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The Small RNA IstR Inhibits Synthesis of an SOS-Induced Toxic Peptide

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Summary

More than 60 small RNAs (sRNA) have been identified in *E. coli* [1–7]. The functions of the majority of these sRNAs are still unclear. For the few sRNAs characterized, expression and functional studies indicate that they act under stress conditions [8–14]. Here, we describe a novel *E. coli* chromosome locus that is part of the SOS response to DNA damage. This locus encodes two sRNAs, IstR-1 and IstR-2, and a toxic peptide, TisB, encoded by *tisAB* mRNA. Transcription of *tisAB* and *istR-2* is SOS regulated, whereas IstR-1 is present throughout growth. IstR-1 inhibits toxicity by base-pairing to a short region in the *tisAB* mRNA. This antisense interaction entails RNase III-dependent cleavage, thereby inactivating the mRNA for translation. In the absence of the SOS response, IstR-1 is present in high excess over its target. However, SOS induction leads to depletion of the IstR-1 pool, concomitant with accumulation of *tisAB* mRNA. Under such conditions, TisB exerts its toxic effect, slowing down growth. We propose that the inhibitory sRNA prevents inadvertent TisB synthesis during normal growth and, possibly, also limits SOS-induced toxicity. Our study adds the SOS regulon to the growing list of global regulatory circuits controlled by sRNA genes.

Results and Discussion

Two sRNAs, IstR-1 and IstR-2, Are Encoded in the *ilvB–ysdAB* Intergenic Region

Inspection of the upstream region of *psr19*, an sRNA-encoding gene predicted in the intergenic region between *ilvB* and *ysdAB* [1], indicated the presence of a LexA binding site [15, 16] that previously had been shown to control the clockwise-oriented, bicistronic *ysdAB* operon during the SOS response. The function of this operon was unknown [17]. Because LexA sites can be bidirectional, we analyzed whether *psr19* was upregulated upon exposure to Mitomycin C (MMC), a trigger of the SOS response. Figure 1A shows that while an RNA species of ~75 nt, here denoted IstR-1 (inhibitor of SOS-induced toxicity by RNA), was present throughout growth, a second RNA of ~140 nt, IstR-2, was specifically induced by MMC. Synthesis of the divergently transcribed *ysdAB* mRNAs (here denoted *tisAB* for toxicity-induced by SOS) increased concomitantly with that of IstR-2, indicating that LexA controls both transcripts. Simultaneously, full-length IstR-1 signals decreased, and a shorter RNA appeared (Figure 1A).

Mapping of the 5′ ends of IstR and *tisAB* RNAs indicated *tisAB* mRNA and both IstR-1 and IstR-2 to be primary transcripts originating from separate promoters. We also identified two processing sites in *tisAB* mRNA and one in IstR RNAs (Figures 1B and 1C; see also Figure S1 in the Supplemental Data available with this article online).

In the Absence of IstR RNAs, *tisAB* Confers Toxicity

To gain insight into the function of the *istR-tisAB* locus, we generated deletions in an *E. coli* K12 strain by replacing either the *istR* genes, the *tisAB* region, or the entire locus with a chloramphenicol resistance cassette. None of these deletions affected growth. When we attempted to transfer these three deletions by phage P1 transduction into an SOS-on strain (*lexA51*-Def with a constitutive SOS response; [17]), transductants were obtained only for Δ*istR-tisAB* and *istR*$^+$Δ*tisAB*, but not for Δ*istR-tisAB*$^+$. The Δ*istR-tisAB*$^+$ mutant locus could, however, be moved into the SOS-on strain when the *istR* locus was provided on a plasmid (data not shown). This suggests that *tisAB* encodes an SOS-induced toxic function that is repressed in the presence of the *istR* locus.

