Molecular Microbiology (2008) 70(5), 1076–1093 ■
doi:10.1111/j.1365-2958.2008.06394.x
First published online 15 August 2008

Repression of small toxic protein synthesis by the Sib and OhsC small RNAs

Elizabeth M. Fozo,[1] Mitsuoki Kawano,[1†] Fanette Fontaine,[1] Yusuf Kaya,[2] Kathy S. Mendieta,[1] Kristi L. Jones,[2] Alejandro Ocampo,[2] Kenneth E. Rudd[2*] and Gisela Storz[1]**

[1]*Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD, USA.*

[2]*Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, Miami, FL, USA.*

Summary

The sequences encoding the QUAD1 RNAs were initially identified as four repeats in *Escherichia coli*. These repeats, herein renamed SIB, are conserved in closely related bacteria, although the number of repeats varies. All five Sib RNAs in *E. coli* MG1655 are expressed, and no phenotype was observed for a five-*sib* deletion strain. However, a phenotype reminiscent of plasmid addiction was observed for overexpression of the Sib RNAs, and further examination of the SIB repeat sequences revealed conserved open reading frames encoding highly hydrophobic 18- to 19-amino-acid proteins (Ibs) opposite each *sib* gene. The Ibs proteins were found to be toxic when overexpressed and this toxicity could be prevented by coexpression of the corresponding Sib RNA. Two other RNAs encoded divergently in the *yfhL-acpS* intergenic region were similarly found to encode a small hydrophobic protein (ShoB) and an antisense RNA regulator (OhsC). Overexpression of both IbsC and ShoB led to immediate changes in membrane potential suggesting both proteins affect the cell envelope. Whole genome expression analysis showed that overexpression of IbsC and ShoB, as well as the small hydrophobic LdrD and TisB proteins, has both overlapping and unique consequences for the cell.

Accepted 31 July, 2008. For correspondence. *E-mail krudd@miami.edu; Tel. (+1) 305 2436 055; Fax (+1) 305 2433 955; **E-mail: storz@helix.nih.gov; Tel. (+1) 301 4020 968; Fax (+1) 301 4020 078. †Present address: Omics Science Center, RIKEN Yokohama Institute, Kanagawa 230-0045, Japan.

Introduction

The identification of small, non-coding RNAs (sRNAs) in all organisms has increased dramatically in recent years (reviewed in Altuvia, 2007; Livny and Waldor, 2007). Computational as well as experimental evidence suggests that at least 70 sRNAs are transcribed from the *Escherichia coli* MG1655 genome (reviewed in Storz and Gottesman, 2006). A few of the characterized sRNAs bind proteins and modify their activities. The other characterized sRNAs function by base pairing with target mRNAs. Base pairing leads to changes in the stability and/or translation of the target. The majority of the base-pairing sRNAs in *E. coli* act on targets not encoded in the same genetic region and only have limited complementarity with their targets. However, two sRNAs, RdlD and SymR, control the expression of proteins encoded on the opposite strand and thus are perfectly complementary to the target mRNA (Kawano *et al*., 2002; 2007). In addition, one sRNA, IstR, is encoded in the same intergenic region as its *tisB* mRNA target and shares a 23-nucleotide stretch of complementarity with this target (Vogel *et al*., 2004). Interestingly RdlD, SymR and IstR all control the translation of proteins (LdrD, SymE and TisB respectively) whose overexpression is toxic to the cell.

Most genes encoding sRNAs in *E. coli* are unique sequences; however, the sequences encoding the QUAD1 RNAs were initially identified as four repeat elements in the intergenic regions of *E. coli* (Rudd, 1999). These sequences were predicted to encode sRNA molecules as they contained potential –35 and –10 promoter elements, but no ribosome binding site or open reading frame (ORF). Subsequent studies demonstrated transcription of these genes in MG1655 and identified homologous sequences in *Shigella* and *Salmonella* species (Argaman *et al*., 2001; Rivas *et al*., 2001; Wassarman *et al*., 2001; Hershberg *et al*., 2003). Two of the QUAD repeats, QUAD1a and QUAD1b, are located in tandem in the intergenic region between *yegL-mdtA*. The other two QUAD1 sequences, QUAD1c and QUAD1d, are located in the *ygfA-serA* and *yqiK-rfaE* intergenic regions, respectively, distant from each other and the QUAD1ab region.

We were interested in determining the function of the sRNAs encoded by the QUAD1a, QUAD1b, QUAD1c and QUAD1d repeats as well as a fifth repeat identified in this

study. As the number of these homologous sequences can vary in other *E. coli* strains, we propose to rename the repeats SIBs for short, intergenic, abundant sequences. We confirmed that all five Sib RNAs are expressed in MG1655. Furthermore, although the deletion of all *sib* genes was not detrimental under any of the conditions tested, we found multicopy plasmids carrying the SIB sequences could not be introduced into the corresponding deletion strain unless the plasmid-encoded Sib RNA was expressed at high levels. Upon re-examination of the *sib* gene sequences we discovered the presence of a small ORF encoded opposite each Sib RNA. These genes, termed '*ibs*' for induction brings stasis, encode very hydrophobic 18- to 19-amino-acid proteins. The Ibs are highly conserved in all species that contain the *sib* genes. Overexpression of the Ibs proteins is lethal to the cell, but this can be prevented by expression of the corresponding antisense Sib RNA.

We also examined another pair of RNAs, previously referred to as RyfB and RyfC, which are encoded divergently in the same intergenic region but share a 19-nucleotide region of complementarity (Kawano *et al*., 2005). We found the RyfB RNA also encodes a short hydrophobic ORF (26 amino acids in length) that is lethal upon overproduction and thus we renamed this gene *shoB*. The RyfC RNA regulates levels of ShoB in the cell, and is now denoted OhsC for oppression of hydrophobic ORF by sRNA.

High levels of both IbsC and ShoB lead to changes in membrane potential suggesting that both act at the membrane, consistent with the hydrophobic nature of the proteins. In addition, whole genome expression analysis upon overexpression of IbsC and ShoB as well as LdrD and TisB, two other short hydrophobic proteins that are toxic upon overexpression, revealed that these small proteins all induce a common set of genes. However, additional genes are strongly induced or repressed by subsets of these proteins indicating that the small, hydrophobic proteins do not act in an identical fashion.

Results

The numbers of SIB repeats vary between species

The QUAD1 repeats were initially identified as four highly homologous sequences located in three separate intergenic regions of the *E. coli* chromosome (Rudd, 1999). Expression of the four QUAD1 RNAs was reported in separate studies (Argaman *et al*., 2001; Rivas *et al*., 2001; Wassarman *et al*., 2001). As most sRNAs of *E. coli* are unique sequences, we sought to determine the function of these duplicated sRNA genes. First, we re-examined the number of QUAD1 repeats encoded by *E. coli*. A revision of the MG1655 sequence (Hayashi *et al*., 2006) revealed a 375-nucleotide region had been omitted in the *yqiK-rfaE* intergenic region, where the QUAD1d sequence is located. A fifth sequence homologous to the QUADs was identified in this region (Fig. 1). As there are five of these repeat sequences in MG1655, the original name QUAD1 is a misnomer. We propose to rename these sequences SIBs for short intergenic abundant sequences with the individual repeats designated as SIBa, SIBb, SIBc, SIBd and SIBe following the original QUAD1a, QUAD1b, QUAD1c and QUAD1d nomenclature.

Upon searching for homologous sequences in other *E. coli* strains, we noted that the number of SIB repeats varies between strains (Table 1). Two sequenced EHEC strains, *E. coli* 0157:H7 EDL933 and *E. coli* 0157:H7 VT-2 Sakai, contain seven SIB repeats, with one additional repeat in the *yegL-mdtA* intergenic region and the other additional repeat in the *ygfA-serA* region. On the other hand, the genomes of *E. coli* CFT073 and other UPEC/APEC strains are predicted to have only four SIB repeats, with a single repeat in the *yqiK-rfaE* region. It is worth noting though that the 'missing' *sib* genes of UPEC/APEC strains occur in the *yqiK-rfaE* intergenic region, the same region where the original sequencing error occurred in MG1655. The genomes of *Shigella*, *Salmonella* and *Citrobacter* also contain SIB repeats of varying numbers (Table 1). Repeats in the same intergenic region are more homologous to each other than with the repeats in other

Table 1. Number of *sib* genes in various enteric strains.

Strain	Number of genes	Genes present[a]
E. coli K-12 MG1655 *E. coli* K-12 W3110	5	A, B, C, D, E
E. coli 0157:H7 EDL933 *E. coli* 0157:H7 VT-2 Sakai	7	A, B, B2, C, C2, D, E
E. coli CFT073 (UPEC)[b] *E. coli* 01:K1 (APEC)	4	A, B, C, D
Shigella boydii Sb227 *Shigella sonnei* Ss046	7	A, B, B2, C, C2, D, E
Shigella flexneri 2a 2457T[c]	5	C, C2, D, D2, E
Shigella dysenteriae Sd197	3	C, D, E
Salmonella typhimurium LT2[d]	3	A, C, D
Citrobacter koseri ATCC BAA-895	1	A

a. The A, B, C, D, E designation comes from the original QUAD nomenclature with A and B flanked by *yegL* and *mdtA*, C flanked by *ygfA* and *serA* and D and E flanked by *yqiK* and *rfaE*. '2' refers to a duplicated gene.
b. Two additional UPEC strains, 06:K15:H31 (str.536) and UT189, contain the same four genes.
c. Two additional *S. flexneri* strains, 2a str. 301 and 5 str. 840f1, contain the same five genes.
d. *Salmonella enterica* serovar Typhi CT18, *S. enterica* serovar Typhi LT2, *S. enterica* Paratypi and *S. enterica* Choleraesuis contain the same three genes.

