such broadened substrate specificity often comes at a mutagenic cost, as these enzymes display lower fidelities on undamaged DNA compared to their replicative counterparts (13, 19, 25). Organisms in all domains of life have therefore developed elaborate regulatory

systems to restrict the access of Y-family DNA polymerases to the appropriate termini where TLS function is required (1, 2, 5, 17, 19, 21, 25, 27, 28, 32, 50, 63). *E. coli* has two Y-family DNA polymerases, DinB (polymerase IV) and UmuD'$_2$C (polymerase V), both of which are transcriptionally induced as part of the SOS response to DNA damage (14). In addition to transcriptional regulation through the SOS response, TLS polymerases are regulated by their interactions with various other cellular proteins. Notably, regulation of both DinB and UmuD'$_2$C are strongly influenced by interactions with the β processivity clamp of DNA polymerase III (2, 3, 66), the *umuD* gene products UmuD$_2$ and UmuD'$_2$ (16, 18, 52, 63, 64), and RecA (53, 55, 56). In eukaryotes, TLS is similarly controlled by association with the PCNA processivity clamp (1, 32, 43, 50, 69) and interactions with recombination proteins (53, 55, 56).

DinB (termed DNA polymerase kappa in eukaryotes) is the only Y-family polymerase to be found in all domains of life (46) and in *E. coli* is the most abundant DNA polymerase in the cell after DNA damage (29). *E. coli* DinB is known to be involved in the phenomena of λ untargeted mutagenesis (7) and adaptive mutagenesis (22). More recently it has been discovered that Δ*dinB* strains are sensitive to the DNA-damaging agents nitrofurazone (NFZ) and 4-nitroquinolone-1-oxide (4-NQO), which mainly form adducts on the N^2 position of guanine. Strikingly, *in vitro* DinB preferentially and accurately bypasses certain N^2-dG adducts (26, 30, 39, 67). This ability to preferentially bypass these N^2-dG adducts is conserved evolutionarily (26), suggesting a possible reason for the conservation of DinB across all domains of life.

I have recently reported that DinB physically interacts with the essential transcriptional modulator NusA and that *nusA* interacts genetically with both the *dinB*

and the *umuDC* gene products (11). NusA is an essential protein that functions in both termination and antitermination of transcription and is associated with the RNA polymerase (RNAP) throughout the elongation and termination phases of transcription (10, 12, 20, 31, 33, 34, 40, 57). Originally identified in 1974, NusA forms an antitermination complex with the λN protein that is required for successful λ phage infection (15). NusA is an essential gene in *E. coli* and is conserved in even the smallest of bacterial genomes, however *nusA* function has been found to be dispensable in specific situations. In particular, it has been reported that, in the presence of *rho* alleles that reduce transcription termination, deletion of *nusA* results in viable cells (68). More recently it has been reported that a viable Δ*nusA* mutant can be constructed in reduced genome background (9), in which 14 percent of the *E. coli* genome has been removed by the targeted deletion of horizontally acquired DNA (49). Analysis of this Δ*nusA* mutant revealed that *nusA* function was required for the suppression of cryptic prophage expression, specifically the *rac* prophage. NusA is highly conserved throughout bacterial and archaeal domains of life; however, to date, no eukaryotic sequence or functional homolog has been identified. I have previously shown that the temperature sensitivity of the *nusA11* allele (41) can be suppressed by overexpression of either *dinB* or *umuDC*, in a manner requiring the catalytic activities of these proteins (11). Furthermore, I have shown that *nusA11* mutants are defective in the *dinB*-dependent phenomenon of adaptive mutagenesis (Chapter 3).

Here I report that *nusA,* in addition to roles in transcription elongation and termination, plays a previously unrecognized role in the DNA repair/damage tolerance pathways in *E. coli*. I show that mutants of *nusA*, both the *nusA11* and Δ*nusA* mutants,

are sensitive to DNA damaging agents. Additionally the *nusA11* mutant, at the permissive temperature, displays phenotypes reminiscent of mutants that have defects in DNA processing. I present evidence that the role for *nusA* in promoting survival after challenge with DNA damage, in addition to participating in a pathway involving *dinB*, likely extends to a role for *nusA* in nucleotide excision repair/transcription coupled repair. I have obtained genetic evidence for a role of RNA polymerase in such a process through the isolation of RNA polymerase mutants that display altered cellular survival after exposure to nitrofurazone. Specifically I show that the altered ability of these RNA polymerase mutants to deal with NFZ is dependent upon $nusA^+$ and $uvrA^+$. Taken together, my results suggest that NusA is important for allowing cells to cope with the lethal effects of DNA damage by two mechanisms. I propose that NusA is required to couple the process of transcription to the process of TLS to enable transcription-coupled translesion synthesis, and also that NusA may also be involved in a new class of NusA-dependent transcription-coupled nucleotide excision repair in *E. coli*.

RESULTS

nusA mutants are sensitive to DNA damaging agents

Intrigued by my initial observations linking *nusA* to translesion DNA synthesis, I looked to see if *nusA* mutants shared any phenotypes with TLS polymerase mutants, i.e. sensitivity to DNA damaging agents. Strikingly, I found that at the permissive temperature, *nusA11* strains were specifically sensitive to the DNA damaging agents NFZ and 4-NQO (Figure 1A), which are the same DNA damaging agents to which a Δ*dinB* strain is sensitive (26). These experiments were carried out at the permissive temperature (30°C), a condition under which the sensitivity of the Δ*dinB* strain to NFZ is less than the degree of sensitivity observed at 37°C. I noted that the *nusA11* strain was considerably more sensitive than the Δ*dinB* strain, implying that NusA may play a role in NFZ/4-NQO resistance that is independent of DinB. Furthermore, the sensitivity of the *nusA11* strains to NFZ and 4-NQO can be complemented in *trans* at the restrictive temperature (42°C) (Figure 1C-D). Efforts to construct a *nusA11*Δ*dinB* double mutant yielded what are likely to be strains carrying suppressors since some isolates of the double mutants displayed a synergistic phenotype, being much more sensitive than either single mutant, while other isolates displayed an epistatic phenotype, where the double mutant resembled a *nusA11* single mutant. Although this phenomenon prevented me from being able to unambiguously define the phenotype of the *nusA11*Δ*dinB* double mutant, it is clear from all these data that NusA is important for cellular survival after exposure to the DNA damaging agents NFZ and 4-NQO, thus making *nusA* a novel gene involved in this response. I can also conclude that the *nusA11* mutation at 30°C very specifically affects a

function related to withstanding DNA damage that is genetically separable from the *nusA* functions required for viability.

Due to the complications I encountered in my efforts to determine the phenotype of the *nusA11ΔdinB* double mutant, I decided to look at the viable Δ*nusA* mutant constructed by Cardinale et al. (9) in a reduced genome background (MDS42) where approximately 14% of the genome has been removed (9, 49). Working in this genetic background allowed me to analyze the effects of a complete deletion of *nusA* in addition to the *nusA11* point mutant. I find that the Δ*nusA*Δ*dinB* double mutant is much more sensitive to nitrofurazone than either of the single mutants (Figure 1B), suggesting that perhaps it is the second class of *nusA11ΔdinB* strain described above that carries the suppressor mutation. Interestingly, the Δ*dinB* single mutant does not display the same degree of NFZ sensitivity as is seen in wild-type strains of *E. coli*, which possess the original 14% of the chromosome that is missing in the reduced genome strain. These data imply that NusA likely acts in a pathway with DinB as well as in a separate pathway to allow cells to survive NFZ treatment.

In the reduced genome background, I find that both the *nusA11* and Δ*nusA* mutants are sensitive to NFZ (Figure 2A and D). However, the Δ*nusA* mutant is more sensitive than the *nusA11* mutant in this background. These data lend support to the notion that the *nusA11* mutant is a partial loss of function mutant with respect to NFZ and 4-NQO survival. Additionally, I noticed that the Δ*nusA* strain is sensitive to wider variety of DNA damaging agents compared to the *nusA11* strain. The Δ*nusA* strains are sensitive to both ultraviolet (UV) radiation and to methyl methanesulfonate (MMS), whereas the *nusA11* mutant is not (Figure 2 B-C and E-F). These data suggest that *nusA*

is generally required for survival after exposure to genotoxic stress, and suggest that there are specific parts of *nusA* that are required for cellular survival after DNA damage. Specifically, these analyses reveal that functions of *nusA* required for survival after exposure to NFZ are genetically separable from those required for survival after UV irradiation.

Peptide array analysis reveals that NusA preferentially binds to one surface of DinB

Previous analysis of the NusA peptides that interact with DinB identified a potential DinB binding region on the surface of NusA that encompassed the residue mutated in the *nusA11* allele (11). It was this observation that had originally stimulated me to discover that overexpression of DinB results in suppression of the temperature sensitivity of the *nusA11* temperature sensitive allele, in a manner that required the catalytic activities of DinB (11). I performed the reciprocal experiment by using cellulose filter peptide arrays to search for peptides of DinB that potentially interact with NusA. The membrane was probed with NusA and visualized with an anti-NusA antibody. Interacting peptides were identified and mapped onto a structural model of DinB (26). Interestingly, I found that the NusA-interacting peptides on DinB localize mostly to a single face of the protein (Figure 3A). The binding of NusA to DinB maps to the "back" surface of DinB, which is distinct from the interaction surfaces on the "front" of DinB that interact with RecA and UmuD (18). These data suggest that the binding of all of these potential modulators of DinB might be able to occur at the same time.

In an effort to design a DinB variant that is unable to interact with NusA, I mutagenized several residues present in a group of strongly interacting peptides identified

from the DinB peptide array. These mutants were scored for failure to serve as a multicopy suppressor of the *nusA11* temperature sensitivity, implying a lack of genetic interaction with *nusA*. I was able to identify one mutant of DinB, in which a surface exposed lysine was mutated to a glutamic acid (K217E) (Figure 3A). When this variant was overexpressed, it failed to suppress the temperature sensitivity of the *nusA11* strain (Figure 3B).

I then looked to see whether the *dinB(K217E)* mutant, which no longer exhibits a functional genetic interaction with *nusA,* complemented the survival of a Δ*dinB* strain after exposure to NFZ. I found that the expression of *dinB(K217E)*, from a low copy number plasmid under its native promoter, can restore the NFZ sensitivity of a Δ*dinB* mutant at lower doses of NFZ, but is approximately 5-fold reduced for complementation at higher doses of NFZ (2.25μg/mL-2.5μg/mL) (Figure 3C). These data imply that, with respect to survival after exposure to NFZ, DinB likely acts in a *nusA*-dependent as well as *nusA*–independent manner. A possible explanation might be that DinB may act at stalled transcription complexes in addition to at replisomes. Similarly, the greater sensitivity of the *nusA* mutants to NFZ and 4NQO compared to a Δ*dinB* mutant implies that NusA likely acts in a DinB-dependent as well as DinB–independent pathways with respect to survival after DNA damage.

nusA mutants display phenotypes of altered DNA processing

Because of the phenotypes displayed in the *nusA11* mutant at the permissive temperature, I decided to look at the morphology of *nusA11* mutant strains. At the permissive temperature, I found that the *nusA11* cells were elongated compared to wild-

type (Figure 4A). Filamentation is a hallmark of SOS induction, due to in part to induction of *sulA* as part of the SOS regulatory network, which blocks cell division. The purpose of this filamentation is thought to be to keep the daughter chromosomes together so that repair strategies based on homologous recombination can be employed. Quantification of this filamentation phenotype revealed that *nusA11* cells were somewhat elongated compared to wild-type, with a smaller population of cells displaying extreme filamentation, more than 30 times the size of wild-type cells (Figure 4A). Since *nusA* deletion mutants are viable in a background that has been deleted for cryptic prophages (9), I considered the possibility that the filamentation seen in the *nusA11* background might be due to partial induction of these cryptic prophages. In order to test this hypothesis, I measured cell length of Δ*nusA* cells in this reduced genome background. In this background, which is deleted for cryptic phages, the Δ*nusA* mutant similarly results in filamentation (Figure 4B). These results suggest that the filamentation seen in the *nusA11* background is not likely due to induction of cryptic prophages, but rather to another cellular process.

The similarities between the *nusA11* and *lexA*(Def) cells (which are deleted for *lexA* as well as *sulA* so that cell division can occur) (Figure 4A) led me to question if the filamentation seen in the *nusA11* background was *sulA*-dependent. Further analysis of this mutant revealed that the filamentation seen in the *nusA11* background is *sulA*-independent (Figure 4C). However, it is unclear as to what this may mean as there exist examples of *sulA*-independent filamentation that is dependent on induction of the SOS response (23). SOS induction is important for survival after exposure to NFZ or 4-NQO, as is seen by the reduced survival of a *lexA3*(Ind$^-$) strain, a background in which the SOS

genes are constitutively repressed (Figure 5). Furthermore, addition of either a Δ*dinB* or *nusA11* mutation does not increase the sensitivity of a *lexA3*(Ind$^-$) strains, indicating that NusA is only able to help cells cope with the lethal effects of DNA damage if the cells are capable of inducing the SOS response. Nevertheless, the filamentation seen in the *nusA11* background, led me to consider the possibility that the *nusA11* cells were displaying a phenotype of chronic partial SOS induction.

SOS induction can occur in cultures of strains carrying certain mutations even without the addition of exogenous DNA damaging agents, a phenomenon known as chronic partial SOS induction. Such chronic partial induction can occur either because all cells are partially induced for the SOS response or because only a sub-population of cells becomes induced (36, 44). Utilization of a reporter in which GFP is placed under the control of a LexA regulated promoter (P*sulA*), integrated into the *attB* locus on the chromosome (36), allowed me to monitor SOS induction on a single cell level. Analysis of cells grown at the permissive temperature revealed that approximately 2.5% of *nusA11* cells are induced for the SOS response in comparison to wild-type or *lexA*(Def) controls in which SOS induction is ~0.1% or 100% of cells respectively (Figure 6A-D). Thus, at the permissive temperature the *nusA11* strains exhibit chronic partial SOS induction. The chronic partial SOS induction exhibited in the *nusA11* mutant is reminiscent of mutants that have altered DNA processing such as *uvrD* (3'→5' DNA helicase II) (47) and *dam* (GATC-specific DNA methylase) (35, 48) that display this two-population chronic partial SOS induction (36). Thus my observations suggests that NusA plays a role in maintaining chromosomal integrity even in cells that have not been exposed to an exogenous DNA damaging agent.

Is this chronic partial SOS induction seen in a sub-population of *nusA11* cells caused by an accumulation of ssDNA? To answer this question, I monitored the presence of ssDNA through the formation of RecA-GFP foci, using a RecA-GFP translational fusion integrated into the chromosome (51). Wild-type *E. coli* strains grown at the permissive temperature accumulate RecA-GFP foci in approximately 2% of the population with 72% of those foci located at the poles (Figure 6 E and H). Polar RecA-GFP foci have been observed before and are thought to be RecA storage structures (51). If wild-type strains are irradiated with UV, all cells then have RecA-GFP foci (Figure 6F and H), an indication that this RecA-GFP fusion is responding to DNA damage. Approximately 19% of *nusA11* cells, grown at the permissive temperature without the addition of exogenous DNA damaging agents, have RecA-GFP foci with 64% of those foci being present at the poles (Figure 6 G-H). These data are consistent with *nusA11* cells having more ssDNA present, which results in induction of the SOS response. Also there is precedent for RecA-GFP foci forming without SOS induction in response to DNA damage (60), which may explain why there are more RecA-GFP foci than SOS induced cells in the *nusA11* strain. Together, these data show that the *nusA11* mutation displays phenotypes reminiscent of mutations in genes involved in DNA processing and suggest a role for $nusA^+$ in the maintenance of DNA. The inferred presence of ssDNA in the *nusA11* background lends support to my proposed model of transcription-coupled translesion synthesis (11), in which NusA recruits TLS polymerases to fill in gaps opposite lesions on the transcribed strand potentially allowing for the continuation of transcription (Figure 10).

Interactions between *nusA* and nucleotide excision repair

My data show that *nusA*$^+$ is required to promote survival after exposure to DNA damaging agents and suggested a role for NusA beyond its involvement with DinB/TLS polymerases. Intriguingly, a high-throughput screen looking at the *E. coli* protein interactome identified NusA as an interaction partner of the nucleotide excision repair (NER) factor UvrA (8). These results suggested to me that NusA might play a role in a type of transcription-coupled repair (TCR). In *E. coli*, as well as in other organisms, UV photoproducts are preferentially repaired on the transcribed compared to the non-transcribed strand (37, 38), a phenomenon known as transcription coupled repair. In *E. coli* TCR is initiated by the stalling of RNAP by a lesion in the transcribed strand, which is then recognized by the transcription coupling repair factor, Mfd (59). Mfd is capable of translocating the stalled RNAP, exposing the damaged DNA, and recruiting NER to repair the lesion (58). Thus, the interaction between NusA and UvrA was of particular interest as UvrA is the first NER protein recruited to stalled RNAP's during Mfd-dependent transcription-coupled repair.

To determine if the physical interaction between NusA and UvrA was relevant *in vivo*, I performed epistasis analysis for the *nusA11,* Δ*dinB* and Δ*uvrA* alleles. These analysis revealed that the Δ*uvrA* strain is much more sensitive to NFZ than the *nusA11* or Δ*dinB* single mutants (Figure 7A), suggesting that the major pathway for dealing with NFZ-induced damage is through NER. Intriguingly, I discovered that addition of either the *nusA11* or Δ*dinB* mutation to a Δ*uvrA* background did not further sensitize the cells to NFZ, with *uvrA* displaying an epistatic relationship to both *nusA* and *dinB*. These data support the notion that NusA acts in the NER pathway in addition to the TLS pathway.

The epistatic relationship between *dinB* and *uvrA* may imply a role for DinB in gap filling step of NER as has been implicated for pol kappa in eukaryotes (45).

One possible explanation for the genetic interaction between *nusA* and *uvrA* is that NusA is involved in a pathway of transcription coupled repair. To test this hypothesis, I similarly performed epistasis testing with *nusA11*, Δ*dinB* and Δ*mfd*. To my surprise, I found that for both NFZ and 4NQO *mfd* displayed an additive phenotype with Δ*dinB* and *nusA11*, implying that they function in separate pathways (Figure 7B-C). While the additive phenotype between the *nusA11* and Δ*mfd* alleles could imply that NusA is not involved in TCR, it could also implicate NusA in the process of transcription coupled repair but in a manner that is Mfd-independent, comprising a novel class of the transcription coupled repair pathway. A novel class of NusA-dependent TCR would be of particular interest as transcription-coupled repair has been thought to be less important in bacteria than in eukaryotes. However this inference could be incorrect if there exists a second class of TCR, NusA-dependent TCR, which has not been previously recognized.

Screen for RNA polymerase mutants with altered ability to deal with nitrofurazone

The roles for *nusA*$^+$ in transcription elongation and termination have been appreciated for many years, but my data suggest a role for *nusA* in helping cells to cope with the lethal effects of DNA damage. The sensitivity of Δ*nusA* and *nusA11* mutants to DNA damaging agents indirectly suggest a role for transcription/RNAP in the NusA-dependent events that help promote survival. However, I was interested in establishing a direct link of RNAP to DNA damage repair/damage tolerance through NusA. Using mutagenized plasmid libraries of *rpoB* (54), the β catalytic subunit of RNAP, I screened

for mutants of *rpoB* that displayed altered ability to deal with NFZ-induced damage, compared to an $rpoB^+$ plasmid control. These mutant variants of *rpoB*, generated by random PCR mutagenesis, are regulated by an IPTG inducible promoter and, under induced conditions, it has been estimated that approximately 85-90% of cellular RNAP's have incorporated the plasmid-encoded His_6-tagged subunit, with the wild-type genomic copy of *rpoB* still expressed (54).

Mutagenized plasmid libraries were introduced into wild-type *E. coli* cells (AB1157) and screened at 37°C for either NFZ sensitivity (NFZ^S) or NFZ resistance (NFZ^R), compared to $rpoB^+$ plasmid control. Out of ~800 mutants screened, 36 mutants were isolated that, when expressed in wild-type *E. coli,* displayed altered cellular survival after NFZ exposure. 6 NFZ^R mutants and 30 NFZ^S mutants were isolated in this screen. This was not a full coverage screen. I decided to further characterize existing mutants rather than continue to screen for more, since I isolated such a high percentage (~4.5%) of mutants which, when expressed in wild-type *E. coli,* displayed an altered response to NFZ. NFZ sensitive or resistant phenotypes were confirmed by a second round of screening and mutations were identified by sequencing. All types of mutations from missense, nonsense and silent base substitutions to frameshift mutations were isolated (Table 1), and several carried more than one mutation. Interestingly, all the mutations mapped to the N-terminal half of *rpoB*. While this may mean that not enough mutants were screened in order to be able to isolate mutations in the C-terminal half, it suggests that there is a specific function in the N-terminal portion of *rpoB* required for dealing with NFZ related damage.

By eliminating mutants with many amino acid changes, nonsense mutants and frameshift mutants, in consultation with Bob Landick (U.W. Madison) I then selected mutants that displayed robust $NFZ^{S/R}$ phenotypes for further characterization. NFZ^{S} mutant *rpoB(D185Y)* and NFZ^{R} mutants *rpoB(V287A)*, *rpoB(D320N)* displayed either 10-fold sensitivity or 10-fold resistance to NFZ compared to the $rpoB^{+}$ plasmid control (Figure 9A). None of these mutants showed altered cellular survival after UV irradiation nor did they display rifampicin resistance (data not shown). Several of the NFZ^{R} mutations including, *rpoB(V287A)* and *rpoB(D320N)*, affect residues that are located on the surface of the RNAP when mapped onto the crystal structure of the *T. thermophilus* RNAP elongation complex (65). The *rpoB(D185Y)* (NFZ^{S}) mutation affects a residue that could potentially make contact with the DNA (Figure 8).

How is it that these mutant RNAP's are able to alter cellular survival after exposure to NFZ? In order to address this question I expressed these variants in a variety of mutant backgrounds, and scored cellular survival after exposure to NFZ. Strikingly, I found that NFZ^{S} mutant, *rpoB(D185Y)*, loses this sensitive phenotype in either a *nusA11* background at 30°C or a Δ*uvrA* background at 37°C (Figure 9A-C). Similarly, NFZ^{R} mutants *rpoB(V287A)* and *rpoB(D320N)*, confer the same survival after NFZ exposure as the $rpoB^{+}$ plasmid control in a Δ*uvrA* background (Figure 9C). Analysis of the class of NFZ^{R} mutants in the *nusA11* background were complicated by the $rpoB^{+}$ plasmid control, which is not as sensitive to NFZ at 30°C as is seen at 37°C (data not shown), thereby making it difficult to determine if these variants displayed a resistant phenotype in the wild-type background. Nevertheless in the *nusA11* background, the NFZ^{R} mutants, *rpoB(V287A)* and *rpoB(D320N)*, displayed similar levels

of sensitization as the *rpoB*$^{+}$ plasmid control (Figure 9B), implying that these effects were *nusA*-dependent. These data indicate that the ability of these RNAP mutant variants to display altered NFZ effects is dependent upon *nusA*$^{+}$ and *uvrA*$^{+}$. The dependence of the *rpoB* NFZ^{R} and NFZ^{S} phenotypes on *nusA*$^{+}$ and *uvrA*$^{+}$ lends support to the model of alternative NusA-dependent transcription-coupled repair described above and in discussion. In contrast, in a Δ*dinB* mutant background, *rpoB(D185Y)* (NFZ^{S}), retains some sensitivity, while the NFZ^{R} mutants, *rpoB(V287A)* and *rpoB(D320N)*, continue to display NFZ^{R} compared to the *rpoB*$^{+}$ plasmid control, even though the resistance of these mutants in a Δ*dinB* background is slightly greater than the NFZ^{R} seen in the wild-type background (Figure 9D).

DISCUSSION

My previous observation that the essential RNA polymerase modulator NusA physically and genetically interacts with the translesion DNA polymerase DinB led me to investigate the potential role for NusA in the DNA damage response. In so doing, I discovered that NusA plays an important role in helping cells deal with the lethal effects of DNA damage in *Escherichia coli*.

I report that *nusA* mutant strains are sensitive to DNA damaging agents. Specifically I show that *nusA11*(ts) strains, at the permissive temperature, are specifically sensitive to NFZ and 4-NQO, while Δ*nusA* strains, in a reduced genome background, are sensitive to a wider variety of DNA damaging agents, including UV radiation. I have previously suggested a model of transcription-coupled translesion synthesis (TC-TLS) to explain the genetic interactions between *dinB* and *nusA* (11) (Figure 10). While the sensitivity to DNA damaging agents in strains with altered *nusA* function is consistent with this model, my results also suggest that NusA acts in another pathway independent of DinB that helps cells to survive after DNA damage.

Peptide array analysis of the surfaces of DinB that interact with NusA allowed me to generate mutants that could potentially disrupt this interaction. Characterization of one such mutant suggests that DinB likely functions in *nusA*-dependent as well as *nusA*-independent pathways. These separate pathways might possibly involve i) DinB acting at the replisome and ii) DinB being recruited by NusA to sites of RNA polymerases that have been stalled by gaps in the transcribed strand, as is suggested by my model of TC-TLS.

Furthermore, I report that, at the permissive temperature, *nusA11* strains are elongated and display chronic partial SOS induction. These phenotypes are often seen in strains that have mutations in genes that are involved in DNA processing. These data suggest that, in addition to its roles in transcription elongation, NusA may also be involved in maintaining the integrity of the chromosome. Analysis of RecA-GFP foci in a *nusA11* background showed an accumulation of RecA-GFP foci at the permissive temperature, compared to $nusA^+$ strains. These observations suggest that *nusA11* cells have a higher frequency of single stranded gaps in their DNA. Such single stranded gaps could arise from the inability of the mutant NusA11 protein to recruit TLS polymerases to gaps that have resulted from DNA damage by endogenous DNA damaging agents.

Roles for NusA in promoting cellular survival after DNA damage also extend beyond a pathway involving DinB. I propose an alternative model of transcription-coupled repair, in which NusA participates in a previously unrecognized class of transcription-coupled repair (Figure 10). Data supporting this model include the epistatic relationship between *nusA* and *uvrA*, the gene encoding the first NER protein to be recruited during Mfd-dependent TCR. Also, sensitivity to a wider variety of DNA damaging agents observed in the Δ*nusA* mutant support that NusA is involved in the repair of a variety of DNA lesions. These data imply that the functions of NusA required for survival after NFZ exposure are genetically separable from those required for survival after UV irradiation, suggesting the possibility of isolating a mutant of *nusA* that displayed the phenotype of UV^S but NFZ^R.

The additive phenotype between the *nusA11* and Δ*mfd* alleles implies that NusA is functioning separately to Mfd, the known transcription-coupling repair factor in *E. coli*.

While these data could imply that NusA is not involved in TCR, it could also implicate NusA in a previously unrecognized class of transcription-coupled repair. This is an attractive model considering that TCR is thought to be a more prominent pathway for the removal of DNA lesions in eukaryotes compared to bacteria, a rationale based on the view that eukaryotic cells spend more time in a quiescent state compared to bacteria. However, in the environment bacteria are not always growing under lab conditions of plentiful nutrients. Thus the existence of a novel class of hitherto unrecognized TCR could imply that TCR is as important in bacteria as in eukaryotes.

Additionally, I have genetic evidence for a role for RNA polymerase in the *nusA*-dependent events that help promote survival after DNA damage. A screen for mutants of *rpoB*, the β catalytic subunit of RNAP, revealed several *rpoB* mutants that either displayed NFZ resistance or sensitivity compared to an $rpoB^+$ plasmid control. This ability to confer NFZ resistance or sensitivity when expressed in a wild-type background is dependent upon $nusA^+$ and $uvrA^+$, as these mutants act as the $rpoB^+$ plasmid control in a *nusA11* or Δ*uvrA* background. When expressed in a Δ*dinB* background, NFZ resistant mutants still confer NFZ resistance, although to a greater extent than displayed in a wild-type background. These data suggest that *dinB* is either not involved in the NFZ sensitivity/resistance displayed by these *rpoB* mutants or could also suggest that DinB somehow interferes with the NFZ resistance of these mutants. These data support a model of alternative NusA-dependent transcription-coupled repair, as the effects exerted in strains expressing *rpoB* variants, is dependent upon $nusA^+$ and $uvrA^+$.

This is the first report of $nusA^+$ involvement in DNA repair/damage tolerance pathways in *E. coli*. Taken together my data suggests roles for $nusA^+$ in DNA damage

tolerance (through TLS DNA polymerases) as well as in DNA repair (through NER or TCR). Furthermore, at the permissive temperature, *nusA11* strains display phenotypes of altered DNA processing, suggesting that in addition to roles in transcription elongation/ termination, $nusA^+$ is required to maintain chromosomal integrity.

