Supplementary Material

The ancestral SgrS RNA discriminates horizontally acquired *Salmonella* mRNAs through a single G-U wobble pair

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This supplement contains:

Figures S1 to S7
Tables S1-4
Supplementary methods
Supplementary References

Supplementary Figures

Figure S1

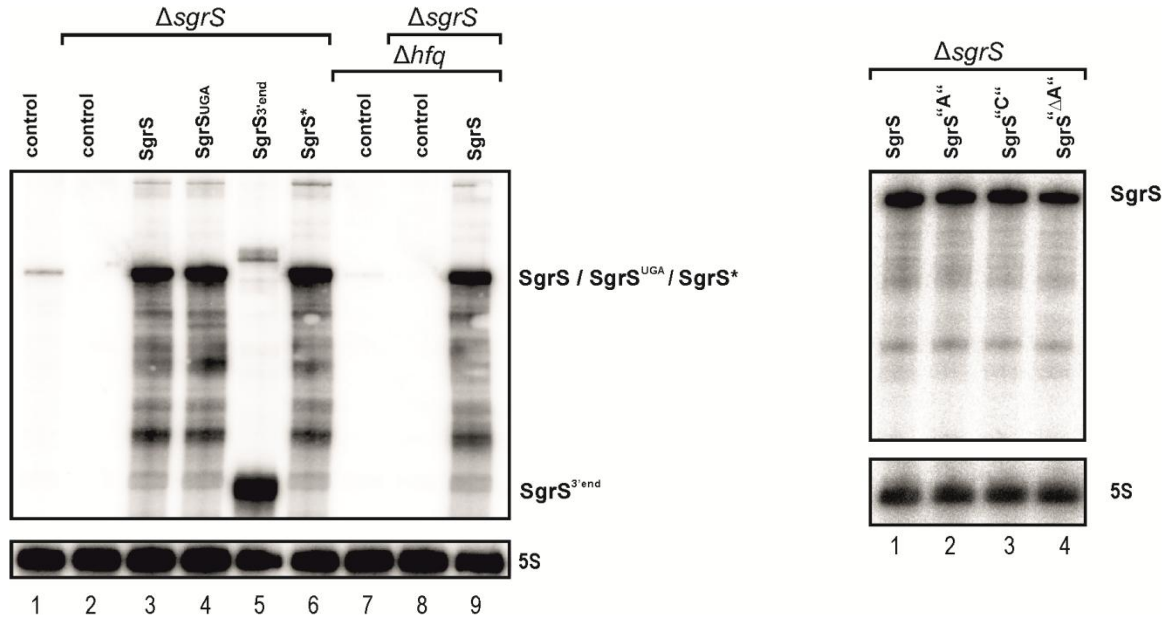

Figure S1: Expression of SgrS variants. Left: Northern blot analysis of plasmid borne SgrS and SgrS mutant expression corresponding to Fig. 2C. Right: Expression analysis of additional SgrS variants employed in Fig. 6B. Probing of 5S rRNA served as loading control.