To test this hypothesis, we introduced plasmids carrying different segments of the *istR-tisAB* region (Figure 1B) into wild-type (*lexA*$^+$), SOS-on (*lexA51*-Def), and SOS-off (*lexA3*-Ind$^-$) strains that had been deleted for the *istR-tisAB* locus [17]. Introduction of the *tisAB*-encoding plasmid (in the absence of *istR*) into the SOS-on strain failed to yield any transformant colonies, whereas SOS-off and wild-type cells were transformable at high efficiency (Table 1, row 2). All three strains formed colonies with any of the other plasmids tested. Taken together, these results demonstrate that SOS-induced expression of *tisAB*—both at single and elevated copy number—confers growth arrest or lethality. Because the introduction of the entire *istR-tisAB* locus resulted in normal growth, *istR* must encode an inhibitor of toxicity.

The 29 Amino Acid TisB Peptide Is Toxic

The *tisAB* operon encodes two putative peptides, TisA (37 aa) and TisB (29 aa) [17], of which only TisB is conserved in enterobacteria (Figure S2). To define the toxic effector, we mutated the initiation codons of *tisA* and

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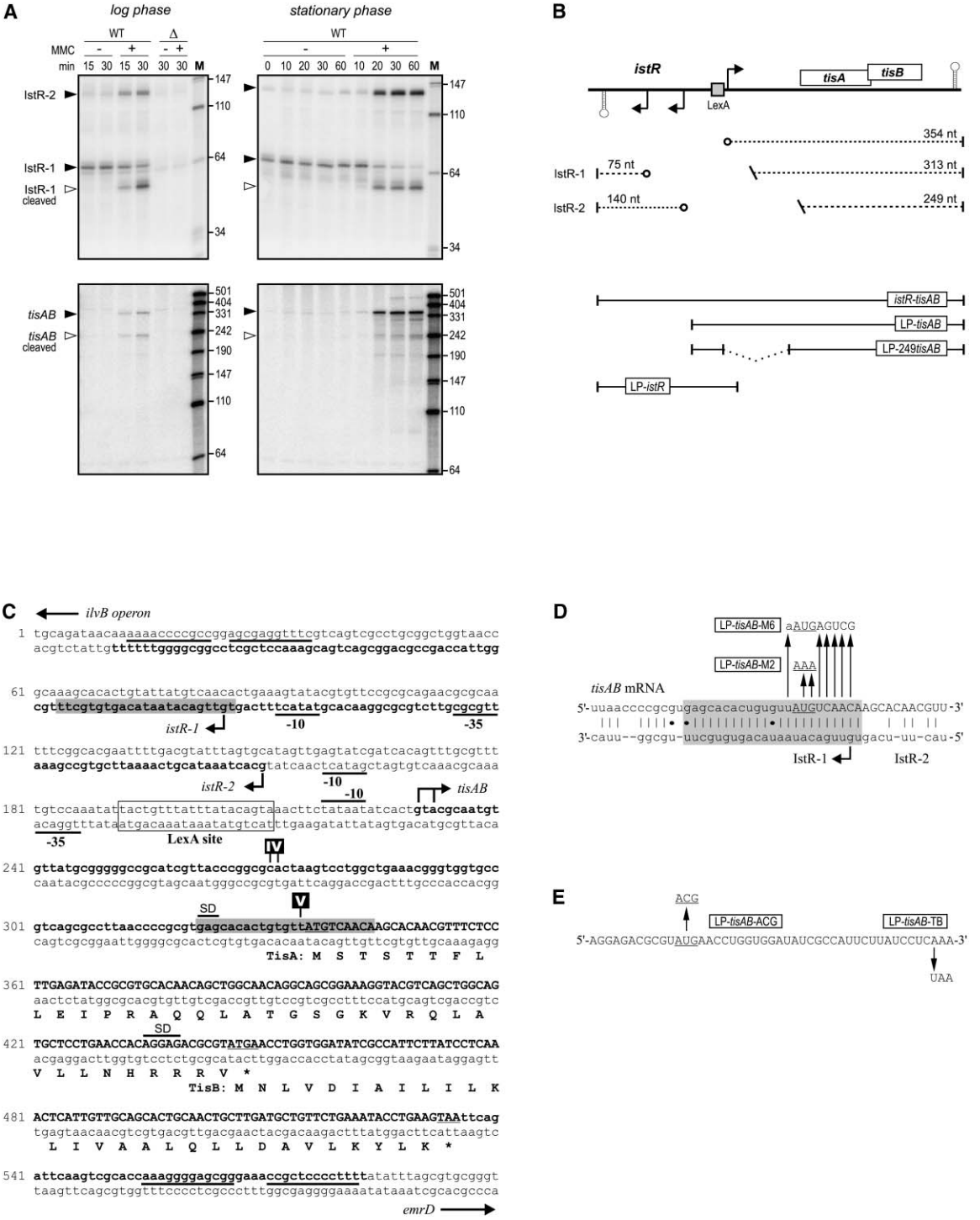


