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Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in *Escherichia coli*

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ABSTRACT

Short regulatory RNAs are widespread in bacteria, and many function through antisense recognition of mRNA. Among the best studied antisense transcripts are RNA antitoxins that repress toxin mRNA translation. The *hok/sok* locus of plasmid R1 from *Escherichia coli* is an established model for RNA antitoxin action. Base-pairing between *hok* mRNA and Sok-antisense-RNA increases plasmid maintenance through post-segregational-killing of plasmid-free progeny cells. To test the model and the idea that sequestration of Sok-RNA activity could provide a novel antimicrobial strategy, we designed anti Sok peptide nucleic acid (PNA) oligomers that, according to the model, would act as competitive inhibitors of *hok* mRNA::Sok-RNA interactions. In *hok/sok*-carrying cells, anti Sok PNAs were more bactericidal than rifampicin. Also, anti Sok PNAs induced ghost cell morphology and an accumulation of mature *hok* mRNA, consistent with cell killing through synthesis of Hok protein. The results support the sense/antisense model for *hok* mRNA repression by Sok-RNA and demonstrate that antisense agents can be used to out-compete RNA::RNA interactions in bacteria. Finally, BLAST analyses of ≈200 prokaryotic genomes revealed that many enteric bacteria have multiple *hok*/*sok* homologous and analogous RNA-regulated toxin–antitoxin loci. Therefore, it is possible to activate suicide in bacteria by targeting antitoxins.

INTRODUCTION

Non-coding regulatory RNAs are widely expressed in many genomes (1,2). A large number of non-coding RNAs are complementary to active open reading frames, yet there is only limited evidence for direct sense/antisense interactions. Antisense transcripts are encoded both *in-cis* and *in-trans* and are believed to modulate RNA processing, decay and translation through direct pairing with complementary target sequences (3). Bacterial genomes and plasmids contain a number of annotated as well as predicted sense and antisense genes. Despite predictions of widespread sense/antisense pairing in several species (4–7), there have been few attempts to experimentally probe these structures and test the effects of disrupted interactions (8).

A paradigm for sense/antisense RNA pairing is the *hok*/*sok* toxin–antitoxin (TA) plasmid stabilization locus of the R1 plasmid in *E. coli* (9). The *hok*/*sok* locus codes for three genes: *hok* (host killing) encodes a highly toxic *trans*-membrane protein that irreversibly damages the cell membrane (10). The *mok* (modulation of killing) reading frame overlaps with *hok* sequences and is required for *hok* expression and translation. Finally, the *sok* (suppression of killing) gene encodes a small antisense RNA *in-cis* that blocks translation of the *mok* reading frame and thus inhibits expression of *hok* mRNA (11). Pairing between Sok and *hok* transcripts is supported by *in vitro* and phylogenetic studies (12–14). Sok-RNA is very unstable (half-life in the order of 30 s) but driven by a strong promoter. In contrast, the full-length *hok* transcript is heavily structured, stable (half-life in the order of 30 min) and inaccessible to either ribosome initiation or Sok-RNA binding. Slow 3′ end processing of *hok* mRNA results in mature transcripts that are accessible for translation and Sok-RNA binding. In the presence of a *hok*/*sok*-carrying

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plasmid, Sok-RNA binds to the mature form of *hok* mRNA and represses translation by preventing ribosome entry (15–17). In addition, formation of the *hok* mRNA::Sok-RNA duplex leads to rapid RNase III-mediated degradation (scavenging) of the mature and translatable form of *hok* mRNA (18). In cases where the plasmid is lost, the Sok-RNA pool is depleted through rapid decay and this frees *hok* mRNA for translation and toxin production kills the cell (19). Hok toxin causes dramatic changes in cell morphology, resulting in characteristic 'ghost cells', where the cell poles appear dense relative to the cell centre (9,10). Therefore, the *hok*/*sok* system provides plasmid stability through post-segregational killing of plasmid free progeny. This mechanism of controlled expression of Hok toxin confers increased plasmid maintenance, and this may provide a net benefit to cells by maintaining plasmid encoded virulence or stress resistance traits. A simplified model of the *hok*/*sok* system is shown in Figure 1. Additional details of the *hok*/*sok* system and other TA systems were reviewed by Gerdes and coworkers (20).

We aimed to test the sense/antisense pairing model for *hok* mRNA::Sok-RNA interactions in cells and also test possibilities to induce Hok-mediated killing using an antisense agent. Short synthetic peptide nucleic acid (PNA) oligomers complementary to Sok sequences were designed to act as competitive inhibitors of sense/antisense interactions. Anti Sok PNAs added to growing cells caused growth arrest and were bactericidal. Also, PNA-treated cells showed the 'ghost' cell morphology and RNA maturation changes characteristic of Hok toxin-mediated cell killing. Finally, using database searches, we revealed a large number of *hok*/*sok* and another family of antisense RNA-regulated toxin genes, *ldr*, in enteric bacterial genomes.