Figure S2

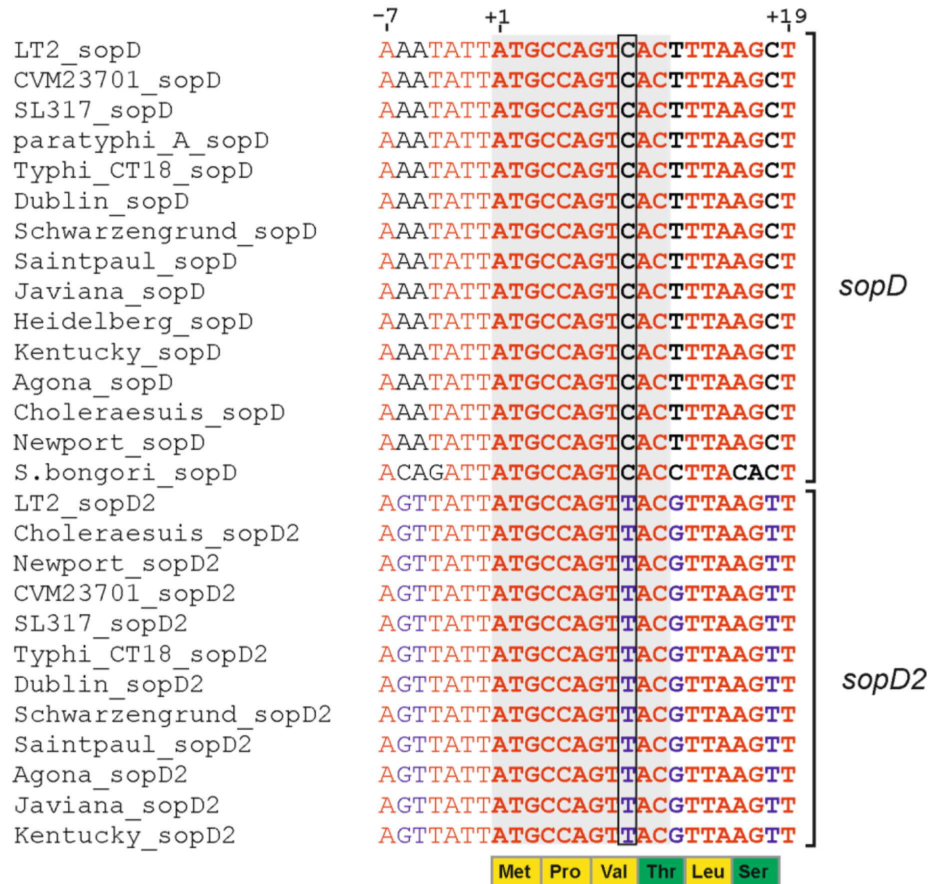

Figure S2: Alignment of *sopD* and *sopD2* 5' UTR and proximal coding sequence. Sequences relevant for interaction with SgrS are shaded in grey. Translation of the first six codons is shown below. The single nucleotide relevant for *sopD* vs. *sopD2* discrimination (position +9) is boxed.

Figure S3

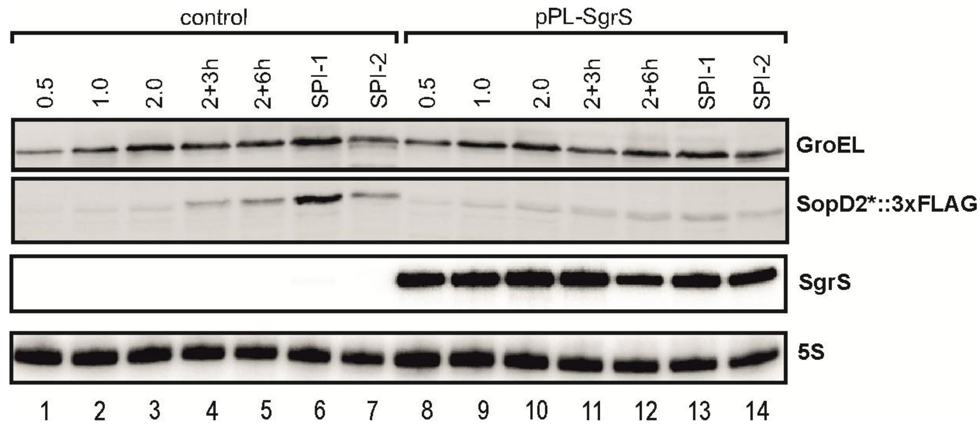

Figure S3: A chromosomal *sopD2 allele is repressed by SgrS.** Western blot analysis of SopD2*::3xFLAG in presence of a control plasmid (pJV300, lanes 1-7) or pPL-SgrS (pKP41-1, lanes 1-7). Samples were collected at the indicates time-points of growth in rich media or under SPI-1 and SPI-2 inducing conditions and subjected to western and northern blot analysis. Probing for GroEL and 5S rRNA were used as loading controls.

