

Overexpression of the LexA-regulated *tisAB* RNA in *E. coli* inhibits SOS functions; implications for regulation of the SOS response

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ABSTRACT

The DNA damage induced SOS response in *Escherichia coli* is initiated by cleavage of the LexA repressor through activation of RecA. Here we demonstrate that overexpression of the SOS-inducible *tisAB* gene inhibits several SOS functions *in vivo*. Wild-type *E. coli* overexpressing *tisAB* showed the same UV sensitivity as a *lexA* mutant carrying a noncleavable version of the LexA protein unable to induce the SOS response. Immunoblotting confirmed that *tisAB* overexpression leads to higher levels of LexA repressor and northern experiments demonstrated delayed and reduced induction of *recA* mRNA. In addition, induction of prophage λ and UV-induced filamentation was inhibited by *tisAB* overexpression. The *tisAB* gene contains antisense sequences to the SOS-inducible *dinD* gene (16 nt) and the *uxaA* gene (20 nt), the latter encoding a dehydratase essential for galacturonate catabolism. Cleavage of *uxaA* mRNA at the antisense sequence was dependent on *tisAB* RNA expression. We showed that overexpression of *tisAB* is less able to confer UV sensitivity to the *uxaA dinD* double mutant as compared to wild-type, indicating that the *dinD* and *uxaA* transcripts modulate the anti-SOS response of *tisAB*. These data shed new light on the complexity of SOS regulation in which the *uxaA* gene could link sugar metabolism to the SOS response via antisense regulation of the *tisAB* gene.

INTRODUCTION

Exposure of *Escherichia coli* to agents that damage DNA or interfere with DNA replication induces expression of a set of genes termed the SOS response (1,2). Many of

the SOS-inducible genes encode proteins that are involved in DNA repair, recombination, DNA replication and cell division. If *E. coli* contains a λ prophage, the lytic cycle of phage λ is also induced as part of the SOS response (3,4). RecA protein and the LexA repressor are the key regulators of SOS induction. Following DNA damage RecA and single-stranded DNA form a nucleoprotein filament, which catalyzes autocleavage of LexA repressor, leading to induction of more than 30 genes including *recA* and *lexA* (2). The kinetics of SOS induction in *E. coli*, measured by monitoring LexA cleavage after UV irradiation, is understood in great detail. The induction starts 1 min after UV irradiation, reaches a maximum after 4 min and stays fully induced for at least 20 min (1,5). Genes containing operators that bind LexA repressor weakly, such as the promoter of the *uvrAB* genes, are first to be turned on during SOS induction. As the level of LexA declines, genes with operators binding LexA more strongly, like *recA* and *sulA*, will be induced (1). This rapid induction of SOS functions reflects the need for increased capacity to tolerate and to repair DNA damage.

Less is known about how the SOS response is turned off. It is believed that repairing DNA damage removes or reduces the amount of RecA nucleoprotein filament leading to accumulation of uncleaved LexA repressor and repression of the SOS genes. Turning off the SOS response can also be an active process and several factors inhibiting SOS induction have been described. The plasmid encoded PsiB protein inhibits SOS induction during transfer of conjugative plasmids (6–8), and overexpression of the chromosomally encoded DinI and RecX proteins directly interferes with the formation of the RecA nucleoprotein filament inhibiting LexA cleavage (9–11).

In addition to the more than 30 genes under direct control of LexA repressor, the transcriptional level of more than 1000 genes in *E. coli* are affected by treatment with DNA damaging agents (12). The translational levels

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of several proteins are also known to be upregulated by DNA damage in a LexA-independent but RecA-dependent manner. One such example is the Eda protein, the last enzyme in the Entner–Doudoroff pathway, whose function is necessary for the recovery of respiration following the SOS response (13,14).

The SOS-inducible *tisAB* gene (synonym gene name *ysdAB*), was first described as an SOS-inducible transcript containing the two open reading frames YsdA and YsdB (2), and later by mutational analysis shown to encode the toxin TisB (15). Recently it has been shown that *in vitro* translation of TisB is mediated by a shorter endonucleolytically processed *tisAB* transcript, termed +42 *tisAB*. Translation of +42*tisAB* RNA is initiated by upstream ribosome binding to a ribosome standby site and may involve sliding of the ribosome to a downstream translational initiation region. Binding of the sRNA *istR-1* to the ribosome standby site prevents loading of ribosomes and inhibits translation of +42 *tisAB* RNA (16).

In this article we describe how overexpression of plasmid encoded *tisAB* inhibits and slows down induction of the SOS response. In addition, we find that overexpression of *tisAB* is necessary for cleavage of the *uxaA* mRNA, encoding altronate dehydratase, an enzyme specific for feeding the sugar acid galacturonate into the Entner–Doudoroff pathway (14). The presence of antisense sequences to *uxaA* and also to the SOS-inducible gene, *dinD*, suggests additional regulatory complexity in the *tisAB* function.

MATERIALS AND METHODS

Strains, plasmids and media

The experiments were carried out in strains AB1157 (*arg*, *his*, *leu*, *pro*, *thr*, *ara*, *gal*, *lac*, *mil*, *xyl*, *thi*, *tsx*, *rpsL*, *supE* and *kdgK*) (17), MG1655 (18,19), DM49 (*lexA3* derived from AB1157) (20) and ER2566 (*lacZ::T7 gene1*) (New England Biolabs, Ipswich, MA, USA). The *uxaA*, *dinD* and *tisAB* chromosomal mutants were made in strain BW25113-pKD46 (21) and introduced into AB1157 and MG1655 via T4GT7 transduction (22) using primers *uxaAP1/uxaAP2*, *dinDP1/dinDP2* and *tisABP1/tisABP2*, respectively (Table 1). In mutant BK4012 (*tisAB::kan^r*) 294 nt of the *tisAB* gene (position 3851350–3851644 in the *E. coli* genome, accession nr NC_000913) was replaced with a Kan^r cassette. BK4012 have the whole *uxaA* and the 15 first nucleotides of the *dinD* antisense regions deleted, leaving the six last amino acids of TisB intact. The SOS box was not deleted in order not to interfere with *istR* induction.

Vector pKK232-8 contains a promoterless *cat* gene allowing for selection of DNA fragments containing promoter activity (23). Plasmid pBK410 was selected from a genomic library and contains a 759-bp fragment from the intergenic region between *ilvBL-emrD* inserted at the BamHI site of pKK232-8. Expression plasmids pET28b-TisA and pET28b-TisB contains the TisA and TisB open reading frames inserted in the NcoI–BamHI restriction sites of the pET28b(+) vector (Novagen, Madison, WI,

USA) using primers TisA F, TisA R, TisB F and TisB R (primers listed in Table 1).

pBK410 mutations M1I, D5stop, K12stop, *tisABuA1* and *tisABuA2* were made using QuikChange site directed mutagenesis with primers TisB1(M1I) (F and R), TisB2(D5stop) (F and R), TisB3(K12stop) (F and R), *tisBuA1* (F and R) and *tisABuA2* (F and R), respectively.

Cells were grown in LB media or K-medium and washed in M9 buffer (24).

Generation of lysogens

Lysogenic *E. coli* was made by infecting the strain with bacteriophage λ and picking and purifying bacteria from the center of a plaque. Lysogeny was confirmed by demonstrating plaque formation on an indicator strain, or demonstrating immunity against reinfection with bacteriophage λ .

Mutagenesis

Exponentially growing bacteria were washed twice with M9 buffer and irradiated with UV light. From the irradiated cells 5 ml were mixed with 5 ml 2 \times K-medium and grown overnight. Appropriate dilutions of the culture were plated on ampicillin containing LB agar plates for determining the total number of bacteria and on rifampicin containing LB agar plates (100 μ g/ml) for determination of the number of rif^R mutants.