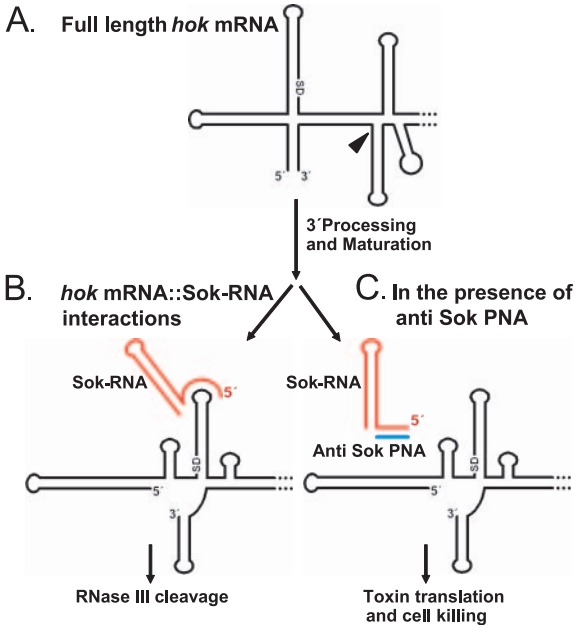

Figure 1. Schematic model of the *hok*/*sok* TA system and the Sok-RNA inhibition strategy used in this study. (**A**) Full-length *hok* mRNA folds into a compact form in which the 5′ and 3′ ends of the molecule pair. The ends of the molecule make an exact match, thus generating a highly folded and 'blunt-ended' RNA structure. The sequestering of the mRNA 3′ end reduces the rate of 3′-processing by polynucleotide phosphorylase and ribonuclease II (15). However, the 3′ exoenzymes removes the terminal 39 nt at the 3′ end of *hok* mRNA at a low rate. The arrow-head points to the bottom of the stem–loop structure, which acts as a 'road-block' for the 3′ end trimming. (**B**) Via its 5′ end single-stranded tail, Sok-RNA (shown in red) recognises a single-stranded stem–loop present only in the truncated, refolded *hok* mRNA. This is because the 3′ trimming of full-length *hok* mRNA releases the very 5′ end of the mRNA and this release triggers a major refolding of the mRNA 5′ end that results in the formation of the antisense RNA binding stem–loop structure (26,27). The refolded isoform of the mRNA is metabolically very stable and binds Sok-RNA avidly, but can also be bound by ribosomes and therefore be translated (26). In the presence of excess Sok-RNA rapid binding of the antisense RNA prevents ribosome binding to *hok* mRNA and thus prevents its translation (12,26). Eventually, the *hok* mRNA::Sok-RNA duplex is formed and then rapidly cleaved (scavenged) by RNase III (18). Therefore, the truncated form of *hok* mRNA does not accumulate in Sok-RNA containing cells. The RNase III cleavage of the *hok* mRNA::Sok-RNA duplex is not required for inhibition of *hok* mRNA translation (41). (**C**) Sok-RNA inhibition strategy. PNAs complementary to the single-stranded 5′ end of Sok-RNA (shown in blue) prevent Sok-RNA binding to *hok* mRNA and thereby induce *hok* translation, synthesis of the lethal Hok protein and cell killing.

MATERIALS AND METHODS

Bacteria, plasmids and PNAs

Escherichia coli K-12 strain CSH50 was used as host and transformed with two plasmids with and without *hok*/*sok* genes (pPR95 and pOU82, respectively) (Table 1). Anti Sok and control PNAs were synthesized and high-performance liquid chromatography purified by Oswel Ltd (UK) (21) (Table 1). All PNAs were synthesized with an attached peptide (KFFKFFKFFK) to enhance cell uptake (22).

Bacterial growth and minimal inhibitory concentration (MIC) measurement

Bacteria were grown in Mueller Hinton (MH) broth containing ampicillin (30 μg/ml) under aeration to mid-log phase of growth. Inoculums from mid-log phase cultures were used to initiate cultures at 10^5 c.f.u./ml in the wells of ultra low attachment 96-well microplate (Costar 3474, Corning incorporated, NY) and incubated with PNA at 37°C for 20 h. The lowest PNA concentrations that prevented measurable cell growth after 20 h were scored as the MIC. C.f.u. were determined by serial dilutions of cell culture with PBS followed by plating on LB plates.

Microscopy

Cells morphology was analysed by phase-contrast microscopy (Zeiss) at 1000× total magnification. Prior to microscopy, cultures were treated with 8 μM PNA or 150 μg/ml rifampicin for 3 h and then collected by centrifugation. Digital images were captured using a CCD camera and Openlab imaging software, version 3.1.5. (Improvision Ltd).