In order to explain my observations I have suggested two models; a model of transcription-coupled translesion synthesis (TC-TLS) and a model of NusA-dependent transcription-coupled repair (NusA-TCR). In the model of TC-TLS, I hypothesize that if an RNA polymerase encounters a gap in the transcribed strand opposite a lesion, it would stall. In this case NusA, which is associated with the RNA polymerase throughout the elongation phase of transcription, might then recruit a TLS polymerase to fill in the gap in the template strand. Repairing the gap would permit transcription of the gene by subsequent RNA polymerases, possibly even by the original RNA polymerase if it is retained during process as in TCR. TC-TLS would provide a way of prioritizing the use of the cells translesion DNA synthesis resources to benefit maximally transcription, the same way TCR prioritizes nucleotide excision repair resources to maximally benefit transcription. My model of NusA-TCR suggests that NusA, independently of Mfd, is also able to recruit NER machinery to sites of RNAP stalled by a lesion in the transcribed strand. However the molecular determinants of this model are unclear. Why the existence of a second class of TCR? One intriguing possible explanation involves a particular class of DNA lesion, such as an N^2-dG lesion, which can be efficiently bypassed by DinB and thus is not a hindrance during replication. However, such lesions would cause a problem when transcription is attempted. In this case, a novel class of TCR involving NusA could be important for the recognition and removal of these DNA

lesions. Since some of these minor groove lesions are not efficiently recognized by normal NER (6, 24), NusA-TCR could help a cell remove this class of lesions from its DNA.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 2 and constructed using standard molecular biology techniques. Plasmids were maintained with ampicillin (100μg/mL) when necessary.

DNA damage sensitivity assays

Sensitivity to DNA damaging agents was determined essentially as described previously (4, 26). Briefly, independent overnight *E. coli* cultures grown in LB medium were diluted in M9 minimal starts and plated on LB agar containing DNA damaging agent nitrofurazone, 4-NQO, or MMS. For UV survival assays, cells were plated on LB agar and then irradiated with UV light (0-40 J/m^2) by using a G15T8 UV lamp (General Electric) at 254 nm, then incubated in the dark. A concentrated stock solution of each DNA damaging agent was first made in N,N-dimethylformamide (for NFZ and 4-NQO), stored at -20°C, and diluted appropriately for each experiment. Percent survival was determined relative to growth in the absence of DNA damaging agent.

Live cell microscopy

Live-cell microscopy was performed as described previously (61, 62). Aliquots of cells were then stained with the membrane dye FM4-64 (240 ng/ml to 1 μg/ml; Molecular Probes). Cells were then placed on a pad of 4% low melt agarose in a solution of M9 minimal salts and covered with a coverslip. The following Chroma filter sets were used: 41002b (TRITC) for FM4-64 and 41012 for GFP. Images were acquired using a Nikon E800 microscope with a charge-coupled device camera (Hamamatsu; model C4742-95)

and OpenLab software (Improvision). Images were colorized in OpenLab and then transferred to Photoshop (Adobe) for figure assembly.

Protein purification

Purification of NusA was achieved by expression of NusA from pNusA in BL21(DE3) pLysS cells and induction with 100μM IPTG at an OD_{600} of 0.5 for 4 hours at 30°C. Cultures were harvested by centrifugation and subjected to lysozyme treatment. The resulting lysate was treated with DNAse and purified using Ni-NTA resin from QIAGEN.

Peptide array mapping

Cellulose filter peptide array consisting of 10-mer peptides scanning the primary sequence of DinB, each peptide offset by 2 residues from the previous (MIT CCR Core Facility) were probed with 5μM purified recombinant His-NusA and developed with an anti-NusA antibody (Neoclone) as described in (42). Control peptide array was performed as described above except without a NusA incubation step.

RNA polymerase mutant screen

Mutagenized libraries of pRL706 (54) transformed into AB1157 were grown in LB medium supplemented with ampicillin induced with 1mM IPTG. Cultures were diluted in M9 minimal salts and 10-fold dilutions stamped onto LB agar containing either 0 or 2μg/mL NFZ with a 96 well pin replicator. Plates were incubated at 37°C and scored for NFZ sensitivity or resistance the next day.

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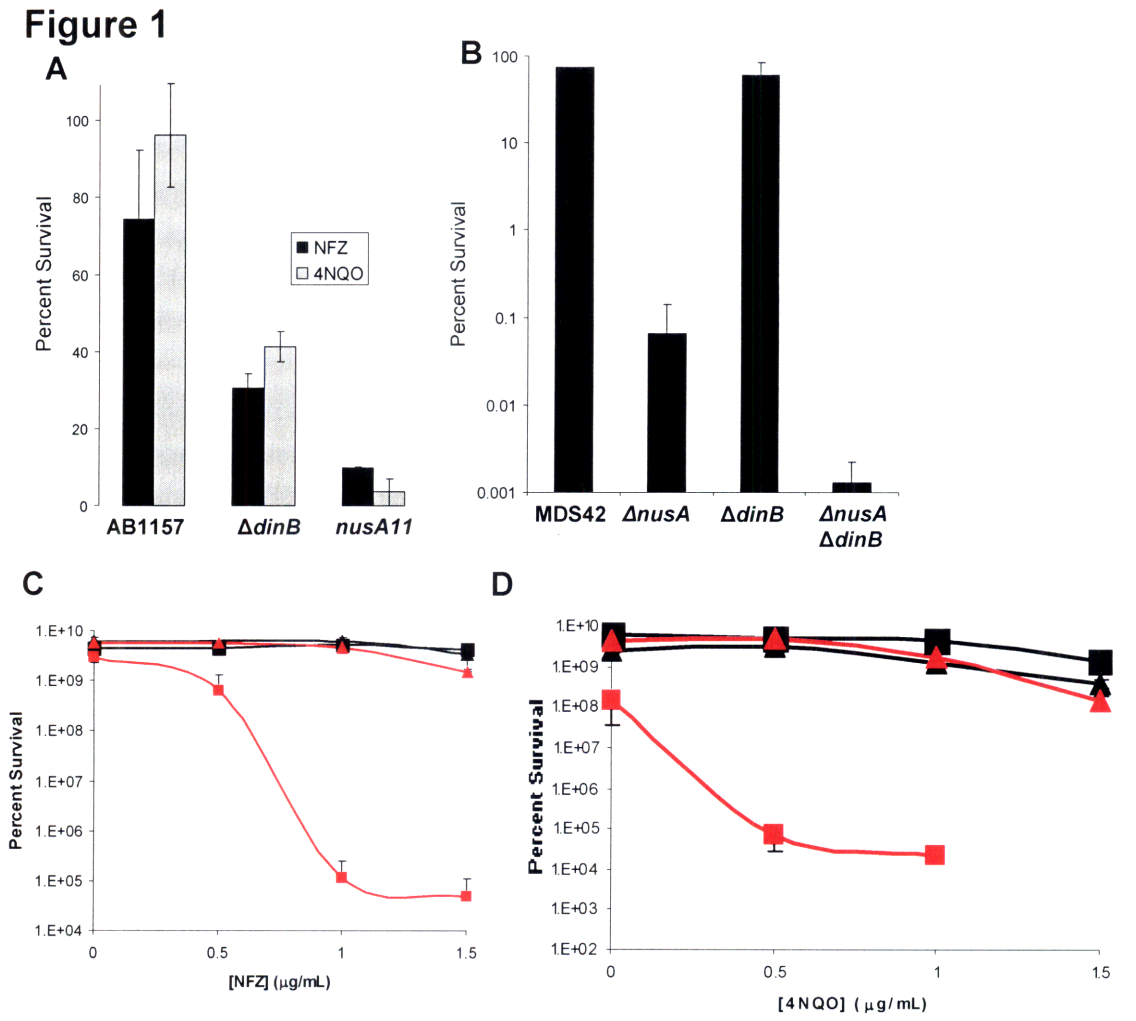


Figure 1. *nusA* mutant cells are sensitive to DNA damaging agents. A) Percent survival of strains (AB1157 derivatives) treated with either 2μg/mL NFZ (black bars) or 2μg/mL 4-NQO (gray bars) at 30°C, shows that at the permissive temperature *nusA11* strains are sensitive to the same DNA damaging agents as Δ*dinB*. In this and all graphs in this figure, error bars represent the standard deviation determined from three independent cultures. B) Percent survival of strains treated with 1μg/mL NFZ, shows that in the reduced genome background (MDS42) Δ*nusA* strains are also sensitive to NFZ and the Δ*nusA*Δ*dinB* double mutant is more sensitive than either of the single mutants. C-D) Complementation of *nusA11* sensitivity to C) NFZ and D) 4NQO in *trans* at the restrictive temperature 42°C. Black lines/symbols represent wild-type (AB1157) and red lines/symbols represent *nusA11* strains. The empty vector (pBR322) is shown as squares and pNusA (pNAG2010) is shown as triangles.

Figure 2

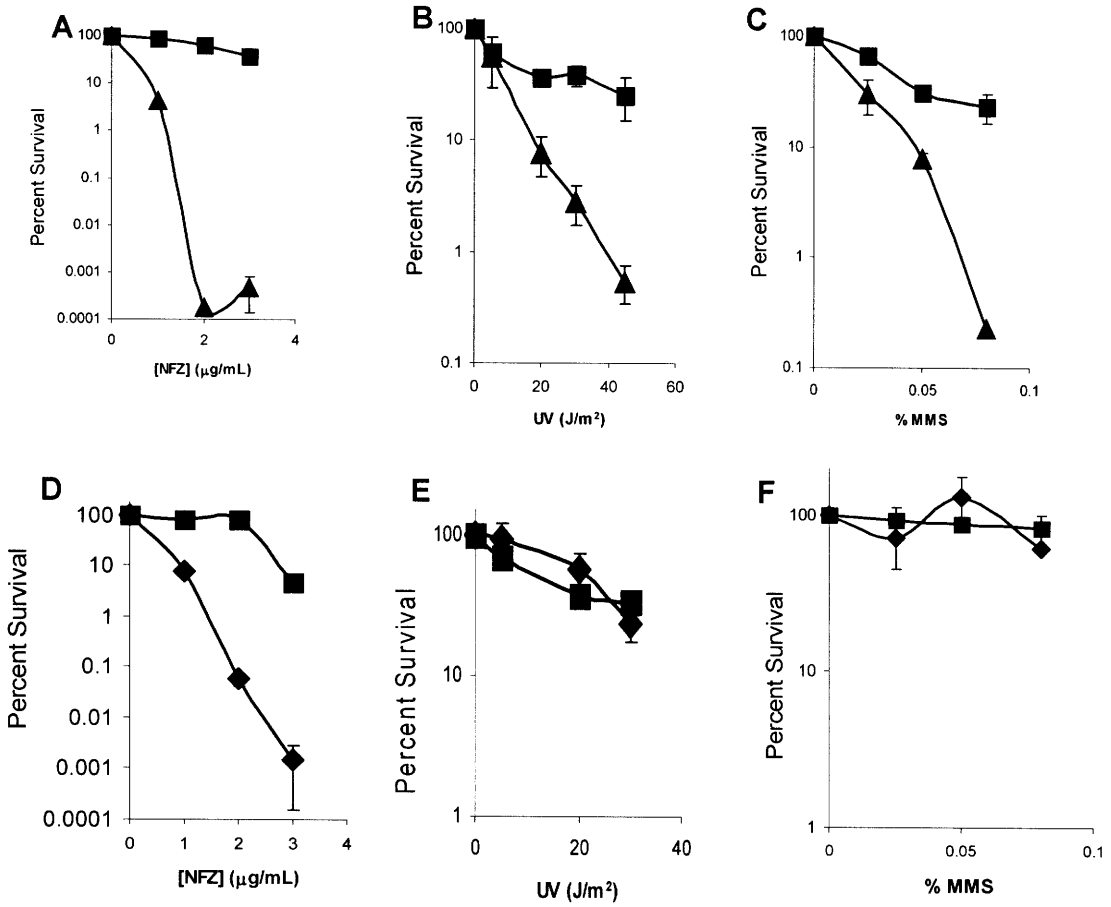


Figure 2. Comparison of the *nusA11* and Δ*nusA* mutation in the reduced genome background (MDS42). A-C) Percent survival of MDS42 (squares) and Δ*nusA* (triangles) to the DNA damaging agents NFZ, UV and MMS respectively at 37°C, show that Δ*nusA* strains are sensitive to a wide variety of DNA damaging agents. D-F) Percent survival of MDS42 (squares) and *nusA11* (diamonds) to the DNA damaging agents NFZ, UV and MMS respectively at 30°C, show that *nusA11* strains are specifically sensitive to NFZ. Error bars represent the standard deviation determined from three independent cultures.

Figure 3

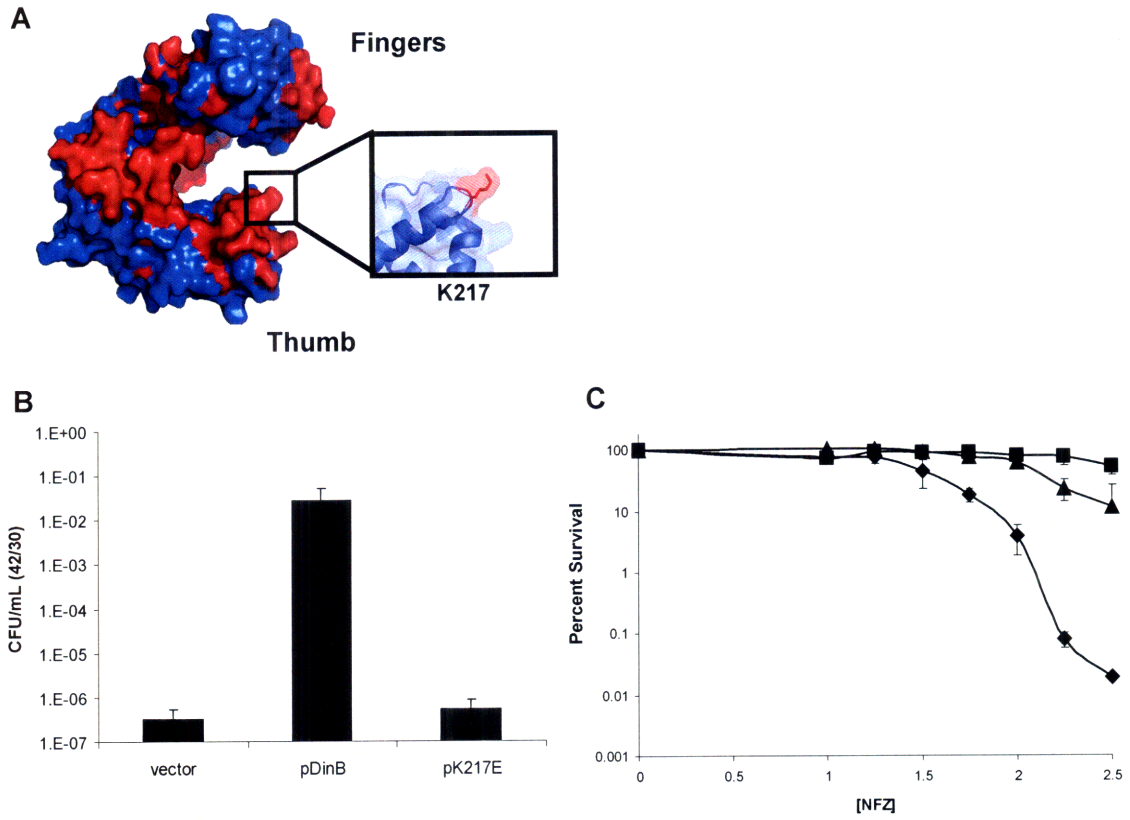


Figure 3. Mapping peptides of DinB that interact with NusA. A) Peptide array mapping of the NusA binding surface on DinB reveals that NusA preferentially binds to only one side of DinB. DinB homology model (in blue) and NusA interacting peptides (shown in red). Inset displays a magnification of the thumb region of DinB with the K217 residue shown in red. B) Overexpression of the *dinB(K217E)* (pYG782-K217E) mutant in fails to suppress the temperature sensitivity of the P90C *nusA11* temperature sensitive strain (SEC29) compared to *dinB*$^{+}$ (pYG782). C) Complementation of nitrofurazone sensitivity of the AB1157 Δ*dinB* strain. Reveals that expression of *dinB(K217E)* (triangles) from a low copy number plasmid under its native protomer (pGY768-K217E) does not completely complement the nitrofurazone sensitivity of a Δ*dinB* strain, as compared to *dinB*$^{+}$ (pYG768) (squares) and the empty vector/pWSK29 (diamonds). In graphs B) and C), error bars represent the standard deviation determined from three independent cultures.

Figure 4

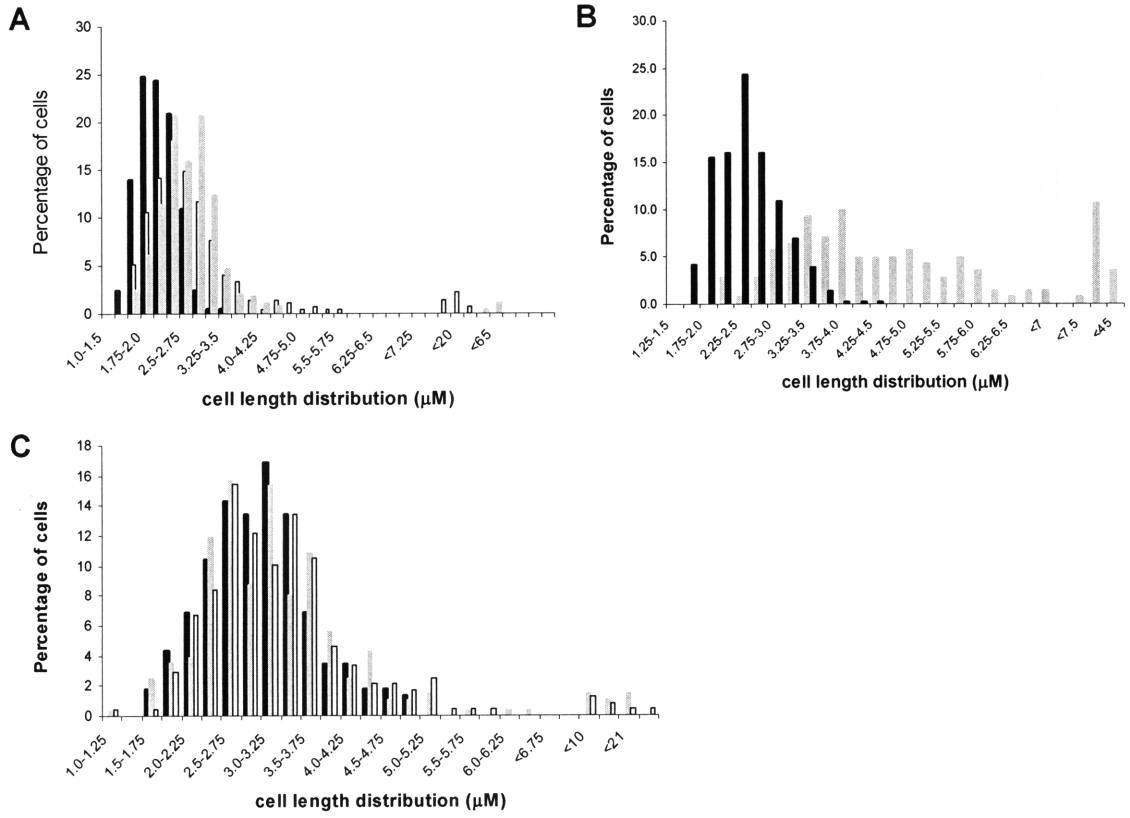


Figure 4. *nusA* mutant cells are elongated. A) Cell length distributions of wild-type/AB1157 (black bars), *lexA*(Def) (open bars) and *nusA11* (SEC164) (grey bars), show that *nusA11* strains are elongated compared to wild-type and similar to *lexA*(DEF). B) Cell length distributions of wild-type/MDS42 (black bars) and Δ*nusA* (grey bars), shows that Δ*nusA* strains are also elongated compared to a wild-type control. C) Cell length distributions of wild-type/AB1157 (black bars), Δ*sulA* (TP651) (open bars), and *nusA11*Δ*sulA* (SEC524)(grey bars), show that filamentation seen in the *nusA11* strains is *sulA*-independent.

Figure 5

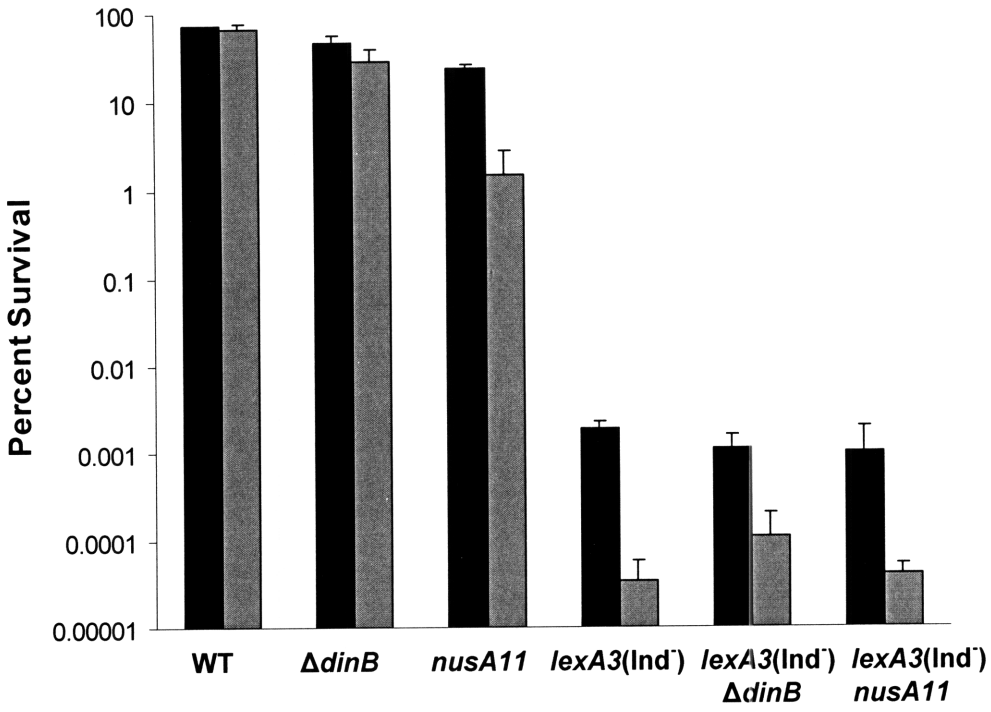
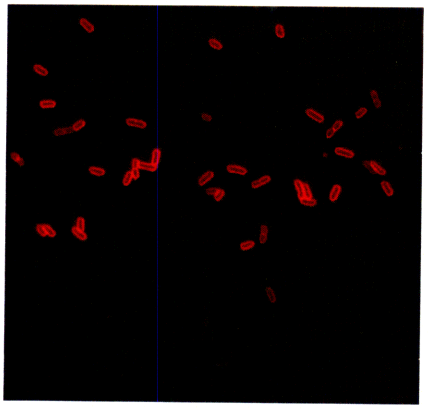
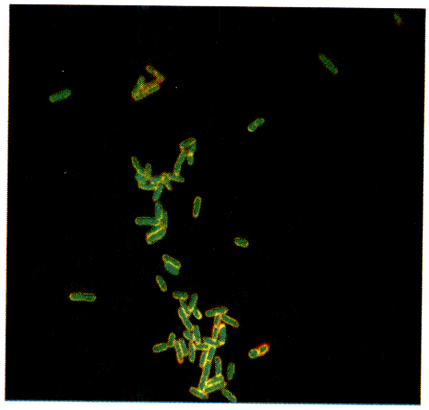
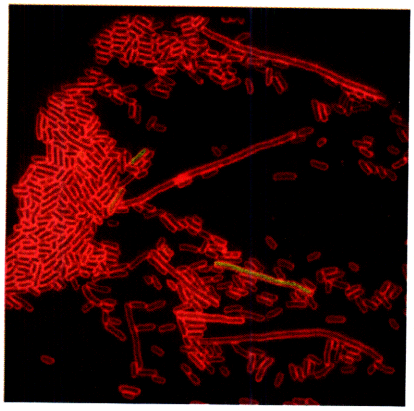


Figure 5. A role for the SOS response in survival after exposure to nitrofurazone. At a concentration of 2μg/mL nitrofurazone, *lexA3*(Ind$^-$) (DM49) mutants are very sensitive to nitrofurazone and this sensitivity is not exaggerated by the addition of either Δ*dinB* (SEC900) or *nusA11* (SEC813) mutation. These experiments were done at the permissive temperature (30°C). Error bars represent the standard deviation of three independent cultures.

Figure 6

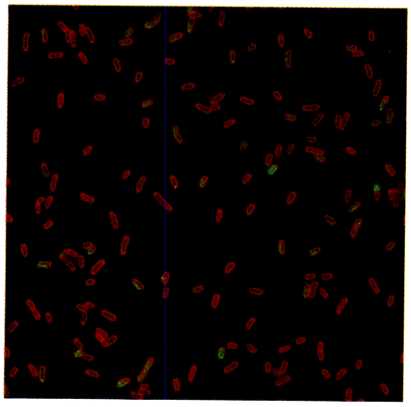
A 

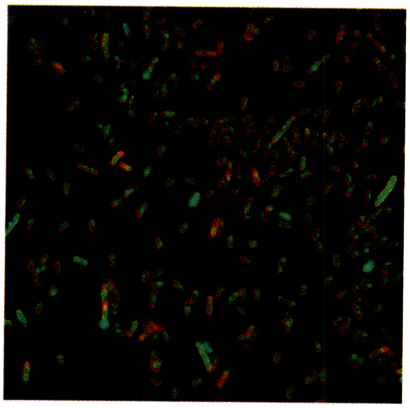
B 

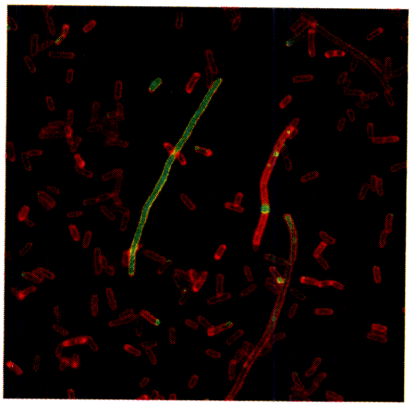
C 

D

	wild type	*lexA*(Def)	*nusA11*
No. cells counted	731	330	402
% SOS induced	0.1	100	2.5

E 

F 

G 

H

	wild type	wild type + UV	*nusA11*
No. cells counted	2000	509	362
% foci	2	>99	19
% of foci at poles	72		64

Figure 6. *nusA11* cells display phenotypes of altered DNA processing. A-C) Representative micrographs of A) wild-type (AB1157) (SEC677), B) *lexA*(Def) (SEC678) and C) *nusA11* cells (SEC679). Cell outlines (shown in red) were visualized with the vital membrane stain FM4-64, and SOS induction as monitored from P_{sulA}-GFP fusion (shown in green). D) Quantification of SOS induced cells show that at the permissive temperature *nusA11* cells display chronic partial SOS induction. E-G) Representative micrographs of E) wild-type/AB1157 cells, F) wild-type/AB1157 cells irradiated with 25 J/m^2 UV, and G) *nusA11* cells. Cell outlines (shown in red) were visualized with the vital membrane stain FM4-64, and RecA-GFP foci are shown in green. H) Quantification of RecA-GFP foci, show that at the permissive temperature *nusA11* cells have more RecA-GFP foci compared to *nusA*$^+$.