Northen blot

RNA was isolated as previously described (25). RNA samples (5 μ g) were denatured at 80°C in formaldehyde loading dye (55% formamide, 0.015% SDS, 0.015% Bromophenol Blue, 0.015% Xylene cyanol, 0.3 mM EDTA, 26.8% formaldehyde and 1.5 \times MOPS) buffer, separated on a 1% agarose, MOPS, formaldehyde gel and transferred to nylon membrane (Hybond N+, Amersham, Little Chalfont, Buckinghamshire, UK) (26).

Radiolabeled RNA probes were synthesized with MAXIscript (Ambion, Austin, TX, USA) (primers for synthesis of T7 template listed in Table 1) and hybridized using ExpressHyb Hybridization Solution (BD biosciences, San Jose, CA, USA). Hybridization signals were visualized using a Typhoon 9410 phosphor imager (Amersham).

Immunoblotting

Overnight cultures of *E. coli* were diluted 1:50 in K-medium and grown for the indicated time periods. Cells were harvested and lysed by plasmolysis (24) and protein extracts were separated on a SDS–polyacrylamide gel (27) and transferred to a PVDF (polyvinylidene difluoride) membrane by electroblotting. The membranes were incubated for 1 h at room temperature with a 1:1000 dilution of rabbit anti-LexA serum. The bound antibodies were visualized by incubating with a 1:5000 dilution of anti-rabbit IgG(Fc)-AP conjugate (Promega, Madison, WI, USA) and staining with BCIP/NBT (Promega). The LexA blot was scanned with a hp scanjet 4500c and the intensities of the protein bands analysed

Table 1. Oligonucleotides used in this study

Name	Sequence
Construct generation	
TisA F	5'-CATCCATGGATGTCAACAAGCACAAACG-3'
TisA R	5'-CGCGGATCCTCATAACGCGTCTCCTGTGG-3'
TisB F	5'-CATCCATGGATGAACCTGGTGGATATCG-3'
TisB R	5'-CGCGGATCCTTACTTCAGGTATTCAGAACAGC-3'
Mutant generation (sequence specific for Kan ^r marker in italic and mutated bases in bold)	
TisB1(M11) F	5'-ACGGAGACGCGTATAAACTGGTGGATATCGCCAT-3'
TisB1(M11) R	5'-GCGATATCCACCAGGTTTATACGCGTCTCCTGTGG-3'
TisB2(D5stop) F	5'-CGTATGAACCTGGTGTAAATCGCCATTCTTATCC-3'
TisB2(D5stop) R	5'-TAAGAAATGGCGATTACACCAGGTTACATACGCGTC-3'
TisB3(K12stop) F	5'-ATATCGCCATTCTTATCCTCTAACTCATTGTTGC-3'
TisB3(K12stop) R	5'-AGTGCTGCAACAATGAGTTAGAGGATAAGAATGG-3'
uxaAP1	5'-TTAACAATGCGCGTACTACTTCGCCATTGAACTGAACTAAGGTCTGGGGTTGATGTAGGCTGGAGCTGCT-3'
uxaAP2	5'-TGCGCCAAACATCTCCGCACTTCGGTACAGTACGGTAGTACCGCCGTTAGCAATCTGAATATCCTCCTTAGTTC-3'
dinDP1	5'-CACAACGTATATAAATACAGTTACAGATTTACTTTCTTTGCAATTGATATCACTGTAGGCTGGAGCTGCT-3'
dinDP2	5'-CGTCCAGTCGTAGTTCGTCAACCTTTGTACATTTTTAATGTCTCTTCTTATCTGAATATCCTCCTTAGTTC-3'
tisABP1	5'-TTGTCCAAATATTACTGTTATTTATACAGTAACTTCTAGTGTAGGCTGGAGCTGCT-3'
tisABP2	5'-GGTGCAGCTTGAATCTGAATTACTTCAGGTATTCAGAACTGAATATCCTCCTTAGTTC-3'
tisABuA1 F	5'-CGGGGGCCGCATTGTTATCTGGCGCATTAAAGTCTGG-3'
tisABuA1 R	5'-CCAGGACTTAATGCGCCAGATAACAATGCGGCCCCCG-3'
tisABuA2 F	5'-CGGGGGCCGCATAGTTAACAGGCGCAATAAGTCTGG-3'
tisABuA2 R	5'-CCAGGACTTATTGCGCCTGTTAACTATGCGGCCCCCG-3'
Ribop robes (T7 promoter in italic)	
tisAB F	5'- <i>TAATACGACTCACTATAGGTGCGACTTGAATCTGA</i> -3'
tisAB R	5'-TGTACGCAATGTGTTATG-3'
recA F	5'- <i>TAATACGACTCACTATAGGTCTGCTACGCCTTCGC</i> -3'
recA R	5'-GCGTTGGCGGCAGCACTGG-3'
uxaA1 F	5'-GGTCAACGACTTCAAACAGTA-3'
uxaA1 R	5'- <i>TAATACGACTCACTATAGGTGTCGATAAAATCTTCCA</i> -3'

Bold bases in TisB1, TisB2, TisB3, tisABuA1 and tisABuA2 nucleotides are mutated. Italic sequences in uxaAP, dinDP and tisABP primers are specific for the kanamycin cassette. T7 promoter sequence in the riboprobes is shown in italic.

with the program ImageJ (Rasband, W.S. and ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997–2007).

Prophage λ reactivation

Strains lysogenic to prophage λ were grown to OD₆₀₀ of 0.4–0.5 in LB media at 37°C. The cells were pelleted by centrifugation, washed three times in 1 × M9 buffer to remove free bacteriophage, exposed to different UV doses (0–45 J/m²) and spread on LB agar plates with a non-lysogenic indicator strain to measure induction of prophage λ .

RESULTS

Isolation of plasmid pBK410

In a search for DNA damage inducible promoters in *E. coli*, digested chromosomal DNA from AB1157 was ligated in front of the promoter less chloramphenicol gene of promoter probe vector pKK232-8, allowing a positive selection of DNA fragments containing promoters because of the chloramphenicol^R phenotype. The library was transformed into wild-type *E. coli* strain AB1157 and plated on media containing chloramphenicol, or chloramphenicol and 3 mM of the alkylating agent methyl methanesulfonate (MMS). MMS is an inducer of