Figure 1. Expression Analysis of the *istR-tisAB* Locus

(A) Northern analysis of *istR* and *tisAB* transcripts. Total RNA was extracted from log and stationary phase cells before and after exposure to MMC (0.5 μg/ml). WT represents wild-type RW118. Δ represents RW118 deleted for the entire *istR-tisAB* locus. M represents size marker, with length in nt indicated. Filled arrowheads indicate full-length IstR-1, IstR-2 (upper panel), or *tisAB* RNAs (lower panel); open arrowheads indicate processed RNAs.

(B) Schematic representation of the transcripts detected in the *istR-tisAB* region and of the plasmids constructed. Drawing is to scale. Circles indicate transcription start points. The 313 and 249 nt *tisAB* RNAs are processed products and, thus, carry 5′ monophosphates (see sites IV and V in [C]). The position of the LexA site is shown. Solid lines represent DNA fragments of *istR-tisAB* carried by the specific plasmids. Plasmid LP-*tisAB* carries the *tisAB* operon including the LexA site and promoter. In plasmid LP-249*tisAB*, the region between the cleavage site upstream of the initiation codon of *tisA* and the original start site is deleted (dashed line). LP-*istR* carries the *istR* genes including the LexA site upstream of the *istR*-2 promoter and *istR*-1 internal promoter.

Table 1. Expression of *tisB* Is Toxic in the Absence of *istR*[a]

	tisAB plasmids[b]	Genotype	WT	SOS off	SOS on	SOS on *rnc14*
1	*lacZ*	–	1.0×10^6	1.0×10^6	0.7×10^6	1.7×10^6
2	LP-*tisAB*	$tisA^+tisB^+$	1.0×10^6	1.1×10^6	**0**	**0**
3	*istR-tisAB*	$istR^+/tisA^+tisB^+$	0.9×10^6	0.9×10^6	0.7×10^{6c}	**0**
4	LP-*istR*	$istR^+$	0.9×10^6	0.9×10^6	0.7×10^6	ND
5	LP-*tisAB*-M2	$tisA\ tisB^+$	4.2×10^5	3.2×10^5	**0**	ND
6	LP-*tisAB*-ACG	$tisA^+tisB$	2.0×10^5	2.3×10^5	3.9×10^5	ND
7	LP-*tisAB*-TB	$tisA^+tisB$	1.8×10^5	5.6×10^5	4.4×10^5	ND
8	LP-*tisAB*-M6	$tisA^+tisB^+$	2.9×10^5	2.6×10^5	**0**	ND
9	LP-249*tisAB*	Δ5′-*tisAtisB*	ND	ND	1.0×10^6	1.1×10^6

[a] Transformation efficiency. Colony-forming units of Δ*istR-tisAB lexA*$^+$ (WT), Δ*istR-tisAB lexA51*-Def (SOS on), and *lexA3*-Ind$^-$ (SOS off) transformed with the indicated plasmids.
[b] *lacZ* carries an internal *lacZ* fragment; LP-*tisAB* carries the *tisAB* operon including the LexA site and promoter; *istR-tisAB* carries the entire locus; and LP-*istR* carries the *istR* genes including the LexA site and the promoters. In LP-*tisAB*-M2 and LP-*tisAB*-ACG, the AUG codons of *tisA* and *tisB* were replaced by AAA and ACG, respectively. LP-*tisAB*-TB encodes a truncated TisB. In LP-*tisAB*-M6, 6 nt in the region of complementarity were changed, and in plasmid LP-249*tisAB*, the region between the cleavage site upstream of the initiation codon of *tisA* and the original start site is deleted. See Figures 1D and 1E for mutations.
[c] Tiny colonies.