Figure 7

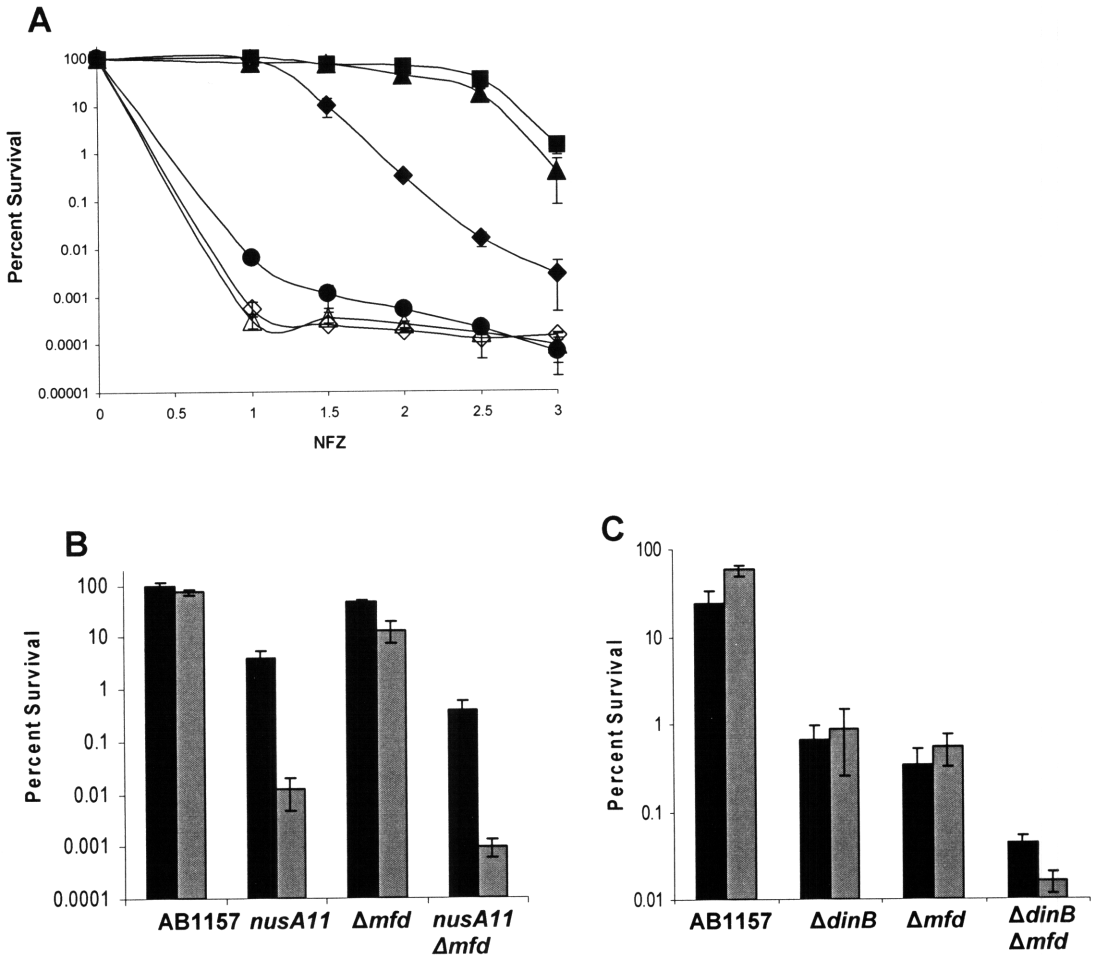


Figure 7. Interactions between *nusA* and nucleotide excision repair. A) Nitrofurazone killing curve, shows that Δ*uvrA* (closed circles) (SEC 316) strains are more sensitive to NFZ than wild-type/AB1157 (squares), Δ*dinB* (closed triangles) or *nusA11* (closed diamonds) (SEC164) strains. Furthermore Δ*dinB*Δ*uvrA* (open triangles) (SEC317) or *nusA11*Δ*uvrA* (open diamonds) (SEC318) strains are not any more sensitive and a Δ*uvrA* alone. Doses of nitrofurazone are in μg/mL. In this and all graphs in this figure, error bars represent the standard deviation determined from three independent cultures. B) At 30°C *nusA11* (SEC164) and Δ*mfd* strains show an additive phenotype with respect to nitrofurazone (2 μg/mL) sensitivity. C) Δ*dinB* and Δ*mfd* strains show an additive phenotype with respect to nitrofurazone (2 μg/mL) sensitivity at 37°C.

Figure 8

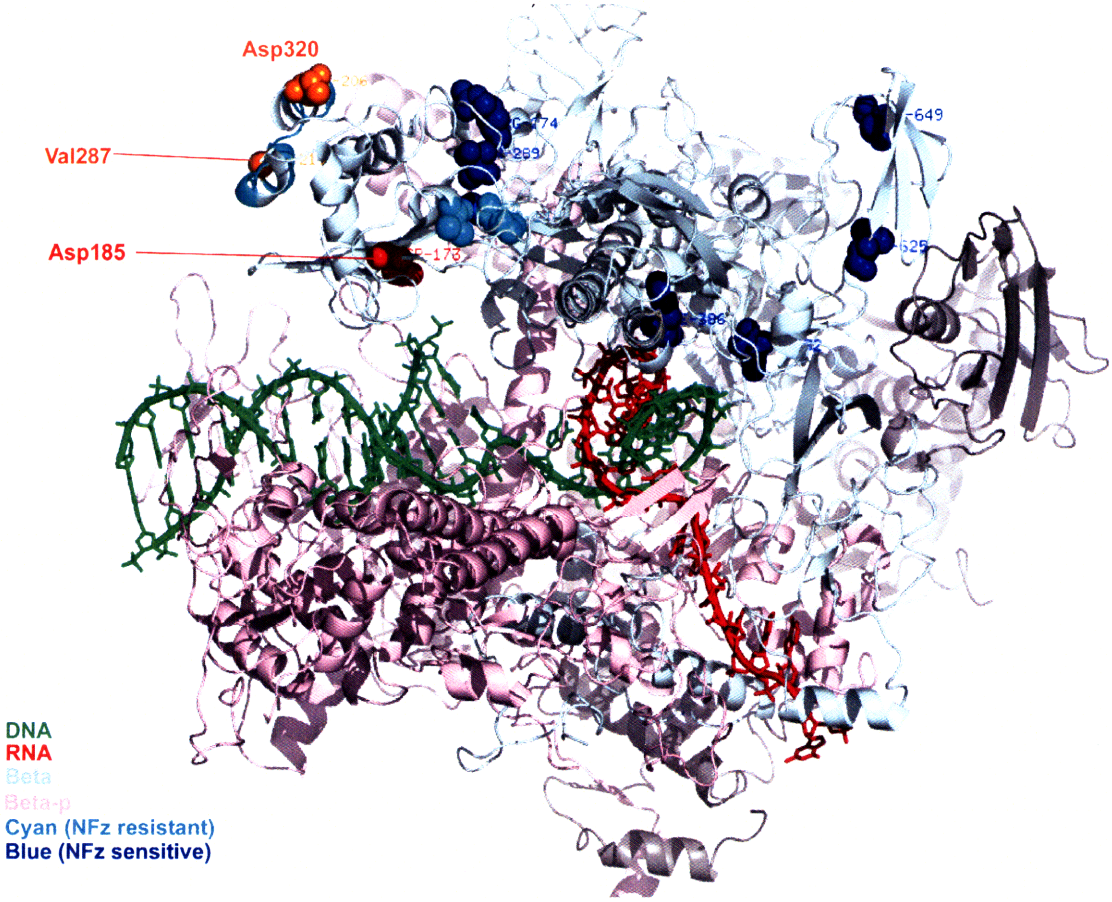


Figure 8. Position of residues affected by NFZ^S or NFZ^R RNA polymerase mutants on the crystal structure of *T. thermophilus* RNAP elongation complex. DNA is shown in green. RNA is shown in red. *rpoB*, β catalytic subunint, shown in grey and the β' catalytic subunit, encoded by the *rpoC* gene, is shown in pink. NFZ resistant mutants are shown in cyan and NFZ sensitive mutants are shown in blue. Position of mutants characterized in figure 9 (Asp320, Val287 and Asp 185) are labeled.

Figure 9

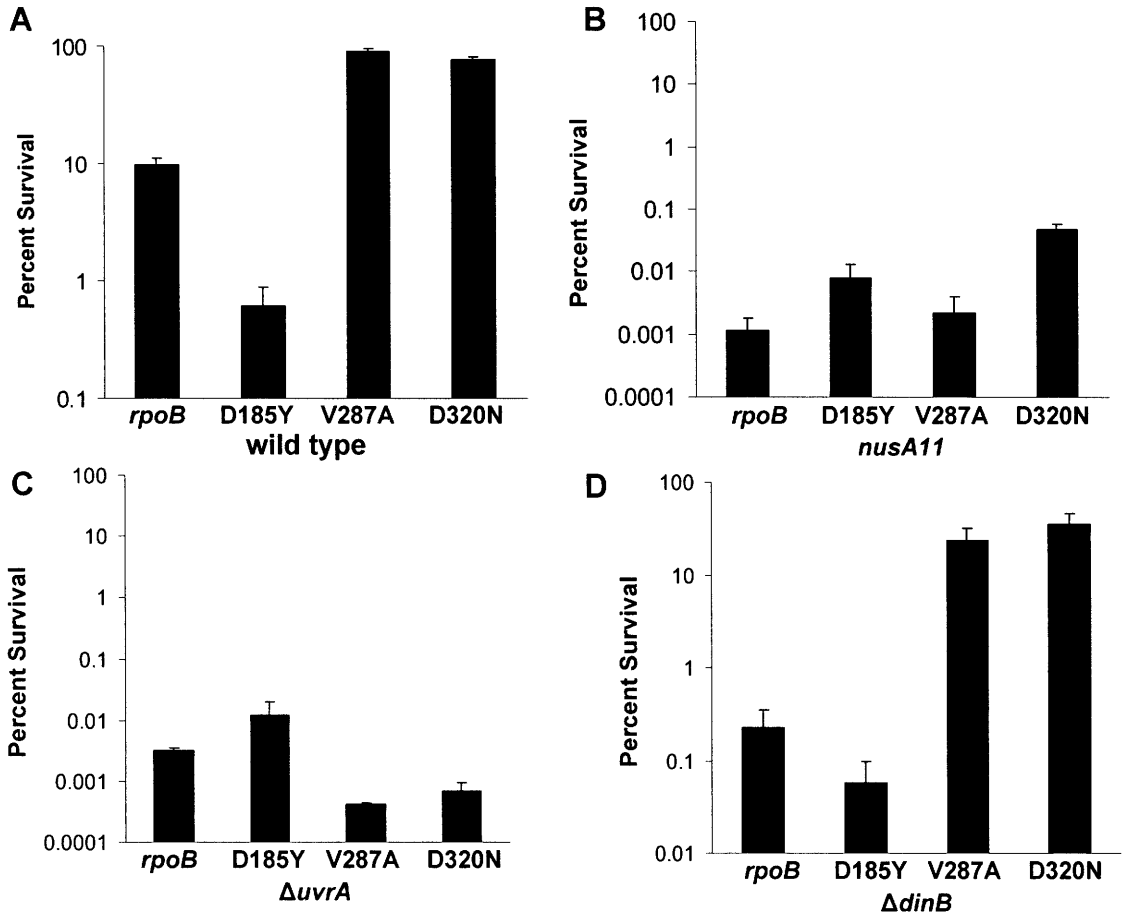


Figure 9. *rpoB* mutants show altered survival after nitrofurazone treatment. A-D) Quantification of selected *rpoB* mutants in a variety of backgrounds. A) wild-type/AB1157, B) *nusA11*, C) Δ*uvrA*, and D) Δ*dinB*. All experiments were done at 37°C except for those done in a *nusA11* background, which were performed at 30°C. Concentration of nitrofurazone is 2 μg/mL. Error bars represent the standard deviation determined from three independent cultures.

Figure 10

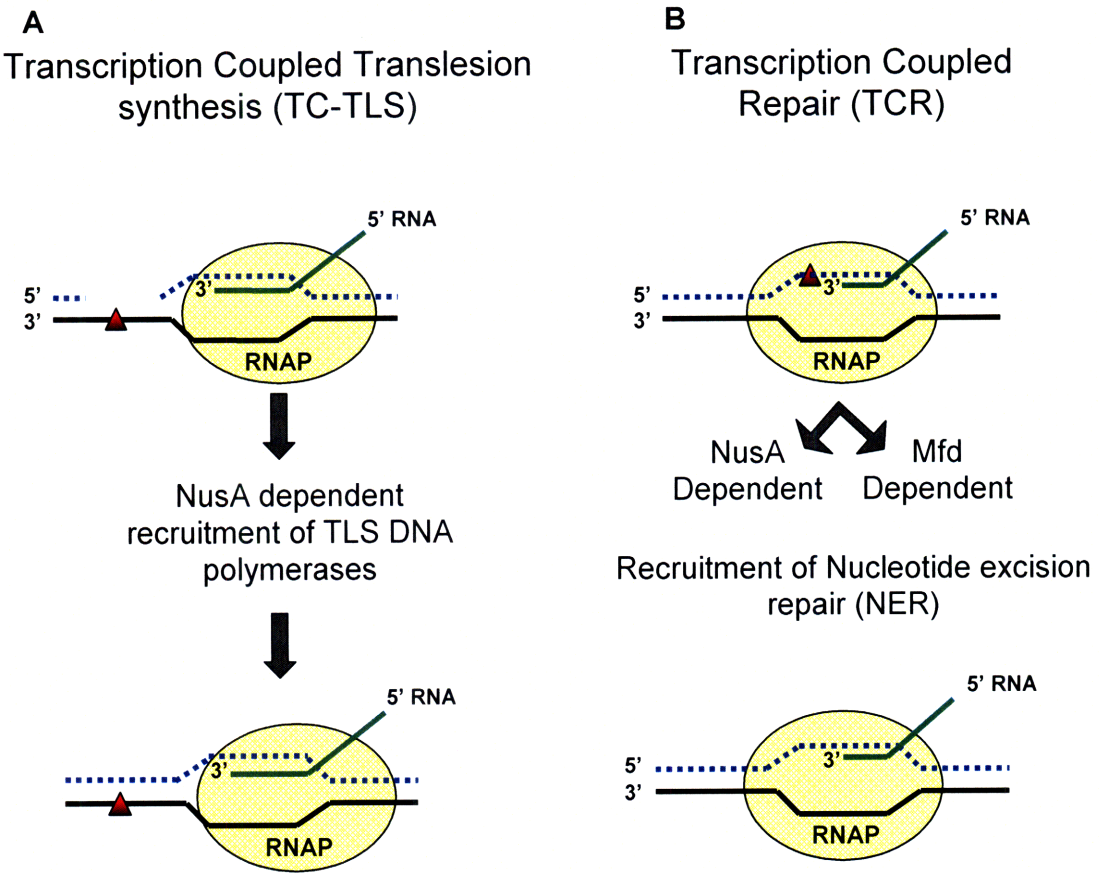


Figure 10. Models of transcription-coupled translesion synthesis (TC-TLS) and alternative transcription-coupled repair (TCR). A) In the model of TC-TLS NusA, associated with elongating RNA polymerases, can recruit TLS polymerases to fill in gaps opposite to lesions in the transcribed strand in order to allow for the continuation of transcription. B) A new class of TCR, NusA-dependent TCR, where NusA participates in a previously unrecognized branch of the TCR pathway. NusA is capable of recruiting NER to sites of stalled RNAP's to repair DNA lesions on the transcribed strand.

Table 1. RNA polymerase mutants isolated as nitrofurazone resistant or sensitive

Plasmid name	Mutation DNA position: Change	Amino acid resuide number	Amino Acid change	Phenotype
1A6	847:A→T	283	Lys→Stop	Sensitive
	867: T→A	289		
	1174: G→A	392		
1A10	*			Sensitive
1F5	801: C→A	267	Arg→Arg	Sensitive
	1221: T→C	407	Arg→Arg	
	1280: A→G	427	Asp→Glu	
1G12	495: T→C	166	Ser→Pro	Sensitive
	607: -1 frameshift			
1H7	460: G→A	154	Gly→Ser	Sensitive
	920: C→A	307	Gly→Gly	
	1179: C→T	393	Asn→Asn	
1B1	*			Sensitive
1C7	*			Resistant
2A7	553: G→T	185	Asp→Tyr	Sensitive
2F4	*			Sensitive
2E9	31: A→G	11	Ile→Val	Sensitive
	55:C→T	19	Pro→Ser	
	352:A→G	118	Lys→Glu	
2F8	*			Sensitive
4A8	860: T→C	287	Val→Ala	Resistant
4B8	*			Sensitive
4F2	1153: T→C	385	Phe→Leu	Resistant
	457: C→T	153	Pro→Ser	
	727:A→T	244	Glu→Val	
4H3	108: G→A			Resistant
	901: T→A			
	931: T→A			
	1032: -1 frameshift			
5A2	*			Sensitive
5E11	772: A→T	258	Asn→Tyr	Sensitive
5G3	958:G→A	320	Asp→Asn	Resistant
6D1	*			Resistant
7A3	1637: A→G	546	Glu→Gly	Sensitive
	1179: -1 frameshift			
7D9	1529: A→G	510	Gln→Arg	Sensitive
	1561:C→T	521	Leu→Leu	

	1679: -1 frameshift			
7D12	1792:G→T	598	Val→Phe	Sensitive
	1973:A→G	658	Gln→Arg	
	2377:G→T	793	Glu→Stop	
7F3	1964:T→C	655	Val→Ala	Sensitive
	2278:A→G	760	Asn→Asp	
7F7	2258:T→C	753	Leu→Pro	Sensitive
7F10	1157:T→G	506	Phe→Cys	Sensitive
9A7	1315:A→G	439	Lys→Glu	Sensitive
9A10	1606: G→A			Sensitive
	1979: -1 frameshift			
9B10	1596: A→T	532	Ala→Ala	Sensitive
	1898: T→A	633	Leu→His	
	1974: G→T	658	Gln→His	
9B11	*			Sensitive
9C5	1702/1703: AA→GG	568	Asn→Gly	Sensitive
	1875: A→T	625	Glu→Asp	
9C7	1317: A→G	439	Lys→Arg	Sensitive
	1493: T→C	498	Ile→Thr	
9D3	1845: T→C	615	Val→Val	Sensitive
	2235: A→G	745	Glu→Glu	
	2372: T→C	791	Leu→Pro	
9E2	1328: A→T	443	Asp→Val	Sensitive
9F7	2228: C→T	743	Pro→Leu	Sensitive
	2333: A→G	778	Glu→Gly	
	2482: T→C	828	Phe→Leu	
9G10	1836:C→T	612	Gly→Gly	Sensitive
	1948: G→A	650	Val→Ile	
	1985: C→A	662	Ser→Thr	
	2016: A→T	672	Glu→Asp	

*Sequencing of these mutants either failed completely or was not able to identify mutation

Table 2. Strains and plasmids used in this study

Strains	**Genotype**	**Reference**
AB1157	*thr-1 leuB6 proA2 hisG4 thi1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*	Lab Stock
AB1157 Δ*dinB*::frt (SEC95)	As AB1157 except Δ*dinB*::frt	This work
SEC164	As AB1157 except *nusA11 zha0132*::Tn*10*	This work
TP651	As AB1157 except Δ*sulA*::Tet	Murphy et al., 2000
CAG12127	*Lam*⁻ *sfsB3198*::Tn*10*kan rph-1	CGSC
SEC302	As AB1157 except *nusA11 sfsB3198*::Tn*10*kan	This work
SEC524	As SEC302 except Δ*sulA*::Tet	This work
AB1157 *lexA*(Def)	As AB1157 except *lexA300*::Spc *sulA11*	Lab Stock
SS996	F⁻ *lacMS286 argE3 his-4 thi-1 xyl-5 mtl-1 sulB103* del(attB):P*sulA*-gfp	McCool et al., 2004
CAG18341	*nadA3052*::Tn*10*kan, *rph-1*	CGSC
SS996 *nadA*	As SS996 except *nadA3052*::Tn*10*kan	This work
SEC677	As AB1157 except del(attB):P*sulA*-gfp	This work
SEC678	As AB1157 except *lexA300*::Spc *sulA11* del(attB):P*sulA*-gfp	This work
SEC679	As SEC164 except del(attB):P*sulA*-gfp	This work
SS3041	F⁻ *lacMS286 argE3 his-4 thi-1 xyl-5 mtl-1 sulB103 ygaD1*::kan *recA∘1403 recA4136*::*gfp901*	Renzette et al., 2005
SEC477	As AB1157 except *ygaD1*::kan *recA∘1403 recA4136*::*gfp901*	This work
SEC604	As SEC164 except *ygaD1*::kan *recA∘1403 recA4136*::*gfp901*	This work
JW4019-2	F⁻, *Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *LAM*⁻, *rph-1*, *Δ(rhaD-rhaB)568, ΔuvrA753::kan, hsdR514*	Baba et al., 2006
SEC316	As AB1157 except Δ*uvrA753::kan*	This work
SEC317	As SEC95 except Δ*uvrA753::kan*	This work
SEC318	As SEC164 except Δ*uvrA753::kan*	This work
AB1157 Δ*mfd*::kan	As AB1157 except Δ*mfd*::kan	Selby and Sancar,
AB1157 Δ*dinB*::Cm	As AB1157 except Δ*dinB*::Cm	Jarosz et al., 2006
SEC194	As AB1157 except Δ*mfd*::kan Δ*dinB*::Cm	This work
SEC199	As SEC164 except Δ*mfd*::kan	This work
DM49	As AB1157 except *lexA3*(Ind⁻)	Lab stock
SEC900	As DM49 except Δ*dinB*::frt	This work

SEC813	As DM49 except *nusA11 zha0132*::Tn*10*	This work
MDS42	MG1655 with ~14% of genome deleted	Posfai et al., 2006
MDS42Δ*nusA*	As MDS42 except Δ*nusA*::Cm	Cardinale et al., 2008
MDS42 Δ*dinB*	As MDS42 except Δ*dinB749*::kan	This work
MDS42 *nusA11*	As MDS42 except *nusA11 zha0132*::Tn*10*	This work
SEC29	Same as P90C except *nusA11 zha0132*::Tn10	Cohen et al., 2009
JW0221-1	F⁻, *Δ(araD-araB)567*, *ΔdinB749::kan*, *ΔlacZ4787*(::rrnB-3), *LAM⁻*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba et al., 2006

Plasmid		
pWSK29	Vector, pSC101-like replicon, Amp^R	Jarosz et al., 2006
pYG768	DinB under its native promoter in a low copy number pWSK29 plasmid	Kim et al., 1997
pYG782	*dinB* gene cloned in the pWSK30 plasmid (Amp^R) which is the same as pWSK29 but with the MCS cloned in the opposite orientation under the lac promoter	Kim et al., 1997
pYG768(K217E)	As pYG768 with indicated mutation introduced using quickchange site-directed mutagenesis	This work
pYG782(K217E)	As pYG782 with indicated mutation introduced using quickchange site-directed mutagenesis	This work
pRL706	Encodes C-terminal His6-tagged version of the *rpoB* gene, under control of a *lac*-repressor-regulated *trc* promoter cloned into pBR322, Amp^R	Severinov et al. 1997
mutagenized pRL706	As pRL706 except mutagneized by error prone PCR	Santangelo et al., 2003
pDFJ1	DinB expression plasmid under T7 promoter in pET11t backbone; Amp^R	Jarosz et al., 2006
pDFJ1(K217E)	As pDFJ1 with indicated mutation introduced using quickchange site-directed mutagenesis	This work
pNusA	N-terminal His_6-tagged full length NusA cloned into pET11d	Mah et al., 1999
pBR322	Empty vector, Amp^R	New England Bio labs
pNAG2010	NusA cloned into pBR322 under its own promoter	Craven et al., 1994

Chapter 5

A Novel DinB Variant Reveals Diverse Physiological Consequences of Incomplete Extension by a Y-family DNA Polymerase

This Chapter has been submitted for publication as: Daniel F. Jarosz, Susan E. Cohen, James C. Delaney, John M. Essigmann, and Graham C. Walker. A novel DinB variant reveals diverse physiological consequences of incomplete extension by a Y-family DNA polymerase. My contributions include Figures 4, 5, 6 and Supplemental Figure 3.

ABSTRACT

DinB and its mammalian ortholog pol κ catalyze proficient bypass of damaged DNA in translesion synthesis (TLS). DinB is also the only Y-family DNA polymerase conserved among all domains of life. Y-family DNA polymerases, including DinB, have been implicated in diverse biological phenomena ranging from adaptive mutagenesis in bacteria to several human cancers. Complete TLS requires dNTP insertion opposite a replication blocking lesion and subsequent extension with several dNTP additions. Here we report the first example of preferential TLS extension by any Y-family DNA polymerase. We also describe a new class of TLS DNA polymerase mutant resulting from mutation of an evolutionarily conserved tyrosine (Y79), generating a DinB variant that catalyzes formal lesion bypass, but cannot finish the subsequent extension steps that are required to complete TLS, stalling three nucleotides after an N^2-dG lesion. Expression of this variant *in vivo* dramatically enhances killing during challenge with DNA damaging agents, transforming a bacteriostatic drug nitrofurazone into a bactericidal drug. Diverse gene products modulate this toxicity associated with incomplete TLS extension, including the toxin-antitoxin module MazEF and the iron import protein TonB. Together, these results not only indicate that DinB is specialized to perform remarkably proficient TLS insertion and extension, but also expose unexpected connections between the products of incomplete TLS and cell fate.

INTRODUCTION

Y-family DNA polymerases are found in virtually all organisms and possess the remarkable ability to copy over lesions that would otherwise stall DNA replication, a process termed translesion synthesis (TLS) (45). Such broadened substrate specificity often comes at a mutagenic cost, however, as these enzymes display lower fidelities on undamaged DNA templates than their replicative counterparts (9, 17, 20). Organisms have therefore developed elaborate regulatory systems to restrict the access of Y-family DNA polymerases to primer termini where TLS is required (1, 2, 4, 11, 12, 25, 26, 33, 55).

Studies of Y-family DNA polymerase function in prokaryotes, eukaryotes, and archea have revealed that diverse mechanisms contribute to regulation of their function (17, 18, 20, 47, 60). In *E. coli*, the function of both DinB (DNA pol IV) and $UmuD'_2C$ (DNA pol V) is strongly influenced by interactions with the β processivity clamp of DNA polymerase III (2, 3, 59) and the *umuD* gene products, $UmuD_2$ and $UmuD'_2$ (10, 15, 49, 55, 57). Other protein-protein interactions, notably with single stranded DNA binding protein (SSB) (10, 49), RecA (50-52), and RecFOR (13) also play critical roles in regulating TLS. In eukaryotes, TLS is similarly controlled in numerous ways including by association with the PCNA processivity clamp (1, 33, 44, 47, 64), interaction with recombination proteins (40, 48), and by ubiquitylation of both PCNA and the polymerases themselves (1, 4, 19, 26, 32, 44, 47).

Several Y-family DNA polymerases display a remarkable intrinsic preference for catalytic action on particular damaged substrates. Pol η from both *S. cerevisiae* and *H. sapiens* copies preferentially over cyclobutane pyrimidine dimers (23, 37, 39), whereas *E. coli* DinB and its mammalian ortholog *M. musculus* DNA polymerase κ (pol κ) each display strikingly elevated catalytic proficiencies for dC insertion across from certain N^2-dG lesions (21). Numerous diverse structural features of Y-family DNA polymerases contribute to their novel catalytic capabilities. For example, relatively accommodating active sites (35, 53, 63) and divergent C-terminal 'little finger' domains (5) have been shown to facilitate TLS by several Y-family DNA polymerases. Specialized TLS function does not arise exclusively from open active sites, however. Indeed, the active sites of both pol κ (36) and DinB (15) are somewhat closed under many conditions, and this may occur at least in part through interaction with other proteins.

Although some of the multiple protein-protein interactions that govern access of Y-family DNA polymerases to primer termini are understood, comparatively little is known about their intrinsic biochemical preference for activity on particular damaged substrates. Specific active site residues in both pol η and DinB are critical for TLS function. In *E. coli* DinB and *S. cerevisiae* pol η, mutation of F13 or F34, respectively, generates polymerase variants that are proficient for normal DNA synthesis but unable to catalyze TLS (21, 43). Similarly, mutation of Y52 in human DNA polymerase η profoundly affects UV-induced mutagenesis, but does not alter its ability to perform canonical DNA synthesis (14). Other such molecular determinants of TLS function assuredly exist (5), some of which may be common to all Y-family DNA polymerases

and others of which may be restricted to certain such polymerases according to their distinct substrate specificities.

Complete TLS involves insertion of a nucleotide opposite an adducted base (i.e. lesion bypass) and extension from that position by several subsequent nucleotide additions. In eukaryotes, these steps are often catalyzed by at least two distinct DNA polymerases (47). Replication by the TLS polymerase must continue for several nucleotides beyond the site of DNA damage, greater than 5 nucleotides in the case of pol V in *E. coli*, in order to avoid reversal by the proofreading activity of the replicative DNA polymerase (11). Consistent with a role in surveying the products of TLS, the *E. coli dnaQ*$^+$ gene, which encodes the ε proofreading subunit of DNA polymerase III (pol III), affects several mutagenic phenomena that depend on *dinB*$^+$ (29-31). Consequently, regulating the length of the products formed during TLS appears to be of critical importance, as those that are too small risk being removed by proofreading and those that are too long may carry undue mutagenic potential.

More broadly, recent studies hint that TLS may be connected to cellular stress response networks in unexpected ways (16, 41, 46). Curiously, lethality induced by hydroxyurea (HU)-mediated dNTP depletion can be abrogated by UmuC variants that lack several regulatory motifs, and this rescue requires *dinB*$^+$ (16). Cytotoxicity from HU exposure also requires the toxin-antitoxin modules *mazEF* and *relBE* (16); these genes act synergistically to effect HU resistance in a wild-type background (6).