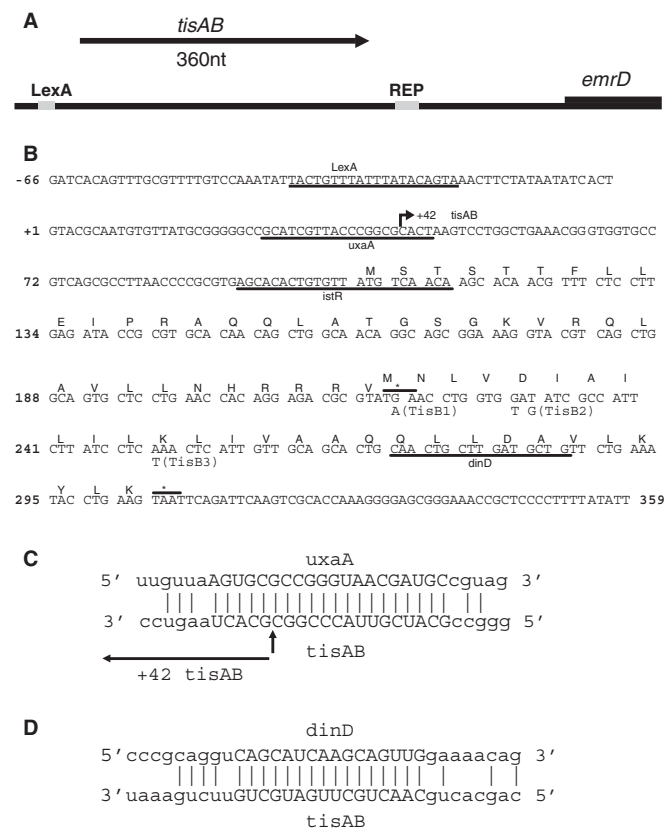


Figure 1. Overview and details of the 759-bp pBK410 insert. (A) Direction of transcription of *tisAB* is shown by arrow and the *emrD* start is indicated. LexA-binding site and the single repetitive extragenic palindromic (REP) sequence is shaded. (B) Nucleotide sequence of the first 425 bp of the pBK410 insert up to transcriptional stop of *tisAB*. TisA and TisB open reading frames are labelled. The underlined *istR* sequence is the complement to the *istR-1* and *istR-2* sequence, which is described elsewhere (15). Sequence homology to *uxaA* and *dinD* is underlined. Numbering is relative to transcriptional startpoint of the 359-nt *tisAB* RNA. Arrow directed towards right +42 *tisAB* indicates the cleavage site of the endonucleolytically processed *tisAB* transcript. TisA and TisB translational stops are indicated with asterisk and line. Base changes leading to mutations TisB1, TisB2 and TisB3 are indicated below the nucleotide sequence. (C) Potential base pairing between *uxaA* and *tisAB* RNA is indicated, 20-nt core homology is capitalized. Information on *tisAB* RNA cleavage is from elsewhere (15). (D) Potential basepairing between *dinD* and *tisAB* RNA is indicated, 16-nt core homology is capitalized.

the adaptive response to alkylating agents in *E. coli* and also a potent inducer of the SOS response. Unexpectedly, one plasmid clone, pBK410, was selected on the basis of conferring MMS sensitivity to AB1157. Sequencing of pBK410 revealed a 759-bp insert from the intergenic region between *ilvBL-emrD*. This intergenic region has been shown to contain two overlapping sRNAs termed *istR-1* and *istR-2*, and the divergently transcribed SOS-inducible gene termed *ysdAB/tisAB* (2,15). The pBK410 clone contains the *ysdAB/tisAB* gene, a single repetitive extragenic palindromic (REP) sequence and the first 37 amino acids of the *emrD* gene, while the *istR* genes are not contained in this clone (Figure 1A). A PARALIGN (28) search of the pBK410 insert revealed three sequences

repeated in other regions of the *E. coli* genome, the 20-nt sequence GCATCGTTACCCGGCGCACT is antisense to a sequence at the 3'-end of the *uxaA* gene (Figure 1C), the 16-nt sequence CAACTGCTTGA TGCTG is antisense to a sequence at the 3'-end of the SOS-inducible *dinD* gene (Figure 1D), and the 22-nt sequence AGCACACTGTGTTATGTCAACA is antisense to the *istR* sequences. The role of the 22-nt *istR* repeat in antisense regulation of *tisAB* has been previously described (15,16).

TisAB is overexpressed from plasmid pBK410

The insert sequence of pBK410 starts 66 nt upstream of the *tisAB* transcription start (Figure 1B). The functionality of the SOS operator in pBK410 was confirmed by northern analysis using a *tisAB* specific probe (Figure 2A). Total RNA for northern analysis was isolated from unirradiated and UV irradiated BK4012 (*tisAB::kan^r*) and wild-type AB1157 carrying plasmid pBK410 or the cloning vector pKK232-8. A band corresponding to the 359-nt full-length *tisAB* transcript is clearly UV inducible in AB1157 carrying the pKK232-8 or pBK410 (Figure 2A, lanes 6 and 9). The band is totally absent in the *tisAB* deletion mutant BK4012 (*tisAB::kan^r*).

The northern experiments also illustrate that unirradiated AB1157 expresses significant amounts of *tisAB* transcript from pBK410 compared to the apparent absence of *tisAB* signal in AB1157 carrying only the cloning vector, pKK232-8. The level of *tisAB* transcript expressed from pBK410 in unirradiated AB1157 (Figure 2A, lane 7), is comparable to the fully induced level of *tisAB* in AB1157 carrying the cloning vector, pKK232-8 (Figure 2A, lane 6). Primer extension and 3'-RACE confirmed the transcriptional start and stop of the *tisAB* transcript reported earlier (data not shown) (15).

UV sensitization of wild-type *E. coli* by overexpression of *tisAB*

Several of the SOS-inducible genes in *E. coli* have a direct role in DNA repair, and mutants of SOS genes are often more sensitive to UV irradiation. To investigate a potential role of the *tisAB* gene in repair of UV induced DNA lesions, we tested the BK4012 (*tisAB::kan^r*) mutant for UV resistance. We found no detectable difference in UV survival of BK4012 relative to AB1157, suggesting that the *tisAB* gene does not have a direct role in repair of UV mediated DNA damage (data not shown).

Survival experiments showed that the UV sensitivity of AB1157 carrying pBK410 was similar to the UV sensitivity of the *lexA3* strain DM49 (Figure 2B), which is unable to induce the SOS response due to defective LexA cleavage (20). In addition to the *tisAB* gene, plasmid pBK410 also contains a repetitive extragenic palindromic (REP) sequence and the first 37 aa of the *emrD* coding sequence (Figure 1A). To investigate if the presence of the repetitive extragenic palindromic sequence and the *emrD* sequence in pBK410 affects the UV sensitisation of AB1157, plasmid pBK411 was generated. In plasmid pBK411 the