Table 2. Inhibition of *tisAB*-Dependent Toxicity by IstR Provided In Trans[a]

	Inhibitor *istR* genes[b]			
Target[b]	LP-*istR*	P_L-*istR*-1	P_L-*istR*-1-M6′	P_L-*istR*-1-M2′
lacZ	3.9×10^5	4.4×10^5	4.0×10^5	1.6×10^5
LP-*tisAB*	2.8×10^5	3.5×10^5	**0**	1.4×10^5
LP-*tisAB*-M2	**0**	**0**	**0**	2.4×10^5
LP-*tisAB*-M6	**0**	**0**	2.5×10^5	**0**

[a] Transformation efficiency. Colony-forming units of SOS-on Δ*istR-tisAB lexA51*-Def, transformed with the indicated plasmids.
[b] *lacZ* carries an internal *lacZ* fragment and LP-*tisAB* carries the *tisAB* operon including the LexA site and promoter. In LP-*tisAB*-M2, the AUG codon of *tisA* was replaced by AAA to disrupt possible base-pairing with *istR*. In LP-*tisAB*-M6, six nucleotides in the region of complementarity were changed. LP-*istR* carries the *istR* genes including the LexA site and the promoters. In P_L-*istR*-1, the *istR* gene is driven by the Lambda P_L promoter. P_L-*istR*-1-M6′ and P_L-*istR*-1-M2′ carry base pair changes complementary to *tisAB*-M6 and *tisAB*-M2, respectively. See Figures 1D and 1E for mutations.

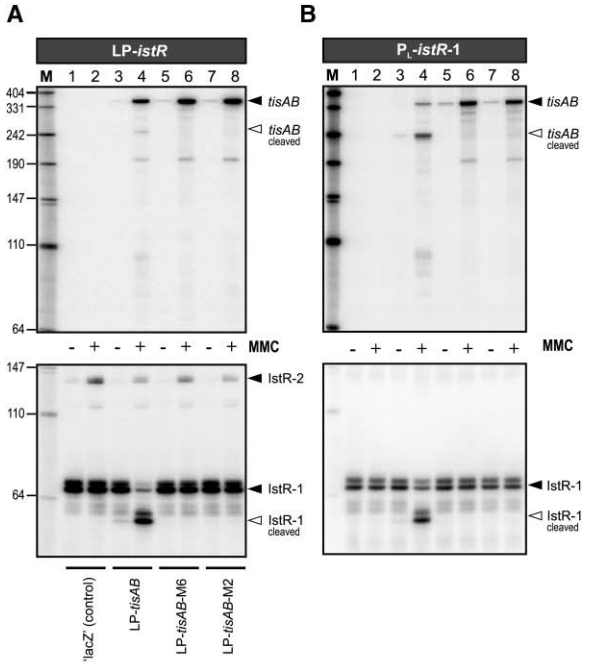
tisB from AUG to AAA and ACG, respectively (LP-*tisAB*-M2 and LP-*tisAB*-ACG; Figures 1D and 1E). Transformation assays with plasmids carrying mutated *tisAB* operons indicated that translation of TisB, but not of TisA, was required to confer toxicity (Table 1, rows 5 and 6). Changing the 12th (AAA) codon of *tisB* to a UAA stop codon, a change predicted to truncate TisB (LP-*tisAB*-TB; Figure 1E; Table 1, row 7), further confirmed that toxicity nests in *tisB*.