Figure S4

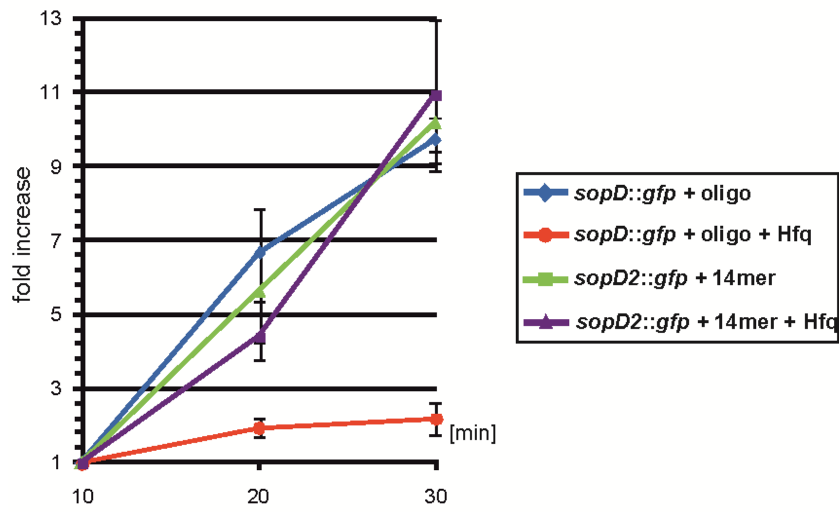

Figure S4: Translational control of *sopD::gfp* and *sopD2::gfp* by the SgrS 14mer. Analogous to Fig. 5B. Experiments have been performed in triplicates and average rates of translation have been plotted. The error bars indicate the SD of three independent experiments.

Figure S5

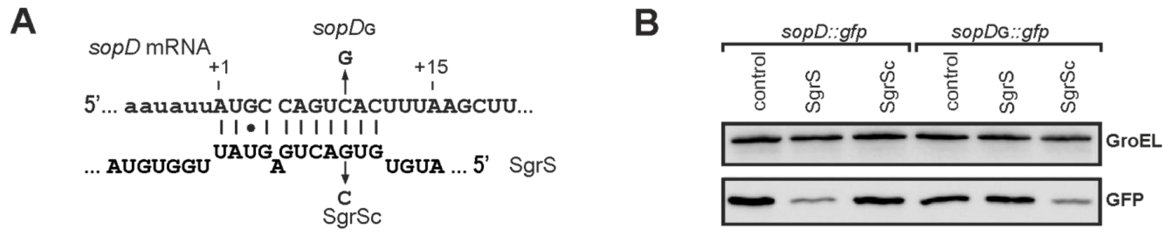

Figure S5: Base orientation does not account for discrimination between *sopD* and *sopD2*. Left: Graphical presentation of predicted SgrS-*sopD* interaction. Positions relevant for *sopD* vs. *sopD2* discrimination have been flipped. Right: Western blot analysis of the mutants depicted on the left. *Salmonella* (Δ*sgrS*) double-transformants were grown in rich media to OD_{600} of 2.0 and total protein samples were subjected to western blot analysis of GFP protein. Probing of GroEL served as loading control.

Figure S6

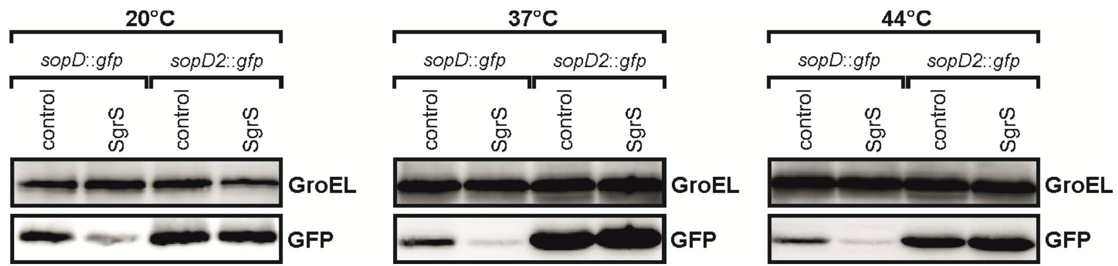

Figure S6: Discrimination between *sopD* and *sopD2* is not sensitive to temperature. Western blot analysis of cells harboring the *sopD::gfp* or *sopD2::gfp* translational reporter in combination with the control plasmid (pJV300) or pPL-SgrS (pKP41-1). Cells were cultivated at 20°C, 37°C or 44°C. Probing of GroEL served as loading control.

Figure S7

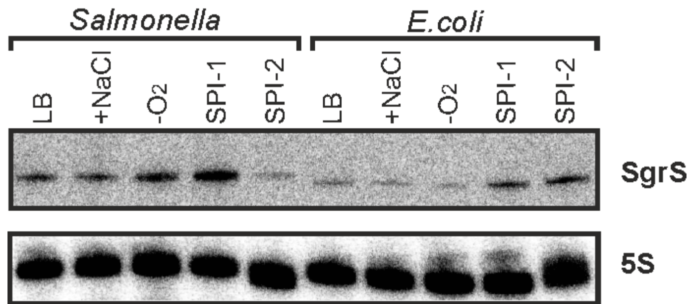

Figure S7: Comparison of SgrS expression between *E. coli* and *Salmonella*. *Salmonella* (SL1344) and *E. coli* (MG1655) were grown for 12h (37°C) in LB, LB containing 0.3M NaCl, LB with low oxygen, and SPI-1 or SPI-2 conditions, and compared for SgrS expression. The SgrS RNA was detected by Northern blot with an oligonucleotide (JVO-1366) complementary to SgrS of both species. Probing of 5S rRNA served as loading control.