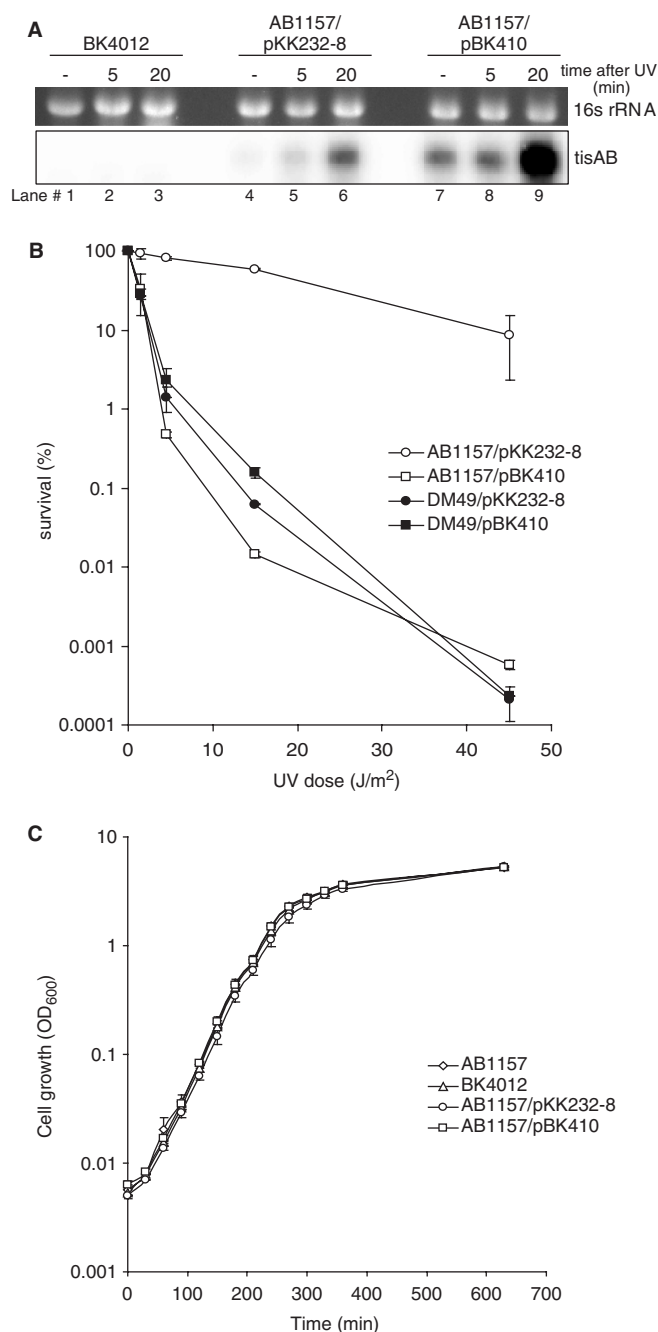


Figure 2. *tisAB* expression. (A) Expression of *tisAB* RNA in BK4012 (*tisAB::Kan^r*), AB1157 transformed with pKK232-8 and AB1157 transformed with pBK410 (*tisAB* overexpression). Total RNA was extracted from the strains and 5 μ g RNA obtained from unexposed cells (–) and cells exposed to UV (50 J/m²) at two different time points (5 and 20 min after UV-exposure) was separated on formaldehyde-1% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a *tisAB* riboprobe. (B) UV survival was measured by irradiating exponentially growing AB1157 and DM49 (*lexA3*) transformed with the cloning vector pKK232-8 or plasmid pBK410 (overexpressing *tisAB*) to different doses of UV in M9 buffer and plating the cells on LB agar plates. Error bars showing the standard deviations are included. (C) 1:1000 dilutions of overnight cultures were grown in LB medium for 630 min and OD₆₀₀ was measured every 30 min.

repetitive extragenic palindromic sequence and *emrD* sequences downstream of the *tisAB* terminator were removed and the ability of pBK411 to confer sensitivity to AB1157 was measured. Plasmids pBK410 and pBK411 were found to confer the same UV sensitivity to AB1157 (data not shown). Plasmid pBK410 expresses significant levels of *tisAB* from uninduced cells at a level comparable to fully induced AB1157 carrying only the cloning vector pKK232-8. To examine the toxicity of *tisAB* expression from plasmid pBK410 we compared the growth of AB1157 carrying pBK410 or pKK232-8 and the *tisAB* deletion mutant BK4012. The growth curve shown in Figure 2C clearly demonstrates that uninduced expression of *tisAB* from pBK410 is not toxic. The data suggest that the reduction in UV resistance of AB1157 result from overexpression of the plasmid encoded *tisAB* gene product, and that a chromosomal deletion of *tisAB* does not affect any cellular functions contributing to UV survival.

Overexpression of *tisAB* leads to higher levels of LexA

Activation of the SOS regulon depends on cleavage of the LexA repressor, in which the level of LexA before induction and the kinetics of LexA cleavage after induction is well characterized (1,29). The DM49 (*lexA3*) like UV sensitivity of wild-type AB1157 carrying pBK410 could be explained by a delay in SOS induction caused by higher levels of *tisAB*. We therefore tested the effect of *tisAB* overexpression on LexA repressor cleavage in UV irradiated cells. The level of LexA in protein extracts from strain AB1157 carrying either pBK410 or the cloning vector pKK232-8 were followed for 60 min after UV irradiation by immunoblotting with LexA antibody (Figure 3A). The level of uncleaved LexA repressor in unirradiated cells (Figure 3A, lane 2) is higher in AB1157 in the presence of pBK410 compared to AB1157 carrying only pKK232-8. A scan of the LexA immunoblot suggests that the level of LexA repressor in AB1157 is approximately twice the amount in cells carrying pBK410 compared to normal cells (Figure 3B). Figure 3A and B suggest that in cells carrying pBK410 the amount of LexA repressor 5 min after irradiation is comparable to the basal level of LexA in unirradiated cells carrying only pKK232-8, indicating a 5-min delay of SOS induction due to *tisAB* overexpression. Both the kinetics of LexA degradation after UV irradiation and the accumulation of LexA 10–20 min after UV irradiation appears to be parallel in cells carrying pBK410 compared to normal cells (Figure 3A and B), and indicates that only the endpoint levels of LexA is higher. Our immunoblotting experiments could not detect any differences in the amount of crossreacting (CR) proteins appearing on the western blot, suggesting that the effect from pBK410 is specific to LexA (Figure 3A). The results demonstrate that the constitutive overexpression of *tisAB* from pBK410 elevates the level of LexA in uninduced AB1157 resulting in higher LexA levels throughout the induction period and a shorter phase of full induction.

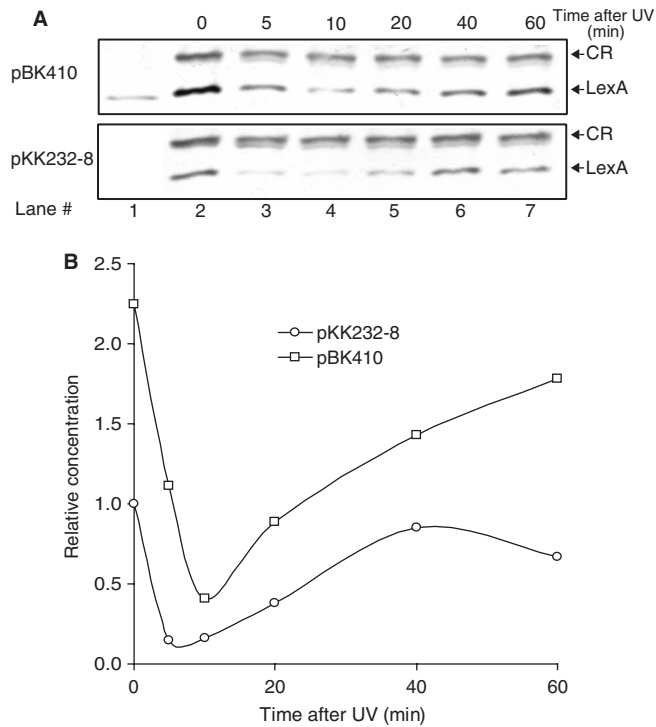


Figure 3. Immunoblotting and quantitation of LexA repressor in AB1157 transformed with pKK232-8 (cloning vector) or pBK410 (*tisAB* overexpression). (A) Reaction mixtures containing 25 μ g plasmolyzed protein extracts from unexposed and cells exposed to UV for indicated times, separated by SDS-polyacrylamide gel electrophoresis, electroblotted to PVDF membrane and visualized by BCIP/NBT via rabbit anti-LexA serum. Lane 1 contains 3 ng of purified LexA protein. CR indicates a cross-reacting protein of unknown origin, shown here for normalization. (B) The bands in (A) were scanned and quantified as indicated in Materials and methods section. Intensities of LexA were normalized against the intensity of the cross-reacting protein (CR). All values are relative to LexA in uninduced AB1157/pKK232-8.