IstR-1 Is an Antisense Inhibitor of *tisAB* Expression

The presence of IstR RNAs rescued cells from the toxic effects of *tisAB*. *istR-1* and *istR-2* sequences were cloned downstream of the Lambda P_L promoter under the control of LacI repressor [18] to define which one of the RNAs repressed TisB toxicity. Plasmid P_L-*istR*-1 carries the *istR*-1 gene, whereas P_L-*istR*-2 carries both *istR*-1 and *istR*-2. The SOS-on/Δ*istR-tisAB* strain carrying the inhibitor *istR* plasmids was transformed with a second compatible plasmid carrying the *tisAB* operon to estimate toxicity. Transformation rates demonstrated that both plasmids, P_L-*istR*-1 expressing IstR-1 and P_L-*istR*-2 expressing IstR-2 as well as IstR-1—from its internal promoter—rescued cells from the toxic effect, as did the plasmid carrying the natural *istR* locus (Table 2). Thus, under the conditions tested, IstR-1 alone sufficed to fully abolish toxicity.

BLAST (Basic Local Alignment Search Tool) searches identified a 23 nt region, shared by IstR-1 and IstR-2, that is complementary to the predicted translation initiation region of *tisA* (Figure 1), suggesting that IstR-1/IstR-2

(C) Sequence of the *istR-tisAB* region. Horizontal arrows indicate the transcription start sites of IstR-1, IstR-2, and *tisAB* mRNA. The −35 and −10 hexamers of the σ^{70} consensus of the two *istR* promoters are underlined. The −10 hexamer of the *tisAB* promoter is shown. This promoter has no obvious −35 sequence. Lines between top and bottom strands indicate the inverted repeat sequences of the flanking Rho-independent terminators. Sequences of complementarity between *istR* and *tisAB* are shaded. SD indicates putative Shine-Dalgarno sequences of *tisA* and *tisB*, and IV and V show the processing sites in *tisAB* mRNA.
(D) Potential base-pairing between IstR RNAs and *tisAB* mRNA in the region of the *tisA* start codon. The *tisA* start codon is underlined, and the region of uninterrupted complementarity is shaded. Short and long arrows indicate mutational changes in LP-*tisAB*-M2 and LP-*tisAB*-M6, respectively. Amino acid changes in TisA-M6 are MSTS to MSRS.
(E) *tisB* sequence mutations. The region around the *tisB* start codon, as well as changes in RNA, is shown.



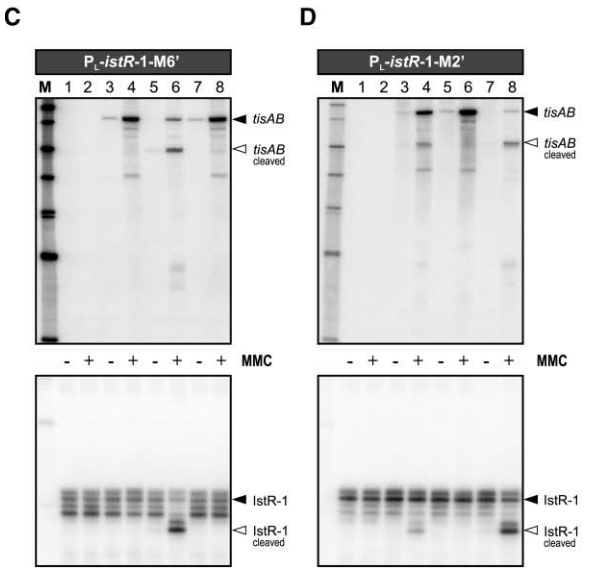


Figure 2. Cleavages in IstR-1 and *tisAB* mRNA Are Dependent on Sequence Complementarity: Northern Analysis