Here we report that DinB catalyzes strikingly proficient and preferential extension after dC incorporation opposite an N^2-furfuryl-dG DNA lesion. Moreover, a cluster of aromatic hydrophobic residues surrounding DinB's 'steric gate' dictate its function in

TLS extension. Specifically, we demonstrate that Y79, which is invariant among DinB orthologs, critically influences the extension steps of DinB-catalyzed TLS. Our data also indicate that cellular survival is coupled to completion of TLS and that the toxicity of abortive TLS is mediated by both the toxin-antitoxin module MazEF and the iron import protein TonB. These observations strongly indicate that the detailed execution of TLS is coupled to fundamental aspects of cellular physiology.

RESULTS

DinB catalyzes preferential extension on an N^2-dG damaged template

We systematically analyzed the activity of wild-type DinB around a site-specific N^2-furfuryl-dG lesion by performing a series of staggered primer extension assays. Individual standing start assays were performed using primers with 3' termini starting four base pairs before the site of the lesion and ending four base pairs after the lesion (Figure 1A; Supplemental Table I). The apparent catalytic proficiency of DinB (V_{max}/K_m) on the damaged template is comparable to an undamaged control for the first several additions tested, and as we previously reported, DinB is exceptionally proficient at catalyzing dC insertion opposite this lesion due to a reduced K_m for dCTP on the damaged substrate (21). Additionally, we found that DinB is even more proficient at extending from the dC opposite the N^2-furfuryl-dG lesion, showing a 25-fold increased catalytic proficiency on the damaged template for this reaction (Figure 1B). This striking difference in activity arises primarily from a low K_m for dATP addition at the +1 position immediately following the the N^2-furfuryl-dG lesion (Figure 1B). Subsequent extension steps proceed with comparable efficiencies on both damaged and undamaged templates. These observations reveal that in addition to highly proficient insertion opposite an N^2-furfuryl-dG lesion, efficient extension immediately after the lesion is also a specialized function of DinB. The exceptional proficiency with which DinB catalyzes such TLS supports our hypothesis that at least one of its key biological roles is to replicate over ubiquitously occurring classes of N^2-dG adducts (21, 22). Intriguingly, as described below, an independent line of investigation pointed to the further physiological importance of later extension steps that proceed with more modest proficiency.

Aromatic residues surrounding DinB's steric gate are critical for its function

We previously reported that, in addition to its well established role in discrimination against improper rNTP incorporation (7), the steric gate residue of DinB (F13) is indispensable for its TLS function (21). This insight grew out of our efforts to develop a structural model of DinB encountering an N^2-furfuryl-dG lesion. We subsequently undertook a more detailed examination of this model to ascertain whether additional residues might contribute to the preferential activity of DinB on a damaged substrate. We noted that F13 is surrounded by a cluster of aromatic residues: F12, F76, and Y79 (Figure 2A). In our homology model, Y79 stacks with DinB's F13 steric gate, while F76 and F12 are oriented with a rotation of roughly 90 degrees relative to it (Figure 2A). Provocatively, conservation of F12 is universal among Y-family DNA polymerases (Figure 2B) and this residue corresponds to F34 of *S. cerevisiae* Pol η, mutation of which selectively eliminates its TLS function (43). F76, in contrast, is comparatively weakly conserved even among DinB orthologs. Y79 is invariant among DinB sequences from diverse organisms. Intriguingly, *E. coli* UmuC also shares a tyrosine residue at this position, whereas the related but functionally distinct enzymes Rev1 and Rad30 possess a phenylalanine instead (Figure 2B).

To examine the effect of these amino acids on DinB function, we mutated each of F12, F76, and Y79 to valine and examined the ability of low copy number plasmids encoding these variants under the control of the wild-type *dinB* promoter to complement the sensitivity of a *ΔdinB* strain to the DNA damaging agents nitrofurazone (NFZ) and 4-nitroquinoline-1-oxide (4-NQO) (21). Mutation of F12 generates a DinB variant that is unable to complement NFZ sensitivity, but shows a far less dramatic phenotype than the

extreme sensitivity conferred by the steric gate mutant *dinB*(F13V) (Figure 2C). This observation indicates that the equivalent phenylalanine residues in *S. cerevisiae* DNA polymerase η may have a unique function in that subfamily of TLS DNA polymerases. Mutation of F76 also impairs complementation (Figure 2C), but again does not confer similarly increased NFZ sensitivity to *dinB*(F13V). Strikingly, mutations that alter Y79 (Y79A, Y79V, Y79L) have a profound effect on sensitivity to both NFZ and 4-NQO (Figure 2D-E). For example, a plasmid expressing DinB(Y79L) is not only unable to complement the NFZ sensitivity of a *ΔdinB* strain, but also confers a further 10-50-fold sensitivity beyond that of an empty vector control. These observations are consistent with a critical role for Y79 in the *in vivo* bypass of N^2-dG adducts, and reveal that its mutation produces catastrophic effects when cells are exposed to NFZ or 4-NQO.

DinB(Y79) mutant proteins catalyze proficient lesion bypass

We initially suspected that the inability of DinB(Y79L) to restore NFZ or 4-NQO resistance to a *ΔdinB* strain (Figure 2C-E) would be due to an inability to catalyze TLS over the N^2-dG lesions produced by these agents, just as the with DinB(F13V) mutant protein we previously described (21). Unexpectedly, when we examined the TLS activity of DinB(Y79L) *in vitro*, we discovered that it is entirely able to bypass an N^2-furfuryl-dG lesion and has comparable activity to wild-type DinB on undamaged DNA (Figure 3A). However, the products synthesized by DinB(Y79L) are much shorter than those produced by wild-type DinB and the majority appear to arise from stalled synthesis three nucleotides past the site of the lesion. This phenomenon may arise from exacerbation of a natural pause site at the +3 position, as wild-type DinB also shows modest

accumulation of this product at lower enzyme concentrations. Notably, wild-type DinB also stalls three nucleotides following the site of an N^2-dG DNA-protein crosslink in independent primer extension assays (42).

Expression of DinB(Y79L) does not result in an increased frequency of mutation to rifampicin resistance upon NFZ treatment ($4.2 \pm 2.0 \times 10^{-8}$ for pDinB(Y79L) vs. $5.5 \pm 3.0 \times 10^{-8}$ for pDinB; average mutation frequency and standard deviation from five independent cultures), suggesting that any TLS it carries out *in vivo* is relatively accurate. Thus, DinB(Y79L) defines a new class of Y-family DNA polymerase mutants that are able to catalyze formal lesion bypass but are unable to finish the subsequent extension steps that are required physiologically to complete TLS. These combined observations strongly indicate that the extension steps of TLS have at least as much physiological importance as lesion bypass itself.

To precisely recapitulate the TLS extension defect of DinB(Y79L) we examined its activity on a primer synthetically elongated three base pairs beyond the site of the N^2-furfuryl-dG lesion. Whereas DinB(Y79L) retains roughly half of the activity of wild-type DinB for this addition on an undamaged template, it is impaired by more than 20-fold on the damaged substrate (Figure 3B). This result reveals that the stalling behavior of DinB(Y79L) likely arises from communication between the lesion and the enzyme's active site at the +3 position. There is an emerging precedent for such interactions with the template past the site of synthesis dictating polymerase activity. Structural studies of the archaeal DinB ortholog Dbh have revealed that its ability to catalyze –1 frameshift mutagenesis in polyG regions requires specific interactions between the enzyme and the template at the +3 position that stabilize an extrahelical base (62). Similarly, specific

contacts between enzyme and template allow replicative DNA polymerases to couple distal mismatch recognition to synthesis in the active site (24). Our observations regarding DinB(Y79L) indicate that Y79 plays a critical role in catalysis at the +3 position during TLS.

We also tested whether the function of Y79 requires its hydroxyl group by constructing a Y79F mutation. A plasmid expressing DinB(Y79F) fully complements the NFZ sensitivity of a *ΔdinB* strain over the dose regime used in our initial experiments (Supplemental Figure 1A). Unexpectedly, pDinB(Y79F) confers remarkable resistance to a *ΔdinB* strain when treated with a *ca.* 10-fold higher dose of NFZ (Supplemental Figure 1B). The reason for this effect is unclear, but it may indicate that Y79 either participates in a specific hydrogen-bonding interaction(s) or is posttranslationally modified under conditions of elevated NFZ exposure. Phosphorylation of Y79 is unlikely, however, as deletion of either of the tyrosine kinases in *E. coli* (*etk* or *wzc*) does not phenocopy the effects of pDinB(Y79F) at high NFZ doses (data not shown). Interestingly, deletion of *dinB* itself confers relatively little sensitivity under these conditions, suggesting that DinB(Y79F) likely either gains a function that promotes survival or interferes with a process that mediates cell death.

Genetic interactions between *dinB* and the ε proofreading subunit of DNA Pol III

Although DinB(Y79L) and DinB(F13V) are both unable to carry out complete TLS, it is unclear why their expression causes such a profound increase in killing by NFZ in a *ΔdinB* strain (Figure 2C,D) and even strikingly in a wild-type *dinB*$^+$ background (Figure 4A). In contrast, a plasmid expressing the catalytically deficient DinB(cat-)

mutant protein, which also cannot carry out TLS, does not cause a similar increase in NFZ sensitivity (Supplemental Figure 2). These observations, along with the biochemical properties of DinB(Y79L) and DinB(F13V), hinted that these mutant proteins may exert their effects during NFZ exposure by inducing a futile cycle in which the immature TLS intermediates they produce are constantly destroyed by the action of the ε proofreading activity of DNA polymerase III, encoded by the *dnaQ*$^+$ gene. A similar behavior has been observed for pol V (11). Indeed, recent studies have revealed that extension of a TLS product by a single base pair is sufficient to rescue it from ε-mediated degradation (12).

We therefore examined the effects of DinB(Y79L), DinB(F13V), and DinB(Y79F) expression in a *ΔdnaQ dinB*$^+$ background. Deletion of *dnaQ* does not prevent DinB(Y79L) or DinB(F13V) expression from conferring increased sensitivity to NFZ in a *dinB*$^+$ background (Figure 4A-B). *E. coli* strains bearing *dnaQ* deletions commonly acquire suppressor mutations in *dnaE*, referred to as *spq-2* (34, 54); the *spq-2* suppressor mutation does not affect the phenotypes of any of the *dinB* alleles used in this study (data not shown). We also employed the *dnaQ903* allele, which encodes an ε variant that lacks one its three exonuclease domains as well as the C-terminal amino acids that are required for interaction with the α catalytic subunit of DNA polymerase III (54, 56). The striking mutator frequency of strains bearing *dnaQ903* indicate that it is defective in proofreading function (56). Expression of either DinB(F13V) or DinB(Y79L) still enhances NFZ killing in a *dnaQ903* strain (Figure 4C) compared to a vector control. Curiously, we observed a strikingly reduced transformation efficiency of pDinB in the *dnaQ903* strain (Figure 5C) and the infrequent pDinB transformants that we

did obtain displayed highly variable phenotypes, suggesting that they had acquired suppressor mutations. These results indicate that the NFZ killing seen upon DinB(F13V) or DinB(Y79L) expression is not primarily due to a futile cycle of abortive TLS followed by proofreading. Indeed, further experiments following up on these unexpected findings revealed considerably increased complexity (see below).

We were surprised to find that mutation of *dnaQ* has no effect on the NFZ sensitivity conferred by DinB(Y79L) or DinB(F13V) expression given the abundance of data suggesting genetic interactions between $dnaQ^+$ and TLS polymerases, including $dinB^+$ (11, 29-31). We therefore examined the consequences of *dinB* deletion combined with *dnaQ* deletion or the *dnaQ903* proofreading-deficient allele. Both of the *dnaQ* alleles confer sensitivity to NFZ, although the degree of sensitivity differs (Figure 5A-B). Like a *ΔdnaQ* mutation, the *dnaQ903* allele abolishes proofreading (54, 56). However, the N-terminal portion of the DnaQ protein is present, providing a possible explanation for the differing magnitudes of NFZ sensitivity. We observed an epistatic relationship with respect to NFZ sensitivity for each *dnaQ* allele and *ΔdinB* (Figure 5A-B) in which the double mutants behave most similarly to the *dnaQ* mutants alone. These observations confirm that $dnaQ^+$ affects $dinB^+$ function under conditions of NFZ exposure. However, the data presented in Figure 4B-C indicate that this interaction does not mediate the NFZ sensitivity observed upon DinB(F13V) or DinB(Y79L) expression.

Unexpectedly, we observe a diversion from this paradigm in the case of DinB(Y79F), which is transformed into an enhancer of cytotoxicity during NFZ exposure in a *ΔdnaQ* strain (Figure 4A-B). This striking reversal may reflect an increased importance of $dnaQ^+$ in regulating the function of this DinB variant. We observed that

the transformation efficiency of both *dnaQ* mutant strains is reduced relative to wild-type. However this phenomenon is particularly striking for pDinB and pDinB(Y79F) (Figure 5C). Furthermore, we were unable to transform pDinB(Y79F) into a *dnaQ903* strain, reinforcing our conclusion that there is an interaction between *dinB* and *dnaQ* that is independent of DnaQ's biochemical proofreading activity. Together these phenomena underscore the importance of $dnaQ^+$ in relieving the consequences of a deleterious DinB function that may be exacerbated in the DinB(Y79F) mutant.

Toxin-antitoxin pairs and metal homeostasis modulate toxicity of abortive TLS

The extreme effects of DinB(Y79L) and DinB(F13V) expression in a $dinB^+$ background led us to investigate the mechanism of NFZ sensitivity by examining recovery of viable bacteria from regions between colonies on NFZ agar plates. Consistent with NFZ being a bacteriostatic rather than bactericidal agent (38), we were able to recover an equivalent number of colony forming units from strains expressing either DinB or DinB(Y79F). Remarkably, we were unable to recover bacteria from strains expressing DinB(Y79L) or DinB(F13V) (data not shown), suggesting that NFZ is transformed into a bactericidal agent in these mutants.

To further characterize the mechanisms of DinB(Y79L)- and DinB(F13V)-mediated cytotoxicity, we considered their effects in a variety of mutant backgrounds. The *mazEF* and *relBE* genes encode toxin-antitoxin modules that alter cellular physiology and metabolism in response to environmental stress, such as antibiotic exposure (8, 28). Inactivation of *mazEF* or *relBE* increases resistance to replication inhibition by hydroxyurea (Godoy et al. 2006). We considered whether toxin-antitoxin pairs might similarly mediate the lethality of replication intermediates produced by

DinB(Y79L) or DinB(F13V). Strains bearing deletions of *mazEF*, *relBE*, or an alternative toxin-antixoin pair *hipAB* are not themselves sensitive to NFZ (Supplemental Figure 3). Although the effects of DinB(Y79L) and DinB(F13V) expression are unchanged in *ΔrelBE* and *ΔhipAB* strains, DinB(Y79L) expression confers increased cell death in a *ΔmazEF* strain (Figure 6A), indicating that the *mazEF* gene products may protect the cell from the abortive TLS intermediates it produces. In contrast, DinB(F13V) expression still promotes NFZ-mediated killing in a *ΔmazEF* strain (Figure 6B).

We also examined the effect of deleting *tonB* on cytotoxicity associated with DinB(Y79L) and DinB(F13V) expression. The *tonB* gene encodes a ferric iron import protein that mediates cellular lethality due to oxidative stress (58) and HU exposure (6), but not due to bactericidal drugs (27). Strikingly, we observed that deletion of *tonB* partially suppresses the lethality associated with expression of DinB(Y79L) and to a greater extent DinB(F13V) (Figure 6C-D), suggesting that incomplete TLS induces cellular toxicity that is linked to metal homeostasis. While the precise mechanism of TonB-mediated cytotoxicity remains to be elucidated, these observations suggest that diverse cellular stress responses are coupled to the faithful execution and completion of TLS.

DISCUSSION

Our previous observation that mutation of DinB's F13 'steric gate' abolishes its TLS activity stimulated us to investigate other possible determinants of DinB function. In so doing, we discovered that DinB is also specialized to catalyze the first extension step of TLS over an N^2-dG lesion with a striking catalytic proficiency that is more than 25-fold greater than on an undamaged substrate. Thus, in contrast to two polymerase models proposed in eukaryotes (47, 61, 64) in which distinct polymerases promote the insertion and extension steps of TLS, *E. coli* DinB appears capable of catalyzing both steps with remarkable proficiency during TLS over N^2-dG lesions.

In an effort to explain these findings and identify structural determinants of DinB's specialized TLS function, we revisited our model of DinB encountering a minor groove N^2-dG lesion and noted that F12, F76, and Y79 along with F13 comprise an evolutionarily conserved core of aromatic residues within the active site. Mutational analysis reveals that each of them contribute differently to *dinB*$^+$-dependent NFZ resistance. Mutation of F76 or F12 results in a *dinB* variant that is unable to fully complement the NFZ sensitivity of a *ΔdinB* strain. Mutation of Y79 (A, V, L) results in DinB variants that are entirely unable to complement NFZ sensitivity, and furthermore increase NFZ sensitivity by as much as 50-fold. Based on our previous observation of similar behavior for the DinB(F13V) steric gate mutant, we anticipated that DinB(Y79L) might behave similarly in a lesion bypass assay. To our complete surprise, DinB(Y79L) is entirely proficient at dNTP insertion opposite an N^2-furfuryl-dG adduct and the immediately subsequent extensions, but appears unable to carry out the final extension steps of TLS. Strikingly, the toxicity of DinB(F13V) and DinB(Y79L) expression

indicate that cellular survival is coupled to successful completion of TLS. Moreover, the comparable NFZ sensitivities in each case suggest that the final extension steps of TLS are at least as physiologically important as insertion of a dNTP opposite an adducted base.

This surprising result offered a potential model for the striking cytoxicity in NFZ induced by DinB(Y79L) expression. Insightful and detailed studies have revealed that short TLS intermediates produced by pol V (<5 nucleotides) are subject to the proofreading function of DNA pol III, thereby establishing a futile cycle of abortive DNA synthesis and exonucleolytic reversal (11). This phenomenon initially seemed a possible explanation for the effects of DinB(Y79L) expression. However, this does not appear to be the case as *dnaQ* mutants do not suppress the effects of DinB(Y79L) expression.

Further investigations suggested that a complex set of genetic interactions mediates the lethality observed upon DinB(Y79L) or DinB(F13V) expression. Inactivation of the toxin-antitoxin pair *mazEF* enhances lethality brought about by expression of DinB(Y79L) but not DinB(F13V), suggesting that it may serve to protect cells from the consequences of certain types of incomplete TLS. Moreover, the production of immature TLS intermediates by either DinB(Y79L) or DinB(F13V) leads to cell death through a mechanism involving the TonB iron import protein. Thus, the mechanisms of lethality differ depending on the nature of the abortive DNA synthesis. These observations underscore the notion that unexpected cellular stress responses are coupled to the precise execution of TLS at several stages.

How are the phenomena we observe related to other types of replication stress? Deletion of *tonB* mitigates sensitivity to nucleotide starvation induced by HU treatment (Davies et al., submitted). Similarly, the effects of DinB(F13V) and DinB(Y79L) expression are suppressed in a *ΔtonB* strain. We have previously observed that *ΔmazEF* strains are also strikingly resistant to HU (16). In contrast, deletion of *mazEF* increases NFZ sensitivity upon DinB(F13V), but not DinB(Y79L) expression. These and other data provide convincing evidence that *mazEF* and *tonB* are linked to replication stress. However, although there are some similarities between lethality caused by inhibition of dNTP synthesis and lethality induced by abortive TLS intermediates, there are clearly also significant differences.

Expression of DinB(Y79L) or DinB(F13V) not only fails to complement the NFZ sensitivity of a *ΔdinB* strain, but also exerts a dominant effect on survival in *dinB*$^+$ backgrounds. In contrast, a catalytically deficient DinB variant fails to complement the NFZ sensitivity of a *ΔdinB* strain but has no dominant effect on survival in a *dinB*$^+$ strain. This unexpected observation could in part be explained by the unique features of each mutant protein. The TLS defect of a catalytically-deficient DinB variant is manifested before dNTP insertion opposite a replication blocking lesion ever takes place. In contrast, both DinB(F13V) and DinB(Y79L) are able to participate in the initial steps of TLS preceding nucleotide insertion opposite the damaged base. The relatively late manifestation of their TLS defects may restrict which alternative mechanisms the cell can employ (Figure 7). Furthermore, the inability of wild-type DinB expressed from the chromosome to negate the effects of DinB(F13V) or DinB(Y79L) expression is especially unexpected. One possible explanation is that once TLS is initiated dynamic

exchange of DinB at the replication fork is considerably reduced, a commitment that necessitates increased regulation.

Together, our data point to the critical importance of the extension phase in DinB-mediated TLS. DinB itself is strikingly proficient at dNTP addition immediately following an N^2-furfuryl-dG lesion, showing a *ca.* 25-fold elevated catalytic proficiency relative to an undamaged template. Failure to complete further extensions results in pronounced lethality upon NFZ exposure. This physiological response is genetically complex and mediated by several unexpected factors. Our observations strongly indicate that TLS is far more nuanced than mere insertion of a dNTP opposite to a damaged base and its subsequent extension. Indeed, the precise execution of TLS is connected to a variety of cellular processes with roles extending well beyond DNA metabolism.

MATERIALS AND METHODS

Bacterial strains and plasmid construction

The strains, plasmids, and primers used in this study, along with strain construction information are described in Supplemental Table I. The plasmid borne *dinB* variants were produced from the low copy number plasmid pYG768 (Kim et al. 1997), which contains $dinB^{+}$ under its own promoter or the DinB expression plasmid pDFJ1 (Jarosz et al. 2006) using a Quikchange site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Plasmids were maintained with ampicillin (100 μg/mL) when necessary.

DNA damage sensitivity, mutation frequency, and transformation efficiency determinations

Sensitivity to DNA damaging agents was determined essentially as described previously (Beuning et al. 2006; Jarosz et al. 2006). Briefly, overnight *E. coli* cultures were diluted 1,000-fold and grown to exponential phase in LB medium before being plated on LB agar containing between 0-50 μM NFZ or 4-NQO. A concentrated stock solution of each DNA damaging agent was first made in N,N-dimethylformamide, stored at -20°C, and diluted appropriately for each experiment. Percent survival was determined relative to growth in the absence of DNA damaging agent. Mutation frequency was measured by reversion to rifampicin resistance at 100 μg/mL as previously described (Beuning et al. 2006; Jarosz et al. 2006). Transformation efficiencies were determined in triplicate by transforming increasing amounts of plasmid DNA into a constant quantity of competent cells and measuring colony forming units.

Oligonucleotide synthesis

The synthesis and post-synthetic derivitization and characterization of the oligonucleotides used as templates in this study was as in (Jarosz et al. 2006). The nested primers used to define the range of preferential DinB TLS activity were purified by gel electropheresis prior to use and are described in Supplemental Table I.

Protein purification and primer extension assays

Purification of wild-type DinB and DinB(Y79L) was performed as previously described (Beuning et al. 2006; Jarosz et al. 2006). Briefly, each protein was expressed from the plasmid pDFJ1 or its DinB(Y79L) expressing derivative in BL21(DE3) pLysS cells by induction with 1 mM IPTG at an OD_{600} of 0.6, and the cultures were incubated at 30°C for the duration of the induction. After four hours, cells were harvested by centrifugation and subjected to lysozyme treatment. The resulting lysate was treated with DNAse and RNAse as described (Jarosz et al. 2006) and purified on monoS and phenylsepharose columns (GE Healthcare). DinB(Y79L) purified virtually indistinguishably from wild-type DinB, except for exhibiting a slightly broader peak during elution from the phenylsepharose column. Similar specific activity is maintained through the entire peak.

Primer extension assays were performed as described previously (Jarosz et al. 2006), using equivalent concentrations of wild-type DinB and DinB(Y79L). Products were separated on a 16% denaturing polyacrylamide gel and quantified using a

phosphorimager (GE Healthcare). In cases where nucleotide addition resulted in multiple products the sum of all products was used to calculate an apparent V_{max} and K_m.

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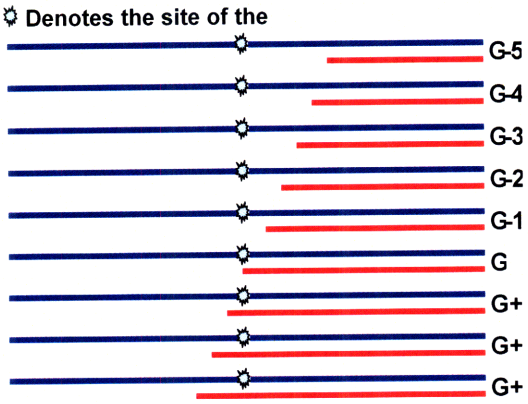
Figure 1

A



B

Primer	V_{max}, G	V_{max}, N^2-FG	K_m, G	K_m, N^2-FG	V_{max}/K_m, G	V_{max}/K_m, N^2-FG	N^2-FG/G Preference
G-5	557.8	668.9	83.2	27.9	6.7×10^6	2.4×10^7	3.6
G-4	755.9	2492.7	173.9	129.3	4.4×10^6	1.9×10^7	4.4
G-3	464.2	304.6	9.6	5.1	4.8×10^7	6.0×10^7	1.2
G-2	1081.7	1149.6	109.0	51.7	9.9×10^6	2.2×10^7	2.2
G-1	894.3	990.5	231.6	16.0	3.9×10^6	6.2×10^7	16.0
G	619.8	886.3	42.2	2.35	1.5×10^7	3.8×10^8	25.7
G+1	240.4	167	67.1	41.7	3.6×10^6	4.0×10^6	1.1
G+2	188.2	150.3	24.9	8.4	7.6×10^6	1.8×10^7	2.4
G+3	446.1	438.2	59.8	19.6	7.5×10^6	2.2×10^7	3.0

Figure 1. Staggered primer extension assays reveal a *ca.* 25-fold catalytic preference for extension from an N^2-furfuryl-dG lesion. A) Schematic of the lesion bearing template (blue) and primers (red) employed. The site of the N^2-furfuryl-dG is indicated by the cyan star. B) Steady state kinetic parameters for primer extension assays using the 5'-^{32}P-labeled primers described above. V_{max} units are pmol min^{-1} mg^{-1}; K_m units are μM; V_{max}/K_m units are pmol min^{-1} mg^{-1} M^{-1}. Reactions were carried out as described in materials and methods. In cases where more than one addition was catalyzed by the addition of a single dNTP substrate, the product is defined as the sum of all radiolabeled oligonucleotides present with a greater length than the primer. Undamaged template designated G; N^2-furfuryl-dG abbreviated as N^2-FG.