Northern analysis of mRNA maturation

Total RNA was extracted from mid-log growth phase cultures using the acid-phenol method as described (23). RNA was fractionated in a 6% polyacrylamide/urea/TBE gel and electrotransferred onto a GeneScreen Plus nylon membrane (Perkin Elmer, USA). Transcripts containing *hok* sequences

Table 1. Plasmids and PNAs used in this study

	Features	References
Plasmids		
pOU82	*bla*$^+$, *hok*/*sok*$^-$, mini-R1	(42)
pPR95	*bla*$^+$, *hok*/*sok*$^+$, mini-R1	(9)
PNAs		
Anti Sok PNA-1	(N)-(KFF)$_3$K-eg1-TATGTCTAGTC	This study
Anti Sok PNA-2	(N)-(KFF)$_3$K-eg1-TATGTCTAG	This study
Anti Sok PNA-3	(N)-(KFF)$_3$K-eg1-TATGTCTAGT	This study
Control SP69	(N)-(KFF)$_3$K-eg1-GTTACATGAT	(21)

were probed using an oligonucleotide that is complementary to the 3′-terminal region of the *hok* open reading frame (Figure 4A): (5′-CTACTTACCGGATTCGTAAGCCATGA-AAGCCGCCACCTCCCTGTGTCCGTCTCTG-3′); labelled using cross-linking alkaline phosphatase and chemifluorescent detection systems (Amersham Biosciences).

Complex formation and binding kinetics analyses *in vitro*

Sok-RNA and the mature, processed form of *hok* mRNA were synthesised *in vitro* using T7 polymerase (Fermentas) and PCR fragment templates generated using *sok* and *hok* primers: sok F, (5′-AAGAAGATAGCCCCGTAGTAAG-3′); sok R, (5′-TGTAATACGACTCACTATAGACTAGACAT-AGGGATGCCTC-3′) T7 promoter region is underlined; hok F, (5′-TGTAATACGACTCACTATAGGGCGCTTGA-GGCTTTCTGC-3′) T7 promoter region is underlined; hok R, (5′-AAGGCGGGCCTGCG-3′). To determine binding kinetics, radioactively labelled Sok-RNA was generated by incorporation of [α-^{32}P]CTP (800 Ci/mmol; Amersham Bioscience) during T7 transcription. Small amounts of labelled RNA (~3000 c.p.m.) were used to spike non-radioactive Sok-RNA. For binding reactions we mixed *hok* mRNA (30 nM) with Sok-RNA (2 nM) or PNA (500 nM) with Sok-RNA (38 nM) in TMN buffer [20 mM Tris–OAc pH 7.5, 10 mM $Mg(OAc)_2$, 100 mM NaCl]. Bound and unbound fractions were separated on an 8% polyacrylamide gel containing 7 M urea at 5 V/cm. The radioactive bands were analysed by autoradiography and densitometry using ImageQuant software (Molecular Dynamics). To minimise complex formation during handling prior to electrophoresis, aliquots were immediately diluted into two volumes of loading buffer (92% formamide, 17 mM Na_2EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue) and loaded onto gels with current applied at each time point. The second-order binding-rate constant (k_2) was calculated as described previously (24).

Annotation of antisense RNA-regulated toxin genes

The fully sequenced genomes of 218 prokaryote organisms were downloaded from the NCBI Web-site and searched for Hok and Ldr protein-encoding genes using the TBLASTN program (25). Hok and Ldr sequences of *E.coli* K-12 were used as seed sequences (Supplementary Table S1). The coordinates for each candidate and the encoded protein sequences are listed in Supplementary Table S1. The toxin genes were annotated on the genomes using Vector NTI (version 7.0).

RESULTS

Design of anti Sok PNAs

The secondary structure of Sok-RNA has been established experimentally (12,14,26,27). The 64 nt long RNA consists of a stable stem–loop structure and a single-stranded 5′-tail (Figure 1). Initially, the 11 nucleotide-long 5′-tail of Sok-RNA recognises a single-stranded loop in the truncated, refolded isoform of *hok* mRNA (12). Therefore, we decided to design antisense PNAs complementary to the 5′ end single-stranded tail of Sok-RNA in the anticipation that they could out-compete Sok-RNA binding to *hok* mRNA and thus induce *hok* mRNA translation. We designed and synthesized PNAs that have 9 (anti Sok PNA-2), 10 (anti Sok PNA-3) or 11 (anti Sok PNA-1) bases complementary to 5′-tail of Sok-RNA.

Anti Sok PNAs inhibit cell growth and are bactericidal

To test whether competitive inhibition of *hok* mRNA::Sok-RNA interaction could trigger Hok synthesis in bacteria, anti Sok PNAs were added to cultures of *E.coli* cells carrying the *hok*/*sok* locus. Growth was assessed by monitoring the growth curve as an indicator for cell survival. All three of the anti Sok PNAs inhibited the growth of a *hok*/*sok*-carrying strain (CSH50/pPR95). Anti Sok PNA-3 was the most potent of the three anti Sok PNAs (Figure 2A) and no growth was detected in the culture with 10 μM PNA after 20 h, indicating an MIC of 10 μM for PNA-3.

To control for possible non-specific effects of PNAs, such as reduced plasmid copy number, two negative control experiments were included. First the *hok*/*sok*-deficient strain (CSH50/pOU82) was grown together with anti Sok PNA and no changes in cell growth were observed, showing that *hok*/*sok* genes are needed for inhibition of cell growth. Second, a scrambled PNA was introduced to both strains for detection of any non-specific effect of PNA chemistry. As expected, cell growth was not affected by these negative controls. Also, previous studies using targeted and non-targeted oligomers indicate that PNAs do not reduce plasmid copy number (22,28). Therefore, the anti Sok PNAs act in a sequence-specific manner to sequester Sok-RNA and activate Hok toxin synthesis.