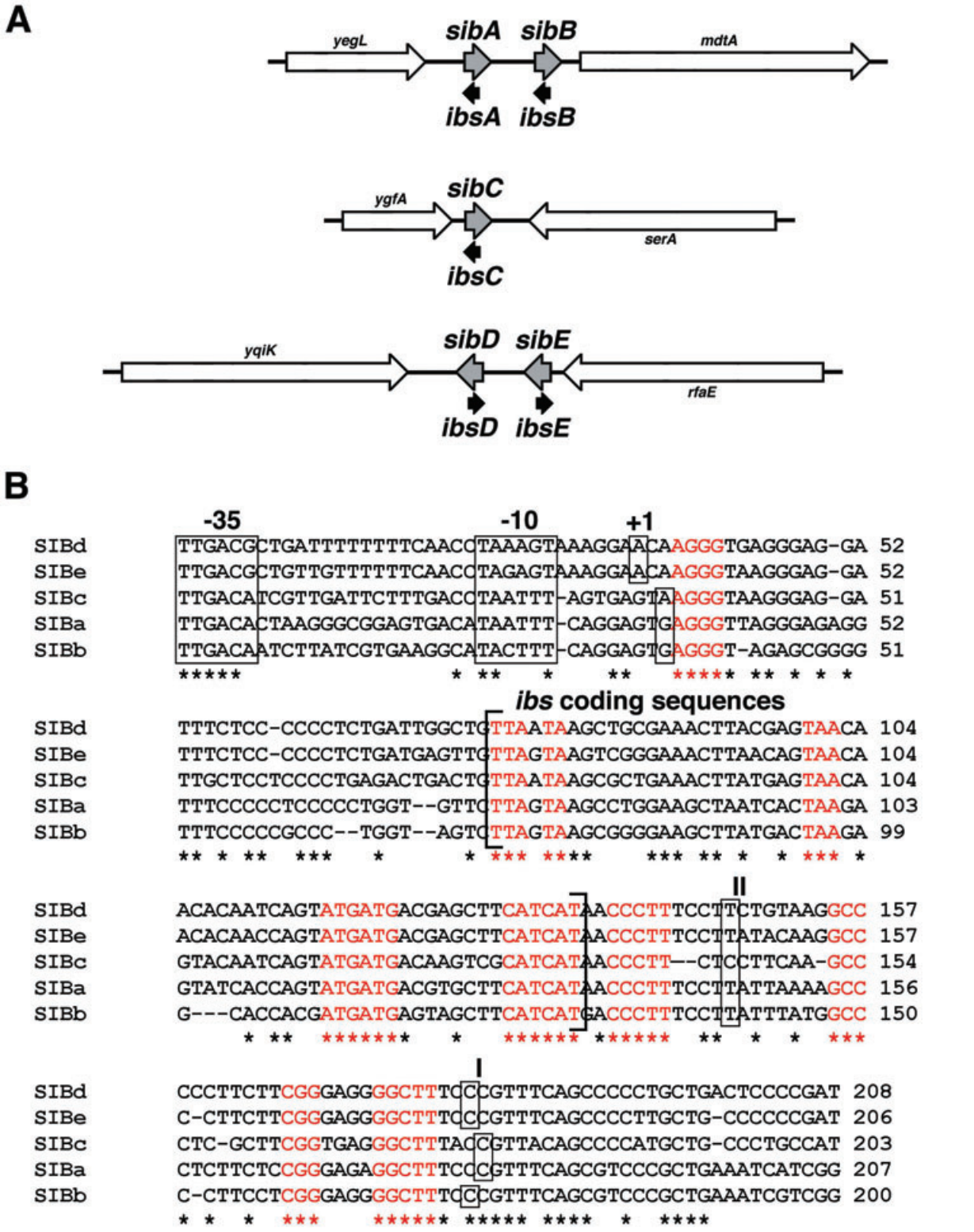

Fig. 1. *sib* gene homology and genetic organization.
A. Genetic organization of the *sib* genes. The Ibs ORFs are indicated by black arrows.
B. CLUSTALW alignment (with minimal manual alignment) of the five SIB repeats from *E. coli* MG1655. Red sequences are those conserved in all SIB repeats found in enteric bacteria. The predicted –35 and –10 promoter elements for the Sib RNAs are shown. The +1 indicates transcriptional start site as determined by 5′ RACE. The two distinct 3′ ends mapped by 3′ RACE are also indicated by I and II. The Ibs ORFs are indicated by brackets.

regions indicating that they are likely to be more recent duplications.

An alignment of the SIB repeats shows that specific nucleotides are absolutely conserved among the enteric species (Fig. 1). Using the M-fold program to predict the secondary structures of the encoded RNAs, many of the conserved nucleotides are predicted to be in stem structures that are conserved across all strains (Fig. S1). Thus, there is substantial sequence and potentially structural conservation for all Sib RNAs.

All five sib *genes in* E. coli *MG1655 are expressed*

Northern analysis was carried out to determine whether *E. coli* MG1655 expressed all five predicted Sib RNAs. To verify that the five oligonucleotide probes were specific to a given *sib* gene, we also probed total RNA isolated from mutant strains carrying single deletions (see below) of each of the *sib* genes. As shown in Fig. 2, transcripts specific to each of the five *sib* genes were detected during growth in rich as well as minimal medium although overall the levels in minimal medium tend to be higher. Two transcripts were observed per *sib* gene. The patterns of expression and the ratio of the shorter and longer transcripts varied to some extent; in general, the longer transcript tended to dominate for RNA isolated from stationary phase cells. In the case of the SibD and SibE RNAs, the longer transcript was expressed more highly in most conditions. Deletion of the one or more *sib* genes did not appear to alter the expression pattern of the other genes (data not shown). Fusions of the *sibC* and *sibE* promoter to the *lacZ* reporter were also constructed. We observed high levels of β-galactosidase activity under all conditions tested, although again the levels were somewhat higher in minimal compared with rich medium (data not shown).

Based on the Northern analysis, each *sib* gene produces two distinct transcripts approximately 150 and 110 nucleotides in length. To map the ends of the two observed transcripts, we carried out 5′ and 3′ RACE analysis. These assays showed that there is a single transcriptional start site and two distinct 3′ ends for each RNA. The lengths range from 136 to 145 nucleotides for the longer transcripts and 104–112 nucleotides for the shorter transcripts (Fig. 1; Tables S1 and S2). There is no obvious terminator corresponding to either of the 3′ ends, although the end corresponding to the longer transcript is adjacent to a long predicted stem-loop. The 3′ end corresponding to the shorter transcript is in a region predicted to be single stranded so we suggest this product may be a result of processing.

Effects of sib *gene deletions*

We generated a series of strains carrying single *sib* gene deletions and various combinations of deletions, such as Δ*sibAB*, Δ*sibDE* and Δ*sibABCDE*, using phage recombination (Court *et al*., 2003). Growth of these deletion strains was monitored in both rich and minimal medium supplemented with various carbon sources and at different temperatures. Under these conditions, there were no discernible differences between the wild-type control and any of the *sib* deletion strains (data not shown).

Effects of Sib RNA overproduction

To examine the effects of Sib RNA overproduction, the individual genes were placed under the control of the P_{BAD} promoter of plasmid pAZ3 (Kawano *et al*., 2007). The wild-type strain was readily transformed with each overexpression construct, and no significant growth differences were observed between strains carrying the empty vector or the plasmids with inserts, with or without the addition of the inducing agent arabinose. This was not the case when the constructs were transformed into their respective *sib* gene deletion strains. For example, successful transformations of a plasmid bearing P_{BAD}-*sibE* into the Δ*sibE* strain occurred only when agar plates were supplemented with 0.1% and 0.2% arabinose (Table 2 and data not shown). No colonies were obtained when cells were plated on 0.02% and 0.002% arabinose even

Table 2. Efficiency of transformation into different *sib* deletion strains.[a]

Vector	Arabinose	MG1655	Δ*sibA*	Δ*sibC*	Δ*sibE*
pAZ3	–	1.6×10^8	5.0×10^7	2.5×10^8	1.6×10^8
	+	1.8×10^8	5.2×10^7	2.9×10^8	1.9×10^8
pAZ3-*sibA*	–	7.8×10^6	3.6×10^7	2.6×10^6	6.0×10^5
	+	8.8×10^6	2.6×10^7	4.1×10^6	5.0×10^5
pAZ3-*sibC*	–	1.3×10^6	3.8×10^6	No colonies	6.0×10^5
	+	1.3×10^6	2.1×10^6	1.0×10^7	1.5×10^6
pAZ3-*sibE*	–	1.1×10^6	4.0×10^6	2.4×10^6	No colonies
	+	2.7×10^6	2.2×10^6	3.8×10^6	2.0×10^6

a. Cells were transformed with 1 ng of DNA. Results similar to those observed for Δ*sibC* and Δ*sibE* were obtained for Δ*sibB* and Δ*sibD*.