Figure 2

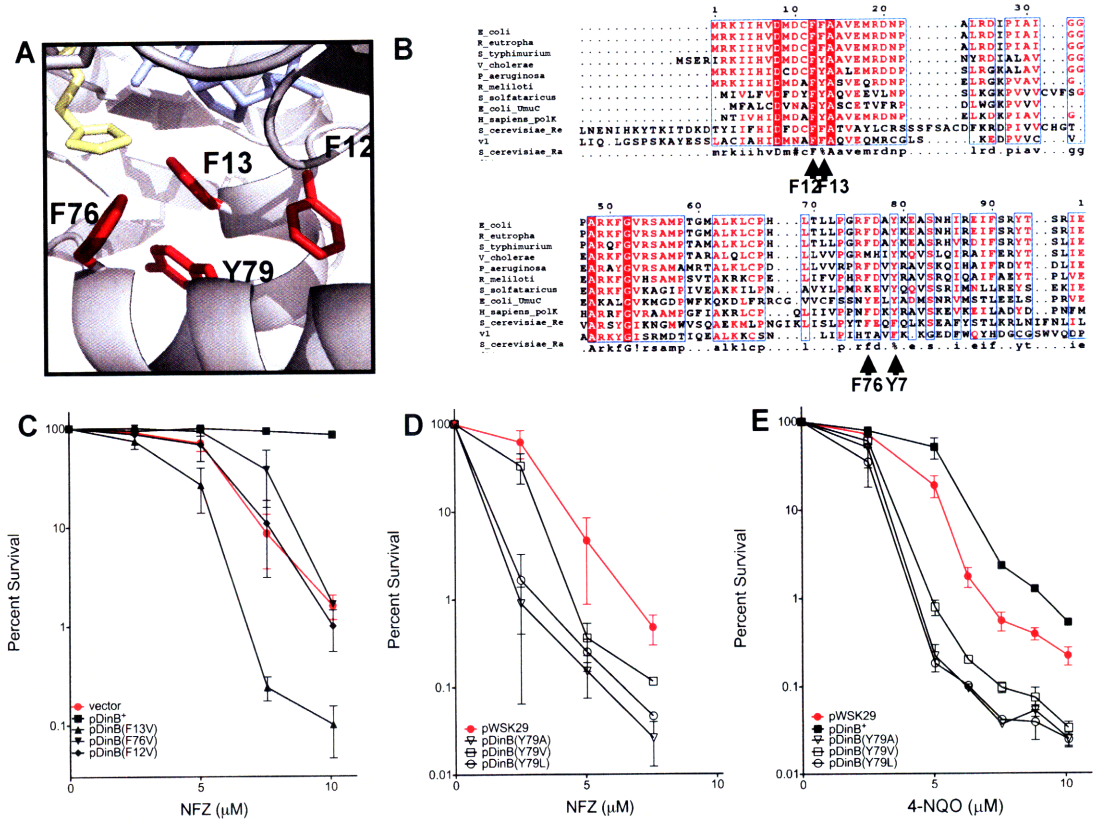
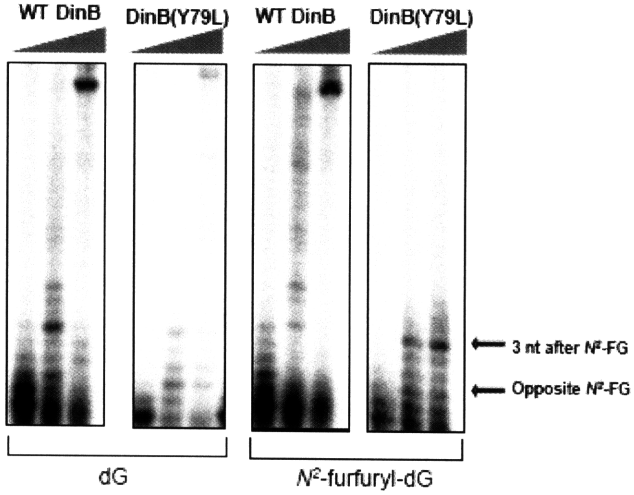


Figure 2. A pocket of aromatic hydrophobic residues in DinB's active site modulates its TLS function. A) Structural model of conserved residues in the active site of DinB suggests potential interactions between the side chains (red), the N^2-furfuryl moiety (yellow), and the incoming nucleotide (blue). B) Alignment of DinB sequences from diverse organisms reveals conservation of F12 and the F/Y13 steric gate residues. Whereas Y79 is universally present among DinB orthologs, F76 shows weaker conservation. Consensus symbols are as follows: (!) represents I/V, (%) represents F/Y, (#) represents N/D/Q/E. C) Mutation of residues in this hydrophobic pocket results in *dinB* variants that are unable to complement the NFZ sensitivity of a *ΔdinB* strain. Chromosomal genotype in all cases is *ΔdinB*. Error bars represent the standard deviation determined from thee independent cultures. D-E) Mutation of Y79 eliminates the ability of DinB to complement the NFZ or 4-NQO sensitivity of a *ΔdinB* strain, respectively. Chromosomal genotype is *ΔdinB*. Error bars represent standard deviations determined from three independent experiments.

Figure 3

A



B

Template	Enzyme	K_M (μM)	V_{max}	V_{max}/K_M	Fold change
G-control/(G+3)	WT DinB	59.8	446.1	7.5×10^6	-
	DinB(Y79L)	330.0	1072.0	3.2×10^6	2.3
N^2-FG/(G+3)	WT DinB	19.6	438.2	2.2×10^7	-
	DinB(Y79L)	1551.0	1416.0	0.9×10^6	24.0

Figure 3. DinB(Y79L) catalyzes lesion bypass. A) DinB(Y79L) bypasses N^2-furfuryl-dG and stalls three nucleotides following the lesion. Primer extension assays with 1, 5, and 50 nM DinB and DinB(Y79L) indicate that DinB(Y79L) is able to catalyze lesion bypass, but cannot complete the extension phase of TLS as the majority of products stall three nucleotides following the site of the lesion. B) Steady state kinetic parameters for extension from the G+3 primer by DinB(Y79L). Reactions conditions are as described in materials and methods. V_{max} units are pmol min^{-1} mg^{-1}; K_m units are μM; V_{max}/K_m units are pmol min^{-1} mg^{-1} M^{-1}.

Figure 4

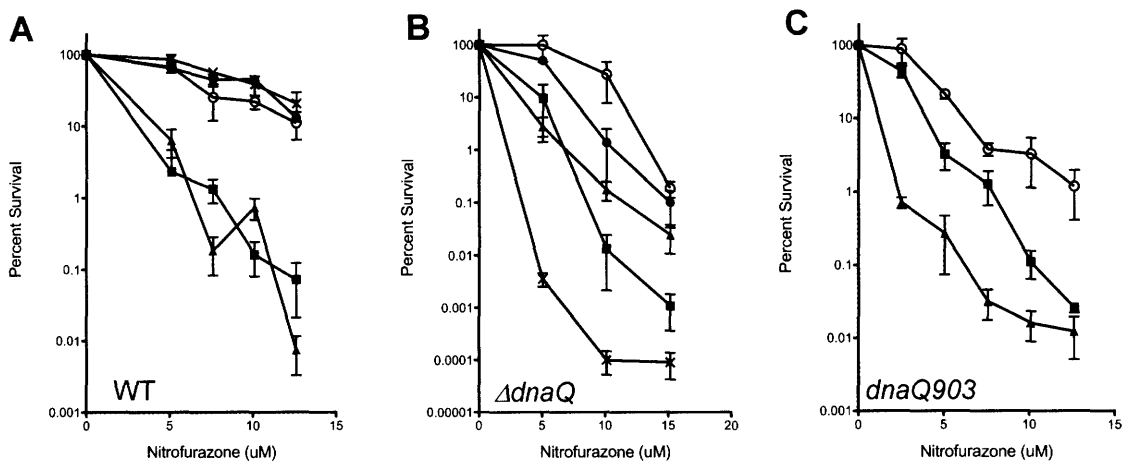
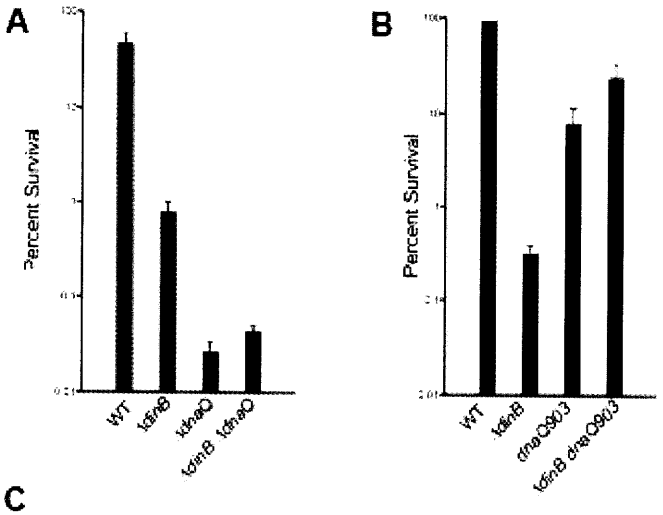


Figure 4. Consequences of DinB variant expression in wild-type and *dnaQ* backgrounds A) Expression of pDinB(Y79L) (closed triangles) or pDinB(F13V) (closed squares) results in profound toxicity to NFZ relative to pDinB (closed circles), an empty vector control (pWSK29; open circles), or pDinB(Y79F) (cross marks). Chromosomal genotype is *dinB*$^{+}$. In this and all graphs in this figure, error bars represent the standard deviation determined from three independent transformants. B-C) Dominance of pDinB(Y79L), pDinB(F13V) and pDinB(Y79F) in *ΔdnaQ* and *dnaQ903* backgrounds, respectively. Symbols and error bars as in A).

Figure 5



C

Strain	Plasmid	Transformation Efficiency per µg	Fold reduction
AB1157	pWSK29	1950000	-
	pDinB	1820000	1
	pDinB(Y79F)	743000	3
ΔdnaQ::kan	pWSK29	461000	-
	pDinB	48500	10
	pDinB(Y79F)	16600	28
dnaQ903::tet	pWSK29	17200	-
	pDinB	166	103
	pDinB(Y79F)	<1	>20600

Figure 5. Evidence for a genetic interaction between *dinB* and *dnaQ*. A-B) The *ΔdnaQ* and *dnaQ903* alleles are epistatic to a *ΔdinB* with respect to NFZ sensitivity at 10 µM NFZ. Error bars represent the standard deviation determined from four independent transductants. C) Transformation efficiencies in colony forming units per µg of DNA for wild-type and *dnaQ* strains.

Figure 6

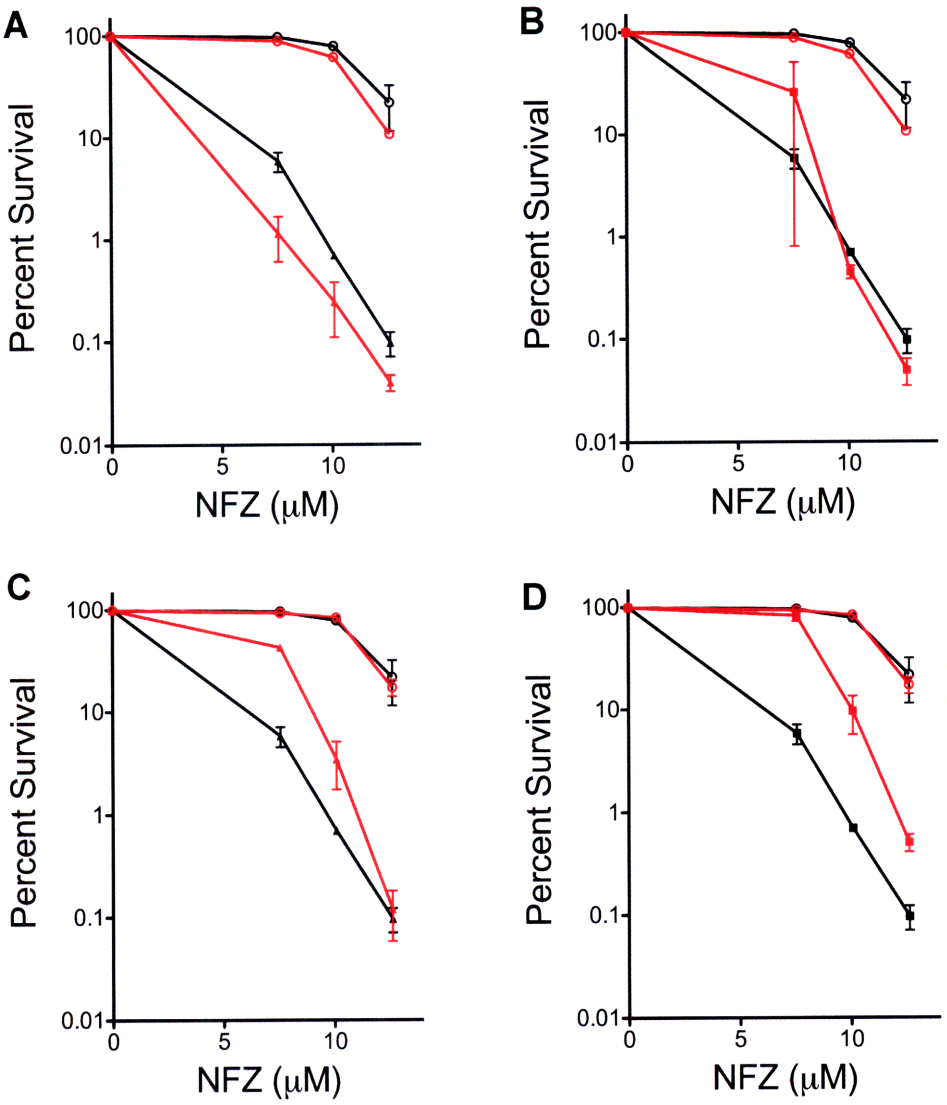


Figure 6. The toxin-antitoxin module *mazEF* and the iron import protein *tonB* modulate the toxicity of DinB(Y79L) and DinB(F13V). A) Deletion of the toxin-antitoxin (TA) pair *mazEF* enhances the killing of DinB(Y79L) expression, consistent with a role for *mazEF* in protection from abortive TLS. Black lines/symbols represent the wild-type strains and red lines/symbols represent *ΔmazEF* strains. Plasmids are pWSK29 (open circles), pDinB(Y79L) (closed triangles). Error bars represent the standard deviation determined from three independent transformants. B) Deletion of *mazEF* does not affect pDinB(F13V) toxicity in NFZ. Lines and symbols as in A); pDinB(F13V) (closed squares). C-D) Deletion of the *tonB* gene, which encodes an iron import protein, mitigates the toxicity of DinB(Y79L) and DinB(F13V) expression. Black lines/symbols represent wild-type strains and red lines/symbols represent *ΔtonB* strains. Symbols otherwise as in A-B). Error bars represent one standard deviation determined from three independent experiments.

Figure 7

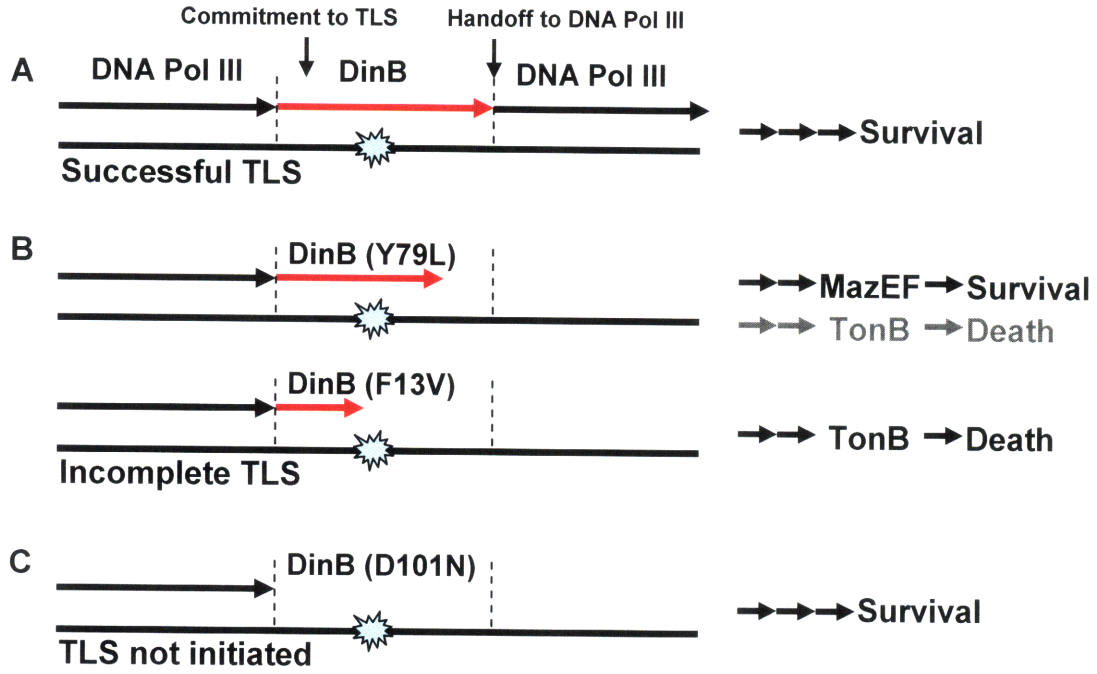
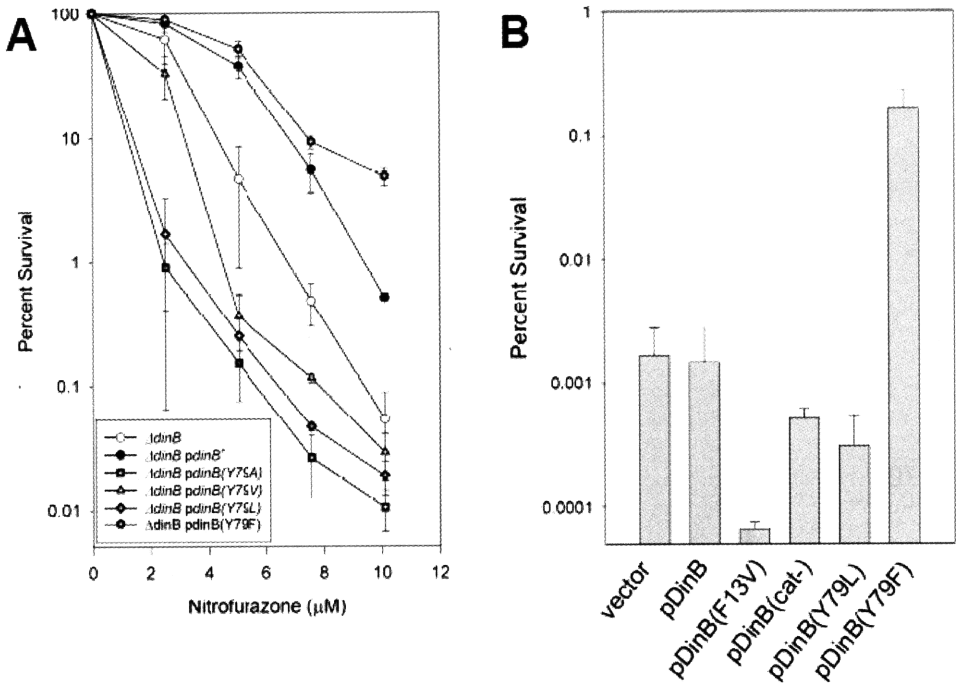


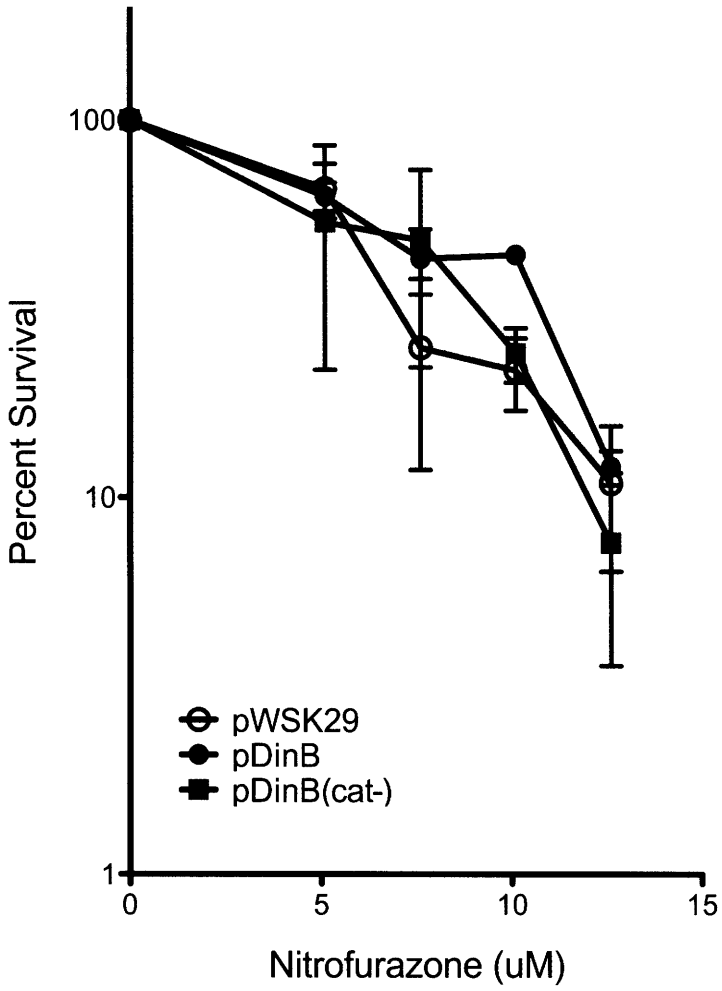
Figure 7. Model for phase separation in TLS. DinB(Y79L) and DinB(F13V) behave normally during the early phases of TLS, manifesting their defects only at or after the point at which the cell has committed to lesion resolution by TLS. The abortive products that they induce diverse physiological responses that affect cell fate. A catalytically deficient DinB variant manifests its phenotype early in TLS, thereby allowing other mechanisms for lesion resolution. Figure adapted from (Fujii and Fuchs, 2004).

Supplemental Figure 1



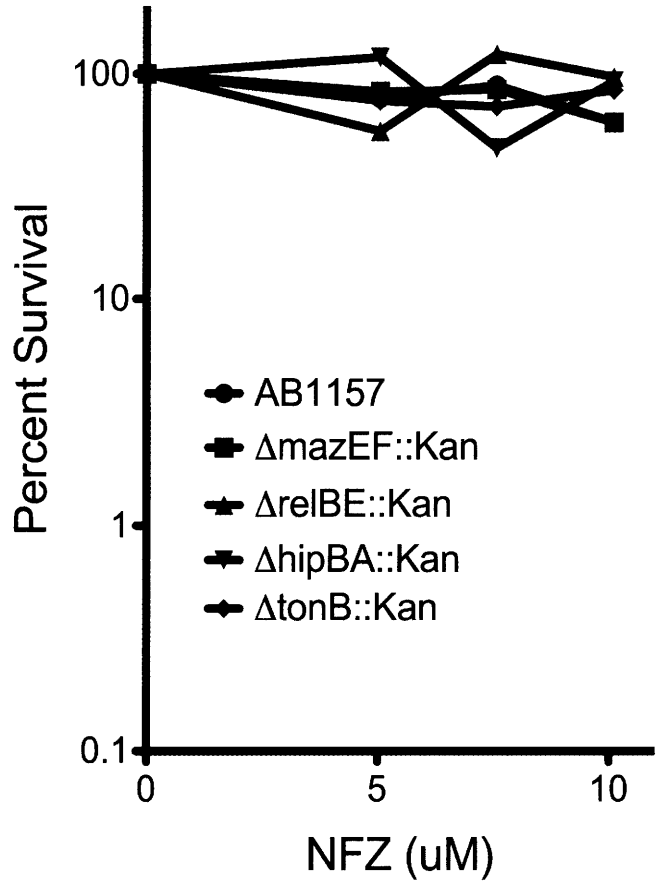
Supplemental Figure 1. A) DinB(Y79F) complements the NFZ sensitivity of a *ΔdinB* strain. Error bars represent the standard deviation determined from three independent experiments. B) Expression of DinB(Y79F) confers *ca.* 200-fold resistance to killing at high doses of NFZ. Plasmids are as described in Supplemental Table I and expressed in a *ΔdinB* background. Strains were treated on plates containing 40 μM NFZ.

Supplemental Figure 2



Supplemental Figure 2. Expression of a catalytically deficient DinB variant does not affect resistance of NFZ in a wild-type background. Expression of pDinB(cat-) (filled squares) does not affect NFZ resistance in a wild-type background relative to pDinB (closed circles) or an empty vector control (pWSK29; open circles).

Supplemental Figure 3



Supplemental Figure 3. Deletion of *mazEF*, *relBE*, *hipAB* and *tonB* do not affect killing upon NFZ treatment.

Strains	Genotype	Reference
BL21 (DE3) pLysS	*E.coli* B. Standard strain used for protein overproduction	GCW lab stock
AB1157	*thr-1 araC-14 leuB-6(Am) Δ(gpt-proA)62 lacY1 tsx-33 qsr'-0 glnV44(AS) galK2(Oc) LAM⁻ Rac-0 hisG4(Oc) rfbC1 mgl-51 rpoS396(Am) rpsL31 kdgK51 xylA5 mtl-1 argE3(Oc) thi-1*	GCW lab stock
AB1157 *ΔdinB*	As AB1157 but *ΔdinB::Cm*	(3)
AB1157 *ΔdnaQ*	As AB1157 but *ΔdnaQ::kan*	This work. Transduction from Keio collection
AB1157 *ΔdnaQ ΔdinB*	As above but *ΔdinB::Cm*	This work
AB1157 *dnaQ903*	As AB1157 but *dnaQ903::tet*	(6)
AB1157 *dnaQ903 ΔdinB*	As above but *ΔdinB::Cm*	This work
AB1157 *dnaE(spq-2)*	As AB1157 but *dnaE(spq-2)*	(5)
AB1157 *ΔhipBA*	As AB1157 but *ΔhipBA*	This work; (2)
AB1157 *ΔrelBE*	As AB1157 but *ΔrelBE*	This work; (2)
AB1157 *ΔmazEF*	As AB1157 but *ΔmazEF*	This work; (2)
AB1157 *ΔtonB*	As AB1157 but *ΔtonB::kan*	Transduced from Keio collection (1)
AB1157 *Δetk*	As AB1157 but *Δetk::kan*	Transduced from Keio collection (1)
AB1157 *Δwzc*	As AB1157 but *Δwzc::kan*	Transduced from Keio collection (1)
Plasmids		
pDFJ1	DinB expression plasmid under T7 promoter in pET11T backbone; Ampicillin resistance	(3)
pDFJ1(Y79L)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work
pDFJ1(Y79F)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work
pDinB	pYG768. DinB under its native promoter in a low copy number pWSK29 plasmid	(4)
pDinB(F12V)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work

pDinB(F76V)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work
pDinB(Y79A)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work
pDinB(Y79V)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work
pDinB(Y79L)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work
pDinB(Y79F)	As pDFJ1 with indicated mutation introduced using quikchange site-directed mutagenesis	This work
pWSK29	Empty vector for pDinB and derivatives	(4)
pDinB(cat-)	As pDinB with D104N mutation	(7)
Primers		
G-5	GCATATGATAGTACAGCTGCAGCCGGA	
G-4	GCATATGATAGTACAGCTGCAGCCGGAC	
G-3	GCATATGATAGTACAGCTGCAGCCGGACG	
G-2	GCATATGATAGTACAGCTGCAGCCGGACGC	
G-1	GCATATGATAGTACAGCTGCAGCCGGACGCC	
G	GCATATGATAGTACAGCTGCAGCCGGACGCCC	
G+1	GCATATGATAGTACAGCTGCAGCCGGACGCCCA	
G+2	GCATATGATAGTACAGCTGCAGCCGGACGCCCAG	
G+3	GCATATGATAGTACAGCTGCAGCCGGACGCCCAGG	

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Chapter 6

Conclusions

Summary of Results

The observation that mutations in the *Escherichia coli* genes *umuC*$^+$ and *umuD*$^+$ abolish mutagenesis induced by UV-light strongly supported the counterintuitive notion that the introduction of mutations into the genome was an active rather than passive process (9). Biochemical studies have revealed that the *umuC*$^+$ and homolog *dinB*$^+$, encode for low to moderate fidelity DNA polymerases, with the ability to catalyze synthesis on imperfect/damaged DNA templates in a process termed translesion synthesis (TLS) (6, 13). Many of these translesion DNA polymerases are members of the Y-family of DNA polymerases and are present in all domains of life. The existence of such a class of DNA polymerase raises a series of questions including, i) how is that these polymerases are regulated, as they display reduced fidelity on undamaged DNA and ii) out of so many polymerases available, how is it that cell knows which one to used? Here I discuss the identification of a potential regulatory factor, NusA. I report for the first time a function for the transcriptional modulator NusA in promoting survival after DNA damage. I suggest that NusA is required to couple the processes of both TLS and nucleotide excision repair to transcription, allowing cells to cope with the lethal effects of DNA damage.

In Chapter Two, we show that a DinB affinity column used to search for potential DinB-interacting proteins within lysates of cells that constitutively express the SOS response, bound the transcriptional modulator NusA. This interaction is a direct interaction as determined by far-western analysis. Furthermore, the use of cellulose filter peptide arrays to search for peptides of NusA that might potentially interact with DinB revealed that one potential DinB binding region of NusA encompasses several surface

residues around the site of the temperature sensitive mutation of the *nusA11*(ts) allele (12). The peptide array data led us to consider the possibility that elevated levels of DinB might stabilize the NusA11 protein, resulting in *dinB*$^+$ serving as a multicopy suppressor of the temperature sensitivity of a *nusA11* strain. I found that increased expression of DinB indeed suppresses the temperature sensitivity of the *nusA11* strain, in manner requiring the catalytic activities of DinB. This multicopy suppression of the *nusA11* temperature sensitivity extends to the other TLS polymerase in *E. coli* encoded by the *umuDC* gene products, similarly requiring the catalytic activities of both *umuD* and *umuC*. In order to explain these data I have suggested a model of transcription-coupled translesion synthesis (TC-TLS), where NusA associated with elongating RNA polymerases (RNAP) can recruit TLS DNA polymerases to fill in gaps opposite to lesion in the transcribed strand.

In Chapter Three, I describe a set of experiments that show a requirement for *nusA*$^+$ in the phenomenon of adaptive mutagenesis. Adaptive mutagenesis describes the accumulation of mutations that occur in non-growing cells over time. In the widely used system devised by Cairns and Foster a *lacI33-lacZ* fusion reporter is used to monitor the appearance of adaptive mutants from a Lac$^-$ to a Lac$^+$ phenotype in *E. coli* (3). While many cellular processes are implicated in the adaptive response, loss of DinB (DNA pol IV) function results in loss of 50 to 85 percent of adaptive mutants (5, 10). I show that strains containing the *nusA11*(ts) mutation, at the permissive temperature, are completely incapable of adaptive mutagenesis. I present evidence that the defect in adaptive mutagenesis seen in the *nusA11* strain, likely involves a role for NusA in the adaptive response that extends beyond an interaction with DinB. Furthermore, the defect in the

nusA11 strains is not due to a transcriptional defect of the adaptive mutagenesis *lacI33-lacZ* reporter, but rather an inability to adapt and mutate in response to environmental stress under growth limiting conditions.