Delayed induction of *recA* mRNA by overexpression of *tisAB*

To determine if inhibition of LexA cleavage by overexpression of *tisAB* also influences the induction of other SOS-inducible genes, we measured the induction of *recA* mRNA in AB1157 in the presence of plasmid pBK410. The *recA* mRNA level indicates the kinetics and amount of induction of the RecA protein, which is a key recombinational and SOS regulatory protein. Exponentially growing cultures of BK4012 (*tisAB::kan^r*) and AB1157 carrying either pBK410 or the cloning vector pKK232-8, were irradiated with 50 J/m² and the amount of *recA* mRNA was determined prior to, and 5 and 20 min after irradiation by northern hybridization against a strand specific *recA* probe (Figure 4). The level of *tisAB* RNA was also determined by rehybridizing the northern blot with a probe against *tisAB*. As shown in Figure 4, *recA* mRNA is rapidly induced both in AB1157 and in BK4012 (*tisAB::kan^r*), reaching a relatively high level after 5 min and is further increased after 20 min. In contrast, induction of *recA* mRNA after 5 min is barely detectable in AB1157 when pBK410 is present (Figure 4, lane 8). The uninduced level of *recA* mRNA also appears to be lower in the presence of pBK410 (Figure 4, lane 7). The result

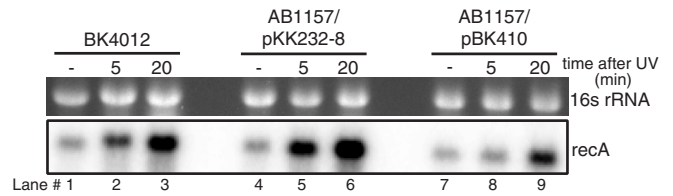


Figure 4. Expression of *recA* mRNA in BK4012 (*tisAB::Kan^r*), AB1157 and AB1157 transformed with pBK410 (*tisAB* overexpression). Total RNA was extracted from the strains and 5 μ g RNA obtained from unexposed cells (-) and cells exposed to UV (50 J/m²) at two different time points (5 and 20 min after UV-exposure) was separated on formaldehyde-1% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a *recA* riboprobe.

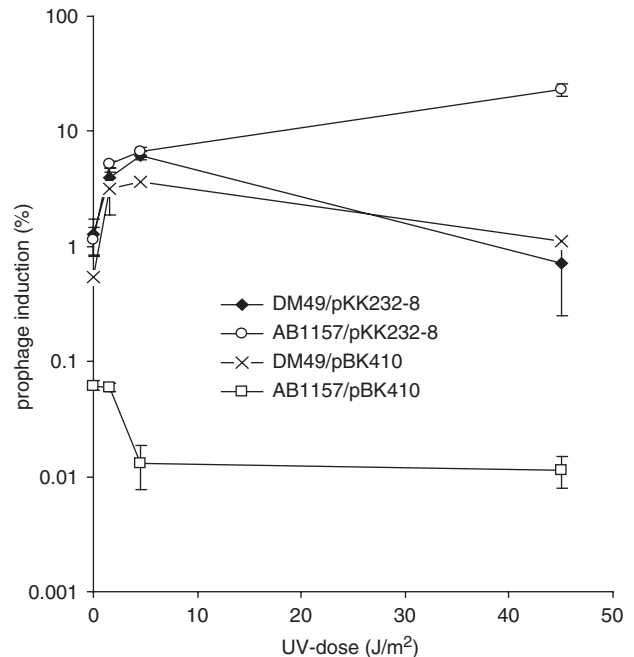


Figure 5. Inhibition of prophage λ induction. Lysogenic AB1157 (λ) and DM49 (*lexA3*) (λ) were grown in K-medium to mid-exponential phase, washed, resuspended in M9 buffer and exposed to UV as indicated. The datapoints shown is an average of three experiments. Error bars showing the standard deviations are included.

implies that overexpression of *tisAB* from plasmid pBK410 delays *recA* mRNA induction.

Overexpression of *tisAB* inhibits UV induction of prophage λ

Induction of prophage λ in lysogenic *E. coli* cells requires RecA coprotease activity for cleavage of the λ CI repressor in a manner similar to LexA cleavage (30,31). To test if overexpression of *tisAB* from pBK410 inhibits induction of prophage λ we made λ -lysogens of AB1157 and DM49 (*lexA3*) and transformed the strains with either pBK410 or the cloning vector pKK232-8. The lysogenic strains were irradiated with UV and appropriate dilutions plated on AB1157 indicator strain to measure induction of prophage λ (Figure 5). Wild-type AB1157 in the presence of pBK410 shows a 20-time lower proportion of lysogenic cells spontaneously inducing prophage λ compared to AB1157

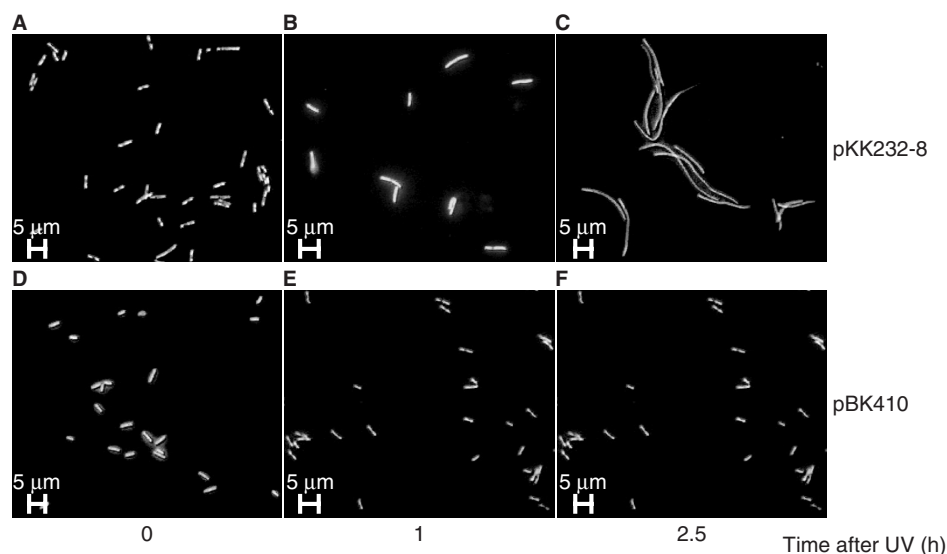


Figure 6. Fluorescence microscopy of UV exposed (50 J/m^2) cells carrying the cloning vector pKK232-8 or plasmid pBK410 (*tisAB* overexpression) followed by growth for 1 and 2.5 h. Cells were grown in K-medium and stained with acridine orange. (A) AB1157/pKK232-8, unexposed; (B) AB1157/pKK232-8, 1 h after UV; (C) AB1157/pKK232-8, 2.5 h after UV; (D) AB1157/pBK410, unexposed; (E) AB1157/pBK410, 1 h after UV; (F) AB1157/pBK410, 2.5 h after UV.

carrying only the cloning vector (0.06% and 1.2%, respectively) (Figure 5). In the presence of pBK410 and after UV irradiation the proportion of lysogenic AB1157 inducing λ decreases approximately six times. Induction of prophage λ in DM49 (*lexA3*) appears to be unaffected by the presence of pBK410. Lysogenic DM49 gave rise to small plaques during prophage λ induction while AB1157 produced bigger plaques. The size of the plaques formed by AB1157 in the presence of pBK410 was similar to those formed by AB1157 carrying the cloning vector, implying that the relatively low number of cells inducing λ in AB1157 carrying pBK410 exhibited wild-type kinetics of λ -induction. It thus appears that *tisAB* inhibits UV induction of prophage λ supporting a role for *tisAB* in regulation of the SOS response.

Overexpression of *tisAB* inhibits cell filamentation

After SOS induction *E. coli* form long filaments as a consequence of activation of the septation inhibitor Sula (32). To test if overexpression of *tisAB* from plasmid pBK410 affected filamentation, exponentially growing cultures of AB1157 carrying pBK410 or the cloning vector pKK232-8 were UV irradiated with 50 J/m^2 and the cells were examined at intervals with fluorescent microscopy to determine the degree of filamentation (Figure 6). In the presence of the cloning vector, strain AB1157 displayed short filaments 1 h after UV exposure and produced long filaments after 2.5 h (Figure 6B and C). AB1157 carrying pBK410 was not generating filaments at all after UV exposure (Figure 6E and F), indicating a role of *tisAB* in modulation of filamentation via regulation of the SOS response.