Table S1: predicted stabilities of selected RNA duplexes

<table>
<tr><th>SgrS</th><th>target</th><th>MFE (kcal/mol)[a]</th><th>MFE (kcal/mol)[b]</th><th>5’-mRNA-3’
3’-sRNA-5’[C]</th></tr>
<tr><td>WT</td><td>sopD</td><td>-18.6</td><td>-14.3</td><td>AUUAUGC–CAGUCACUUU
||•| |||||||
UAUGAGUCAGUGUG</td></tr>
<tr><td>WT</td><td>sopD2</td><td>-17.4</td><td>-13.1</td><td>AUUAUGC–CAGUUACGUUA
||•| ||||•||•
UAUGAGUCAGUGUG</td></tr>
<tr><td>SgrS "A"</td><td>sopD</td><td>-13.4</td><td>-9.0</td><td>AUUAUGC–CAGU––CACUUU
||•| |||| |||
UAUGAGUCAAUGUG</td></tr>
<tr><td>SgrS "A"</td><td>sopD2</td><td>-17.3</td><td>-13.0</td><td>AUUAUGC–CAGUUACGUUA
||•| ||||•||•
UAUGAGUCAAUGUG</td></tr>
<tr><td>SgrS "C"</td><td>sopD</td><td>-20.6</td><td>-16.3</td><td>AUUAUGC–CAGUCACUUU
|||| |||||||
UACGAGUCAGUGUG</td></tr>
<tr><td>SgrS "C"</td><td>sopD2</td><td>-19.4</td><td>-15.1</td><td>AUUAUGC–CAGUUACGUUA
|||| ||||•||•
UACGAGUCAGUGUG</td></tr>
<tr><td>SgrS "ΔA"</td><td>sopD</td><td>-22.4</td><td>-18.1</td><td>AUUAUGCCAGUCACUUU
||•||||||||
UAUGGUCAGUGUG</td></tr>
<tr><td>SgrS "ΔA"</td><td>sopD2</td><td>-21.2</td><td>-16.9</td><td>AUUAUGCCAGUUACGUUA
||•|||||•||•
UAUGGUCAGUGUG</td></tr>
<tr><td>WT</td><td>ptsG</td><td>-22.7</td><td>-18.2</td><td>AAAGCACAAAUACUCAGGAG
•||| |||||||||
GGAUGUGGUUAUGAGUCAGU</td></tr>
</table>

a. The MFE of the RNA duplexes was calculated using the *RNAhybrid* webserver (1).

b. The MFE of the RNA duplexes was calculated using the *RNAcofold* webserver (2).

c. RNA duplexes as predicted by the *RNAhybrid* webserver.