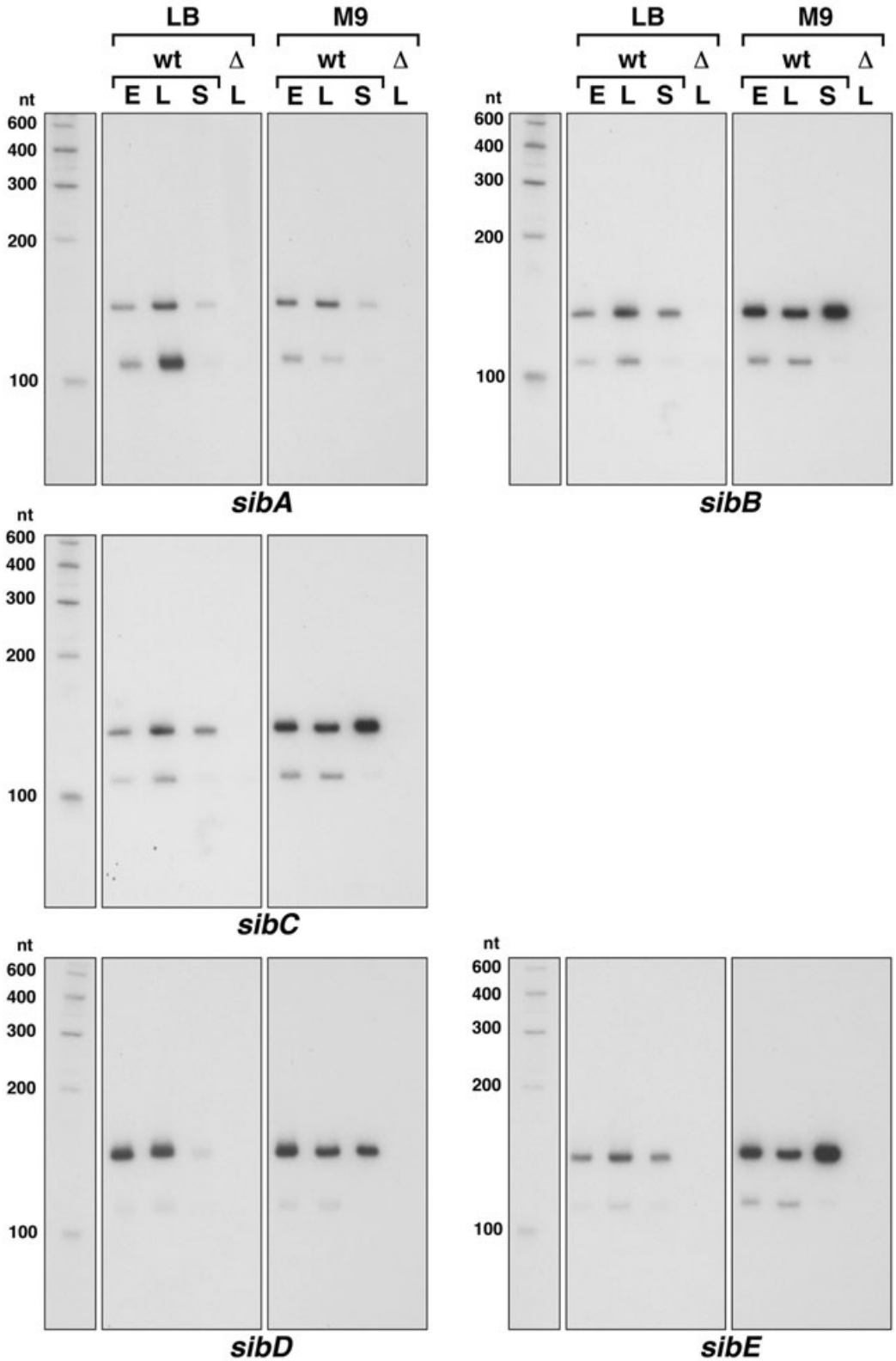

Fig. 2. Expression of the *sib* genes. Total RNA (5 μg) isolated from MG1655 cells grown to $OD_{600} \approx 0.4$ (E), $OD_{600} \approx 1.8$ (L) and $OD_{600} \approx 5.0$ (overnight, S) in LB medium and from MG1655 cells grown to $OD_{600} \approx 0.4$ (E), $OD_{600} \approx 1.8$ (L) and $OD_{600} \approx 2.5$ (overnight, S) in M9 media supplemented with 0.2% glucose were loaded in each lane. The fourth lane in each corresponds to the specific *sib* deletion strain. No additional bands were observed even upon overexposure.

though these concentrations could induce expression of SibE from the plasmid (data not shown). In contrast, P_{BAD}-*sibA*, P_{BAD}-*sibB*, P_{BAD}-*sibC* and P_{BAD}-*sibD* plasmid transformants of the same Δ*sibE* strain could be obtained with and without the inducing agent (Table 2 and data not shown). This was found to be the case for every *sib* deletion strain except Δ*sibA*; transformants of Δ*sibA*, as well as Δ*sibAB*, with the P_{BAD}-*sibA* plasmid could be obtained without the addition of arabinose (Table 2 and data not shown).

To test whether these observations were plasmid-specific, *sibE* was placed under control of the $P_{LlacO-1}$ promoter of plasmid pBR-plac (Guillier and Gottesman, 2006). As was the case with the P_{BAD} plasmids, successful transformation into Δ*sibE* required high (0.1, 0.5 or 1 mM) concentrations of IPTG even though lower (less than 0.1 mM) concentrations of IPTG could induce expression of the SibE RNA (data not shown). This finding suggests that plasmid features such as the antibiotic resistance genes are not responsible for the inability to transform a specific Sib overexpression plasmid into the corresponding deletion strain.

Synthesis of toxic Ibs proteins

The need for high levels of the Sib sRNAs for plasmid transformation into the deletion strains was reminiscent of what has been reported for some plasmid addiction modules. In these cases, cells carrying plasmids encoding a stable toxin and unstable antitoxin are killed when the plasmid is lost and the antitoxin can no longer be synthesized (reviewed in Hayes, 2003; Gerdes *et al*., 2005). For the well-characterized Hok-Sok plasmid addiction module of plasmid R1, the toxin is the 52-amino-acid Hok protein and the antitoxin is the Sok antisense RNA (reviewed in Gerdes and Wagner, 2007). The requirement for high levels of *sib* gene expression from plasmids in the deletion strains led us to hypothesize that the Sib RNAs might regulate the expression of a toxic protein. Upon closer examination of the *sib* genes, we noted a conserved small ORF encoded opposite each *sib* gene (Fig. 1). Whole genome expression analysis using tiled arrays for *E. coli* showed that indeed transcripts were expressed at low levels from the strand opposite the *sib* genes (data not shown). The antisense genes were predicted to encode proteins of 18–19 amino acids containing many hydrophobic residues (Fig. 3A). The small hydrophobic proteins are also conserved in *Shigella*, *Salmonella* and *Citrobacter*, and when the protein sequences are used in TBLASTN searches homologues can even be found in *Haemophilus* and *Mannheimia*.

To determine whether the small ORFs might be toxic and thus be responsible for the plasmid transformation defect observed with the Δ*sib* strains, we cloned the ORFs for the SIBa, SIBc and SIBe repeats together with approximately 70 nucleotides upstream of the predicted start codons behind the arabinose-inducible promoter of pAZ3. In liquid cultures with low concentrations of arabinose, we observed a cessation of growth with slow recovery for strains carrying these plasmids (data not shown). We thus propose the name *ibs* (induction brings stasis) for the genes, with *ibsA* encoded opposite *sibA*, etc. At higher concentrations of the inducing agent, there was an irreversible stop in growth with a significant drop in the ability of the strains to form colonies (Fig. S2 and data not shown). In addition, strains carrying these plasmids were unable to growth on agar plates supplemented with 0.2% arabinose (Fig. 3B). To confirm that overexpression of the Ibs proteins was responsible for the lack of growth, the fourth codon of each clone was mutated to TAG. Overexpression of these mutant constructs did not impair growth in a wild-type strain; however, a *supF* suppressor strain was still susceptible to the toxicity of the mutant proteins (Fig. 3B). In contrast to the case with the P_{BAD}-*sibA* clone, the P_{BAD}-*ibsA* clone gave a phenotype that was similar to the other clones.

We propose that the toxicity of the P_{BAD}-*sibB*, P_{BAD}-*sibC*, P_{BAD}-*sibD* and P_{BAD}-*sibE* plasmids in the respective deletion strains is due to transcription of the *ibsB*, *ibsC*, *ibsD* and *ibsE* genes encoded on the respective plasmids. Without expression of the corresponding *sib* gene, the cells were unable to grow. In the wild-type strains, expression of the chromosomal copy of *sib* genes was sufficient to repress the *ibs* mRNA expressed from the plasmid.

Synthesis of the toxic ShoB protein

The realization that the *sib-ibs* regions encoded both a small toxic protein and the corresponding antitoxin sRNA prompted us to examine other intergenic regions encoding two RNAs. One such region is the *yfhL-acpS* interval encoding the 280- to 320-nucleotide RyfB and 60- to 63-nucleotide RyfC RNAs (Kawano *et al*., 2005). These two RNAs are encoded divergently on opposite strands but share 19 nucleotides of complementarity suggesting possible regulation by base pairing (Fig. 4A). Upon examination of the *ryfB* sequence, we predicted that it could encode a hydrophobic protein of 26 amino acids (Fig. 4B). As was carried out for the IbsA, IbsC and IbsE proteins, the region corresponding to the entire RyfB RNA was cloned in pAZ3. A strain harbouring this plasmid was unable to grow with arabinose (Fig. 4C and Fig. S2), indicating that, like IbsA, IbsC and IbsE, high levels of the 26-amino-acid protein are toxic. Upon mutation of the sixth codon of the ORF to a stop codon, the strain could survive overexpression. Again, a *supF* suppressor strain restored the toxicity of the mutant clone under conditions of arabinose induction (Fig. 4C). As *ryfB* encodes a short hydrophobic ORF, we have renamed

A

```
IbsD     MMKLVIILIVLLLVSFAAY 19
IbsC     MMRLVIILIVLLLISFSAY 19
IbsE     MMKLVIILVVLLLLSFPTY 19
IbsA     MMKHVIILVILLVISFQAY 19
IbsB     MMKLLII-VVLLVISFPAY 18
         **: :** ::**::** :*
```

B

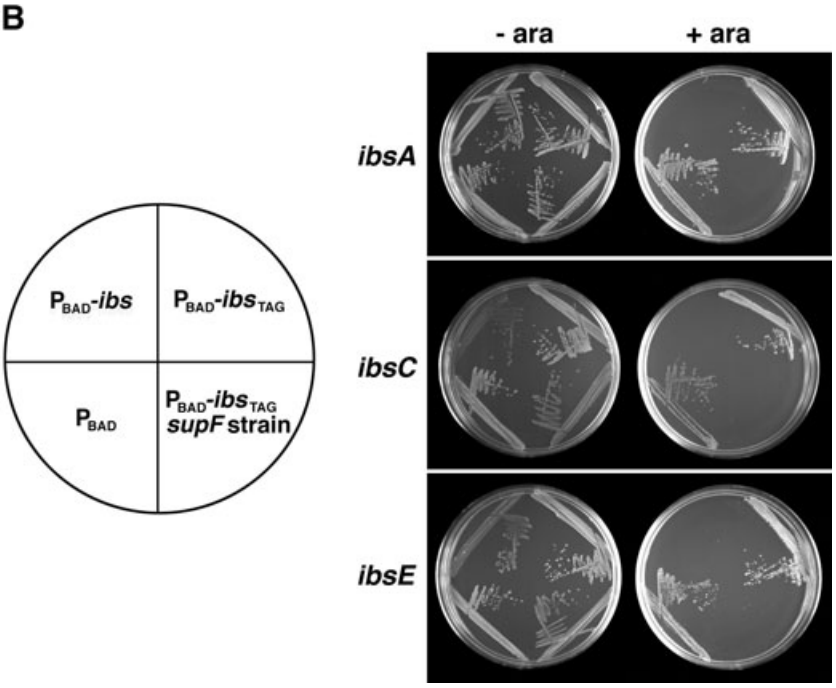

Fig. 3. Overexpression of the Ibs proteins is toxic.
A. CLUSTALW alignment of the Ibs sequences from *E. coli* MG1655.
B. Growth of *E. coli* harbouring either empty vector (pAZ3) pAZ3-*ibsA*, pAZ3-*ibsA*$_{TAG}$, pAZ3-*ibsC*, pAZ3-*ibsC*$_{TAG}$, pAZ3-*ibsE* or pAZ3-*ibsE*$_{TAG}$ grown in the presence or absence of 0.2% arabinose. The orientation of the strains is shown at left.