Moderate decrease in UV mutagenesis by the presence of pBK410

An important SOS response is the increase in mutation frequency as a result of the action of error-prone

Table 2. Mutation frequencies in cells carrying the cloning vector pKK232-8 and plasmid pBK410 (*tisAB* overexpression)

Strain	Plasmid	UV (J/m^2)	Survival (%)	Mutation frequency (10^{-7})
AB1157	pBK410	0	100	0.28
		5	7	3.9
		15	0.005	9.1
	pKK232-8	0	100	0.56
		5	80	7.1
		15	36	23.0
DM49 (<i>lexA3</i>)	pBK410	0	100	0.33
		5	12	2.6
		15	4.6	6.3
	pKK232-8	0	100	0.33
		5	13	1.5
		15	2.4	3.5

translesion polymerases, PolIV (dinB/P) and PolV (umuDC), which are both induced as part of the SOS response (1). To examine the influence of plasmid pBK410 on SOS mutagenesis in wild type *E. coli*, we UV irradiated AB1157 carrying the pBK410 and compared the frequency of *rif^R* forming colonies with AB1157 carrying the cloning vector pKK232-8. The results summarized in Table 2 indicate that among the surviving fraction of AB1157 carrying pBK410 there appears to be a moderate reduction in UV-induced mutation frequency compared to AB1157 carrying only the vector.

The role of the YsdA/TisA and YsdB/TisB open reading frames

The extreme UV sensitivity plasmid pBK410 inflicts on wild-type *E. coli* provides a sensitive assay to investigate

the functional role of different parts of the *tisAB* gene. An obvious candidate for the anti-SOS phenotype we observe from plasmid pBK410 is the TisB protein which is translated from +42 *tisAB* RNA *in vitro* (16). To study the effect of the two open reading frames, TisA and TisB, in sensitizing *E. coli* to UV, we cloned TisA and TisB into the T7 polymerase regulated expression vector pET28b(+). The two clones were transformed into the T7 polymerase expressing strain ER2566 and sensitivity to increasing doses of UV was measured. ER2566 transformed with pBK410 was included as a control, and the results in Figure 7A demonstrate that strain ER2566 carrying pBK410 display UV sensitivity comparable to AB1157 carrying pBK410 (Figure 2B). Moreover, ER2566 carrying pET28b-TisA or pET28b-TisB is not significantly more sensitive to UV exposure than the vector control (Figure 7A). We also observed that after UV irradiation ER2566 carrying the pET28b-TisA and pET28b-TisB constructs formed colonies with similar size to ER2566 carrying only the cloning vector pET28b(+). The lack of UV sensitization from the TisB expression construct can be explained by the special way TisB is translated (16), and to further investigate the role of TisB we constructed mutations TisB1, TisB2 and TisB3 (Figure 1B) in the pBK410 plasmid and assayed for UV sensitivity. Figure 7B shows that mutations TisB2 (D5stop, GAT to TAG) and TisB3 (K12stop, AAA to TAA) eliminates the sensitizing phenotype from pBK410 thereby confirming the role of TisB in the phenotype (15). However, the pBK410 mutation TisB1 (M11, ATG to ATA) which was made in order to mutate the TisB start-codon without destroying the overlapping TisA stop-codon still confers substantial UV sensitivity to AB1157 (Figure 7B). A northern experiment confirmed that these mutant plasmids produced similar amounts of *tisAB* transcript (data not shown). The results show that the role of TisB translation *in vivo* necessitates a closer examination.

Overexpression of *tisAB* mediates SOS-induced cleavage of the *uxaA* mRNA

The presence of both *uxaA* and *dinD* antisense sequence in the *tisAB* transcript suggest antisense regulation between the *tisAB-uxaA* and *tisAB-dinD* transcripts. The size of the core *tisAB-uxaA* antisense region is 20 nt (Figure 1C). Notably, *tisAB* RNA isolated from stationary phase is cleaved in the *tisAB-uxaA* antisense region (15). The *uxaA* gene is the last gene in an operon with *uxaC*, forming an *uxaCA* mRNA of 2900 nt, where the *tisAB-uxaA* antisense region is 300 nt upstream of a terminator sequence in the 3'-end of *uxaA* (Figure 8A). To determine whether the *uxaA* mRNA is cleaved in the antisense region, total RNA was isolated from unirradiated and UV irradiated exponentially growing cultures of BK4012 (*tisAB::Kan^r*) and AB1157 carrying either pBK410 or the cloning vector pKK232-8. A 400 nt strand specific RNA probe (probe 1, Figure 8A) spanning 200 nt to each side of the *tisAB* antisense region of *uxaA* was hybridized to a northern blot of isolated total RNA (Figure 8B, lower panel). As shown in Figure 8B, lane 9, in the presence of pBK410 a band corresponding in size to a