RNA was extracted prior to (−) and at 30 min of MMC treatment (+) from Δ*istR-tisAB* cells, each carrying two plasmids: a p15A plasmid with either a *lacZ* segment (as control) or *tisAB* as indicated below (A) (same order of lanes throughout) and a ColE1 plasmid encoding wild-type or mutant *istR* genes (indicated above the panels). In plasmid P$_L$-*istR*-1, the *istR* gene is driven by the Lambda P$_L$ promoter. P$_L$-*istR*-1-M6′ and P$_L$-*istR*-1-M2′ carry base pair changes complementary to *tisAB*-M6 and *tisAB*-M2, respectively (see Figure 1D). Note that *istR*-1-M2′ restores cleavage as well as colony formation of both *tisAB*-M2 and wild-type *tisAB*-carrying cells (lanes 4 and 8 in [D]; Table 2). The mutation in P$_L$-*istR*-1-M2′ results in only one mismatch to a wild-type target (UUU/AUG), whereas the wild-type IstR-1/*tisAB*-M2 combination has two mismatches (UAC/AAA).

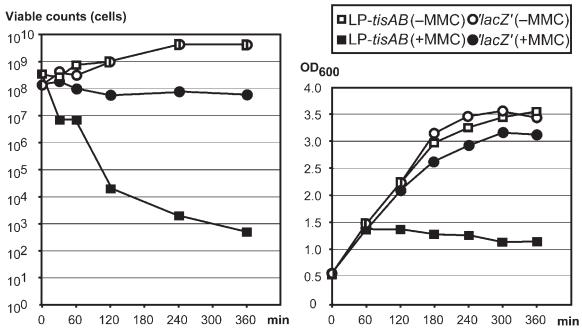


Figure 3. Effects of *tisAB* Expression on Growth and Viable Counts

Experiments were conducted as described in the Experimental Procedures. At each time point, OD (right graph) and viable counts (left graph) were determined from the same samples. The insert above the figure indicates symbols used for the two strains. Squares represent the Δ*istR-tisAB* strain with a plasmid containing the *tisAB* operon including promoter and LexA site. Circles represent the same strain carrying the *lacZ* control plasmid. Open symbols denote no MMC. Filled symbols denote MMC addition at time 0.

might be antisense regulators of *tisAB*. To test the significance of this complementarity, we examined RNA levels and cleavage patterns in Δ*istR-tisAB* strains that carried both *istR* and *tisAB* alleles on compatible plasmids. The Northern blots show that the presence of wild-type *tisAB* mRNA together with any one of the wild-type *istR* RNAs resulted in the appearance of characteristic IstR-1 cleavage products (Figures 2A and 2B, lane 4). Simultaneously, full-length *tisAB* mRNA was converted to an approximately 250 nt RNA. RNA analysis of *tisA* mutant alleles designed to disrupt the putative base-pairing to IstR RNAs (*tisAB*-M2; *tisAB*-M6; Figure 1D) and of *istR*-1 alleles carrying complementary base pair changes (*istR*-1-M6′; *istR*-1-M2′) indicated that mismatches induced by any of the wild-type/mutant combinations eliminated processing of both RNAs (Figures 2A and 2B, lanes 6 and 8; Figures 2C and 2D, lane 4), but compensatory mutations fully restored processing (Figure 2C, lane 6; Figure 2D, lane 8). Thus, cognate interactions between the two complementary sequences in IstR-1 and *tisAB* mRNA entail cleavage of both RNAs. Rapid amplification of cDNA ends (RACE) and primer extension experiments (Figure S1; data not shown) demonstrated that both RNAs were cleaved centrally within the 23 nt region of sequence complementarity; the 5′ end of the processed 249 nt-long *tisAB* mRNA was mapped to the nucleotide preceding the AUG start codon of *tisA* (Figure 1C).