To assess the extent of cell killing by Hok activity, we treated cells with anti Sok PNAs and determined the number of viable cells over time by scoring the number of c.f.u. The results show that Hok induction using anti Sok PNA almost completely eliminated c.f.u. (Figure 2B). Therefore, *hok* induction by competitive inhibition of Sok-RNA binding can eliminate the vast majority of viable bacteria within a large *E.coli* population. For comparison, rifampicin treatment was included in the experiment. Rifampicin was significantly less inhibitory than the anti Sok PNAs, even at much higher concentrations (100–200 μM) (Figure 2B). We cannot explain this efficiency; however, the results are similar to those reported by two other laboratories (28,29), which show that PNA and morpholino (PMO) antisense oligomers are more potent than ampicillin in *E.coli* killing assays, both *in vitro* and *in vivo*. Also, unpublished and published data (30) indicate that PNA oligomers accumulate in *E.coli* and are not removed by transporters which efflux

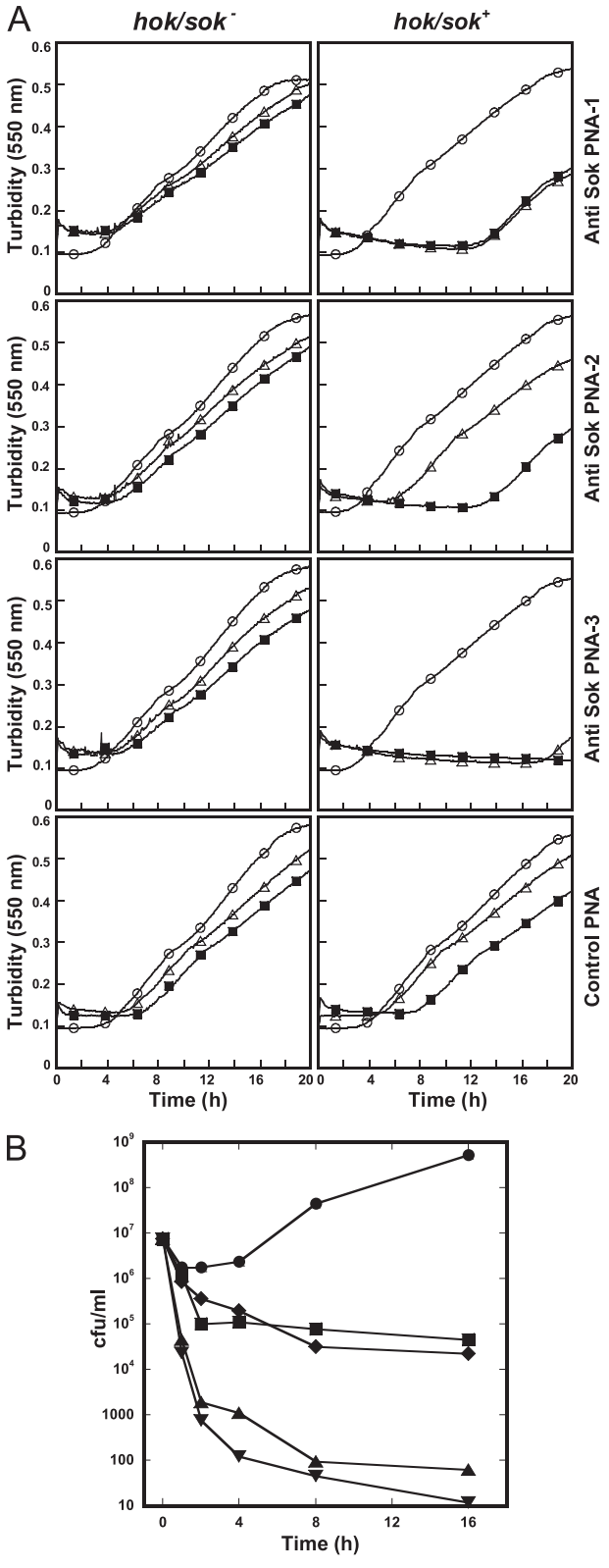

Figure 2. Effects of anti Sok PNAs on the growth of *E. coli* carrying *hok*/*sok* system. (**A**) Turbidity in an untreated culture (open circle) and cultures including anti Sok PNAs added at 8 μM (open triangle) and 10 μM (closed square). (**B**) C.f.u. in an untreated culture (closed circle) and cultures containing anti Sok PNA 5 μM (closed triangle) and 10 μM (closed inverted triangle) and rifampicin at 100 μM = 122 μg/ml (closed square) and 200 μM = 244 μg/ml (closed diamond).

conventional antimicrobials. Therefore, we can speculate that cell accumulation and retention of PNA provides the potent killing activity observed in the c.f.u. assay, whereas rifampicin and ampicillin are rapidly removed from cells after plating.