A

B

```
ShoB     MTDCRYLIKRVIKIIIAVLQLILLFL 26
```

C

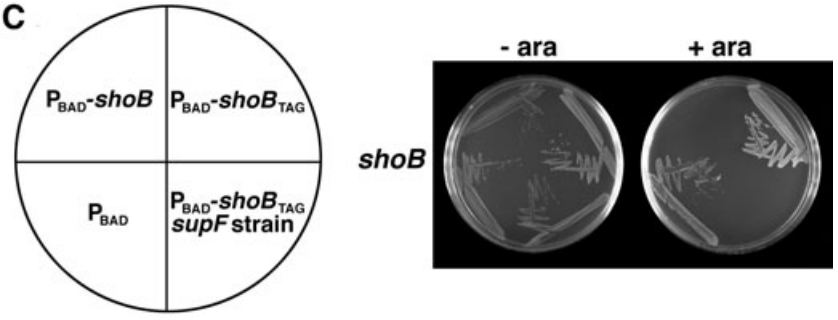

Fig. 4. Overexpression of the OhsC protein is toxic.
A. Genetic orientation of *shoB* (formerly *ryfB*) and *ohsC* (formerly *ryfC*). The ShoB ORF is indicated by the black arrow and the 19-nucleotide regions of complementarity between *shoB* and *ohsC* are indicated by the white boxes.
B. Sequence of ShoB from *E. coli* MG1655.
C. *E. coli* harbouring either pAZ3, pAZ3-*shoB*, pAZ3-*shoB*$_{TAG}$ grown in the presence or absence of 0.2% arabinose. The orientation of the strains is shown at left.

this gene *shoB* and *ryfC* is denoted *ohsC* (oppression of hydrophobic ORF by sRNA). Unlike the more broadly conserved *ibs-sib* loci, the *shoB* and *ohsC* genes appear to be confined to *E. coli* and *Shigella*.

SibC and OhsC RNA repression of IbsC *and* ShoB *expression*

The complete complementarity between the *sib* and *ibs* transcripts and the 19 nucleotides of complementarity between the *ohsC* and *shoB* transcripts led us to propose that the Sibs and OhsC RNAs repress expression of the potentially toxic Ibs and ShoB proteins by base pairing with their respective mRNAs. We examined the effects of the SibC RNA on *ibsC* expression by deleting the *sibC* promoter and monitoring the levels of the *ibsC* mRNA (Fig. 5A). SibC clearly has a negative effect on *ibsC* transcript levels. In fact, by Northern analysis, we were only able to detect the *ibsC* mRNA in a *sibC* promoter deletion strain. The levels of the *ibsC* transcript were similar under the four growth conditions tested discounting the higher background for the sample isolated from cells grown to stationary phase in minimal medium. We also deleted the *ohsC* gene and examined *shoB* mRNA levels. In contrast to what we observed for the *ibsC* mRNA, we did not detect a difference in *shoB* mRNA levels in the presence or absence of the OhsC RNA (Fig. S3).

To further test OhsC RNA regulation of *shoB* expression, we constructed several translational fusions of the *shoB* promoter and 5′ untranslated sequence with the *lacZ* reporter gene (Fig. 5B). Multiple 5′ ends have been mapped for the ShoB transcript (Kawano *et al*., 2005). When the *shoB* promoter together with the longest 5′ sequence was fused to *lacZ*, no β-galactosidase activity was detected (data not shown), possibly due to inhibitory secondary structures formed by the long 5′ untranslated region. We also constructed a fusion in which the sequence between the first and second mapped 5′ ends was deleted (nucleotides 2 698 358–2 698 399) leaving the region of complementarity (nucleotides 2 698 294–2 698 313). The corresponding fusion gave low, but measurable, levels of β-galactosidase activity. Under all growth conditions tested, the levels of β-galactosidase activity were more than twofold higher in strains deleted for *ohsC* compared with the wild-type strain (Fig. 5C), consistent with the hypothesis that the OhsC RNA negatively regulates translation of the ShoB message.

SibC and OhsC RNA repression of IbsC and ShoB toxicity

Our data suggested that the Sib and OhsC RNAs repress Ibs and ShoB protein synthesis, respectively, thereby limiting the toxicity of the small hydrophobic proteins. This was tested directly by cloning the *ibsC* and *shoB* genes behind the P_{BAD} promoter of a derivative of pBAD33 (Guzman *et al*., 1995) and cloning the corresponding SibC and OhsC RNA genes behind the IPTG-inducible $P_{LlacO-1}$ promoter on the compatible plasmid pBR-plac. The *ibsC* stop codon mutation described above was also introduced into the *sibC* clone to eliminate potential expression of the IbsC protein. Cells carrying both the small protein-expression plasmid and the corresponding sRNA-expression plasmid were grown to early exponential phase ($OD_{600} \approx 0.1$). The culture was split and IPTG was added to half of each culture to induce expression of the SibC and OhsC RNAs. After 30 min, arabinose was added to all cultures to induce expression of the IbsC and ShoB proteins. As shown in Fig. 5D, induction of IbsC and ShoB led to cell stasis of the cultures to which no IPTG was added, while the cultures treated with IPTG continued to grow. Thus the SibC and OhsC RNAs, respectively, prevent the toxic effects of IbsC and ShoB overproduction. The results also show that the SibC and OhsC RNAs do not need to be encoded *in cis* in order to repress IbsC and ShoB synthesis.

Reduction in membrane potential upon IbsC and ShoB overexpression

The features of the *ibs* and *shoB* genes are very similar to the *hok* gene of plasmid R1 (Gerdes and Wagner, 2007); all encode small hydrophobic proteins that are toxic upon overexpression and whose synthesis is repressed by sRNAs that have extensive complementarity with the corresponding mRNA. The Hok protein has been suggested to form pores in the membrane given that high levels of the protein dissipate the proton motive force (Gerdes *et al*., 1986). To examine whether high levels of the IbsC and ShoB proteins are having similar detrimental effects on the membrane, we tested the ability of cells to take up the dye $DiBAC_4(3)$ [bis-(1,3-dibarbituric acid)-trimethine oxanol]. This dye enters cells, leading to increased fluorescence, upon membrane depolarization (Wickens *et al*., 2000). Cells taken 0, 5 and 20 min after induction of IbsC or ShoB by the addition of arabinose were incubated with $DiBAC_4(3)$ for 20 min and then analysed by flow cytometry (Fig. 6). No changes were observed for the plasmid control pAZ3. In contrast, induction of IbsC or ShoB had rapid and dramatic effects on the membrane integrity; after 5 min of induction, 50% and 98% of cells, respectively, had depolarized membrane and after 20 min of induction 97% and 99% of all cells were depolarized.

Global effects of IbsC, ShoB, LdrD and TisB overexpression

Remnants of the five *hok-sok* modules have been detected on the *E. coli* K-12 genome, although all appear