In Chapter Four, I describe a set of experiments that establish NusA as a critical factor in allowing cells to cope with the lethal effects of DNA damage. My results are the first to implicate NusA, which is associated with RNA polymerase throughout elongation and termination of transcription, in the processes of DNA repair/damage tolerance. Mutation of *nusA* results in reduced viability after exposure to DNA damaging agents and even without the addition of exogenous DNA damaging agents, *nusA* mutants display phenotypes of altered DNA processing. Furthermore, I can genetically link transcription/RNA polymerase to the *nusA*-dependent events that help promote survival after DNA damage, through the isolation of RNA polymerase mutants with altered ability to deal with the lethal effects of DNA damage. Together, my results suggest that NusA, in addition to its roles in transcription elongation and termination, is important for coordinating the cellular responses to DNA damage by coupling the processes of translesion DNA synthesis and nucleotide excision repair to transcription.

In Chapter Five, in work done in collaboration with Daniel Jarosz, we report the first example of preferential TLS extension by any Y-family DNA polymerase. We also describe a new class of TLS DNA polymerase mutant resulting from mutation of an evolutionarily conserved tyrosine (Y79), generating a DinB variant that catalyzes formal lesion bypass but cannot finish the subsequent extension steps that are required to complete TLS, stalling three nucleotides after an N^2-dG lesion. Expression of this variant *in vivo* dramatically enhances killing during challenge with DNA damaging agents, and

in fact, transforms the bacteriostatic drug nitrofurazone into a bactericidal drug. Diverse gene products modulate this toxicity associated with incomplete TLS extension, including the toxin-antitoxin module MazEF and the iron import protein TonB. Together, these results not only indicate that DinB is specialized to perform remarkably proficient TLS insertion and extension, but also expose unexpected connections between the products of incomplete TLS and cell fate.

Implications of transcription-coupled translesion synthesis (TC-TLS)

I have proposed that, by binding to DinB or some complex involving UmuC and the *umuD* gene products, NusA can couple the process of transcription to the process of translesion synthesis to enable transcription-coupled translesion synthesis (TC-TLS), in a manner analogous to the coupling of transcription to nucleotide excision repair during transcription-coupled repair (TCR). In principle, a process of TC-TLS could be helpful when transcription becomes stalled by gaps in the transcribed strand that are opposite lesions in the non transcribed strand. If a process of transcription-coupled translesion synthesis does exists, and if it is universal as most DNA repair and DNA damage tolerance processes tend to be, it might be of particular importance in mammals where some mRNAs can take many hours to transcribe (16) so that the consequences of encountering a template gap late in the transcriptional process would be severe. I propose the use of gapped plasmids containing a site-specific lesion to test this model of TC-TLS. Such plasmids have been generated to test the bypass frequency for particular DNA lesions (1, 17, 18). I suggest that construction of such a plasmid with a N^2-furfuryl-dG) lesion, opposite a gap in a transcribed gene under the control of a strong promoter or

without any promoter, would allow me to determine the role of transcription in plasmid maintenance, where the repair of the gap would allow for propagation of the plasmid. This type of experiment would allow me to test the model of TC-TLS *in vivo*, and avoid the complications of excluding several possible unknown factors (see below). If a mechanism of TC-TLS were to exist, it would raise more questions than it would answer (see below).

How would NusA recruit DinB/TLS polymerases to sites of stalled RNAP? NusA is associated with all elongating RNAP (11), so how could DinB be specifically recruited to RNAP that have been stalled by a gap in the transcribed strand. It is possible that NusA undergoes a conformational change upon the stalling of RNAP, and it is this conformation that DinB/TLS polymerases can now be recruited. One could also envision a situation where DinB/TLS polymerases are always associated with elongating RNA polymerases, through an interaction with NusA. In this case, every time the RNAP encountered a gap opposite a lesion DinB would already be present to fill in this gap. However, there is not likely to be enough DinB/TLS polymerases to always be associated with all elongating RNA polymerases.

What are the other factors involved in TC-TLS? If the proposed mechanism of TC-TLS were to exist, there would likely need to be several other proteins in addition to RNAP, NusA and TLS polymerases involved in this process. In order for TLS polymerases to fill in a gap opposite a lesion, it would need to reach the opposite end of the gap relative to the RNA polymerase. These gaps could be anywhere from 12-1000

nucleotides in length (discussed in Chapter Two), and thus there would likely need to be a hand-off of the TLS polymerase from NusA/Stalled RNAP to the appropriate primer/termini end. Possibilities for this hand-off factor include RecA, a ssDNA binding protein that can also interact with TLS polymerases DinB and UmuD'$_2$C. Additionally, the histone-like protein HupA, which preferentially binds to gapped DNA (4), was also identified as binding to a DinB affinity column (8), suggesting another hand-off possibility.

What role would NusA have in TC-TLS and recruiting TLS polymerases?

NusA is classified as a general transcription elongation factor and has been found to be associated with all elongating RNA polymerases (11), therefore one can come up with two ideas for the potential role for NusA in the model of TC-TLS. One idea is that since NusA is associated with all elongating RNA polymerases it is a platform, allowing for recruitment and/or binding of TLS polymerases to the site of the stalled RNAP. This idea would not require NusA's ability to function as a transcriptional terminator/ antiterminator. Alternatively, one could hypothesize that NusA, using its abilities to pause or stall elongating RNA polymerases, could sense the upstream gap in the transcribed strand and pause the RNAP before it encounters a situation in which it has run out of template. In this situation, NusA would also function as a "platform" by recruiting TLS DNA polymerases, however it would also utilize its own functions as a transcriptional terminator/antiterminator. In collaboration with Bob Landick at the University of Wisconsin Madison, I will be testing the activity of RNAP activity on a

gapped substrate *in vitro*, where I can then asses the role of NusA in RNAP pausing/stalling.

NusA-dependent TCR, an alternative to Mfd-dependent TCR

My model for a new class of transcription-coupled repair suggests that NusA can recruit NER machinery to sites of RNAP stalled by a lesion in the transcribed strand in a fashion that is independent of Mfd. Why the existence of a second class of TCR? One intriguing possible explanation involves a particular of a class of DNA lesion, such as an N^2-dG lesion, which can be efficiently bypassed by DinB and thus is not a hindrance during replication. However, such lesions would then cause a problem when transcription is attempted. In this case, a novel class of TCR involving NusA could be important for the recognition and removal of these DNA lesions. Since some of these minor groove lesions are not efficiently recognized by normal NER (2, 8), NusA-TCR could help a cell remove this class of lesions from its DNA.

As in the case of my model of TC-TLS, if there were to exist a new class of TCR mediated by NusA, there would most likely be several yet-to-be-determined molecular determinants. For example, the C-terminal half of Mfd is comprised of seven helicase domains and a translocase domain, which are important for the translocation of the stalled RNA polymerase (14, 15). NusA, does not contain such motifs and thus it is likely that one/or more factors would need to involved in translocating the stalled RNAP in this new class of transcription coupled repair.

What about other organisms/domains of life?

NusA is universally conserved throughout bacterial and archaeal domains of life however no sequence or functional homolog has been identified in eukaryotes. Would the functions that I have implicated for *E. coli* NusA in DNA repair/damage tolerance, hold true for NusA in archaea. Similarly, would there be transcription-coupled translesion synthesis or a second class of transcription-coupled repair in eukaryotes. If these mechanisms were to exist, what would be the factors involved. Mfd, like NusA, is extremely conserved throughout bacteria and archaea however a sequence homolog in eukaryotes does not exist (7). However, the human transcription repair coupling factor (TRCF) gene has been identified as ERCC6/CSB (19). Despite the fact that ERCC6/CSB and *mfd* share no significant sequence homology, they are structurally and functionally homologous. Thus it is possible that there exists a function homolog of NusA in eukaryotes.

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Appendix A

Lethal Combinations: synthetic lethality of *nusA11* and *lexA*(Def) mutants in *Escherichia coli*

Authors for this work are Susan E. Cohen and Graham C. Walker

INTRODUCTION

The *Escherichia coli* SOS network coordinates the expression of more than 50 genes (21) in response to DNA damage which is regulated by the LexA transcriptional repressor that binds to operator sequences inhibiting transcription of downstream genes (6). In addition to roles for the *recA*$^+$ gene product in homologous recombination, RecA also plays a central role in controlling the SOS response. Upon DNA damage RecA forms a nucleoprotein filament with ssDNA, which interacts with LexA and induces its own proteolytic digestion, inactivating LexA as a transcriptional repressor. Here I report that the mutation of *nusA* (*nusA11*) is lethal when in combination with a *lexA*(Def) allele in *E. coli*. The results presented in Chapter Four have suggested that the essential transcriptional modulator NusA is also important in promoting survival after challenge with DNA damage. NusA is an essential protein that functions in both termination and antitermination of transcription, and is associated with the RNA polymerase throughout the elongation and termination steps of transcription (3, 5, 8, 12-14, 16, 20). Several genetic links between *nusA* and the SOS response to DNA damage were discussed in Chapter Four, including partial chronic SOS induction observed in sub-population of *nusA11*(ts) cells at the permissive temperature. These results lead me to question how survival after exposure to NFZ would be altered in a *nusA11* strain which is constitutively expressed for the SOS response. The *lexA*(Def) strain, which is deleted for *lexA*, results in the constitutive expression of the SOS response (9, 11, 17, 19). However, for this mutation to result in viability, a mutation in *sulA* must also be present, as constitutive expression of the LexA-repressed *sulA*$^+$ gene otherwise leads to lethal filamentation (7).

Here I show that the mutation of *nusA* (*nusA11*) is synthetically lethal when put in combination with a *lexA*(Def) allele. In addition, the synthetic lethality can be prevented by plasmid-borne $nusA^+$ or $lexA^+$. Synthetic lethality is defined as when the combination of two otherwise non-lethal mutations results in an inviable cell. Although there have been recent efforts to identify such synthetic genetic interactions in *E. coli* (22), a synthetic lethality involving *nusA* would not have been identified since *nusA* is an essential gene. Synthetic lethality seen with loss-of-function alleles has been proposed to arise from at least four different mechanisms (10): i) one gene can buffer the effects of the other, that is it can compensate for the effects of the other. ii) they can function in uniquely redundant roles with respect to an essential function iii) they might be two subunits of an essential multi-protein complex iv) they might be two interconnected components in an essential linear pathway such that each mutation decreases the flow through the pathway.

RESULTS AND DISCUSSION

Independent of which allele was used as donor or recipient, attempts to construct a *lexA*(Def) *nusA11* mutant by $P1_{vir}$ transduction were unsuccessful (Tables 1 and 2). As noted in Table 1, on rare occasion a viable mutant was recovered when the *nusA11* mutation (linked to a Kan^R marker) was transduced in to the *lexA*(Def) background. However, this is likely due to a secondary suppressor mutation. Although this viable strain was temperature sensitive (indicative of *nusA11*) and deleted for *lexA* as confirmed by PCR, these strains did not express the correct antibiotic resistance markers and displayed a mucoid morphology. This phenomenon is not due to a lethal interaction between *nusA* and *sulA,* as the *nusA11*Δ*sulA* mutant was constructed, nor is it a reflection of the failure of *nusA11* mutant strains to express spectinomycin resistance, as other spectinomycin markers could be expressed. Moreover, the lethality of the *nusA11* *lexA*(Def) can be prevented by plasmid-borne $nusA^+$, expressed under its native promoter (data not shown).

In an attempt to understand the physiology underlying this phenomenon I conducted a suppressor screen, looking for genes that when expressed in multicopy allowed for the construction of the *nusA11 lexA*(Def) mutant. An *E. coli* genomic library, present in a multicopy plasmid (23), was transformed into *nusA11*Δ*sulA* strains and 140 isolates screened for viability of a *nusA11 lexA300*(Def) mutant. Plasmids harboring *lexA* were isolated nine times, showing that this phenotype can be complemented by *lexA* in *trans* as well. Interestingly, a plasmid harboring *nusA* was not isolated, implying that perhaps more plasmids should be screened. Five other plasmids were isolated that permitted construction of the *nusA11 lexA*(Def) strain (Table 3). However, the identity of

these genes did not reveal any obvious reason why this otherwise lethal combination now results in viability. Perhaps expression of these gene products allows the cells to deal with or tolerate a toxic intermediate that accumulates in this background. Additionally, a screen based on stabilization of an otherwise unstable plasmid (1, 2) was used to screen for additional mutations that resulted in lethality when in combination with the *lexA*(Def) mutation. This screen yielded two transposon insertion mutations that when in combination with the *lexA*(Def) allele, stabilized the otherwise unstable plasmid carrying *lexA* (S. Cohen and L. Addae, unpublished). However, the identity of these mutants was never resolved.

At the permissive temperature, a sub-population of the *nusA11* cells constitutively express the SOS response (Chapter Four), leading me to question why some cells, but not all, can be constitutively SOS induced. Furthermore, *nusA11* strains, at the permissive temperature, do not have a defect in UV-induced mutagenesis (Figure 1), implying that they are able to perform "SOS mutagenesis", as measured by reversion of an *argE3* mutant to Arg^+. This is in comparison to an un-mutable strain where the *umuDC* genes, which encode the SOS inducible translesion DNA polymerase V, are deleted. Although these data seem to be in conflict with the synthetic lethality observed with the *nusA11* and *lexA*(Def) mutations, they show that this relationship is a complicated one with more experiments required to understand this set of complex interactions. For example, a null mutation of *lexA* could imply that induced expression of any one/or many of the SOS regulated genes could be the reason for the synthetic lethal interaction with *nusA*. Moreover, the fact that SOS can be constitutively induced in a sub-population but not the whole population of cells could imply a role for cellular communication in this

phenomenon.

MATERIALS AND METHODS

Strains and Growth Conditions

All experiments were performed with AB1157 derived strains (*thr-1 leuB6 proA2 hisG4 thi1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*). *nusA11 sfsB3198*::Tn*10*Kan (SEC302), Δ*sulA*::Tet (TP651) (18), *lexA*(Def) (Δ*sulA*::Tet Δ*lexA300*::Spc (9)) (SEC305), and *nusA11 sfsB3198*::Tn*10*Kan Δ*sulA*::Tet (SEC816). For all experiments cultures were grown in Luria-Bertani (LB) medium at 30°C. When necessary plasmids were maintained with 100μg/mL ampicillin. During strain construction antibiotics were used at the following concentrations: 12μg/mL tetracycline, 30μg/mL kanamycin or 60μg/mL spectinomycin.

$P1_{vir}$ transductions

$P1_{vir}$ phage-mediated transduction was performed was previously described (15). All steps of the transduction procedure were performed at 30°C.

Multicopy suppressor screen

An *E. coli* genomic library, present in a multicopy plasmid (23), was transformed into SEC816. This library was then scored for ability to allow for construction of *nusA11 lexA*(Def) mutant. Donor: *lexA300*(Def), Recipient: Δ*sulA*::Tet *nusA11*. All steps were performed at 30°C.

Mutagenesis Assays

SOS mutagenesis assays were performed according to the published method (4). Briefly, equal numbers of cells from cultures growing exponentially in LB were washed with 0.85% saline, exposed to 25 J m^{-2} UV light from a germicidal lamp (General Electric), and then plated on M9 minimal plates with trace arginine (1 μg ml^{-1}). Colony-forming

units were scored after 48 h of growth at 30°C. Survival was determined by plating on equivalent M9 minimal plates with 40 μg ml^{-1} arginine. Non-UV-irradiated cultures were treated identically, but without UV irradiation, to assess the spontaneous mutation frequency. The data represent the average of at least three independent experiments.

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Table 1

	Total number colonies screened	Co-transduction Frequency
Δ*sulA lexA*+	336	16%
Δ*sulA lexA300(Def)*	288	0.7% $Spec^S$

Table 2

	# Transductants	*lexA* locus
Δ*sulA nusA*$^+$	153	*lexA*(Def)
Δ*sulA nusA11 sfsB::Tn10Kan*	0	NA

Table 1 and 2. Transduction frequencies suggest that a *nusA11*, *lexA*(Def) strain is inviable. Table 1. Donor: *nusA11 sfsB*::Tn*10*Kan. Recipient: Δ*sulA* (*lexA*$^+$ or *lexA300*(Def)). Co-transduction determined as transductants that were both Kan^R and temperature sensitive. Table 2. Donor: *lexA300*(Def). Recipient: Δ*sulA* (*nusA*$^+$ or *nusA11*). *lexA* locus column denotes that deletion of *lexA* was confirmed by PCR. In the first row, 16 transductants were checked for deletion of *lexA* by PCR. In the second row, there were not any transductants to check.

Table 3

Isolate	Gene Name	Description
SC13	*ynfF*	Oxidoreductase subunit
SC14	*fdoGHI*	Formate dehydrogenase-O large subunit Formate dehydrogenase-O Fe-S subunit Cytochrome b556 subunit
SC15	*yhjDE*	Conserved inner membrane protien Predicted transporter
SC17	*elaC (rbn) elaD*	Binuclear zinc phosphodiesterase Hypothetical protein
SC19	*yjhG*	Predicted dehydratase

Table 3. Plasmids that when in multicopy allows for construction of the *nusA11 lexA*(Def) mutant.

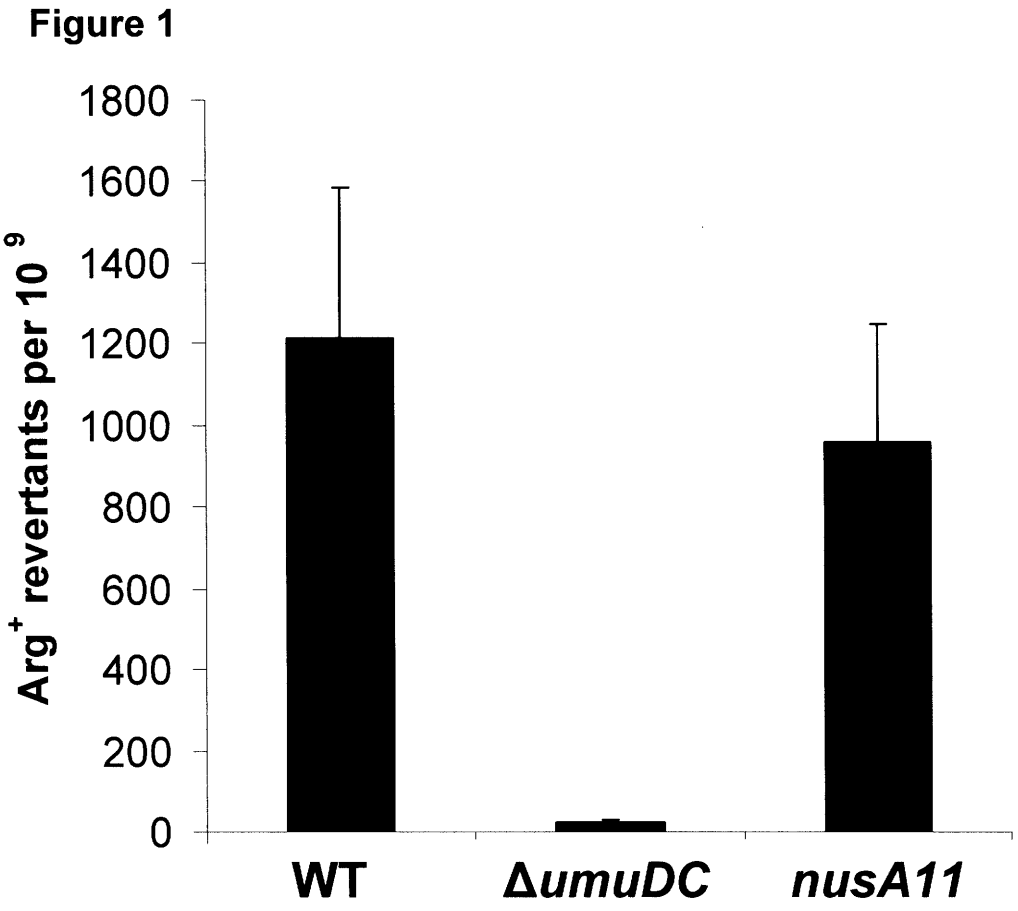

Figure 1. *nusA11* mutants, at the permissive temperature, are not defective in UV-induced mutagenesis (scored as Arg^- to Arg^+), compared to a wild-type (AB1157) and Δ*umuDC* controls.

Appendix B

Overlapping physiological responses of *Escherichia coli* to mutation of the essential transcriptional modulator *nusA* and exposure to the DNA damaging agent nitrofurazone

Authors for this work are Susan E. Cohen, Michael A. Kohanski, James J. Collins and Graham C. Walker

INTRODUCTION

In both prokaryotes and eukaryotes coordinated responses to genotoxic stress includes the induction of various cellular factors, many of which are involved in DNA repair, mutagenesis and cell cycle control (7). In *Escherichia coli*, the SOS response to DNA damage influences the expression of more than fifty genes involved in diverse cellular functions that allow the cell to combat the challenges of DNA damage (4, 7, 20, 21, 23) and is regulated by the $lexA^+$ and $recA^+$ gene products (7).

The SOS network is regulated by the LexA transcriptional repressor which, binds to operator sequences inhibiting transcription of downstream genes (7). In addition to the roles for the $recA^+$ gene product in homologous recombination, RecA also plays a central role in controlling the SOS response. Upon DNA damage RecA forms a nucleoprotein filament with ssDNA, which interacts with LexA and induces its own proteolytic digestion, inactivating LexA as a transcriptional repressor. However, several lines of investigation have underscored the notion that a simplistic view of the SOS response is far from complete. For example, analysis of gene expression in *E. coli* cells that have been irradiated with ultraviolet (UV) light identified genes that became induced in a *lexA* independent manner as well as genes whose expression decreased after UV treatment (4). Furthermore, exposure of *E. coli* to the DNA-damaging agent MMC results in expression changes of >1000 genes (10).

Our previous results have suggested that the essential transcriptional modulator NusA is important for allowing cells to cope with the lethal effects of DNA damage (Chapter Four). NusA is an essential protein that functions in both termination and antitermination of transcription, and is associated with the RNA polymerase throughout

the elongation and termination steps of transcription (2, 6, 9, 12-14, 18, 22). Originally reported in 1974, NusA forms an antitermination complex with λN protein that is required for successful λ phage infection (8). We have reported that the temperature sensitivity of the *nusA11*(ts) allele (19) can be suppressed by overexpression of two SOS induced DNA polymerases DinB (Pol IV) or UmuD'$_2$C (Pol V), in a manner that requires the catalytic capabilities of DinB, UmuD and UmuC (3). Furthermore, *nusA11*(ts) mutants at the permissive temperature (30°C) are: defective for adaptive mutagenesis (Chapter Three), sensitive to the DNA damaging agents nitrofurazone and 4-nitroquinolone-1-oxide, and display chronic partial SOS induction (Chapter 4).

Here we report the changes in mRNA profiles for *E. coli* cells harboring the *nusA11*(ts) mutation as well as cells exposed to the DNA damaing agent nitrofurazone (NFZ). Our analysis show that for either the *nusA11* strains or wild-type *E. coli* exposed to NFZ, the changes are mostly metabolic and have a significant amount of overlap. These data imply that *E. coli* cells respond in a similar manner to either mutation of *nusA* or to NFZ exposure. Furthermore, the SOS response is not induced under either of these conditions. Together these data underscore the notion that the cellular response to DNA damage is far more complex in even the well studied organism such as *E. coli*.

RESULTS AND DISCUSSION

The essential transcription factor NusA, is a key component of all elongating RNA polymerases, however our results suggests that NusA allows cells to cope with the lethal effects of DNA damage. I have shown that at the permissive temperature, *nusA11*(ts) mutants display the phenotype of chronic partial SOS induction, where approximately 2.5% of the population are SOS induced (Chapter 4). This phenotype of chronic partial SOS induction is often seen in strains carrying mutations of genes that are involved in the processing of DNA (17). Furthermore, and increased number of RecA-GFP foci in the *nusA11* mutant, at the permissive temperature, indicate an accumulation ssDNA (Chapter 4). In an attempt to further elucidate the role for $nusA^+$ in modulating the cellular physiology after exposure to DNA damaging agents in *E. coli,* in collaboration with Jim Collins at Boston University we examined the global effects of wild-type or *nusA11* mutants exposed to nitrofurazone using microarray analysis. We chose to look at nitrofurazone because, although the *nusA11* mutation has a broad effect on cellular physiology even at the permissive temperature, the *nusA11* mutation is specifically sensitive to the DNA damaging agents nitrofurazone (NFZ) and 4-nitroquinolone-1-oxide, which can form mostly covalet adducts on the N^2 position of guanine in addition to a variety of other DNA adducts (7).

To our surprise, we found that compared to a $nusA^+$ isogenic parent the changes in mRNA levels in the *nusA11* mutant did not include the SOS regulatory network but rather included genes involved in metabolism (Table 1-2). These data suggested that SOS induction in a small population of cells (2.5%) may not be enough to display an effect as can be detected by microarray analysis. Strikingly, the global changes that

occurred when wild-type (AB1157) cells were treated with nitrofurazone resembled those in the *nusA11* mutant (Table 3-4). Analysis of the mRNA profiles showed that under these two conditions there was a significant amount of overlap with both genes that are up-regulated (Figure 1A) as well as with genes that were down-regulated (Figure 1B). Analysis of the pathways that these genes are involved in revealed that most of the pathways were involved in cellular metabolism (Table 5-8). The up-regulation of genes involved in nitrate metabolism and assimilation, iron ion binding, and oxidoreductase activity and the down-regulation of pathways including glyoxylate metabolism, aerobic metabolism, and the TCA cycle, for example, were not what we would have expected for a DNA damaging agent and mutation of gene involved in DNA repair/damage tolerance.

Though these results were initially surprising, especially for the DNA damaging agent NFZ (24), work done on nitrofurazone and other nitrofurans in the 1970's suggested a possible explanation. It had been reported that mutation frequency increases when *E. coli* cells are treated with 240μM NFZ (1, 15), a much higher concentration than was used for the microarray analysis (5μM). At the concentration used for mutagenesis experiments, it was assumed that the SOS response was induced and error prone mutagenesis can occur. We chose to use a lower dose of NFZ because cells carrying mutations in DNA repair/damage tolerance pathways are sensitive to NFZ in the dose range of 2-10μM NFZ. While these data may be specific to the conditions we tested, it is curious to note that the cellular responses of both the *nusA11* mutation and exposure to NFZ are similar. Moreover, there are not a lot of additional genes that are differentially regulated when *nusA11* cells are treated with NFZ compared to untreated *nusA11* cells, suggesting that the cells may have already been induced for a NFZ-like response.

Taken together these data show that the cellular response to either mutation of *nusA* (*nusA11*) or exposure to the DNA damaging agent NFZ, are similar and involve mostly metabolic processes. However, it is unclear as to what this could all mean. It is possible that the *nusA11* mutant strains suffer from a higher amount of endogenous DNA damage that resembles damage caused by nitrofurazone, or has difficulty dealing with this endogenous DNA damage. The existence of a particular class of endogenously generated N^2-dG lesion, produced by metabolites of lipid peroxidation or the glycolysis pathway (16, 25), which could be dealt with at the replication fork (bypassed by TLS polymerases) but cause problems when transcription is attempted. This type of model could explain why the changes in the *nusA11* mutant resemble those of cells treated with nitrofurazone. However, why is it that cells treated with nitrofurazone are not induced for the SOS response? It is possible that there are two modes in which a cell can deal with the effects of nitrofurazone, one that occurs at low doses (through metabolism) and antoher at high doses (SOS induction). These data uncover the complexity of how cells deal with DNA damage and/or mutation of *nusA*, and support the notion that a complete understanding of the DNA damage response in *E. coli* and the SOS response is far from complete.

MATERIALS AND METHODS

Strains and Growth Conditions

All experiments were performed with AB1157 derived strains (*thr-1 leuB6 proA2 hisG4 thi1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*). Independent cultures of AB1157 (*nusA*$^{+}$) or AB1157 *nusA11 zha0132*::Tn*10* (SEC164) were grown in Luria-Bertani (LB) medium to saturation at 30°C. Cultures were then diluted 1:1000 into fresh LB medium with or without 1μg/mL (5μM) nitrofurazone (stock solution made in N,N-dimethylformamide) and were grown at 30°C for 6 hours.

Gene Expression Analysis

RNA samples were prepared from cultures treated as described above using an RNEasy kit (QIAGEN) according to manufacturer's directions. Contaminating DNA was removed by DNAase treatment (DNA free kit from Ambion). Microarray data collection and analysis was performed as previously described (5, 11).