Anti Sok PNAs induce the characteristic 'ghost cell' morphology

To confirm that the cell killing observed was due to Hok synthesis, we studied cell morphology by phase contrast microscopy (Figure 3). Dead cells with highly condensed material in the poles and a transparent centre 'ghost cells' were observed in cultures of *hok*/*sok*-carrying cells treated with either anti Sok PNA or rifampicin. Again, for comparison, the global transcription inhibitor, rifampicin was included. Rifampicin treatment of *hok*/*sok*-carrying cells leads to a rapid RNase E-mediated degradation of Sok-RNA (31). Since *hok* mRNA is much more stable than Sok-RNA, the decay of Sok-RNA confers derepression of *hok* mRNA translation, and cell killing follows. As expected, rifampicin treatment induced ghost cell morphology (Figure 3). As negative controls, we included *hok*/*sok*-deficient cells and an unrelated PNA. In all cases, the samples

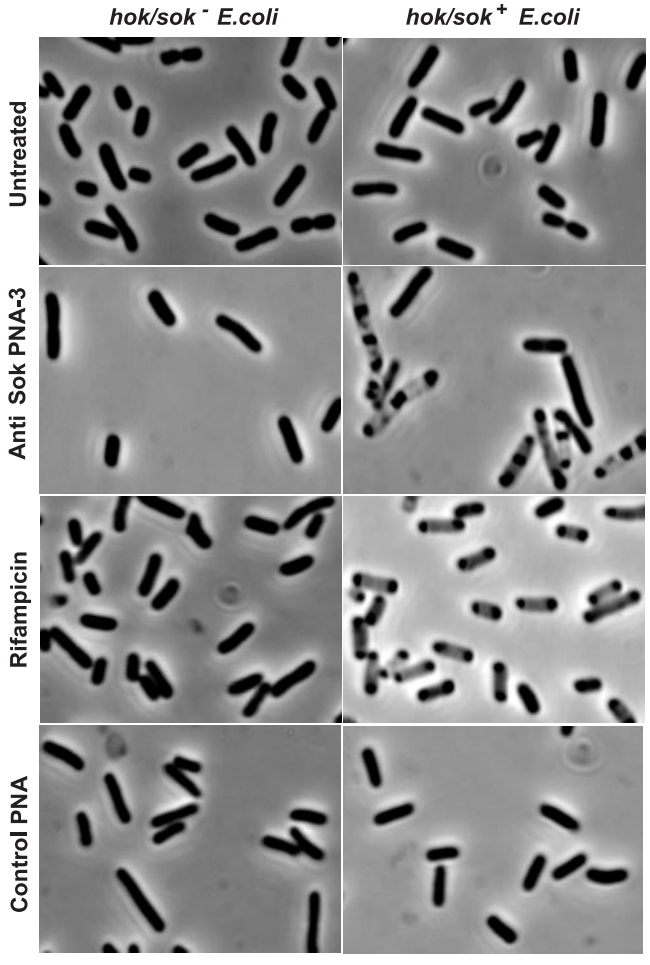

Figure 3. Effect of anti Sok PNAs on cell morphology of *E.coli* carrying *hok*/*sok*. PNAs and rifampicin were added to growing cells carrying control or *hok*/*sok*-containing plasmids.

from the negative controls contained cells with a normal cell morphology. Therefore, anti Sok PNA can inhibit Sok-mediated repression of *hok* mRNA. This result supports the model for direct Sok-RNA pairing with *hok* mRNA.

Anti Sok PNAs cause mature *hok* mRNA accumulation

According to the *hok*/*sok* model (20), blockage of Sok-RNA activity should lead to accumulation of the mature 3′ end truncated *hok* mRNA. Therefore, if the model is correct and the anti Sok PNAs selectively sequester Sok-RNA, it should be possible to detect the truncated, mature isoform of *hok* mRNA in PNA-treated cells. Total RNA was prepared from *hok*/*sok*-carrying cells that were untreated or treated with rifampicin or anti Sok PNA at low micromolar concentrations for 4 h. Northern analysis was performed using a probe that was complementary to a downstream region within the *hok* open reading frame (Figure 4A). The probe clearly detected pre-mRNA transcripts in all samples from *hok*/*sok*-carrying cells (Figure 4B). Importantly, in PNA-treated cells, the mature 3′ end truncated form of *hok* mRNA was detected. The identity of the mature form of *hok* mRNA was indicated by comparison to rifampicin treated cells, which contain detectable amounts of mature *hok* mRNA following transcription arrest (20). Therefore, anti Sok PNA treatment sequesters free Sok-RNA and leads to accumulation of mature and active *hok* mRNA. Sequestered Sok molecules may be protected from 5′ end degradation by RNase E, but it is unlikely that 3′ end degradation by PNPase and RNase II is inhibited (31).