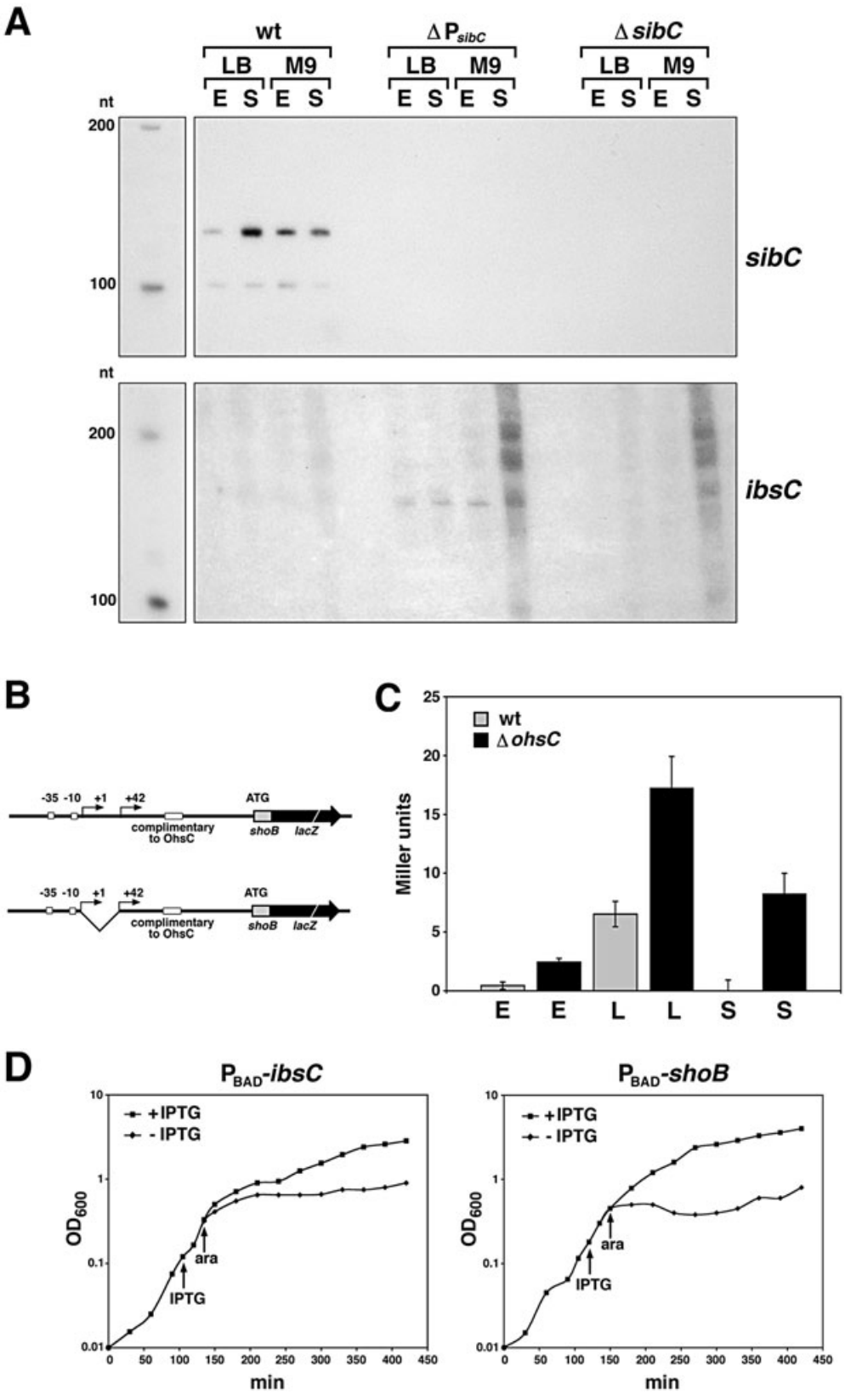

Fig. 5. Repression of IbsC and ShoB synthesis by the SibC and OhsC RNAs.
A. Northern analysis of *ibsC* mRNA levels. Total RNA was isolated from MG1655, ΔP_{sibC} and Δ*sibC/ibsC* cells grown in LB or M9 with 0.2% glucose to $OD_{600} \approx 0.4$ (E) or overnight (S, $OD_{600} \approx 5.0$ in LB and $OD_{600} \approx 2.5$ in M9 glucose).
B. Schematic of *shoB–lacZ* fusions constructed.
C. β-Galactosidase levels of the shoB-lacZ fusion with the short 5′ untranslated region in MG1655 (grey) and a Δ*ohsC* deletion strain (black) grown to $OD_{600} \approx 0.4$ (E), $OD_{600} \approx 1.8$ (L) and overnight (S, $OD_{600} \approx 5.0$) in LB medium. The average (in Miller units) of three independent cultures performed in triplicate is given.
D. Overexpression of the SibC and OhsC RNAs can prevent IbsC and ShoB toxicity. DJ624 (a *mal*::*lacI* derivative of MG1655) was transformed with pBR-plac-*sibC* together with pEF21-*ibsC* or pBR-plac-*ohsC* together with pEF21-*shoB* (shown schematically in Fig. S4). The strains were grown in LB to $OD_{600} \approx 0.1$, the cultures were split and IPTG (1 mM final concentration) was added to half of each culture. After 30 min, arabinose (0.002% final concentration) was added to all samples. OD_{600} was measured over time.

to have degenerated with mutations and transposon insertions (Pedersen and Gerdes, 1999). However, the four *ldr* and the unique *tisB* genes (Kawano *et al*., 2002; Vogel *et al*., 2004) all also encode small hydrophobic proteins whose synthesis is regulated by sRNAs. We confirmed that as for the 19-amino-acid IbsC protein and 26-amino-acid ShoB proteins, ovexpression of the 35-amino-acid LdrD protein and 29-amino-acid TisB protein leads to the inhibition of cell growth and reduction in colony-forming units (cfu) (Fig. S2). We wondered whether the shared properties of the proteins meant that overexpression causes identical changes in the cells. To address this question and also to begin explore the biological functions of these proteins, we carried out whole genome expression analysis after inducing IbsC, ShoB, LdrD or TisB expression for 20 min (the complete data set for three independent experiments is given in Table S3).

Overall, elevated levels of IbsC lead to the largest changes in gene expression; 65 genes were induced more than threefold and 43 genes were repressed more than threefold in three independent experiments. The numbers of genes induced and repressed by ShoB, LdrD and TisB were lower and the fold induction varied more between experiments, especially for ShoB. We do not know the cause of the variation; possibly the levels of ShoB synthesis differed between experiments due to additional levels of regulation. Nonetheless, a number of conclusions can be drawn by examining the expression of operons for which genes are induced ≥ 10-fold (Table 3) or repressed ≤ 5-fold (Table 4) by overexpression of at least one of the proteins. First, a common group of genes is induced by overexpression of all four proteins. Many of these genes encode stress-response or membrane proteins. Most striking among these is the *soxS* mRNA, which encodes a regulator of the superoxide stress response. Northern analysis confirmed that this mRNA indeed is strongly induced by IbsC overexpression as well as to a more limited extent by high levels of ShoB, LdrD and TisB (Fig. 7). Other genes induced by all four proteins encode tryptophanase and proteins involved in maltose transport. These genes are induced by many different stress responses (K.L. Jones and K.E. Rudd, unpublished), and several are regulated by CRP. Some strongly responsive members of the heat shock regulon are also induced by overexpression of all of the small hydrophobic proteins.

Other sets of genes were only induced or repressed by overexpression of subsets of the proteins. For example, overexpression of IbsC and TisB induced many members of the Cpx regulon, such as *degP* and *spy*, which are part of the cell envelope stress response. We also examined *degP* (Fig. 7) and *spy* expression (data not shown) by Northern analysis. While the *degP* and *spy* mRNA levels are clearly induced by IbsC and TisB overexpression, the levels appear to be repressed by LdrD and ShoB overexpression (although this is not reflected in the array data). Among the genes repressed by high levels of both IbsC and ShoB is the *dusB-fis* operon. Some sets of genes were affected by overexpression of only one protein. The *pspABCDE* operon as well as the *pspG* gene, which all encode phage shock proteins, were only induced by elevated IbsC levels. This specific induction was again confirmed by Northern analysis (Fig. 7) where we detected strong induction of the expected 700- and 2100-nucleotide bands reported for the *pspABCDE* operon (Brissette *et al*., 1991).

Together the whole genome expression analysis shows that while overexpression of IbsC, ShoB, LdrD and TisB leads to the induction of a common set of genes, the small proteins also have unique effects. It is interesting to note that many of the genes whose expression is induced encode membrane proteins or are members of the heat shock or envelope stress responses. We suggest that the induction of these genes is a downstream consequence of the changes – to the membrane or other components of the cell – caused by the small, hydrophobic proteins. We do not think the toxic effects of the proteins are exerted through the induced genes, as, for example, overexpression of IbsC is still toxic in a Δ*pspABCDE* Δ*pspG* deletion strain (data not shown). However, the finding that overexpression of some proteins induces unique genes indicates the proteins are not all acting in an identical manner.

Elevated pspABCDE *expression in the absence of SibC RNA repression of* ibsC

While overexpression of IbsC, ShoB, LdrD and TisB from multicopy plasmids clearly has dramatic consequences

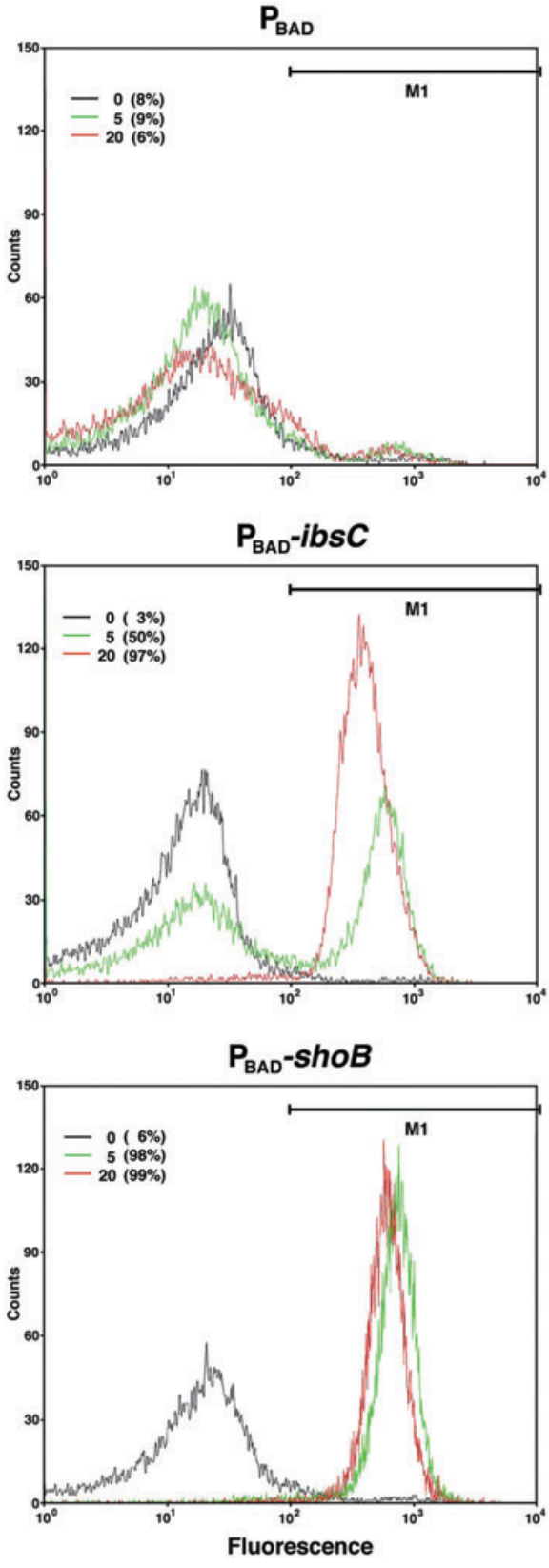

Fig. 6. Changes in membrane polarization upon IbsC and ShoB overproduction. Control cells and cells overexpressing IbsC and ShoB were examined by flow cytometry after staining with $DiBAC_4(3)$, an indicator of membrane depolarization. MG1655 harbouring pAZ3, pAZ3-*ibsC* or pAZ3-*shoB* was grown to exponential phase ($OD_{600} \approx 0.3$). At 0, 5 and 20 min following the addition of 0.2% arabinose, cells were stained with $DiBAC_4(3)$ for 20 min and 15 000 cells were counted. The percentage of cells with a depolarized membrane was calculated from the region denoted M1. The graphs are representative of three repetitions.

for the cell, we wondered whether expression of the proteins from the chromosome could also affect gene expression. To examine the effects of decreased SibC RNA levels and the concomitant increase in *ibsC* mRNA levels, we probed the total RNA samples in Fig. 5A for *pspABCDE* expression. As shown in Fig. 8, the levels of the *pspABCDE* transcript were clearly induced in the *sibC* promoter deletion strain in the LB-stationary-phase culture and even more highly in the M9-exponential culture. No induction was observed for either the wild-type strain or the Δ*sibC* deletion strain in which the *ibsC* gene is also deleted. Thus even low levels of the IbsC protein, which do not have detrimental effects on growth, lead to changes in gene expression.

Discussion

In this work, we characterized the Sib RNAs of *E. coli* MG1655. The sequences encoding these sRNAs were initially referred to as QUAD1a, 1b, 1c and 1d (Rudd, 1999). However, as the number of repeats encoding these sRNAs varies even within *E. coli* strains, we propose to replace the QUAD1 designation with SIB (short, intergenic, abundant sequences). Although we did not observe any growth defects associated with the deletion of the *sib* genes, individually or in combination, we found multicopy plasmids encoding each *sib* gene could not be maintained in a given *sib* deletion strain unless the corresponding Sib RNA was expressed at high levels. Upon closer examination of the *sib* gene regions, we noted the presence of an ORF denoted *ibs* (induction brings stasis) encoding a small hydrophobic protein on the opposite strand of each *sib* gene. We demonstrated that overexpression of Ibs proteins is toxic, and this toxicity is prevented by the complementary Sib RNAs. We also characterized two short transcripts from the *yfhL-acpS* intergenic region. The longer transcript encodes a 26-amino-acid protein, denoted ShoB (short hydrophobic ORF), that is toxic upon overproduction, and the shorter transcript acts as an sRNA, renamed OhsC (oppression of hydrophobic ORF by sRNA), to repress ShoB synthesis by pairing with the *shoB* mRNA.