Supplementary Methods

Plasmid construction

A complete list of all plasmid used in this study can be found in Table S4. To express a plasmid-borne *sgrS* gene from the tightly controlled, L-arabinose inducible P_{BAD} promoter (3) the following strategy was used to clone the *sgrS* gene under P_{BAD} control such that transcription would start precisely at their native +1 site. Plasmid pBAD-His-myc was PCR amplified (cycling parameters: 95°C / 30'', 25x [95°C/ 10", 59°C/ 30", 72°C / 2'], 72°/ 10') with primers JVO-0900/-0901 (JVO-0901 introduces an XbaI restriction site upstream of the *rrnB* terminator sequence) and Phusion DNA polymerase (*Finnzymes*, Finland), the PCR product digested with *Xba*I and *Dpn*I, and purified. To PCR amplify the *sgrS* insert, the sense primer (JVO-0916) was designed such that it starts with the sRNA +1 site and that it carries a 5' phosphate modification. The antisense primer (JVO-0298) binds downstream of the *sgrS* terminator, and adds an XbaI site to its sequence. Following amplification with Phusion DNA polymerase, the PCR product was digested with XbaI and gel-purified. Vector- and sRNA-derived PCR products were ligated with T4 DNA ligase (5' blunt end/ 3' *Xba*I site) and transformed, yielding plasmids pBAD-SgrS (pKP-12-2). Correct inserts were confirmed by sequencing of the plasmids with vector primers, pBad-FW and pBad-REV. The very same insert was used for construction of plasmid pP_L-SgrS (pKP-41-1); here, a *Xba*I-digested PCR product obtained with primers PLlacoB and PLlacoD on plasmid pZE-12-luc (4) served as vector backbone. Plasmid pKP41-1 served as template for the establishment of several SgrS mutant plasmids using the following oligonucleotides: pKP72-1 (JVO-2547/2548), pKP96-2 (JVO-3147/3148), pKP196-1 (JVO-5218/5219), pKP202-2 (JVO-5496/5497), pKP214-1 (JVO-5583/5584) and pKP220-3 (JVO-5581/5582). Plasmid pKP69-2 was constructed by amplification of a truncated *sgrS* allele (JVO-2418/0298) and cloning into the pZE-12-luc backbone using the strategy as described for pKP41-1. Construction of *sopD::gfp* fusion plasmid (pKP67-2) was achieved by amplification of a DNA fragment spanning from -111 (the *sopD* transcriptional start site was mapped by 5'RACE analysis) to the 60 bps of *sopD* coding region using primers JVO-1888/2428. Insert for plasmid *sopD2::gfp* (pKP83-2) was amplified with primer pair JVO-2833/2834. The PCR products were digested with BrfBI and NheI, gel-purified and ligated into pXG-10 (5) backbone digested with the same enzymes. Plasmids pKP67-2 and pKP83-2 served as templates for establishment of *sopD**::*gfp* (pKP80-2), *sopD***::*gfp* (pKP90-1) and *sopD2*::gfp* (pKP87-2) harboring a single nucleotide exchange which was introduced by primers JVS-2553/2554, JVO-2984/2985 and JVO-

2910/2911, respectively. Competent *E. coli* TOP10 or TOP10 F' cells (Invitrogen) were used for all cloning procedures.

Construction of *Salmonella* mutant strains.

A list of all strains used in this study has been summarized in a table below. The mouse-virulent strain SL1344 of *Salmonella enterica* serovar Typhimurium is referred to as the wild-type strain throughout this study. Its derivates were constructed using the lambda red recombinase method (6). The *sgrS* mutant strain was previously published (7), as well as strain JVS-0255 (Δ*hfq*; (8)). Strain JVS-1644 (Δ*sgrS::*FRT) is a marker-less derivate of JVS-0114 using plasmid pCP20 (6). The C-terminal flagged tagged versions of *sopD* (JVS-2082) and *sopD2* (JVS-3902) were constructed by a modified lambda red approach based on (9) using primer pairs JVO-1890/2190 and JVO-3617/3618, respectively. For the chromosomal integration of the SopD2*::3XFLAG mutant (JVS-4514) the *sopD2*::3XFLAG gene of JVS-3902 was sub-cloned into a mid-copy vector (pKP-132-2), a single nucleotide mutation was introduced using primer pair JVO-2910/2911 (resulting in plasmid pKP140-1) and integrated into a Δ*sopD2* strain (generated as described above using lambda red recombination with oligonucleotides JVO-4032/4033). Reintegration of *sopD2* derived from pKP-132-2 yielded a wild-type *sopD2**::3XFLAG allele (JVS-4578), while the *sopD2**::3XFLAG (JVS-4514) was obtained by integration of pKP-140-1. Sequence identity of the *sopD2* allele of both were strains was verified by sequencing. Strain JVS-2715 (Δ*sgrR::*Km^R) was constructed using primer pair JVO-2573/2574 replacing the *sgrR* ORF. All double and triple mutants derived from P22-transductions into the marker-less JVS-1644 (Δ*sgrS::*FRT) strain.

Calculation of RNA duplex energies

We calculated the MFE (minimal free energy) of every RNA/RNA-duplex structure using two independent methods. First, we used the *RNAhybrid* algorithm (1) to predict RNA duplex potentials of SgrS-*sopD*/*sopD2* interactions. This approach does not require concatenation of sRNA and target sequences and does not allow intramolecular hybridizations (base-pairings between target nucleotides or between sRNA nucleotides). Further, *RNAhybrid* identifies the energetically most favorable hybridizations of the sRNA/mRNA duplex and scores potential interactions on the basis of the calculated MFE. The *RNAhybrid* webserver is available at: http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/. Second, we used the *RNAcofold* algorithm, provided by the Vienna

RNA package (http://www.tbi.univie.ac.at/RNA/) as an alternative method to estimate RNA duplex stability. In contrast to the *RNAhybrid* algorithm, *RNAcofold* (10) also accounts for internal structures in both, sRNAs and mRNA. Similar to *RNAhybrid*, scores of duplex stability are made on the basis of MFE calculation. A comparison of both methods is shown in Supplementary Table S1.