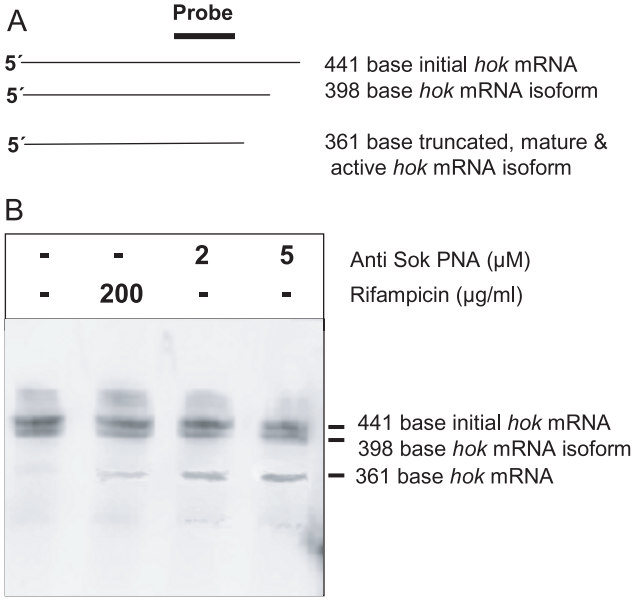

Figure 4. Effect of anti Sok PNA treatment on *hok* mRNA processing. (**A**) *hok* mRNA isoforms. The 441 and 398 nt long RNAs are initial *hok* mRNAs that are processed into the mature, truncated isoform of 361 bp *hok* mRNA (15,19). In the presence of an excess of active Sok-RNA, the truncated version of the *hok* mRNA does not accumulate due to rapid Sok-RNA binding followed by rapid RNase III processing of the *hok* mRNA::Sok-RNA duplex. The long isoforms of *hok* mRNA bind Sok-RNA much slower and therefore accumulate even in the presence of Sok-RNA (26). The long versions of *hok* mRNA cannot bind to ribosomes and be translated and are not harmful to the cell. (**B**) Northern analysis of *hok* mRNAs following exposure to anti Sok PNA or rifampicin. The initial transcripts and 3′ end truncated, mature and active isoforms of *hok* mRNA are indicated.

PNA::Sok-RNA complex formation *in vitro* and interaction kinetics

The phenotypic effects of anti Sok PNAs suggest that they are able to compete with the native interactions between *hok* mRNA and antisense Sok-RNA. If this is the case, it should be possible to determine the binding kinetics *in vitro*. Previously, the binding-rate for Sok-RNA and *hok* mRNA interactions was determined *in vitro* by monitoring complex formation at low concentrations using gel shift analysis of radioactively labelled Sok-RNA (14). To determine the binding-rate for anti Sok PNAs, we first re-established the *hok* mRNA::Sok-RNA interaction assay. Using ^{32}P-labelled Sok-RNA and an excess of the mature form of *hok* mRNA we observed a clear reduction in free Sok-RNA and an increase in a slower migrating *hok* mRNA::Sok-RNA complex over time using PAGE (Figure 5A). By quantifying the reduction in free Sok-RNA we calculated the second-order binding-rate constant (k_2) to be $\sim 1.4 \times 10^5$ M^{-1} s^{-1} (37°C) (Figure 5B), a value that is similar to that reported previously (14). In the same way, we determined the binding-rate of PNA::Sok-RNA complexes. Again, there was a clear reduction in free Sok-RNA (Figure 5A). The reduction in Sok-RNA over time was quantified and the binding-rate constant was $\sim 6.9 \times 10^3$ M^{-1} s^{-1} (37°C) (Figure 5B). Therefore, PNA::Sok-RNA interactions occur less quickly than *hok* mRNA::Sok-RNA interactions *in vitro*. The PAGE analysis did not reveal distinct PNA::Sok-RNA complexes, possibly due to the positively charged peptide attached to PNA and its effects on complex stability and migration during electrophoresis. These *in vitro* results suggest that efficient

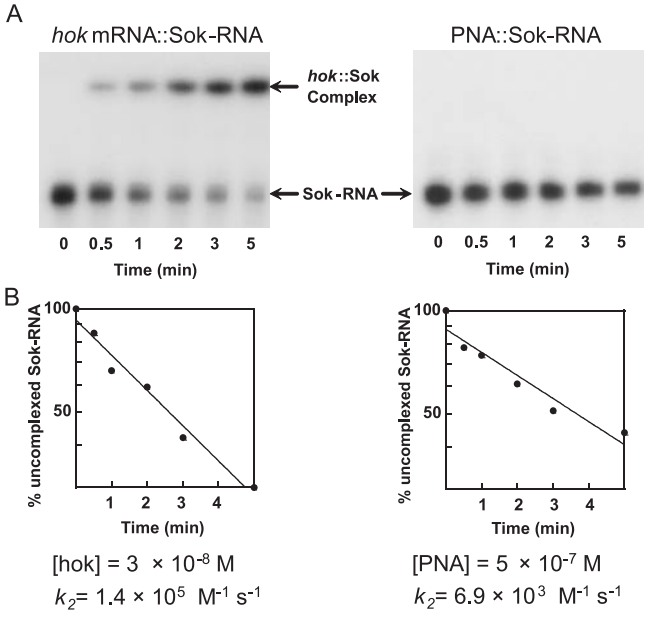

Figure 5. PNA::Sok-RNA complex formation *in vitro* and binding kinetics. (**A**) ^{32}P-Radiolabeled Sok-RNA incubated with a 15-fold molar excess of unlabelled anti Sok PNA (500 nM) or *hok* mRNA (30 nM), fractionated in a polyacrylamide/urea/TBE gel and analysed by autoradiography and densitometry. (**B**) Plots of the fractions of uncomplexed Sok-RNA as a function of time of incubation, with the binding-rate constants indicated.