The organization of the *ibs-sib* genes mirrors that of the *ldr-rdl* genes (Kawano *et al.*, 2002) and the organi-

Table 3. Genes most highly induced upon IbsC, ShoB, LdrD and TisB overexpression.[a]

Gene	Description[b]	Regulation[c]	Average fold induction			
			IbsC	LdrD	TisB	ShoB
soxS	Transcriptional regulator	SoxR, SoxS	67.3	13.7	8.7	8.1
tnaC (*tnaL*)	Tryptophanase	TorR, Crp	26.7	19.6	4.9	23.5
tnaA			34.7	26.1	4.3	20.0
tnaB			4.0	6.2	1.6	3.8
malE	Maltose transport	MalT, Crp	5.8	11.3	3.6	16.3
malF			2.7	4.7	1.3	9.1
malG			2.9	6.0	1.6	12.7
malK	Maltose transport	MalT, Crp	18.6	32.7	2.6	48.6
lamB			5.3	13.3	2.1	19.2
malM			3.3	9.1	1.7	20.0
ibpB	Heat shock	σ^{32}	39.0	24.2	4.6	52.6
ibpA	Heat shock		24.4	20.2	4.1	35.9
htpG	Heat shock chaperone	σ^{32}	11.7	8.2	2.0	16.6
clpB	Protease; chaperone	σ^{32}	11.5	14.3	2.5	24.5
yceP/*bssS*	Regulator		12.9	3.3	2.9	6.8
cpxP (b3913)	Regulator; chaperone	CpxR	17.3	3.2	8.0	5.7
degP (*htrA*)	Protease	CpxR, H-NS	21.0	1.3	2.7	1.7
spy	Periplasmic	CpxR, BaeSR	20.4	1.8	2.9	1.3
ydeH	Unknown	CpxR	49.2	1.1	6.1	1.3
yebE	Unknown	CpxR	44.8	1.6	7.3	2.9
yncJ (b1436)	Unknown; signal peptide		63.1	1.6	7.3	3.1
yhjX	Putative transporter		21.0	44.1	2.6	165.6
pspA	Regulator; phage shock protein	PspF, IHF	30.7	1.1	1.5	1.8
pspB	Regulator; phage shock protein		32.2	1.6	1.6	1.5
pspC	Regulator; phage shock protein		56.9	1.7	2.0	2.1
pspD	Inner membrane		33.1	1.2	1.4	1.2
pspE	Sulphurtransferase		9.5	1.2	1.2	1.5
pspG (*yjbO*)	Inner membrane	PspF, IHF	37.9	1.3	0.7	0.9
ytfK	Unknown	PhoB	22.0	1.9	3.8	3.0
rbsD	Ribose transport	RbsR, Crp	2.6	5.0	1.8	8.2
rbsA	Ribose transport		1.2	2.4	1.0	5.4
rbsC	Ribose transport		1.8	4.7	1.6	21.3
rbsB	Ribose transport		2.3	2.7	1.4	6.6
rbsK	Ribose transport		0.8	2.6	1.2	9.0
rbsR	Ribose transport		1.1	1.4	0.8	2.8
manX	Mannose permease	NagC, DgsA, Crp	4.2	2.1	1.1	5.0
manY	Mannose permease		3.4	2.6	1.1	5.2
manZ	Mannose permease		6.5	5.7	1.0	16.7

a. Genes and operons which are induced ≥ 10-fold in three independent experiments are listed with the average fold induction for the three experiments. Boxes are shaded for those genes and operons induced ≥ 2-fold in all experiments.
b. Descriptions of gene function are taken from EcoGene (http://ecogene.org/).
c. Information about regulation is taken from RegulonDB (http://regulondb.ccg.unam.mx/).

zation of the *shoB-ohsC* genes is similar to that of *tisB-istR* (Vogel *et al*., 2004). In all four of these cases, the mRNA encodes a short hydrophobic protein that is toxic when overexpressed, and the sRNA acts to repress synthesis by base pairing with the mRNA. These features make these loci members of the type I family of toxin–antitoxin where the toxin is a small, hydrophobic protein and the antitoxin is an antisense RNA (reviewed in Gerdes and Wagner, 2007) and more than doubles the number of type I toxin–antitoxin systems identified on the *E. coli* chromosome.

Regulation by the Sib and OhsC RNAs

We suggest that the Sib and OhsC RNAs repress the synthesis of the Ibs and ShoB protein upon base pairing

Table 4. Genes most highly repressed upon IbsC, ShoB, LdrD and TisB overexpression.[a]

Gene	Description[b]	Regulation[c]	Average fold repression			
			IbsC	LdrD	TisB	ShoB
dusB/yhdG	tRNA dihydrouridine synthase B	CRP, IHF, Fis	6.5	0.9	1.1	4.3
fis	Transcriptional activator		7.7	0.9	1.1	3.8
yeeF	Putative transporter		17.8	0.8	1.2	4.7
apt	Adenine phosphoribosyl transferase		6.1	0.9	1.2	2.3
efeU (*ycdN*)	Inactive ferrous iron permease	CpxR	21.2	1.2	2.1	1.2
efeO (*ycdO*)	Inactive ferrous iron transporter		14.3	1.0	1.2	1.2
efeB (*ycdB*)	Inactive ferrous iron transporter		2.6	1.8	1.8	1.2
ompF	Porin	OmpR, EnvY[d]	6.1	1.4	0.9	0.5
yagU	Unknown		26.7	1.7	2.5	1.2

a. Genes and operons which are repressed ≥ 5-fold in three independent experiments are listed with the average fold repression for the three experiments. Boxes are shaded for those genes and operons repressed ≥ 2-fold in all experiments.
b. Descriptions of gene function are taken from EcoGene (http://ecogene.org/).
c. Information about regulation is taken from RegulonDB (http://regulondb.ccg.unam.mx/).
d. Also regulated by Crp, IHF, Lrp, CpxR, RstA, Fur as well as σ^E (Rhodius *et al.*, 2006).

with corresponding mRNAs by interfering with translation as has been found for IstR-1 RNA repression of TisB synthesis (Darfeuille *et al.*, 2007). The sRNAs may also impact the stability of the mRNAs, as we only detected the *ibsC* mRNA in strains carrying a deletion of the *sibC* promoter or the ribonuclease III gene (data not shown). Possibly the shorter Sib transcript is generated by cleavage that occurs upon base pairing with the *ibs* mRNAs. The Sib and OhsC sRNAs have the potential for extensive base pairing with their targets, but the extent of the interaction and how it occurs is not known. The RNA chaperone Hfq, which is required for the regulation by sRNAs that have only limited complementarity with their targets, does not impact the levels of the SibC and *ibsC* RNAs or expression of the *shoB–lacZ* fusion (data not shown).

Several observations about the Sib and OhsC RNAs have consequences for their roles as antisense regulators. The Sib RNAs appear to be expressed constitutively as we detected the *sib* transcripts in *E. coli* MG1655 under all conditions tested, although the levels are generally the highest in minimal media. This constitutive expression is similar to what has been observed for the SymR antisense RNA regulator, which represses toxic SymE protein synthesis (Kawano *et al.*, 2007). Possibly, SymR and the Sib RNAs simply serve as dampers to constitutively maintain low levels of the corresponding toxic proteins. It is also possible that only a fraction of the sRNA molecules is functional for base pairing with the toxin mRNA and that this fraction differs under specific conditions. The Sib RNAs as well as the ShoB and SymR RNAs are predicted to have extensive secondary structures. The structures are likely to impact the stabilities of the sRNAs or their abilities to base pair with the *ibs*, *shoB* and *symE* mRNAs. In addition, the *sib*, *ohsC* and *symR* genes may be subject to transcriptional regulation that we have not yet uncovered.

It is probable that the synthesis of the Ibs and ShoB proteins is subject to regulation beyond repression by the Sib and OhsC RNAs. We suggest the reason why the SibA overexpression plasmid did not require induction in the Δ*sibA* strain, although overexpression of IbsA itself is toxic, is that the IbsA protein is expressed at lower levels due to additional regulation. Further levels of regulation may also explain the observation that no expression was detected for a *lacZ* translational fusion to the *shoB* promoter and full-length 5′ untranslated region, despite the fact that the *shoB* mRNA could readily be detected by Northern analysis (Kawano *et al.*, 2005). In addition, only limited expression was observed when the fusion was made with a shortened 5′ untranslated region. The 5′ portion of the *shoB* mRNA is predicted to fold into an extensive secondary structure which may preclude ribosome binding, as has been observed for the *tisB* mRNA (Darfeuille *et al.*, 2007). Similar to *tisB* and *symE*, which are both repressed by LexA and are induced by DNA damage (Vogel *et al.*, 2004; Kawano *et al.*, 2007), transcription of the *ibs* and *shoB* genes may also be induced by specific growth conditions. Finally, the Ibs and ShoB proteins might be subject to degradation by specific proteases as has been observed for SymE (Kawano *et al.*, 2007).