Both target mutant plasmids LP-*tisAB*-M6 and LP-*tisAB*-M2 conferred toxicity to an SOS-on strain (Table 1, rows 5 and 8) and were therefore used to study interactions with IstR RNAs in the plating assay. The transformation experiments demonstrate that when IstR-1 can base pair to its target site, toxicity is prevented (Table 2). The toxicity effects match the pattern of IstR-1 and *tisAB* mRNA processing, suggesting that cognate base-pairing entails specific cleavage of *tisAB* mRNA, thereby abrogating toxicity. An indication that the cleaved *tisAB* mRNA is inactive was obtained by cloning the DNA fragment encoding this 249 nt RNA to generate an RNA identical to the cleaved species (LP-249*tisAB*). This con-

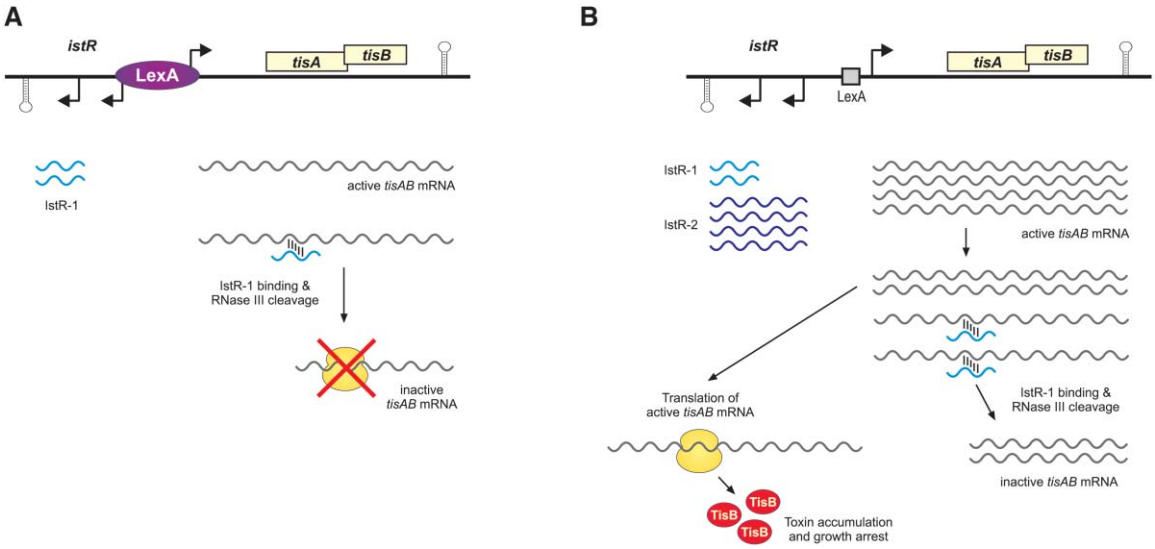


Figure 4. A Model for *tisAB* Control by *istR* Genes

IstR-1 RNA is constitutively transcribed throughout growth, whereas IstR-2 and *tisAB* are under SOS control.

(A) In the absence of SOS, IstR-1 is present in high excess over its target, *tisAB* mRNA, which is expressed at low levels because of incomplete repression by LexA. IstR-1 base-pairing with a 23-nucleotide region in the *tisAB* mRNA entails RNase III-dependent cleavage, which inactivates the mRNA for translation.

(B) SOS conditions induce the transcription of both *tisAB* and *istR*-2. IstR-2 is much less effective than IstR-1 in binding *tisAB* mRNA (our unpublished data), and its role is as yet unclear. Induction of *tisAB* mRNA results in almost complete cleavage of the IstR-1 pool, while *tisAB* gradually accumulates. Under such conditions, the toxic peptide TisB would exert its effect and slow down growth. Thus, we suggest that the inhibitor sRNA functions in preventing inadvertent TisB synthesis during normal growth and possibly in limiting toxicity induced by SOS.

struct failed to confer toxicity in SOS-on cells (Table 1, row 9).