Table 2. Numbers of *hok*/*sok*, *ldr* and protein-regulated TA loci in enteric bacteria[1]

Organism	*hok* genes	*ldr* genes	protein-regulated TA loci[2]
E.coli K-12	5	4	5
E.coli CFT073	5	3	5
E.coli 0157 H7	13	3	8
E.coli 0157 H7 EDL933	13	4	8
S.flexneri 2a 301	10	5	3
S.flexneri 2a 2457T	9	3	2
Salmonella typhi	1	2	6
S.typhi Ty2	0	2	6
Salmonella enterica paratypi	0	2	4
S.typhimurium LT2	0	2	8
Photorhabdus luminescens	3	0	50
E.carotivora atroseptica	1	0	14
Vibrio vulnificus CMCP6 chrII	1	0	1
Total number of genes	61	30	120

[1]*V.vulnificus* is closely related to enterobacteria but not classified as such.
[2]Seven gene-families of protein-regulated TA loci are known: relBE, higBA, parDE, mazEF, phd/doc, vapBC and ccdAB.

competition for Sok-RNA in cells requires an excess of PNA relative to *hok* mRNA. Such an excess is likely to exist as *hok* genes are driven by weak promoters and PNA appears to accumulate in cells and is not removed by drug efflux pumps (30).

Enterobacteria contain a large numbers of *hok*/*sok* and *ldr* toxin loci that are regulated by antisense RNAs

Given the efficient killing of *E.coli* via activation of *hok* described above, we found it interesting to describe in detail the phylogenetic distribution of antisense RNA-regulated toxin-encoding genes. *E.coli* K-12 encodes five *hok*/*sok* loci (32) and four *ldr* (long direct repeats) loci. *ldr* loci are analogous (but not homologous) to *hok*/*sok* loci and encode a small toxin gene of ~30 codons, a small *cis*-encoded antisense RNA that represses translation of the toxin gene. Similar to *hok*/*sok* genes, the toxin-encoding mRNA is stable and the antisense RNA is unstable (33). We searched 218 fully sequenced genomes exhaustively for the presence of *hok* and *ldr* genes using an approach described previously (34). An overview of the results is shown in Table 2. Our analysis shows that *ldr* loci are found only in a narrow spectrum of enteric bacteria, including *E.coli* and *Salmonella* and *Shigella flexneri* species, whereas *hok*/*sok* loci are found in a broader spectrum of enterobacteria, in one *Vibrio* species, and in one of four *Salmonella* species examined. In contrast, protein-regulated TA genes (e.g. *relBE*, *mazEF* etc.) have a much broader phylogeny (34). We conclude that *ldr* and *hok*/*sok* loci are confined to enteric and closely related bacteria.

The genomic localizations of *hok*/*sok* and *ldr* loci on two *E.coli* strains and 10 additional enteric pathogen species are shown in Figure 6 and Supplementary Figure S1. For comparison, the locations of protein-regulated TA loci were also included. The *ldr* loci tend to cluster, often on directly opposite chromosomal locations (i.e. in the four *E.coli* species and in one *S.flexneri* species). In contrast, *hok*/*sok* and protein-regulated TA loci were almost randomly scattered on the chromosomes, except in the case of *Photorhabdus luminiscens*, which has three clustered *hok*/*sok* loci and a bewilderingly large number of protein-regulated TA loci (50), many of which are clustered at the terminus (*terC*) region. In *E.coli* K-12, protein-regulated TA loci function as stress-response elements that regulate cellular metabolism in starvation conditions or as genetic stabilization elements that increase the genetic stability of the chromosome segments on which they reside (35). The cellular functions of the chromosome-encoded *hok*/*sok* and *ldr* loci are not yet known but their presence in large numbers is consistent with a role as genetic stabilization elements (20).

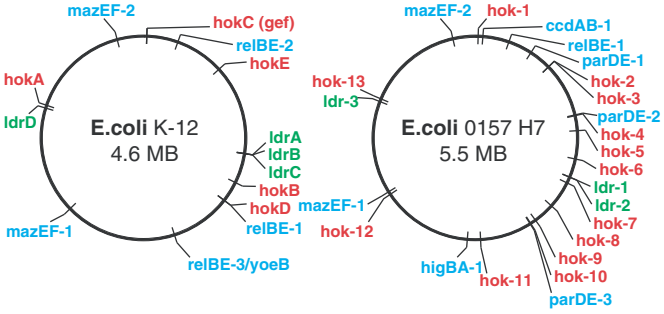

Figure 6. Localization of *hok*/*sok* (red) and the *hok*/*sok*-like *ldr* loci (green) on two *E.coli* chromosomes. Protein-regulated TA loci (blue) were included for comparison (34). The genes were found using tblastn (Supplementary Table S1) and annotated with VectorNTI.