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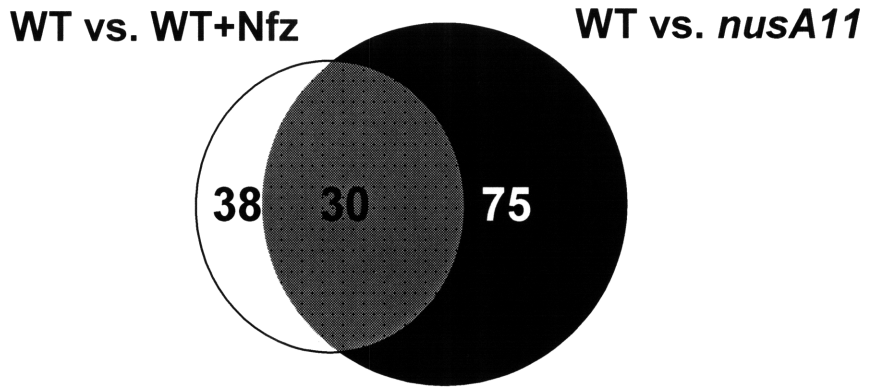
Figure 1

A



B

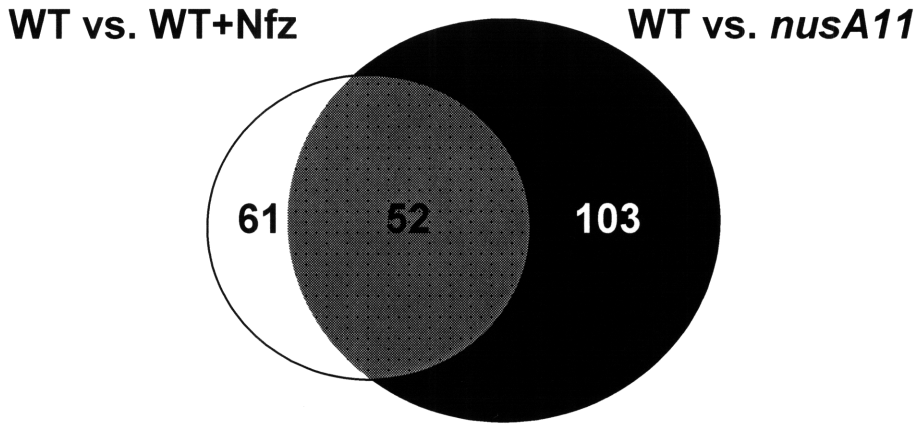


Figure 1. Venn-diagrams show that there exist a significant overlap in induced changes both in the up-regulation (A) and down-regulation (B). Genes that are either up-regulated or down-regulated in the *nusA11* mutant compared to *nusA*$^{+}$ are shown in black, genes up or down-regulated in the wild-type treated with nitrofurazone compared to an untreated control are shown in white, genes that overlap between these data sets are shown in grey.

Table 1. Genes up-regulated in *nusA11* background

gene	fold change (*nusA11*/*nusA*$^+$)	p value (up)	Description
adhE	5.735108049	1.06E-06	enzyme; Energy metabolism, carbon: Fermentation;fused acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase;CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase; pyruvate-formate-ly
adiY	3.149172241	0.00046	putative regulator; Not classified;DNA-binding transcriptional activator;putative ARAC-type regulatory protein
ahpF	2.596788171	5.83E-07	enzyme; Detoxification;alkyl hydroperoxide reductase, F52a subunit, FAD/NAD(P)-binding;alkyl hydroperoxide reductase, F52a subunit; detoxification of hydroperoxides
cadB	3.473838458	0.00016	transport; Transport of small molecules: Amino acids, amines;predicted lysine/cadaverine transporter;transport of lysine/cadaverine
caiF	2.226390514	0.00051	regulator; Central intermediary metabolism: Pool, multipurpose conversions;DNA-binding transcriptional activator;transcriptional regulator of cai operon
cysG	2.361200141	4.21E-09	enzyme; Biosynthesis of cofactors, carriers: Heme, porphyrin;fused siroheme synthase 1,3-dimethyluroporphyriongen III dehydrogenase and siroheme ferrochelatase/uroporphyrinogen methyltransferase;uroporphyrinogen III methylase; sirohaeme biosynthesis
dcuB	1.925820624	0.0005	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;C4-dicarboxylate antiporter;anaerobic dicarboxylate transport
dcuC	6.506081975	3.56E-10	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;anaerobic C4-dicarboxylate transport;transport of dicarboxylates
dmsA	4.211071389	0.0001	enzyme; Energy metabolism, carbon: Anaerobic respiration;dimethyl sulfoxide reductase, anaerobic, subunit A;anaerobic dimethyl sulfoxide reductase subunit A
dmsB	3.337174978	0.0019	enzyme; Energy metabolism, carbon: Anaerobic respiration;dimethyl sulfoxide reductase, anaerobic, subunit B;anaerobic dimethyl sulfoxide reductase subunit B
dmsC	2.158540757	7.98E-05	enzyme; Energy metabolism, carbon: Anaerobic respiration;dimethyl sulfoxide reductase, anaerobic, subunit C;anaerobic dimethyl sulfoxide reductase subunit C
Eno	3.376618838	1.97E-06	enzyme; Energy metabolism, carbon: Glycolysis;enolase
fdnG	2.370388721	0.00072	enzyme; Energy metabolism, carbon: Anaerobic respiration|1.4.1 metabolism; energy production/transport; electron donor|1.3.7 metabolism; energy metabolism, carbon; anaerobic respiration|7.1 location of gene products; cytoplasm;formate dehydrogenase-N, alp
fdnI	2.472314023	0.00011	enzyme; Energy metabolism, carbon: Anaerobic respiration;formate dehydrogenase-N, cytochrome B556 (gamma) subunit, nitrate-inducible;formate dehydrogenase-N, nitrate-inducible, cytochrome B556(Fdn) gamma subunit

fruA	3.200994061	6.17E-11	regulator; Degradation of small molecules: Carbon compounds;fused fructose-specific PTS enzymes: IIBcomponent/IIC components;PTS system, fructose-specific transport protein
fruB	4.076052593	1.42E-09	enzyme; Transport of small molecules: Carbohydrates, organic acids, alcohols;fused fructose-specific PTS enzymes: IIA component/HPr component;PTS system, fructose-specific IIA/fpr component
fruK	4.08609855	2.8E-09	enzyme; Energy metabolism, carbon: Glycolysis;fructose-1-phosphate kinase
glk	2.555277545	8.93E-06	enzyme; Degradation of small molecules: Carbon compounds;glucokinase
gpmM	8.394286284	1.31E-10	putative enzyme; Not classified;phosphoglycero mutase III, cofactor-independent;putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase
hcp	4.231792827	2.18E-12	orf; Not classified;hybrid-cluster [4Fe-2S-2O] protein in anaerobic terminal reductases
hypA	3.314238287	0.00036	phenotype; Energy metabolism, carbon: Anaerobic respiration;protein involved in nickel insertion into hydrogenases 3;pleiotrophic effects on 3 hydrogenase isozymes
hypC	3.568366172	0.00012	phenotype; Energy metabolism, carbon: Anaerobic respiration;protein required for maturation of hydrogenases 1 and 3;pleiotrophic effects on 3 hydrogenase isozymes
hypD	3.042116353	0.0004	phenotype; Energy metabolism, carbon: Anaerobic respiration;protein required for maturation of hydrogenases;pleiotrophic effects on 3 hydrogenase isozymes
malF	5.138480014	1.86E-07	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;maltose transporter subunit;part of maltose permease, periplasmic; membrane component of ABC superfamily
malG	2.879537945	0.0009	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;maltose transporter subunit;part of maltose permease, inner membrane; membrane component of ABC superfamily
malM	5.099412342	6.75E-05	phenotype; Degradation of small molecules: Carbon compounds;maltose regulon periplasmic protein;periplasmic protein of mal regulon
malP	4.817508905	0.00032	enzyme; Degradation of small molecules: Carbon compounds;maltodextrin phosphorylase
malQ	3.790749175	3.58E-06	enzyme; Degradation of polysaccharides;4-alpha-glucanotransferase (amylomaltase)
malS	1.919713033	6.72E-06	enzyme; Degradation of polysaccharides;alpha-amylase
mtlD	2.537978182	0.000457	enzyme; Degradation of small molecules: Carbon compounds;mannitol-1-phosphate dehydrogenase, NAD(P)-binding;mannitol-1-phosphate dehydrogenase
mtlR	2.359258771	7.24E-05	regulator; Degradation of small molecules: Carbon compounds;DNA-binding repressor;repressor for mtl
napB	3.820503195	8.06E-07	carrier; Energy metabolism, carbon: Electron transport;nitrate reductase, small, cytochrome C550 subunit, periplasmic;cytochrome c-type protein
napC	2.88821405	1.36E-06	putative enzyme; Energy metabolism, carbon: Electron transport;nitrate reductase, cytochrome c-type, periplasmic;cytochrome c-type protein
napG	2.21680059	0.000219	carrier; Energy metabolism, carbon: Electron transport;ferredoxin-type protein essential for electron transfer from ubiquinol to periplasmic nitrate reductase (NapAB);ferredoxin-type protein: electron transfer
napH	2.204583824	0.000159	carrier; Energy metabolism, carbon: Electron transport;ferredoxin-type protein essential for electron transfer from ubiquinol to periplasmic nitrate reductase (NapAB);ferredoxin-type protein: electron transfer

narG	14.97353203	2.41E-08	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrate reductase 1, alpha subunit;
narH	16.84230472	2.25E-12	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrate reductase 1, beta (Fe-S) subunit;nitrate reductase 1, beta subunit
narI	3.207194837	5.33E-07	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrate reductase 1, gamma (cytochrome b(NR)) subunit;nitrate reductase 1, cytochrome b(NR), gamma subunit
narJ	8.583132878	7.74E-13	enzyme; Energy metabolism, carbon: Anaerobic respiration;molybdenum-cofactor-assembly chaperone subunit (delta subunit) of nitrate reductase 1;nitrate reductase 1, delta subunit, assembly function
narK	4.740064421	1.02E-05	transport; Transport of small molecules: Anions;nitrate/nitrite transporter;nitrite extrusion protein
narL	3.401172866	7.33E-06	regulator; Energy metabolism, carbon: Anaerobic respiration;DNA-binding response regulator in two-component regulatory system with NarX (or NarQ);pleiotrophic regulation of anaerobic respiration: response regulator for nar, frd, dms and tor genes
narX	1.916479634	0.00089	enzyme; Energy metabolism, carbon: Anaerobic respiration;sensory histidine kinase in two-component regulatory system with NarL;nitrate/nitrate sensor, histidine protein kinase acts on NarL regulator
nikA	2.179043001	0.00058	transport; Transport of small molecules: Cations;nickel transporter subunit;periplasmic binding protein for nickel; periplasmic-binding component of ABC superfamily
nirB	16.19808848	1.02E-11	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite reductase, large subunit, NAD(P)H-binding;nitrite reductase (NAD(P)H) subunit
nirC	5.33028005	1.92E-12	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite transporter;nitrite reductase activity
nirD	7.007253066	1.56E-09	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite reductase, NAD(P)H-binding, small subunit;nitrite reductase (NAD(P)H) subunit
nrdD	3.403250469	1.33E-05	enzyme; 2'-Deoxyribonucleotide metabolism;anaerobic ribonucleoside-triphosphate reductase
nrdG	2.186385302	0.00015	enzyme; Central intermediary metabolism: Nucleotide interconversions;anaerobic ribonucleotide reductase activating protein
nrfA	7.175334058	0	carrier; Energy metabolism, carbon: Electron transport;nitrite reductase, formate-dependent, cytochrome;periplasmic cytochrome c(552): plays a role in nitrite reduction
nrfB	10.18408646	2.18E-14	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite reductase, formate-dependent, penta-heme cytochrome c;formate-dependent nitrite reductase; a penta-haeme cytochrome c
nrfC	5.57579299	0	enzyme; Energy metabolism, carbon: Anaerobic respiration;formate-dependent nitrite reductase, 4Fe4S subunit;formate-dependent nitrite reductase; Fe-S centers
nrfD	2.611775694	3.36E-11	enzyme; Energy metabolism, carbon: Anaerobic respiration;formate-dependent nitrite reductase, membrane subunit;formate-dependent nitrate reductase complex; transmembrane protein
nrfE	2.307190293	8.55E-12	enzyme; Energy metabolism, carbon: Anaerobic respiration;heme lyase (NrfEFG) for insertion of heme into c552, subunit NrfE;formate-dependent nitrite reductase; possible assembly function
nrfF	2.119163157	1.62E-11	enzyme; Energy metabolism, carbon: Anaerobic respiration;heme lyase (NrfEFG) for insertion of heme into c552, subunit NrfF;part of formate-dependent nitrite reductase complex

ompX	2.8276378	8.2E-05	membrane; Outer membrane constituents;outer membrane protein;outer membrane protein X
pfkA	4.247727841	3.95E-10	enzyme; Energy metabolism, carbon: Glycolysis;6-phosphofructokinase I
rsuA	2.535220095	8.28E-06	enzyme; RNA synthesis, modification, DNA transcription;16S rRNA pseudouridylate 516 synthase;16S pseudouridylate 516 synthase
speB	2.079383224	0.000196	enzyme; Central intermediary metabolism: Polyamine biosynthesis;agmatinase
tpiA	2.810790641	7.46E-05	enzyme; Energy metabolism, carbon: Glycolysis;triosephosphate isomerase
treC	7.560421492	9.51E-05	enzyme; Degradation of small molecules: Carbon compounds;trehalose-6-P hydrolase;trehalase 6-P hydrolase
ttdR	2.205861687	2.86E-07	putative regulator; Not classified;predicted DNA-binding transcriptional regulator;putative transcriptional regulator LYSR-type
yagL	2.002763406	0.00094	phenotype; Not classified;CP4-6 prophage; DNA-binding protein;DNA-binding protein
yccM	2.592288583	9.65E-08	predicted 4Fe-4S membrane protein
ydhY	2.140839539	0.000836	putative enzyme; Not classified;predicted 4Fe-4S ferridoxin-type protein;putative oxidoreductase, Fe-S subunit
yeeX	3.654682303	5.035E-05	phenotype; Not classified;conserved protein;putative alpha helix protein
yeiS	2.07610393	4.42E-05	predicted inner membrane protein
yeiT	2.046121289	0.00173	putative enzyme; Not classified;predicted oxidoreductase;putative oxidoreductase
yfbT	1.935859166	0.00016	putative enzyme; Not classified;predicted hydrolase or phosphatase;putative phosphatase
yfbU	2.612685249	0.0002	conserved protein
yfeX	2.423612973	0.00034	conserved protein
yhfL	2.238008734	8.8E-05	conserved secreted peptide
yjjW	2.252942771	1.5E-05	putative enzyme; Not classified;predicted pyruvate formate lyase activating enzyme;putative activating enzyme
ysaA	2.070622658	0.00039	predicted hydrogenase, 4Fe-4S ferredoxin-type component
ytfE	3.99084155	8.4E-07	predicted regulator of cell morphogenesis and cell wall metabolism
zwf	2.193005353	0.000111	enzyme; Energy metabolism, carbon: Oxidative branch, pentose pathway;glucose-6-phosphate dehydrogenase;

Table 2. Genes down-regulated in *nusA11* background

gene	fold change (*nusA11/nusA*$^+$)	p value (down)	Description
aceA	0.110595148	0.000459383	Enzyme; Central intermediary metabolism: Glyoxylate bypass;isocitrate lyase;
aceB	0.089286446	2.02405E-05	Enzyme; Central intermediary metabolism: Glyoxylate bypass;malate synthase A;
aceK	0.403636254	1.76342E-06	Enzyme; Central intermediary metabolism: Glyoxylate bypass;isocitrate dehydrogenase kinase/phosphatase;
acnB	0.390477953	0.000481602	enzyme; Energy metabolism, carbon: TCA cycle;bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase;aconitate hydrase B
acs	0.140472179	1.13673E-06	Enzyme; Fatty acid and phosphatidic acid biosynthesis;acetyl-CoA synthetase;
actP	0.19209728	4.33943E-07	Electrochemical potential driven transporters; Porters (Uni-, Sym- and Antiporters); The Solute:Sodium Symporter (SSS)
aer	0.34916447	8.25391E-05	regulator; Degradation of small molecules: Carbon compounds; motility (incl. chemotaxis, energytaxis, aerotaxis, redoxtaxis); location of gene products; inner membrane
aldB	0.358437048	1.3429E-05	enzyme; Degradation of small molecules: Carbon compounds;aldehyde dehydrogenase B;aldehyde dehydrogenase B (lactaldehyde dehydrogenase)
argT	0.182902834	5.6505E-05	transport; Transport of small molecules: Amino acids, amines;lysine/arginine/ornithine transporter subunit;lysine-, arginine-, ornithine-binding periplasmic protein; periplasmic-binding component of ABC superfamily
astA	0.34927033	0.00064757	arginine succinyltransferase
astB	0.293346632	3.52003E-05	succinylarginine dihydrolase
astD	0.378667279	0.000566122	Putative enzyme; Not classified;succinylglutamic semialdehyde dehydrogenase;
bax	0.442453475	0.000300443	orf; Not classified;conserved protein;putative ATP-binding protein
betA	0.554510109	0.000300744	Enzyme; Osmotic adaptation;choline dehydrogenase, a flavoprotein
betB	0.367049956	0.000695898	enzyme; Osmotic adaptation;betaine aldehyde dehydrogenase, NAD-dependent;NAD+-dependent betaine aldehyde dehydrogenase
csiD	0.221475855	0.000197082	predicted protein
csiR	0.462104486	2.09504E-06	Putative regulator; Not classified;DNA-binding transcriptional dual regulator;putative transcriptional regulator
cstA	0.119351147	2.8756E-09	phenotype; Global regulatory functions;carbon starvation protein;
cycA	0.360648168	1.76137E-07	transport; Transport of small molecules: Amino acids, amines;D-alanine/D-serine/glycine transporter;transport of D-alanine, D-serine, and glycine
cyoA	0.267514748	0.000372546	Enzyme; Energy metabolism, carbon: Aerobic respiration;cytochrome o ubiquinol oxidase subunit II;

cyoC	0.2828992	0.001226949	Enzyme; Energy metabolism, carbon: Aerobic respiration;cytochrome o ubiquinol oxidase subunit III;
cyoD	0.262441601	0.000757147	Enzyme; Energy metabolism, carbon: Aerobic respiration;cytochrome o ubiquinol oxidase subunit IV;
dctA	0.197320534	1.84937E-05	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;C4-dicarboxylic acid, orotate and citrate transporter;uptake of C4-dicarboxylic acids
fadA	0.480167834	7.49294E-06	enzyme; Degradation of small molecules: Fatty acids;3-ketoacyl-CoA thiolase (thiolase I);thiolase I; 3-ketoacyl-CoA thiolase; acetyl-CoA transferase
fadB	0.360665167	1.40367E-05	enzyme; Degradation of small molecules: Fatty acids;fused 3-hydroxybutyryl-CoA epimerase/delta(3)-cis-delta(2)-trans-enoyl-CoA isomerase/enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase;4-enzyme protein: 3-hydroxyacyl-CoA dehydrogenase; 3-hydroxybutyry
fimC	0.380000705	0.000894237	factor; Surface structures;chaperone, periplasmic;periplasmic chaperone, required for type 1 fimbriae
fimD	0.441438694	3.0633E-11	membrane; Outer membrane constituents;outer membrane usher protein, type 1 fimbrial synthesis;outer membrane protein; export and assembly of type 1 fimbriae, interrupted
fimF	0.378665792	4.11158E-10	structural component; Surface structures;minor component of type 1 fimbriae;fimbrial morphology
fimG	0.404960004	3.42728E-09	structural component; Surface structures;minor component of type 1 fimbriae;fimbrial morphology
fimH	0.471194411	1.56479E-07	structural component; Surface structures;minor component of type 1 fimbriae;minor fimbrial subunit, D-mannose specific adhesion
fimI	0.304366328	1.63941E-05	structural component; Surface structures;fimbrial protein involved in type 1 pilus biosynthesis;fimbrial protein
fumC	0.348283251	0.000110149	enzyme; Energy metabolism, carbon: TCA cycle;fumarate hydratase (fumarase C),aerobic Class II;fumarase C= fumarate hydratase Class II; isozyme
gabD	0.134975082	2.45509E-09	enzyme; Central intermediary metabolism: Pool, multipurpose conversions;succinate-semialdehyde dehydrogenase I, NADP-dependent;succinate-semialdehyde dehydrogenase, NADP-dependent activity
gabP	0.206732913	6.63499E-10	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;gamma-aminobutyrate transporter;transport permease protein of gamma-aminobutyrate
gabT	0.140903954	8.26911E-09	enzyme; Central intermediary metabolism: Pool, multipurpose conversions;4-aminobutyrate aminotransferase, PLP-dependent;4-aminobutyrate aminotransferase activity
glcA	0.527723573	2.89262E-05	Putative transport; Not classified;glycolate transporter;putative permease
glcC	0.392796687	0.00067801	regulator; Degradation of small molecules: Carbon compounds;DNA-binding transcriptional dual regulator, glycolate-binding;transcriptional activator for glc operon
glcD	0.225623618	1.32726E-07	enzyme; Degradation of small molecules: Carbon compounds;glycolate oxidase subunit, FAD-linked;glycolate oxidase subunit D
glcF	0.209276613	9.66908E-10	enzyme; Central intermediary metabolism: Pool, multipurpose conversions|1.7.1 metabolism; central intermediary metabolism; unassigned reversible reactions;glycolate oxidase iron-sulfur subunit;
gltA	0.250386092	0.000461589	Enzyme; Energy metabolism, carbon: TCA cycle;citrate synthase;

gltI	0.279485692	0.000103931	putative transport; Not classified;glutamate and aspartate transporter subunit;periplasmic-binding component of ABC superfamily
gltJ	0.601634415	0.001189155	transport; Transport of small molecules: Amino acids, amines;glutamate and aspartate transporter subunit;glutamate/aspartate transport system permease; membrane component of ABC superfamily
gltK	0.421234644	5.25951E-06	transport; Transport of small molecules: Amino acids, amines;glutamate and aspartate transporter subunit;glutamate/aspartate transport system permease; membrane component of ABC superfamily
gltL	0.501808333	0.000131454	transport; Transport of small molecules: Amino acids, amines;glutamate and aspartate transporter subunit;ATP-binding protein of glutamate/aspartate transport system
gudD	0.43271029	2.12847E-14	metabolism; carbon utilization; carbon compounds;(D)-glucarate dehydratase 1;(D)-glucarate dehydratase 1 (L)-idarate dehydratase (L)-idarate epimerase (D)-glucarate epimerase
gudP	0.30503873	1.62807E-14	metabolism; carbon utilization; carbon compounds, transport; Electrochemical potential driven transporters; Porters (Uni-, Sym- and Antiporters); The Major Facilitator Superfamily (MFS)
gudX	0.380512653	2.66966E-13	putative enzyme; Not classified; metabolism; carbon utilization; carbon compounds|10 cryptic genes;predicted glucarate dehydratase;putative (D)-glucarate dehydratase 2
iclR	0.279937047	8.36739E-12	regulator; Central intermediary metabolism: Glyoxylate bypass;DNA-binding transcriptional repressor;repressor of aceBA operon
katG	0.45009299	5.80043E-06	Enzyme; Detoxification;catalase/hydroperoxidase HPI(I);catalase; hydroperoxidase HPI(I)
kgtP	0.311869059	0.000235894	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;alpha-ketoglutarate transporter;alpha-ketoglutarate permease
maeB	0.412545171	7.85334E-05	putative enzyme; Not classified;fused malic enzyme predicted oxidoreductase/predicted phosphotransacetylase;putative multimodular enzyme
melA	0.195863707	2.00044E-08	enzyme; Degradation of small molecules: Carbon compounds;alpha-galactosidase, NAD(P)-binding;alpha-galactosidase
melB	0.178412178	2.59135E-13	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;melibiose:sodium symporter;melibiose permease II
mglB	0.115572629	1.57266E-05	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;methyl-galactoside transporter subunit;galactose-binding transport protein; receptor for galactose taxis; periplasmic-binding component of ABC superfamily
msrB	0.320055573	0.001398159	methionine sulfoxide reductase B
osmY	0.187566185	0.000381873	phenotype; Osmotic adaptation;periplasmic protein;hyperosmotically inducible periplasmic protein
paaA	0.280288087	6.64484E-11	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaB	0.278280489	1.18474E-11	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaC	0.234718425	1.14119E-12	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaD	0.274807827	1.4343E-11	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaE	0.355750577	1.07573E-10	putative enzyme; Not classified;predicted multicomponent oxygenase/reductase subunit for

			phenylacetic acid degradation;
paaF	0.371448988	1.15089E-07	Putative enzyme; Not classified;enoyl-CoA hydratase-isomerase;
paaG	0.304574346	1.86533E-13	Putative enzyme; Not classified;acyl-CoA hydratase;
paaH	0.426133783	1.12526E-06	Putative enzyme; Not classified;3-hydroxybutyryl-CoA dehydrogenase;
paaI	0.355785018	2.32621E-08	predicted thioesterase
paaJ	0.490811968	0.00020562	Putative enzyme; Not classified;predicted beta-ketoadipyl CoA thiolase
paaK	0.49149955	0.000278699	phenylacetyl-CoA ligase
phoH	0.2625074	2.69858E-05	regulator; Central intermediary metabolism: Phosphorus compounds;conserved protein with nucleoside triphosphate hydrolase domain;PhoB-dependent, ATP-binding pho regulon component; may be helicase; induced by P starvation
psiF	0.341049251	0.001166297	phenotype; Central intermediary metabolism: Phosphorus compounds;conserved protein;induced by phosphate starvation
putA	0.251873573	1.02918E-09	enzyme; Degradation of small molecules: Amino acids;fused DNA-binding transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase;proline dehydrogenase, P5C dehydrogenase
putP	0.238599512	2.14126E-09	transport; Transport of small molecules: Cations;proline:sodium symporter;major sodium/proline symporter
puuA	0.43502021	0.000660315	Putative enzyme; Not classified;gamma-Glu-putrescine synthase;putative glutamine synthetase
puuB	0.523912456	0.000501581	Putative enzyme; Not classified;gamma-Glu-putrescine oxidase, FAD/NAD(P)-binding;probable oxidoreductase
rsd	0.376519633	0.000127568	Putative regulator; Not classified;stationary phase protein, binds sigma 70 RNA polymerase subunit;
sdhA	0.194048161	9.1402E-05	Enzyme; Energy metabolism, carbon: TCA cycle;succinate dehydrogenase, flavoprotein subunit;
sdhB	0.2703314	0.000306873	enzyme; Energy metabolism, carbon: TCA cycle;succinate dehydrogenase, FeS subunit;succinate dehydrogenase, iron sulfur protein
sdhC	0.107800093	0.000116112	enzyme; Energy metabolism, carbon: TCA cycle;succinate dehydrogenase, membrane subunit, binds cytochrome b556;succinate dehydrogenase, cytochrome b556
sdhD	0.119944036	1.68936E-05	enzyme; Energy metabolism, carbon: TCA cycle;succinate dehydrogenase, membrane subunit, binds cytochrome b556;succinate dehydrogenase, hydrophobic subunit
sthA	0.330950189	0.001185807	Putative enzyme; Not classified;pyridine nucleotide transhydrogenase, soluble;
trg	0.420885973	0.000604859	regulator; Chemotaxis and mobility|3.1.3.3 regulation; type of regulation; posttranscriptional; inhibition / activation of enzymes|5.3 cell processes; motility (incl. chemotaxis, energytaxis, aerotaxis, redoxtaxis)|6.1 cell structure; membrane|7.3 locatio
xylA	0.313659609	2.868E-09	Enzyme; Degradation of small molecules: Carbon compounds;D-xylose isomerase;
xylB	0.612513409	1.10237E-06	Enzyme; Degradation of small molecules: Carbon compounds;xylulokinase;

xylE	0.42593957	5.87207E-08	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;D-xylose transporter;xylose-proton symport
xylF	0.054094898	4.41422E-33	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;D-xylose transporter subunit;xylose binding protein transport system; periplasmic-binding component of ABC superfamily
xylG	0.211959079	5.05658E-21	putative transport; Not classified;fused D-xylose transporter subunits of ABC superfamily: ATP-binding components;putative ATP-binding protein of xylose transport system
xylH	0.372509468	2.39477E-13	putative transport; Not classified;D-xylose transporter subunit;putative xylose transport, membrane component; membrane component of ABC superfamily
yagG	0.283705233	6.74303E-18	Putative transport; Not classified;CP4-6 prophage; predicted sugar transporter;putative permease
yagH	0.41380969	2.2821E-11	Putative enzyme; Not classified;CP4-6 prophage; predicted xylosidase/arabinosidase;putative beta-xylosidase
ybdD	0.194482287	6.94258E-09	conserved protein
ycgF	0.363185288	1.77842E-05	predicted FAD-binding phosphodiesterase
ydcV	0.351223594	7.65526E-05	putative transport; Not classified;predicted spermidine/putrescine transporter subunit;membrane component of ABC superfamily
ydcW	0.372058917	6.14181E-05	Putative enzyme; Not classified;medium chain aldehyde dehydrogenase;putative aldehyde dehydrogenase
yeaC	0.271203744	0.00036309	conserved protein
yeaH	0.299470542	0.001314735	conserved protein
ygaF	0.166971371	1.15205E-05	predicted enzyme
ygeW	0.446157295	3.28832E-11	Putative enzyme; Not classified;conserved protein;putative carbamoyl transferase
yicI	0.440291531	5.34762E-10	predicted alpha-glucosidase;
yjcH	0.09130251	3.24052E-07	conserved inner membrane protein involved in acetate transport;
ylaC	0.350253086	0.000529873	predicted inner membrane protein;
ytfQ	0.254173642	1.78428E-05	putative regulator; Not classified;predicted sugar transporter subunit: periplasmic-binding component of ABC superfamily;putative LACI-type transcriptional regulator
ytfR	0.528872794	5.02833E-06	putative transport; Not classified|4.3.A.1.a transport; Primary Active Transporters; Pyrophosphate Bond (ATP, GTP, P2) Hydrolysis-driven Active Transporters; The ATP-binding Cassette (ABC) Superfamily + ABC-type Uptake Permeases; ABC superfamily ATP bindi
ytfT	0.597638502	1.20382E-05	putative transport; Not classified;predicted sugar transporter subunit: membrane component of ABC superfamily;putative transport system permease protein
ytjA	0.184682661	5.39328E-05	predicted protein