Interaction between IstR-1 and *tisAB* mRNA Results in RNase III-Dependent Cleavage

The experiments reported above show that base-pairing of IstR-1 to *tisAB* mRNA promoted cleavage of these transcripts. To examine RNase III involvement, we compared RNA levels and cleavage patterns in RNase III-proficient (*rnc*$^+$) and -deficient cells (*rnc14*). The results (Figures S3A and S3B) show that in the presence of high levels of IstR-1, *tisAB* mRNA was processed to the 249 nt RNA in an *rnc*$^+$, but not in an *rnc14* background. IstR-1 RNA was also processed by RNase III when *tisAB* mRNA was present. Transformation experiments support a role of RNase III in IstR-dependent repression of toxicity (Table 1). The introduction of plasmids expressing both *istR* and *tisAB* genes into an *rnc14*/SOS-on strain failed to yield any transformant colonies (Table 1, row 3). Thus, IstR-1 inhibits toxicity by inducing RNase III-dependent cleavage of *tisAB* mRNA and converting it to the inactive 249 nt RNA. Interestingly, *tisAB* mRNA levels in untreated cells (without MMC) were increased when either RNase III or IstR was absent (compare lanes 1 and 3 and 1 and 9 in Figure S3A), suggesting that IstR and RNase III both affect *tisAB* transcript levels during normal growth.

TisB-Dependent Toxicity Results in Growth Arrest and/or Cell Death

Toxicity is here defined as the inability of cells to form colonies upon plating. This assay cannot distinguish between bacteriostatic and killing effects. We examined growth and viable counts of untreated and MMC-treated cultures of Δ*istR-tisAB* strains carrying a control or a *tisAB* plasmid. In the absence of MMC, both the control and the *tisAB* strain reached $OD_{600} = 3.5$ after about 4 hr (Figure 3). MMC treatment slowed down growth of the control strain but had a more profound effect on the *tisAB* strain. The effects of MMC on plating were drastic. Viable counts of the control strain decreased by two orders of magnitude. In the MMC-treated *tisAB*-carrying strain, however, plating values dropped without any lag period, from $\sim 10^8$ to $\sim 10^3$. Thus, MMC treatment and/or the SOS response in itself has a significant effect on plating efficiency, but the presence of plasmid-borne *tisAB* decreases colony formation by an additional five orders of magnitude.

Many toxin-antitoxin systems have been found in bacteria; most often, toxicity appears to be triggered by a number of adverse growth conditions, such as starvation and stationary phase [19, 20]. The work reported here describes the first toxin that is induced by the SOS response, suggesting an important role in the response that protects cells from the effects of DNA damage. This finding also adds this well-defined stress regulon to the growing list of global regulatory circuits that are controlled by sRNA-encoding genes. What is the role of the *istR* RNAs in the SOS regulon? Under normal growth conditions, IstR-1 is present in high excess over its target, *tisAB* mRNA, and will inactivate it. In contrast, SOS induction results in almost complete cleavage of the IstR-1 pool, while *tisAB* mRNA gradually accumulates. Under such conditions, the toxic peptide TisB would exert its effect, slowing down growth (Figure 4). Thus,

IstR-1 functions primarily in preventing inadvertent TisB synthesis during normal growth and, possibly, in limiting toxicity induced by SOS. The role of IstR-2 under SOS conditions is currently unclear.

What is the mechanism by which *tisB* expression is inhibited? The target site of IstR-1 is located around the predicted translation start site of *tisA* and more than 100 nt from that of *tisB*. Thus, assuming translational coupling, one could predict IstR-1 to inhibit *tisB* translation by blocking ribosomal access to the *tisA* start site. This is unlikely because a *tisA* start codon mutation does not abrogate toxicity. Instead, IstR-1 appears to inhibit *tisB* synthesis by promoting *tisAB* mRNA cleavage and thereby converting it to an inactive 249 nt RNA. Why this RNA cannot be translated is at present unknown, but in vitro experiments support this result (our unpublished data). The mechanistic details of *tisAB* regulation by IstR RNAs and the function of TisB in the SOS response are presently under investigation.

Supplemental Data

Supplemental data including detailed Experimental Procedures and several figures and tables are available at http://www.current-biology.com/cgi/content/full/14/24/2271/DC1/.

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