DISCUSSION

Three conclusions can be drawn from the results of these experiments. First, the data support the sense/antisense model for *hok* mRNA::Sok-RNA interactions. Second, anti Sok PNAs are bactericidal to *E.coli* containing a *hok*/*sok* plasmid. Third, the results demonstrate that antisense agents can be used as competitive inhibitors to study RNA::RNA interactions in bacteria. Furthermore we extend the previous knowledge of RNA TA systems distribution in Enterobacteria.

The *hok*/*sok* TA system is a paradigm for sense/antisense RNA interactions (Figure 1). Here we show that anti Sok PNAs selectively kill *hok*/*sok*-carrying *E.coli* cells. Also, anti Sok PNAs induced 'ghost' cell morphology and accumulation of the mature, 3′ end truncated *hok* mRNA. All of these effects are characteristic of Hok toxin-mediated cell killing. Together, the results support the model of sense/antisense RNA interaction in the *hok*/*sok* TA plasmid stability system in cells.

The presence of *hok* and other toxin-encoding genes in many free-living enteric bacteria (Figure 6, Supplementary Figure S1 and Table S1) provides possibilities to induce bacterial suicide. The basic idea is to target the antitoxin inside cells to release expression or activity of the endogenous toxin. The cell-killing activity of Hok toxin is well established, and purified Hok toxin is bactericidal when electroporated into *E.coli* (36). Therefore, *hok* induction should kill *E.coli*. The challenge is to develop molecules that can enter *E.coli* and release *hok* mRNA for translation.

Surprisingly, the anti Sok PNAs caused a greater than five orders of magnitude reduction in viable cell counts at low micromolar concentrations. The high level of cell-killing observed indicates that the anti Sok PNAs are bactericidal and that the vast majority of cells retain the plasmid and capacity to produce Hok toxin during treatment. This suggests that effective competitive inhibitors of antitoxin RNAs can be designed and with further improvement may provide effective new antimicrobials. Recent studies using peritoneal infections in mice suggest that antisense agents based on PNA and morpholino chemistry are effective antimicrobials against *E.coli* when targeting essential genes (28,37). In the case of PNA and other types of antisense oligomers, further progress is needed to improve toxicity, distribution and other drug properties to provide effective inhibitors for the clinic. We speculate that antitoxin RNAs may provide more accessible or sensitive targets relative to growth essential genes. Also, the emergence of drug resistance would seem unlikely as the target antitoxin structure is constrained by a need to maintain recognition with the toxin encoding mRNA. Plasmid loss would provide an obvious resistance mechanism; however, the level of cell killing observed compares well with that of conventional antimicrobials, and targeting chromosome-encoded *hok*/*sok* or *ldr* loci would circumvent the problem of resistance by loss of the TA-carrying plasmid. Also we found that the binding-rate of PNA::Sok-RNA interaction is rapid, but slower than that for *hok* mRNA::Sok-RNA interactions. This result suggests that an excess of PNA relative to *hok* mRNA is needed to out-compete the native interactions in cells; however, a slow dissociation rate for PNA::Sok-RNA complexes and PNA accumulation is expected and this may also aid competition. Therefore, while we have observed *hok* activation in cells with the present inhibitor design, it may be possible to more efficiently trigger Hok-mediated cell death by using antisense agents with faster binding kinetics, although we suspect that cell delivery will remain the major limiting factor in this approach.

Many TA loci are chromosomal in pathogenic *E.coli* (38). Previously, it was shown that the *hok*/*sok* TA gene stability element is widespread in *E.coli* plasmids (39) and that the *E.coli* genome encodes five *hok*/*sok* homologous loci (32). Using database searches we extend here these previous analyses to additional enteric bacteria and the results show a surprising abundance of homologous and analogous TA loci in pathogen genomes. Interestingly, while *hok*/*sok* loci in *E.coli* K-12 are inactive, those in the pathogenic Ochmann ECOR collection are active. However, it is important to consider that single copy chromosome-encoded toxin genes may be more difficult to activate than plasmid copies.

RNA::RNA interactions are inherently difficult to study in cells. One general problem is that sequence mutagenesis alters both sequence and higher order folding, which could affect RNA expression, maturation and RNA half-life. Also, RNA interactions are transient and often involve RNA isoforms, as exemplified by the *hok*/*sok* system. Therefore, additional methods to probe RNA interactions in living cells are needed. Competitive inhibition using antisense agents has been used to sequester microRNAs in mammalian cells (40), and the same basic approach should prove helpful in studies of RNA interactions in bacteria. Here we show that PNA can be used to competitively inhibit RNA::RNA interactions in bacteria, and the results suggest that this general approach using antisense agents and possibly a similar approach using expressed RNAs can be applied to study predicted RNA interactions in bacteria.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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