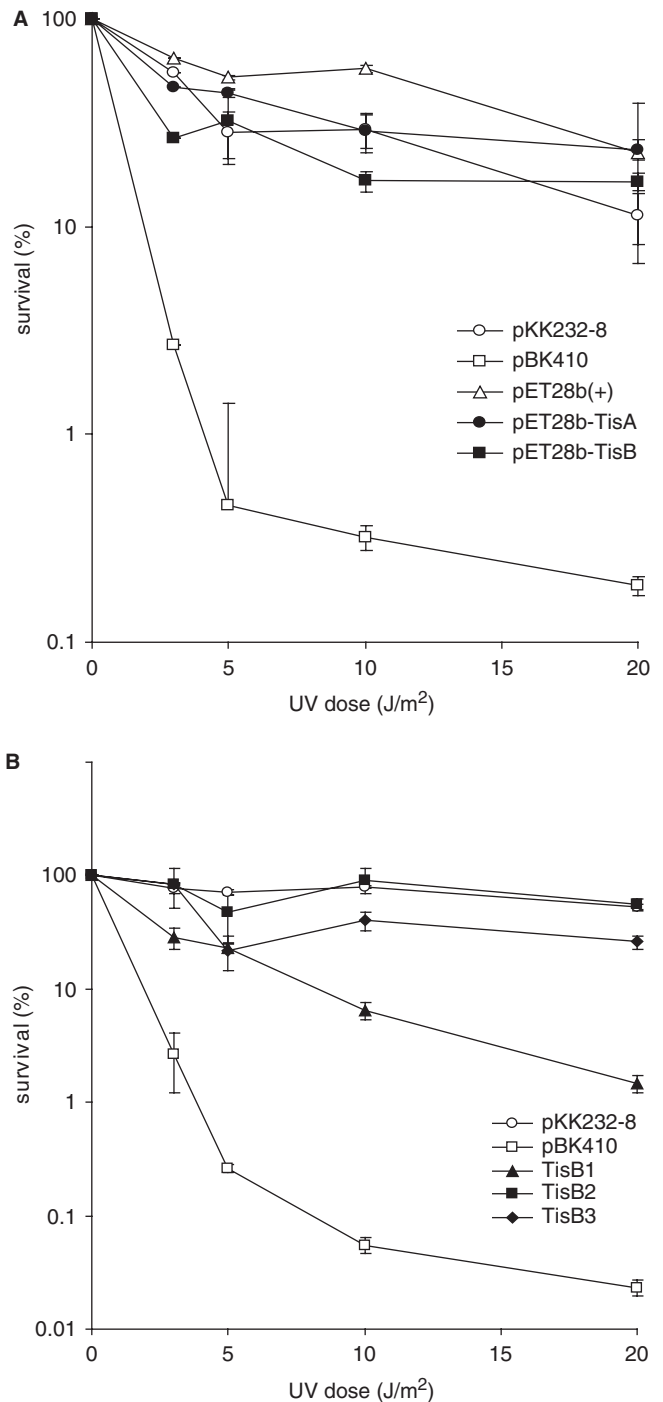


Figure 7. UV survival of TisA and TisB expression plasmids (A) and TisB mutants of pBK410 (B). (A) Cloning of TisA and TisB in expression plasmid pET28b(+) is described in Materials and methods section. Cells were grown to exponentially phase in K-medium added 1mM IPTG for induction. Survival was measured by irradiating exponentially growing ER2566 transformed with pKK232-8, pBK410 (*tisAB* overexpression), pET28b(+), pET28b(+)-TisA and pET28b(+)-TisB to different doses of UV in M9 buffer and plating the cells on LB agar plates. The points shown is average of three experiments, error bars showing standard deviations are included. (B) UV survival of AB1157 transformed with pBK410 and the TisB mutated derivatives TisB1(M11), TisB2(D5stop) and TisB3(K12stop). Cells were grown to exponentially phase in K-medium, washed in M9, irradiated with different doses of UV and plated on LB agar.

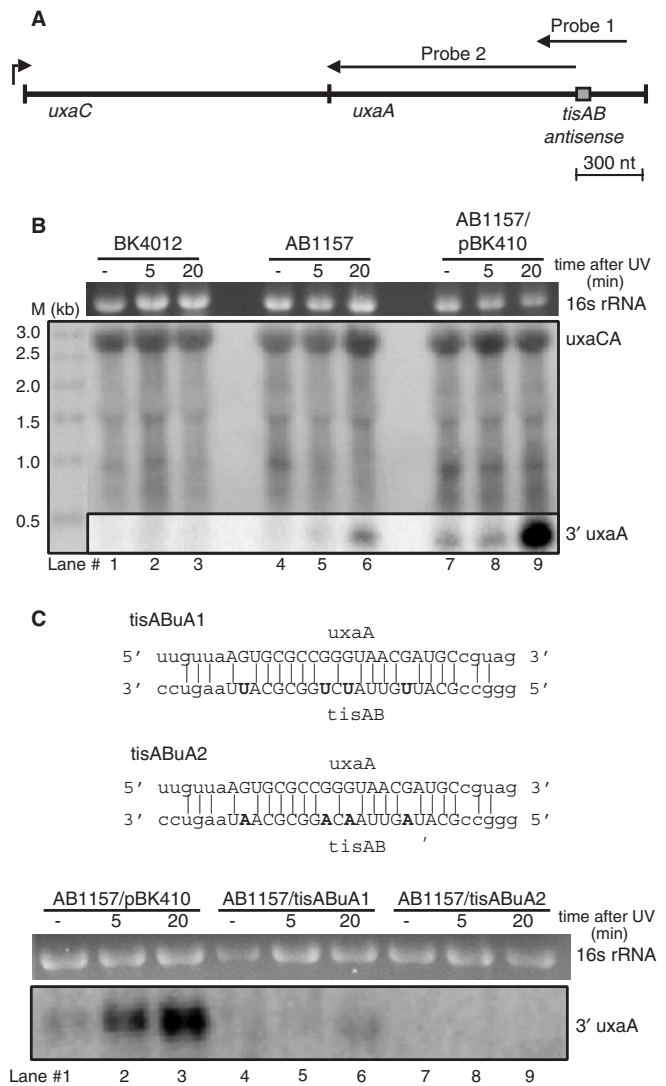


Figure 8. Analysis of *tisAB* mediated *uxaA* cleavage. (A) Map of *uxaCA* operon and position of northern probes and antisense region. Drawing is to scale. (B) Expression and cleavage of *uxaA* mRNA in BK4012 (*tisAB::Kan^r*), AB1157 and AB1157 transformed with pBK410 (overexpressing *tisAB*). Total RNA was extracted from the three isogenic strains and 5 µg RNA obtained from unexposed cells (-) and cells exposed to UV (50 J/m²) at two different time points (5 and 20 min after UV exposure) was separated on formaldehyde-1% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a 400 nt *uxaA* riboprobe (Probe 1) covering the *uxaA/tisAB* antisense sequence (lower panel), and rehybridized with a 1100 nt *uxaA* riboprobe (Probe 2) covering only the sequence upstream of the antisense sequence. Size marker: 4 µg Millennium Markers-Formamide (Ambion). (C) Sequence alignments indicating base changes of the pBK410 derived mutants *tisABuA1* and *tisABuA2* followed by cleavage of *uxaA* mRNA in AB1157 transformed with pBK410 and the antisense mutants *tisABuA1* and *tisABuA2*. Northern was carried out as in (B) except the blot was hybridized only with the 400-nt probe (Probe 1).

300 nt 3'-cleavage product of *uxaA* is clearly present in AB1157 20 min after UV irradiation. A weaker band with similar size is also visible in AB1157 carrying the cloning vector pKK232-8 (Figure 8B, lane 6). The 300 nt band is totally absent in the *tisAB::kan^r* mutant BK4012. The 400-nt probe detected only a faint full-length *uxaCA* mRNA of 2900 nt (data not shown), therefore the blot was

rehybridized to a second probe (probe 2, Figure 8A) covering the first 1100 nt of *uxaA*. This probe produced a signal corresponding to the full-length *uxaCA* transcript, confirming the faint band detected with the 400 nt. To further examine the role of *tisAB* on *uxaA* mRNA processing we made two pBK410 mutant plasmids (*tisABuA1* and *tisABuA2*) with base substitutions in the *tisAB-uxaA* antisense sequence (Figure 8C). The mutant plasmid, *tisABuA1*, contained four C to U substitutions while the other plasmid, *tisABuA2*, contained C to A substitutions in the same positions. The northern experiment demonstrates that the ability to process *uxaA* mRNA is totally abolished with the *tisABuA2* plasmid (lanes 7–9) and strongly impaired with the *tisABuA1* construct (lanes 4–6), indicating that GA basepairing in the antisense region disrupt interference completely while GU basepairing partly maintain *uxaA* mRNA processing. It thus appears that SOS-induced cleavage of *uxaA* mRNA in the 3' antisense region is dependent on *tisAB* expression.

Delayed UV sensitization of the *uxaA*, *dinD* double mutant after overexpression of *tisAB*

The *tisAB*-dependent processing of *uxaA* mRNA raises the reciprocal question if *uxaA* mRNA is able to mediate an antisense effect on *tisAB* activity. In order to investigate any effect of the *uxaA* and *dinD* antisense sequences on UV sensitivity conferred by *tisAB* overexpression we performed survival assays in *uxaA* and *dinD* deletion mutants. The UV sensitivity of isogenic MG1655, BK5060 (*uxaA::kan^r*), BK5131 (Δ *dinD*) and BK5065 (Δ *dinD*, *uxaA::kan^r*) carrying plasmid pBK410 or pKK232-8 suggests that pBK410 is less able to confer sensitivity to BK5065 (Δ *dinD*, *uxaA::Kan^r*) compared to MG1655 when irradiated with 2 and 5 J/m² (Figure 9). The results shows that MG1655 carrying pBK410 reaches a lower plateau of UV sensitivity at around 0.1% survival after irradiation with 5 J/m² while BK5065 (Δ *dinD*, *uxaA::Kan^r*) carrying pBK410 is less sensitive at this UV dose. The results indicate that UV sensitivity conferred by overexpression of *tisAB* is partially dependent on both the *uxaA* and *dinD* genes.