Toxicity of small hydrophobic proteins

The synthesis of the *E. coli* Ibs proteins, Ldr, ShoB and TisB proteins presumably is tightly regulated because high levels of these hydrophobic proteins are toxic to the cell. It is not clear why these proteins in particular are so

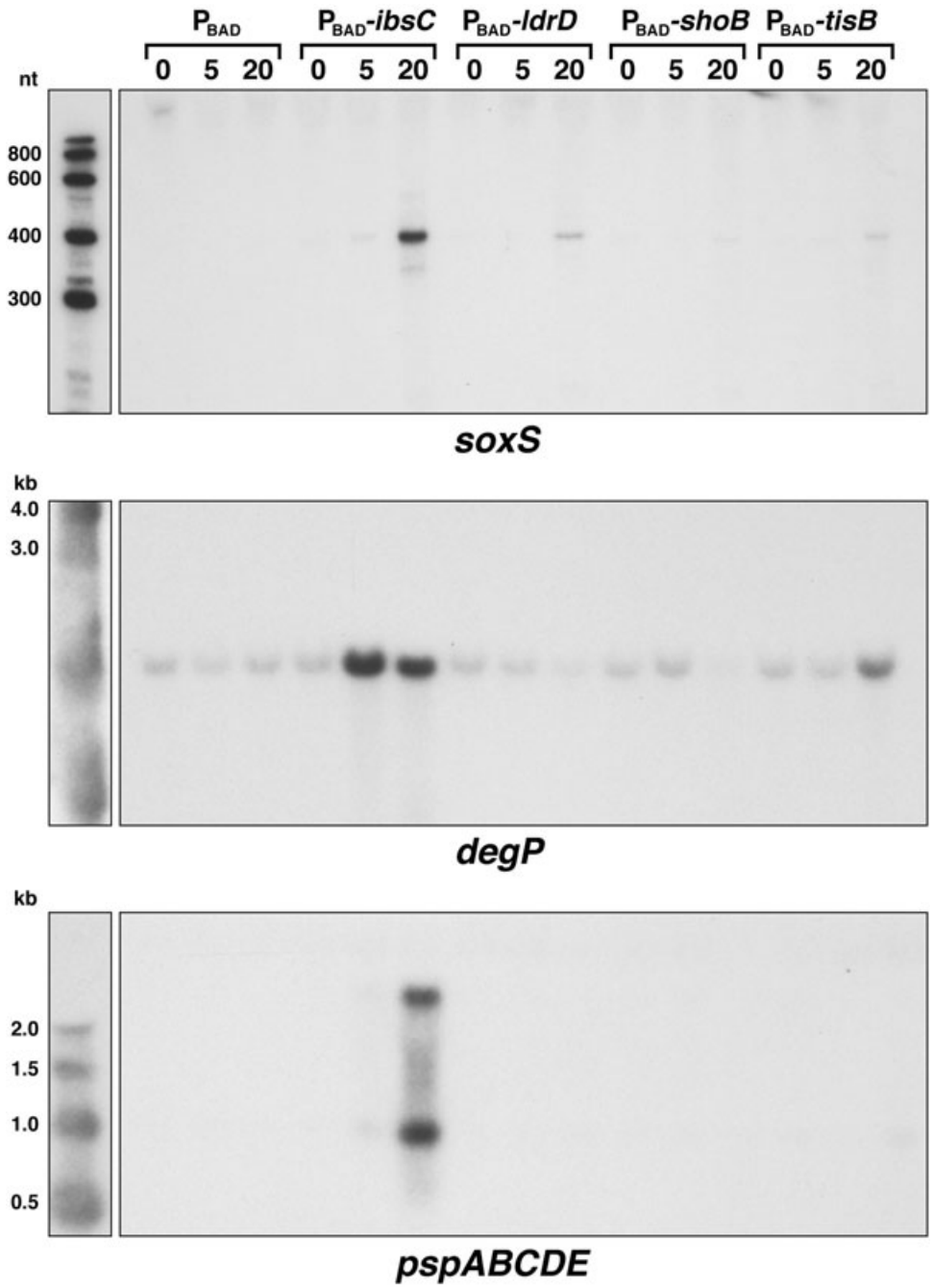

Fig. 7. Transcripts induced by IbsC, LdrD, ShoB and TisB overproduction. Northern analysis of *soxS*, *degP* and the *pspABCDE* mRNA levels after IbsC, LdrD, ShoB and TisB overproduction. Total RNA was isolated from MG1655 harbouring pAZ3, pAZ3-*ibsC*, pAZ3-*ldrD*, pAZ3-*shoB* or pAZ3-*tisB* at 0, 5 and 20 min following the addition of 0.2% arabinose to exponentially growing cultures ($OD_{600} \approx 0.3$).

toxic as we have found that high levels of other small hydrophobic proteins identified by Hemm *et al*. (submitted) do not have similarly detrimental effects (Fig. S2 and data not shown). The exact mechanism of Ibs, Ldr, ShoB and TisB toxicity also is unknown. Given their hydrophobic nature, we suggest that they insert into membranes and/or interact with other proteins. Consistent with this hypothesis, we found that induction of high levels of IbsC and ShoB led to a rapid loss of membrane potential.

IbsC, LdrD, ShoB or TisB overexpression leads to the induction of a core set of genes, indicating some commonalities in the effects of these proteins. The observation that many of the induced genes encode proteins that are localized to the membrane is also consistent with the

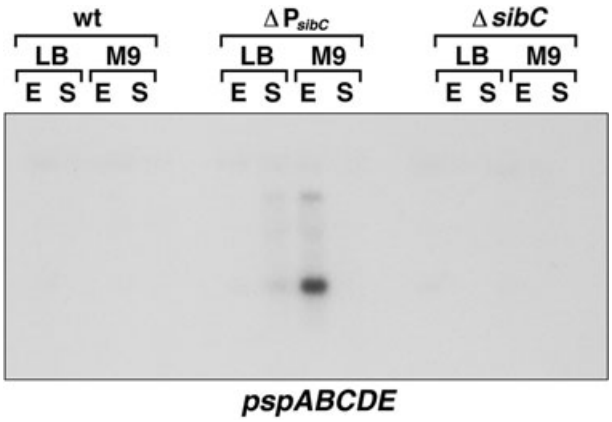

Fig. 8. Transcripts induced by derepressed levels of IbsC. Northern analysis of *pspABCDE* mRNA levels in the same MG1655, ΔP_{sibC} and Δ*sibC-ibsC* RNA samples probed in Fig. 5A.

possibility that all four proteins affect the membrane. Perhaps more interesting, we found that overexpression of subsets of the proteins leads to the induction and repression of unique sets of genes. Only high levels of IbsC induced the phage shock proteins (*pspABCDE*, *pspG*). Induction of this regulon can occur in response to multiple environmental stresses, all of which are believed to dissipate proton motive force (reviewed in Darwin, 2005), and the PspA protein was shown to interact with phospholipids and serve to block movement of protons across damaged membranes (Kobayashi *et al.*, 2007). Perhaps the overproduced IbsC proteins are forming pores in the membrane, leading to induction of the *psp* regulon. However, the observation that only IbsC overexpression leads to the induction of the *psp* genes may also mean that IbsC interacts with specific proteins that are not targeted by LdrD, ShoB or TisB.

The biological roles of the Ibs, Ldr, ShoB and TisB proteins expressed from the chromosome are unknown. In the case of TisB, which is expressed in response to DNA damage, it has been proposed that its expression may not normally kill the cell, but lead to growth arrest, which would allow for DNA repair prior to cell division (Vogel *et al.*, 2004). Although we did not observe any growth defects for a strain with de-repressed IbsC expression, we did find that expression of the *pspABCDE* operon was induced indicating that low levels of IbsC do have consequences for the cell. Future experiments aimed at characterizing the changes in gene expression upon de-repression of the chromosomally encoded *ibs*, *ldr*, *shoB* and *tisB* genes should give insights into why these genes are maintained by the cell.

Possible role of multiple genes

The *ibs-sib* genes, similar to *ldr-rdl* genes, are present as multiple copies on the chromosome. What is the role of the many copies? That fact that we observe constitutive expression of the Sib RNAs suggests that the repeats are not maintained for differential regulation of the *sib* genes, although it is possible that the *ibs* genes are differentially expressed. We did observe exclusive specificity; the lethal effects of the Sib overexpression plasmids were only seen with the strains carrying deletions of the same *sib* gene suggesting that the Sib RNAs do not have overlapping roles in *ibs* regulation. However, given the high degree of homology among the Ibs proteins, it seems unlikely that the proteins have different functions. In addition, the variation in the number of *ibs-sib* genes across even the different species of *E. coli* suggests that different numbers of these genes provide a selective advantage. Ongoing studies to further investigate the regulation by the antisense RNAs, the causes of toxicity upon overexpression of the small proteins, phenotypes associated with endogenous levels of the genes, as well as the genome-wide distribution of these type I toxin–antitoxin modules should begin to provide insight into cellular roles of this large number of small, hydrophobic proteins.

Experimental procedures

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Tables S4 and S5, and the sequences of all oligonucleotides used in the study are listed in Table S6. The mini-λ-Red recombination system was used to generate deletion strains of the *sib* genes (Datsenko and Wanner, 2000; Yu *et al.*, 2000; Court *et al.*, 2003). In all cases, pKD4 (Datsenko and Wanner, 2000), which encodes kanamycin resistance, was used as the template plasmid in PCR reactions together with oligonucleotide primers containing 20 bases of pKD4 sequence and approximately 40 nucleotides of homology to the chromosomal region. Subsequently, the kanamycin cassette was removed using pCP20 (Cherepanov and Wackernagel, 1995).

Plasmids pAZ3 (Kawano *et al.*, 2007), a plasmid with a pBR origin that is a derivative of pBAD18 with an EcoRI site at +1, and pEF21, a plasmid with a pACYC origin that is a derivative of pBAD33 (Guzman *et al.*, 1995) with the introduction of a PstI site at +1, were used to overproduce RNAs from the P_{BAD} promoter. For cloning into pAZ3, the *sibA*, *sibC*, *sibD* and *sibE* genes along with approximately 50 base pairs downstream of the mapped 3′ end were amplified from MG1655 genomic DNA, digested with EcoRI and HindIII and cloned into the corresponding sites of pAZ3. The *ibsA*, *ibsC*, *ibsE* and *yoaJ* genes were cloned into pAZ3 in the same fashion although in these cases the 5′ ends were not known. The *sibB* gene was similarly cloned into pAZ3, except that the fragment was digested with EcoRI and XbaI. For cloning into pEF21, the *ibs* sequences were amplified from the genomic DNA, digested with PstI and HindII and cloned into the corresponding sites in pEF21. Plasmid pBR-plac (Guillier and Gottesman, 2006) was used to express RNAs from the $P_{LlacO-1}$ promoter. Gene sequences were amplified from genomic DNA, digested with

AatI and EcoRI and cloned into the corresponding sites of pBR-plac. All experiments utilizing pBR-plac were performed in the strain DJ624, a Δ*lacX74* derivative of MG1655. Plasmid DNA was always isolated using the Qiagen Mini Plasmid Kit, and PCR purification was performed using either Qiagen PCR Purification Kit or Qiagen Gel Extraction Kit.