Table 3. Genes up-regulated by nitrofurazone treatment

gene	fold change ([wt +nfz]/wt)	p value (up)	Description
adhE	4.179370	5.186E-05	enzyme; Energy metabolism, carbon: Fermentation;fused acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase;CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase; pyruvate-formate-ly
cadB	4.322040	1.248E-05	transport; Transport of small molecules: Amino acids, amines;predicted lysine/cadaverine transporter;transport of lysine/cadaverine
dcuB	2.830658	1E-07	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;C4-dicarboxylate antiporter;anaerobic dicarboxylate transport
focA	4.459106	1.93E-06	putative transport; Degradation of small molecules: Carbon compounds;formate transporter;probable formate transporter (formate channel 1)
fruA	3.00309	5.93E-10	regulator; Degradation of small molecules: Carbon compounds;fused fructose-specific PTS enzymes: IIBcomponent/IIC components;PTS system, fructose-specific transport protein
fruB	4.198856	6.571E-10	enzyme; Transport of small molecules: Carbohydrates, organic acids, alcohols;fused fructose-specific PTS enzymes: IIA component/HPr component;PTS system, fructose-specific IIA/fpr component
fruK	4.023795	4.195E-09	enzyme; Energy metabolism, carbon: Glycolysis;fructose-1-phosphate kinase
glk	2.159431	0.00021	enzyme; Degradation of small molecules: Carbon compounds;glucokinase
gpmM	8.579813	8.59E-11	putative enzyme; Not classified;phosphoglycero mutase III, cofactor-independent;putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase
hcp	2.307125	2.99E-05	orf; Not classified;hybrid-cluster [4Fe-2S-2O] protein in anaerobic terminal reductases
hypA	3.38108	0.00029	phenotype; Energy metabolism, carbon: Anaerobic respiration;protein involved in nickel insertion into hydrogenases 3;pleiotrophic effects on 3 hydrogenase isozymes
hypC	3.89353	4.47E-05	phenotype; Energy metabolism, carbon: Anaerobic respiration;protein required for maturation of hydrogenases 1 and 3;pleiotrophic effects on 3 hydrogenase isozymes
hypD	3.347746	0.00013	phenotype; Energy metabolism, carbon: Anaerobic respiration;protein required for maturation of hydrogenases;pleiotrophic effects on 3 hydrogenase isozymes
malF	6.300699	5.45E-09	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;maltose transporter subunit;part of maltose permease, periplasmic; membrane component of ABC superfamily
malG	3.890495	3.087E-05	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;maltose transporter subunit;part of maltose permease, inner membrane; membrane component of ABC superfamily
malK	19.08041	6.63E-07	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;fused maltose transport subunit, ATP-binding component of ABC superfamily/regulatory protein;ATP-binding component of transport

			system for maltose
malM	6.69887	4.17E-06	phenotype; Degradation of small molecules: Carbon compounds;maltose regulon periplasmic protein;periplasmic protein of mal regulon
malP	5.25455	0.00015	enzyme; Degradation of small molecules: Carbon compounds;maltodextrin phosphorylase;
malQ	3.96927	1.71E-06	enzyme; Degradation of polysaccharides;4-alpha-glucanotransferase (amylomaltase);
malS	3.11986	1.55E-14	enzyme; Degradation of polysaccharides;alpha-amylase;
mtlR	2.38399	6E-05	regulator; Degradation of small molecules: Carbon compounds;DNA-binding repressor;repressor for mtl
narH	5.22495	2.54E-05	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrate reductase 1, beta (Fe-S) subunit;nitrate reductase 1, beta subunit
nirB	10.1864	1.16E-08	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite reductase, large subunit, NAD(P)H-binding;nitrite reductase (NAD(P)H) subunit
nirC	2.93892	3.86E-06	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite transporter;nitrite reductase activity
nirD	4.04102	1.07E-05	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite reductase, NAD(P)H-binding, small subunit;nitrite reductase (NAD(P)H) subunit
nrfA	3.51683	5.3E-08	carrier; Energy metabolism, carbon: Electron transport;nitrite reductase, formate-dependent, cytochrome;periplasmic cytochrome c(552): plays a role in nitrite reduction
nrfB	5.11644	5.46E-08	enzyme; Energy metabolism, carbon: Anaerobic respiration;nitrite reductase, formate-dependent, penta-heme cytochrome c;formate-dependent nitrite reductase; a penta-haeme cytochrome c
nrfC	3.05401	6.59E-10	enzyme; Energy metabolism, carbon: Anaerobic respiration;formate-dependent nitrite reductase, 4Fe4S subunit;formate-dependent nitrite reductase; Fe-S centers
pfkA	4.22563	4.55E-10	enzyme; Energy metabolism, carbon: Glycolysis;6-phosphofructokinase
ravA	2.77391	1.32E-06	putative regulator; Not classified;fused predicted transcriptional regulator: sigma54 activator protein/conserved protein;putative 2-component regulator
tff	2.568639	0.00012	Novel sRNA, function unknown;identified in a large scale screen; function unknown
treC	7.28554	0.000124	enzyme; Degradation of small molecules: Carbon compounds;trehalose-6-P hydrolase;trehalase 6-P hydrolase
yccM	2.235655	5.48E-06	predicted 4Fe-4S membrane protein
ydhY	2.236260	0.00044	putative enzyme; Not classified;predicted 4Fe-4S ferridoxin-type protein;putative oxidoreductase, Fe-S subunit
yjiA	2.192145	0.00032	predicted GTPase
yjiX	3.117484	3.38E-06	conserved protein
yjiY	6.189454	3.87E-05	orf; Not classified;predicted inner membrane protein;putative carbon starvation protein
yjjW	2.2473185	1.65E-05	putative enzyme; Not classified;predicted pyruvate formate lyase activating enzyme;putative activating enzyme

Table 4. Genes down-regulated by nitrofurazone treatment

	fold change ([wt +nfz]/wt)	p value (down)	Description
aceB	0.159	**gene**	enzyme; Central intermediary metabolism: Glyoxylate bypass;malate synthase A
aceK	0.4650	4.54E-05	enzyme; Central intermediary metabolism: Glyoxylate bypass;isocitrate dehydrogenase kinase/phosphatase;
acnB	0.34382	8.92E-05	enzyme; Energy metabolism, carbon: TCA cycle;bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase;aconitate hydrase B
acs	0.2044	6.5E-05	enzyme; Fatty acid and phosphatidic acid biosynthesis;acetyl-CoA synthetase
actP	0.232	6.75E-06	4.2.A.21 transport; Electrochemical potential driven transporters; Porters (Uni-, Sym- and Antiporters); The Solute:Sodium Symporter (SSS) Family|6.1 cell structure; membrane|7.3 location of gene products; inner membrane;acetate transporter
aldB	0.4183	0.000180	enzyme; Degradation of small molecules: Carbon compounds;aldehyde dehydrogenase B;aldehyde dehydrogenase B (lactaldehyde dehydrogenase)
csiR	0.4356	3.63917E-07	putative regulator; Not classified;DNA-binding transcriptional dual regulator;putative transcriptional regulator
cstA	0.209	9.20E-06	phenotype; Global regulatory functions;carbon starvation protein;
cycA	0.398	2.12E-06	transport; Transport of small molecules: Amino acids, amines;D-alanine/D-serine/glycine transporter;transport of D-alanine, D-serine, and glycine
fadA	0.4899	1.27E-05	enzyme; Degradation of small molecules: Fatty acids;3-ketoacyl-CoA thiolase (thiolase I);thiolase I; 3-ketoacyl-CoA thiolase; acetyl-CoA transferase
fumC	0.329	5.07E-05	enzyme; Energy metabolism, carbon: TCA cycle;fumarate hydratase (fumarase C),aerobic Class II;fumarase C= fumarate hydratase Class II; isozyme
gabD	0.175	1.93E-07	enzyme; Central intermediary metabolism: Pool, multipurpose conversions;succinate-semialdehyde dehydrogenase I, NADP-dependent;succinate-semialdehyde dehydrogenase, NADP-dependent activity
gabP	0.223	4.22E-09	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;gamma-aminobutyrate transporter;transport permease protein of gamma-aminobutyrate
gabT	0.174	2.45E-07	enzyme; Central intermediary metabolism: Pool, multipurpose conversions;4-aminobutyrate aminotransferase, PLP-dependent;4-aminobutyrate aminotransferase activity
garK	0.282	2.45E-09	carbon utilization; carbon compounds;glycerate kinase
garL	0.2595	2.68E-05	carbon utilization; carbon compounds;alpha-dehydro-beta-deoxy-D-glucarate aldolase
garP	0.302	2.50E-05	carbon utilization; carbon compounds; Electrochemical potential driven transporters; Porters (Uni-, Sym- and Antiporters); The Major Facilitator Superfamily (MFS)

garR	0.22	2.64E-08	carbon utilization; carbon compounds; central intermediary metabolism; glycolate metabolism;tartronate semialdehyde reductase;tartronate semialdehyde reductase (TSAR)
glcA	0.313	1.53E-13	putative transport; Not classified;glycolate transporter;putative permease
glcB	0.0691	1.05E-17	enzyme; Central intermediary metabolism: Glyoxylate bypass;malate synthase G
glcC	0.471	0.004974	regulator; Degradation of small molecules: Carbon compounds;DNA-binding transcriptional dual regulator, glycolate-binding;transcriptional activator for glc operon
glcD	0.112	2.03E-14	enzyme; Degradation of small molecules: Carbon compounds;glycolate oxidase subunit, FAD-linked;glycolate oxidase subunit D
glcF	0.122	4.03E-16	enzyme; Central intermediary metabolism: Pool, multipurpose conversions; central intermediary metabolism; unassigned reversible reactions;glycolate oxidase iron-sulfur subunit
glcG	0.126	2.86E-14	conserved protein
glnH	0.294	0.000966	transport; Transport of small molecules: Amino acids, amines;glutamine transporter subunit;periplasmic glutamine-binding protein; permease
gltI	0.302	0.00025	putative transport; Not classified;glutamate and aspartate transporter subunit;periplasmic-binding component of ABC superfamily
gltJ	0.576	0.000489	transport; Transport of small molecules: Amino acids, amines;glutamate and aspartate transporter subunit;glutamate/aspartate transport system permease; membrane component of ABC superfamily
gltK	0.445	1.87E-05	transport; Transport of small molecules: Amino acids, amines;glutamate and aspartate transporter subunit;glutamate/aspartate transport system permease; membrane component of ABC superfamily
gudD	0.371	2.35E-19	carbon utilization; carbon compounds;(D)-glucarate dehydratase 1;(D)-glucarate dehydratase 1 (L)-idarate dehydratase (L)-idarate epimerase (D)-glucarate epimerase
gudP	0.298	5.51E-15	carbon utilization; Electrochemical potential driven transporters; Porters (Uni-, Sym- and Antiporters); The Major Facilitator Superfamily (MFS)
gudX	0.397	2.826E-12	putative enzyme; putative (D)-glucarate dehydratase 2
iclR	0.264	9.52E-13	regulator; Central intermediary metabolism: Glyoxylate bypass;DNA-binding transcriptional repressor;repressor of aceBA operon
katG	0.419	9.08E-07	enzyme; Detoxification;catalase/hydroperoxidase HPI(I);catalase; hydroperoxidase HPI(I)
melA	0.384	0.000641	enzyme; Degradation of small molecules: Carbon compounds;alpha-galactosidase, NAD(P)-binding;alpha-galactosidase
melB	0.347	4.74E-06	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;melibiose:sodium symporter;melibiose permease II
mglB	0.163	0.00023	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;methyl-galactoside transporter subunit;galactose-binding transport protein; receptor for galactose taxis; periplasmic-binding component of ABC superfamily
paaA	0.372	2.97E-07	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation

paaB	0.351	2.27E-08	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaC	0.310	7.25E-09	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaD	0.353	4.22E-08	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaE	0.398	8.024E-09	putative enzyme; Not classified;predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation
paaF	0.435	7.17E-06	putative enzyme; Not classified;enoyl-CoA hydratase-isomerase
paaG	0.356	1.52E-10	putative enzyme; Not classified;acyl-CoA hydratase
paaH	0.418	7.009E-07	putative enzyme; Not classified;3-hydroxybutyryl-CoA dehydrogenase
paaI	0.371	8.27E-08	predicted thioesterase
paaJ	0.494	0.000239	putative enzyme; Not classified;predicted beta-ketoadipyl CoA thiolase
phoH	0.298	0.000132	regulator; Central intermediary metabolism: Phosphorus compounds;conserved protein with nucleoside triphosphate hydrolase domain;PhoB-dependent, ATP-binding pho regulon component; may be helicase; induced by P starvation
putA	0.266	4.59E-09	enzyme; Degradation of small molecules: Amino acids;fused DNA-binding transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase;proline dehydrogenase, P5C dehydrogenase
putP	0.277	7.416E-08	transport; Transport of small molecules: Cations;proline:sodium symporter;major sodium/proline symporter
rsd	0.387	0.000192	putative regulator; Not classified;stationary phase protein, binds sigma 70 RNA polymerase subunit;
xylF	0.310	8.748E-07	transport; Transport of small molecules: Carbohydrates, organic acids, alcohols;D-xylose transporter subunit;xylose binding protein transport system; periplasmic-binding component of ABC superfamily
yagG	0.394	1.396E-10	putative transport; Not classified;CP4-6 prophage; predicted sugar transporter;putative permease
yagH	0.495	7.726E-08	putative enzyme; Not classified;CP4-6 prophage; predicted xylosidase/arabinosidase;putative beta-xylosidase
ybdD	0.246	6.187E-07	conserved protein
ycgF	0.441	0.00042	predicted FAD-binding phosphodiesterase
ydcV	0.408	0.00059	putative transport; Not classified;predicted spermidine/putrescine transporter subunit;membrane component of ABC superfamily
ydcW	0.428	0.00050	putative enzyme; Not classified;medium chain aldehyde dehydrogenase;putative aldehyde dehydrogenase
ygaF	0.234	0.000297	predicted enzyme
yqiJ	0.509	1.707E-05	putative enzyme; Not classified;predicted inner membrane protein;putative oxidoreductase
ytfQ	0.238	7.485E-06	putative regulator; Not classified;predicted sugar transporter subunit: periplasmic-binding component of ABC superfamily;putative LACI-type transcriptional regulator
ytfR	0.4915	4.258E-07	putative transport; Not classified|4.3.A.1.a transport; Primary Active Transporters; Pyrophosphate Bond (ATP, GTP, P2) Hydrolysis-driven Active Transporters; The ATP-binding Cassette (ABC) Superfamily + ABC-type Uptake Permeases; ABC superfamily ATP

Table 7. Pathways up-regulated in a *nusA11* background

Pathway	genes in pathway
nitrate metabolism	*narK, nirD, narG, narH, nirB, nirC, narJ, narI, narX, narL*
nitrate assimilation	*narK, nirD, narG, narH, nirB, nirC, narJ, narI, narX, narL*
iron ion binding	*yjjW, hcp, narG, ydhY, ysaA, narH, nirB, narI, hcr, fdnG, nrfB, nrfC, napG, nrfA, napB, fdnI, napH, dmsA, yccM, adhE, napC, nrdG, nrfF*
generation of precursor metabolites and energy	*yjjW, hcp, narG, ydhY, ysaA, narH, nirB, ahpF, narJ, narI, hcr, fdnG, nrfB, pfkA, napG, tpiA, nrfC, napB, nrfA, fdnI, dmsC, napH, yccM, dmsA, glk, treC, eno, napC*
cation binding	*malS, hcp, zraS, yegX, nrdD, nikA, malQ, malZ, nrfB, napG, napH, yccM, dmsA, hypA, adhE, nrdG, yjjW, narG, narH, ysaA, ydhY, nirB, hcr, narI, fdnG, nrfC, nrfA, napB, fdnI, speB, treC, napC, nrfF*
oxidoreductase activity, acting on other nitrogenous compounds as donors	*Hcp, narJ, nirD, narI, nrfA, narG, narH, nirB*
ion binding	*malS, hcp, hypD, zraS, yegX, nrdD, nikA, malQ, malZ, nrfB, pfkA, napG, napH, yccM, dmsA, hypA, adhE, eno, nrdG, yjjW, narG, ydhY, narH, ysaA, nirB, hcr, narI, fdnG, nrfC, nrfA, napB, fdnI, speB, treC, napC, nrfF*
electron transport	*yjjW, hcp, narG, ydhY, ysaA, narH, nirB, ahpF, narJ, narI, hcr, fdnG, nrfB, nrfC, napG, nrfA, napB, fdnI, dmsC, napH, dmsA, yccM, napC*
oxidoreductase activity	*yjjW, hcp, narG, ydhY, ysaA, narH, nrdD, nirB, ahpF, narJ, narI, hcr, fdnG, cysG, zwf, nrfC, napG, nirD, nrfA, fdnI, dmsC, napH, dmsA, yccM, mtlD, ygfF, adhE, nrdG*
iron-sulfur cluster binding	*yjjW, hcp, napG, nrfC, narG, ydhY, ysaA, narH, napH, yccM, nirB, dmsA, hcr, fdnG, nrdG*
metal cluster binding	*yjjW, hcp, napG, nrfC, narG, ydhY, ysaA, narH, napH, yccM, nirB, dmsA, hcr, fdnG, nrdG*
transition metal ion binding	*yjjW, hcp, narG, ydhY, ysaA, narH, zraS, nrdD, nirB, nikA, narI, hcr, fdnG, nrfB, napG, nrfC, napB, nrfA, fdnI, napH, yccM, dmsA, speB, adhE, hypA, napC, nrdG, nrfF*
metal ion binding	*yjjW, malS, hcp, narG, hypD, ydhY, ysaA, narH, zraS, nrdD, nirB, nikA, narI, hcr, fdnG, nrfB, pfkA, napG, nrfC, napB, nrfA, fdnI, napH, yccM, dmsA, speB, adhE, hypA, eno, napC, nrdG, nrfF*
4 iron, 4 sulfur cluster binding	*yjjW, napG, nrfC, narG, ydhY, ysaA, narH, napH, yccM, dmsA, fdnG, nrdG*
nitrate reductase complex	*narJ, narI, narG, narH*
nitrate reductase activity	*narJ, narI, narG, narH*

Table 8. Pathways down-regulated in a *nusA11* background

pathway	genes in pathway
acetyl-CoA metabolism	*aceB, aceK, acs, aceA, acnB, fumC, sdhB, gltA, sdhD, sdhA, sdhC*
tricarboxylic acid cycle	*aceB, aceK, aceA, acnB, fumC, sdhB, gltA, sdhD, sdhA, sdhC*
coenzyme catabolism	*aceB, aceK, aceA, acnB, fumC, sdhB, gltA, sdhD, sdhA, sdhC*
acetyl-CoA catabolism	*aceB, aceK, aceA, acnB, fumC, sdhB, gltA, sdhD, sdhA, sdhC*
Cofactor catabolism	*aceB, aceK, aceA, acnB, fumC, sdhB, gltA, sdhD, sdhA, sdhC*
aerobic respiration	*aceB, aceK, aceA, acnB, fumC, sdhB, gltA, sdhD, sdhA, sdhC*
catabolism	*bax, aceK, yagH, astB, aceA, sdhB, fadB, sdhD, gltA, xylA, aceB, astA, fadA, paaJ, acnB, astC, fumC, astD, putA, sdhA, sdhC, allA, katG*
cellular catabolism	*bax, aceK, yagH, astB, aceA, sdhB, fadB, sdhD, gltA, xylA, aceB, astA, paaJ, acnB, astC, fumC, astD, putA, sdhA, sdhC, allA, katG*
cellular respiration	*aceB, aceK, aceA, acnB, fumC, sdhB, gltA, sdhD, sdhA, sdhC*
main pathways of carbohydrate metabolism	*aceB, aceK, maeB, aceA, acnB, melA, fumC, sdhB, sdhD, gltA, iclR, xylA, sdhA, sdhC*
energy derivation by oxidation of organic compounds	*aceB, aceK, maeB, aceA, acnB, melA, fumC, sdhB, sdhD, gltA, iclR, xylA, sdhA, sdhC*
generation of precursor metabolites and energy	*aceK, maeB, fadH, aceA, ygaF, sdhB, betA, sdhD, gltA, xylA, glcF, aceB, cyoA, cyoD, trxC, glcD, acnB, melA, fumC, paaE, ygfK, iclR, sthA, cyoC, sdhA, puuB, katG, sdhC*
glutamine family amino acid catabolism	*astD, astA, putA, astB, astC*
arginine catabolism to glutamate	*astD, astA, astB, astC*
glyoxylate cycle	*aceB, aceK, iclR, aceA*
glutamate metabolism	*astD, astA, putA, astB, astC*
symporter activity	*dctA, putP, xylE, actP, kgtP, yicJ, melB, yagG*
carboxylic acid metabolism	*aceK, maeB, betB, astB, acs, aceA, fadB, betA, ygeW, paaF, puuA, gabT, asnA, aceB, paaH, fadA, astA, paaJ, astC, fumC, astD, putA, iclR, paaG*
organic acid metabolism	*aceK, maeB, betB, astB, acs, aceA, fadB, betA, ygeW, paaF, puuA, gabT, asnA, aceB, paaH, fadA, astA, paaJ, astC, fumC, astD, putA, iclR, paaG*
coenzyme metabolism	*aceB, aceK, acs, aceA, acnB, fumC, sdhB, sdhD, gltA, sthA, xylA, sdhA, sdhC*
oxidoreductase activity	*msrB, aldB, maeB, betB, fadH, ygaF, gabD, sdhB, fadB, beta, glcF, ydcW, paaH, cyoA, cyoD, trxC, glcD, melA, astD,*

	paaE, putA, ygfK, sthA, cyoC, sdhA, puuB, katG, sdhC
transport	*yejE, ytfR, cycA, kgtP, sdhB, ydcV, gltJ, ytfT, ugpE, sdhD, xylF, garP, ytfQ, cyoA, cyoD, yejF, paaE, sdhA, mglB, sdhC, putP, gltK, actP, gudP, yjfF, gltI, ugpB, xylE, melB, glcF, ugpC, gabP, yicJ, trxC, xylG, yhhJ, yagG, dctA, cyoC, xylH, fimD, gltL, argT*
Arginine catabolism	*astD, astA, astB, astC*
amino acid catabolism	*astD, astA, putA, astB, astC*
amine catabolism	*astD, astA, putA, astB, astC*
nitrogen compound catabolism	*astD, astA, putA, astB, astC*
glyoxylate metabolism	*aceB, aceK, iclR, aceA*
enoyl-CoA hydratase activity	*fadB, paaF, paaG*
localization	*yejE, ytfR, cycA, kgtP, sdhB, ydcV, gltJ, ytfT, ugpE, sdhD, xylF, garP, ytfQ, cyoA, cyoD, yejF, paaE, sdhA, mglB, sdhC, putP, gltK, actP, gudP, yjfF, gltI, ugpB, xylE, melB, glcF, ugpC, gabP, yicJ, trxC, xylG, yhhJ, yagG, dctA, cyoC, xylH, fimD, gltL, argT*
establishment of localization	*yejE, ytfR, cycA, kgtP, sdhB, ydcV, gltJ, ytfT, ugpE, sdhD, xylF, garP, ytfQ, cyoA, cyoD, yejF, paaE, sdhA, mglB, sdhC, putP, gltK, actP, gudP, yjfF, gltI, ugpB, xylE, melB, glcF, ugpC, gabP, yicJ, trxC, xylG, yhhJ, yagG, dctA, cyoC, xylH, fimD, gltL, argT*

Table 9. Pathways up-regulated by nitrofurazone treatment

Pathway	**genes in pathway**
nitrate metabolism	*nirC, narJ, nirD, narH, nirB*
nitrate assimilation	*nirC, narJ, nirD, narH, nirB*
generation of precursor metabolites and energy	*yjjW, nrfC, hcp, nrfA, narH, ydhY, nirB, yccM, narJ, treC, glk, nrfB, pfkA*
oxidoreductase activity, acting on other nitrogenous compounds as donors	*hcp, narJ, nirD, nrfA, narH, nirB*
iron ion binding	*yjjW, nrfC, hcp, nrfA, narH, ydhY, nirB, yccM, adhE, nrfB, nrfF*
nitrite reductase [NAD(P)H] activity	*nirD, nirB*
phosphofructokinase activity	*fruK, pfkA*
oxidoreductase activity, acting on other nitrogenous compounds as donors, with NAD or NADP as acceptor	*nirD, nirB*

Table 10. Pathways down-regulated by nitrofurazone treatment

pathway	genes in pathway
glyoxylate metabolism	*aceB, glcB, garR, aceK, iclR*
aldehyde metabolism	*aceB, glcB, garR, aceK, iclR*
glyoxylate cycle	*aceB, glcB, aceK, iclR*
acetyl-CoA metabolism	*aceB, glcB, aceK, acs, acnB, fumC*
organic acid metabolism	*aceK, astB, acs, garK, betA, ygeW, paaF, gabT, asnA, paaH, aceB, garR, fadA, paaJ, fumC, glcB, putA, paaG, iclR*
catabolism	*aceB, garR, fadA, aceK, yagH, paaJ, astB, acnB, fumC, glcB, putA, allA, katG*
carboxylic acid metabolism	*aceK, astB, acs, betA, ygeW, paaF, gabT, asnA, paaH, aceB, garR, fadA, paaJ, fumC, glcB, putA, paaG, iclR*
tricarboxylic acid cycle	*aceB, glcB, aceK, acnB, fumC*
coenzyme catabolism	*aceB, glcB, aceK, acnB, fumC*
acetyl-CoA catabolism	*aceB, glcB, aceK, acnB, fumC*
cofactor catabolism	*aceB, glcB, aceK, acnB, fumC*
amino acid transporter activity	*gabP, gltI, gltJ, putP, gltK, cycA, glnH, gltL*
amino acid transport	*gabP, gltI, gltJ, putP, gltK, cycA, glnH, gltL*
aerobic respiration	*aceB, glcB, aceK, acnB, fumC*
cellular catabolism	*aceB, garR, aceK, yagH, paaJ, astB, acnB, fumC, glcB, putA, allA, katG*
amine transporter activity	*gabP, gltI, gltJ, putP, gltK, cycA, glnH, gltL*
amine transport	*gabP, gltI, gltJ, putP, gltK, cycA, glnH, gltL*
main pathways of carbohydrate metabolism	*aceB, garR, aceK, acnB, melA, fumC, glcB, iclR*
organic acid transporter activity	*gabP, putP, gltK, cycA, actP, gltJ, gltI, glnH, gltL*
carboxylic acid transporter activity	*gabP, putP, gltK, cycA, actP, gltJ, gltI, glnH, gltL*
physiological process	*fadH, gabD, ydcV, yggG, phoH, xylF, garP, asnA, garR, gudX, glcD, paaE, rsd, mglB, cdaR, actP, yjfF, astB, gltI, ugpB, glcF, ugpC, gabP, gudD, ulaC, yagG, yhhJ, yicI, paaG, xylH, ytfR, aceK, cycA, acs, betT, gltJ, ytfT, betA, garK, paaF, ytfQ, paaJ, cstA, glnH, allA, xylB, putP, gltK, aldB, yagH, gudP, ygaF, ygeW, melB, ydcW, gabT, paaH, aceB, fadA, recD, garL, melA, acnB, fumC, glcB, putA, iclR, gltL, katG*