DISCUSSION

In this study we have demonstrated that overexpression of *tisAB* in wild type *E. coli* interferes with SOS induction. Overexpression of *tisAB* to nontoxic levels leads to higher amount of LexA repressor both in unirradiated AB1157 and throughout the induction period causing a delay in *recA* mRNA induction. AB1157 overexpressing *tisAB* were sensitive to UV, displayed lower level of spontaneous and induced prophage λ induction, and were lacking cell filamentation. In addition we found that expression of *tisAB* is necessary for cleavage of the galacturonate catabolism specific *uxaA* mRNA.

According to the present model for SOS induction (1), the enhanced basal level of LexA repressor in unirradiated AB1157 carrying pBK410 could explain the 5 min delay in *recA* mRNA induction (Figure 4). The significance of the delayed *recA* induction could be severe given the

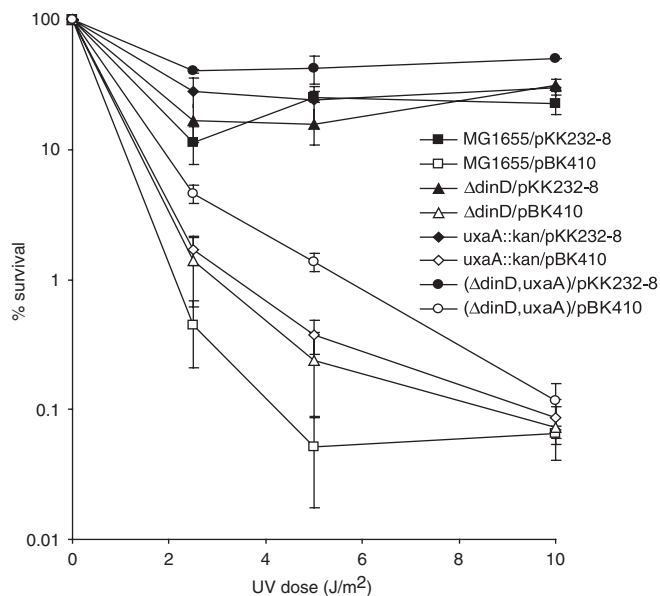


Figure 9. Effect of *uxaA* and *dinD* mutations on UV survival of cells carrying overexpressing *tisAB* (pBK410). Cells were grown in K-medium to exponential phase, washed in M9, UV irradiated with indicated doses and plated on LB agar plates.

important role of the RecA protein in recovery after UV irradiation. The lack of UV induced cell filamentation in AB1157 carrying pBK410 (Figure 6F) also suggests that the higher levels of LexA leads to insufficient induction of SulA, the SOS-inducible inhibitor of septation (33).

Our data demonstrated that UV-induced mutagenesis in AB1157 appears to be normal in the surviving fraction of irradiated cells carrying pBK410. Further, the plaques formed by lysogenic AB1157 carrying pBK410 displayed a size similar to wild-type cells. Most likely, a subpopulation of surviving cells behaves as wild-type cells, and does not express the anti-SOS phenotype.

An important question regarding the inhibition of SOS functions by overexpression of *tisAB* is what functional part of the *tisAB* gene is responsible for the inhibitory effect. An obvious candidate is the TisB peptide which has been reported to encode an SOS-inducible toxin (15,16). However, our protein expression constructs containing the TisB or TisA reading frames under transcriptional control of an IPTG-inducible promoter failed to confer cell death (Figure 2C) or to produce detectable UV sensitivity to the host, as compared to plasmid pBK410 (Figure 7A). Assuming that the TisB peptide is the component mediating inhibition of SOS functions, the lack of phenotype from pET28 constructs could simply be explained by a lack of TisB expression from this system. It has been shown *in vitro* that TisB translation is effective only from the +42 transcript of *tisAB*, suggesting that the RNA structure and context could be important for TisB translation. In addition, our observation that the TisB1(M1I) mutation in pBK410 still confers considerable UV sensitivity to wild-type *E. coli* (Figure 7B) suggests that the role of TisB translation *in vivo* needs to be further investigated. However, our data indicate that the actual function of the *tisAB* gene under normal levels of

induction is not a toxin that kills cells under stress, but a function inhibiting or regulating activities important during SOS induction. Several steps in the sequential events leading to SOS induction could be affected by *tisAB*. Overexpression of *tisAB* could directly or indirectly affect the cleavage or binding properties of LexA repressor and λ cI repressor leading to higher basal levels of the proteins. By example, a change in internal pH affects LexA affinity for operators (34). The RecA filament or the RecA protein itself could be inhibited by *tisAB* overexpression analogous to the function of DinI and RecX proteins (9). The key to understand the target of *tisAB* appears to rest in how and if TisB is translated *in vivo* and the possible target and location of the protein.

The presence of antisense sequences to both the *uxaA* gene and the SOS-inducible *dinD* gene (Figure 1C and D) suggests additional complexity in the way *tisAB* mediates its anti-SOS effect and also the way *tisAB* itself is regulated. Typically, antisense RNA sequences affect RNA stability or translation of mRNA (35). We were able to demonstrate that *tisAB* expression affects processing of *uxaA* mRNA by mediating cleavage of the *uxaA* transcript, giving rise to a 300 nt cleavage fragment from the 3'-end of *uxaA*. Since cleavage occurs in the coding region of *uxaA* it will result in inactivation of the *uxaA* mRNA, and possibly having a polar effect on the upstream *uxaC* on the same mRNA. The *uxaA* gene is specific for galacturonate catabolism in *E. coli*, and the UxaA enzyme (altronate dehydratase) mediates formation of 2-keto-3-deoxy-gluconate (KDG) from galacturonate which is further phosphorylated to a substrate for the Eda enzyme in the Entner–Doudoroff pathway (14). The Eda enzyme is known to play a role in the SOS response, as the Eda protein is induced by DNA-damaging agents in an RecA-dependent manner, and it is hypothesized that Eda is necessary for recovery of respiration following the SOS response (13). We may speculate if overexpression of *tisAB* reduces the level of UxaA and the amount of KDG formed, thereby inhibiting the cells' ability to recover respiration following SOS induction.

We also investigated the opposite possibility that *uxaA* mRNA via the *uxaA/tisAB* antisense region had a role in generating +42 *tisAB* RNA. In our hands the level of +42 *tisAB* RNA from UV exposed log phase cells were found to be below detection level in acrylamide northern experiments (data not shown). However, in agreement with Vogel *et al.* (15) the +42 *tisAB* cleavage product was present in northern experiments with RNA obtained from stationary phase cells exposed to UV (data not shown). Further, wild-type and *uxaA* cells showed the same level +42 *tisAB* RNA after UV exposure indicating that *uxaA* mRNA may not promote cleavage of *tisAB*. Nevertheless, these experiments are not excluding that *uxaA* modulate processing of *tisAB* RNA. In fact we may speculate if *uxaA* mRNA inhibit *tisAB* cleavage under growth conditions in which *uxaA* is upregulated to avoid an SOS inhibitory effect of the +42 *tisAB* transcript.

We were not able to demonstrate any *tisAB*-mediated degradation of *dinD* mRNA, similar to *uxaA* mRNA degradation. It is intriguing that *tisAB* and *dinD*, both

being induced by the SOS response, has sequences with potential of mediating interaction between the two RNA transcripts. The DinD protein function is not known, the gene is dispensable for growth, and the only phenotype known of *dinD* is an *recA*-dependent cold sensitivity of the *dinD* mutation *pcsA68* (36). It should also be noted that SOS-mediated induction of β -galactosidase from the *dinD2::Mud(Ap^R,lac)* mutant is delayed (37), suggesting a possible role for DinD in SOS induction.

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