The translational *lacZ* fusion to *shoB* assayed in Fig. 5C was generated using PCR (Horton *et al*., 1989). PCR product A was the result of amplifying from −75 to −1 of *shoB* message, with additional 15 nucleotides at the 3′ end that overlapped the first 15 nucleotides of PCR product B. PCR product B was from second mapped 5′ end of the *shoB* mRNA (nucleotide 2 698 357) to the seventh codon of ShoB. The two products were then spliced together using the external primers. This product was digested with EcoRI and BamHI and cloned into the corresponding sites of pRS552 (Simons *et al*., 1987). The plasmid was then recombined onto λRS45, which was subsequently used to lysogenize DJ480.

Growth conditions

Escherichia coli K-12 MG1655 was grown in Luria–Bertani (LB with 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per litre) or M9 minimal media supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 μg ml^{-1} thiamine and 0.2% glucose at 37°C. Arabinose was added as indicated to final concentrations of 0.002%, 0.02%, 0.1%, 0.2%, and IPTG was added as indicated to final concentrations of 0.1, 0.5 or 1 mM. Antibiotics were added when needed to the following concentrations: 30 μg ml^{-1} kanamycin, 25 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin.

RNA extraction

Cells for RNA extraction were grown in LB or M9 + 0.2% glucose and harvested at $OD_{600} \approx 0.4$, $OD_{600} \approx 1.8$, or from overnight cultures ($OD_{600} \approx 5.0$ in LB; $OD_{600} \approx 2.2$ in M9). Total RNA was isolated by hot acid-phenol as described previously (Kawano *et al*., 2002).

Northern analysis

For the detection of the Sib, *ibs* and *soxS* transcripts, total RNA (5 μg for Sib and *ibs* and 10 μg for *soxS*) was separated on a denaturing 8% polyacrylamide-8 M urea gel and transferred to a Zeta-Probe Membrane (Bio-Rad). Oligonucleotide probes, specific for the individual Sib RNAs, were labelled with ^{32}P using T4 polynucleotide kinase (New England Biolab). Hybridization and wash steps were as described previously (Opdyke *et al*., 2004). For the detection of the *degP* and *pspABCDE* mRNAs, total RNA (5 μg) was separate on 1× MOPS-1% agarose gel, transferred to a Zeta-Probe Membrane (Bio-Rad) and hybridized and washed as described previously (Opdyke *et al*., 2004).

5′ and 3′ RACE

5′ and 3′ RACE analysis was carried out as described (Argaman *et al*., 2001). Primers used to amplify *sib* cDNA are found in Table S6. The amplified cDNA fragments were then cloned into vector pCRII Topo (Invitrogen) and sequenced. The results of these analyses are found in Tables S1 and S2.

Transformation efficiency

To determine transformation efficiency, overnight cultures were diluted 1:500 into 50 ml of LB and grown to $OD_{600} \approx 0.5$. Cells were harvested and washed twice, once with 50 ml of cold water and once with 2 ml of cold water. Cell pellets were re-suspended in 200 μl of cold water. A total of 40 μl of cells were transformed with 1 ng of plasmid by electroporation. Cells were then re-suspended in a final volume of 1 ml of SOC media and placed at 37°C with shaking for 1 h. Cells were plated onto LB agar plates supplemented as indicated and incubated for 16 h at 37°C.

β-Galactosidase assays

The β-galactosidase assays were carried out as described by Miller (1972).

Flow cytometry

MG1655 harbouring pAZ3, pAZ3-*ibsC* or pAZ3-*shoB* was grown to $OD_{600} \approx 0.4$ in LB and then induced with arabinose at a final concentration of 0.2%. At 0, 5 or 20 min, samples were diluted in 500 μl of filtered M9 + 10 μg ml^{-1} $DiBAC_4(3)$ (Biotium) to approximately 5×10^6 cfu ml^{-1}. After a 20 min incubation at room temperature in the dark, cells were analysed by flow cytometry in a FACSalibur (Beckton Dickinson) with a 488 nm laser. All parameters were collected as logarithmic signals. Samples were run at a low flow rate setting such that the event rate was 500 events per second. Green fluorescence from stained cells was collected in the FL1 channel (530 ± 15 nm). On average, cells with depolarized membrane become 50-fold more fluorescent in the presence of $DiBAC_4(3)$. The two populations of cells were easily separable in each staining experiment.

Microarray analysis

MG1655 harbouring pAZ3, pAZ3-*ibsC*, pAZ3-*shoB*, pAZ3-*ldrD* and pAZ3-*tisB* was grown to $OD_{600} \approx 0.3$ in LB and was induced with arabinose at a final concentration of 0.2%. Samples were harvested at 20 min post induction. RNA was prepared as described in Kawano *et al*. (2002) with these modifications: following extraction with hot acid phenol : chloroform, the supernatant was extracted twice with phenol : chloroform and once with chloroform, whereupon the supernatant was ethanol precipitated. Preparation of the cDNA and hybridization to Affymetrix *E. coli* antisense arrays were performed as described by Massé *et al*. (2005).

Acknowledgements

We thank V. Gallegos, A. Kimchi, Z. Li, J. Saud, G. Tolun and M. Valledor for technical assistance and S. Gottesman and members of the Storz lab for helpful discussions and

comments. This research was supported by NIH Grant No. R01-GM58560 (K.E.R.), the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (E.M.F., M.K., F.F., K.S.M. and G.S.), a research fellowship from the Japan Society for the Promotion of Science (M.K.) and a Research Associateship from the National Research Council (E.M.F.).

References

Altuvia, S. (2007) Identification of bacterial small non-coding RNAs: experimental approaches. *Curr Opin Microbiol* **10:** 257–261.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E.G., Margalit, H., and Altuvia, S. (2001) Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol* **11:** 941–950.

Brissette, J.L., Weiner, L., Ripmaster, T.L., and Model, P. (1991) Characterization and sequence of the *Escherichia coli* stress-induced *psp* operon. *J Mol Biol* **220:** 35–48.

Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158:** 9–14.

Court, D., Swaminathan, S., Yu, D., Wilson, H., Baker, T., Bubunenko, M., *et al.* (2003) Mini-lambda: a tractable system for chromosome and BAC engineering. *Gene* **315:** 63–69.

Darfeuille, F., Unoson, C., Vogel, J., and Wagner, E.G.H. (2007) An antisense RNA inhibits translation by competing with standby ribosomes. *Mol Cell* **26:** 381–392.

Darwin, A.J. (2005) The phage-shock-protein response. *Mol Microbiol* **57:** 621–628.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Gerdes, K., and Wagner, E.G. (2007) RNA antitoxins. *Curr Opin Microbiol* **10:** 117–124.

Gerdes, K., Bech, F.W., Jørgensen, S.T., Løbner-Olesen, A., Rasmussen, P.B., Atlung, T., *et al.* (1986) Mechanism of postsegregational killing by the *hok* gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the E. coli *relB* operon. *EMBO J* **5:** 2023–2029.

Gerdes, K., Christensen, S.K., and Løbner-Olesen, A. (2005) Prokaryotic toxin–antitoxin stress response loci. *Nat Rev Microbiol* **3:** 371–382.

Guillier, M., and Gottesman, S. (2006) Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. *Mol Microbiol* **59:** 231–247.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177:** 4121–4130.

Hayashi, K., Morooka, N., Yamamoto, Y., Fujita, K., Isono, K., Choi, S., *et al.* (2006) Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. *Mol Syst Biol* **2**: 2006.0007.

Hayes, F. (2003) Toxins–antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* **301:** 1496–1499.

Hershberg, R., Altuvia, S., and Margalit, H. (2003) A survey of small RNA-encoding genes in *Escherichia coli*. *Nucleic Acids Res* **31:** 1813–1820.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77:** 61–68.

Kawano, M., Oshima, T., Kasai, H., and Mori, H. (2002) Molecular characterization of long direct repeat (LDR) sequences expressing a stable mRNA encoding for a 35-amino-acid cell-killing peptide and a *cis*-encoded small antisense RNA in *Escherichia coli*. *Mol Microbiol* **45:** 333–349.

Kawano, M., Reynolds, A.A., Miranda-Rios, J., and Storz, G. (2005) Detection of 5′ and 3′ UTR-derived small RNAs and *cis*-encoded antisense RNAs in *Escherichia coli*. *Nucleic Acids Res* **33:** 1040–1050.

Kawano, M., Aravind, L., and Storz, G. (2007) An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Mol Microbiol* **64:** 738–754.

Kobayashi, R., Suzuki, T., and Yoshida, M. (2007) *Escherichia coli* phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. *Mol Microbiol* **66:** 100–109.

Livny, J., and Waldor, M.K. (2007) Identification of small RNAs in diverse bacterial species. *Curr Opin Microbiol* **10:** 96–101.

Massé, E., Vanderpool, C.K., and Gottesman, S. (2005) Effect of RyhB small RNA on global iron use in *Escherichia coli*. *J Bacteriol* **187:** 6962–6971.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Opdyke, J.A., Kang, J.G., and Storz, G. (2004) GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. *J Bacteriol* **186:** 6698–6705.

Pedersen, K., and Gerdes, K. (1999) Multiple hok genes on the chromosome of *Escherichia coli*. *Mol Microbiol* **32:** 1090–1102.

Rhodius, V.A., Suh, W.C., Nonaka, G., West, J., and Gross, C.A. (2006) Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biol* **4:** e2.

Rivas, E., Klein, R.J., Jones, T.A., and Eddy, S.R. (2001) Computational identification of noncoding RNAs in *E. coli* by comparative genomics. *Curr Biol* **11:** 1369–1373.

Rudd, K.E. (1999) Novel intergenic repeats of *Escherichia coli* K-12. *Res Microbiol* **150:** 653–664.

Simons, R.W., Houman, F., and Kleckner, N. (1987) Improved single and multicopy lac-based cloning vectors for protein and operon fusions. *Gene* **53:** 85–96.

Storz, G., and Gottesman, S. (2006) Versatile roles of small RNA regulators in bacteria. In *The RNA World*, 3rd edn. Gesteland, R.F., Cech, T.R., and Atkins, J.F. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 567–594.

Vogel, J., Argaman, L., Wagner, E.G.H., and Altuvia, S. (2004) The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide. *Curr Biol* **14:** 2271–2276.

Wassarman, K.M., Repoila, F., Rosenow, C., Storz, G., and Gottesman, S. (2001) Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev* **15:** 1637–1651.

Wickens, H.J., Pinney, R.J., Mason, D.J., and Gant, V.A. (2000) Flow cytometric investigation of filamentation, membrane patency, and membrane potential in *Escherichia coli* following ciprofloxacin exposure. *Antimicrob Agents Chemother* **44:** 682–687.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* **97:** 5978–5983.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Blackwell Publishing are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.