Table S2: Bacterial strains used in this study.

Strain	Relevant markers/ genotype	Reference/ source
S. typhimurium		
SL1344	Str^R *hisG rpsL xyl*	(11)
JVS-0114	SL1344 Δ*sgrS::*Km^R	(12)
JVS-0255	SL1344 Δ*hfq::*Cm^R	(8)
JVS-1644	SL1344 Δ*sgrS::*FRT	this study
JVS-2081	SL1344 Δ*sgrS::*FRT/*sopD*::3XFLAG	this study
JVS-2082	SL1344 *sopD*::3XFLAG	this study
JVS-2715	SL1344 Δ*sgrR::*Km^R	this study
JVS-3814	SL1344 Δ*sgrS::*FRT/Δ*hfq::*Cm^R/*sopD*::3XFLAG	this study
JVS-4514	SL1344 *sopD2**::3XFLAG	this study
JVS-4515	SL1344 Δ*sgrS::*FRT/*sopD2**::3XFLAG	this study
JVS-4578	SL1344 *sopD2*::3XFLAG	this study
JVS-4579	SL1344 Δ*sgrS::*FRT/*sopD2*::3XFLAG	this study
E. coli		
JVS-5709	MG1655	(13)
TOP10	*mcr*A Δ*(mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ*(ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
TOP10F'	F´{*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ*(ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen

Table S3: Plasmids used in this study.

Plasmid trivial name	Plasmid stock name	Relevant fragment	Comment	Origin, marker	Reference
control	pJV300		Control plasmid, expresses a ~50 nt nonsense transcript.	ColE1, Amp^R	(14)
pBAD	pKP8-35		pBAD control plasmid, expresses the same ~50 nt nonsense RNA as pJV300	pBR322, Amp^R	(12)
pBAD-SgrS	pKP12-2	SgrS	SgrS under the control of the inducible pBAD promotor	pBR322, Amp^R	This study
P_L-SgrS	pKP41-1	SgrS	ColE1 plasmid, based on pZE12-luc, expresses SgrS from a PLlacO promoter	ColE1, Amp^R	This study
P_L-SgrS*	pKP72-1	SgrS	Same as pKP-41-1 but carries a single nt exchange in the conserved 3' end	ColE1, Amp^R	This study
P_L-SgrS^{UGA}	pKP96-2	SgrS	Same as pKP-41-1 but carries a single nt exchange in the SgrT ORF (STOP mutation).	ColE1, Amp^R	This study
P_L-SgrS$^{3'end}$	pKP69-2	SgrS	Derivate of pKP41-1. Truncation of the SgrS 5' end.	ColE1, Amp^R	This study
P_L-SgrS$^{\text{"A"}}$	pKP196-1	SgrS	Same as pKP-41-1 but carries a single nt exchange in the SgrS 3' end.	ColE1, Amp^R	This study
P_L-SgrS^{C}	pKP202-2	SgrS	Same as pKP-41-1 but carries a single nt exchange in the SgrS 3' end.	ColE1, Amp^R	This study
P_L-SgrS$^{\text{"C"}}$	pKP214-1	SgrS	Same as pKP-41-1 but carries a single nt exchange in the SgrS 3' end.	ColE1, Amp^R	This study
P_L-SgrS$^{\text{"}\Delta\text{A"}}$	pKP220-3	SgrS	Same as pKP-41-1 but carries a single nt deletion in the SgrS 3' end.	ColE1, Amp^R	This study
sopD::gfp	pKP67-2	*sopD* 5'UTR	GFP reporter plasmid. Carries the *Salmonella sopD* 5'UTR and 60 bps of the ORF	pSC101*, Cm^R	This study
sopD::gfp*	pKP80-2	*sopD* 5'UTR	Same as pKP-67-2 but carries a single nt mutation in the SgrS interaction sequence.	pSC101*, Cm^R	This study
sopD2::gfp	pKP83-2	*sopD2* 5'UTR	GFP reporter plasmid. Carries the *Salmonella sopD2* 5'UTR and 60 bps of the ORF	pSC101*, Cm^R	This study
sopD2::gfp*	pKP87-2	*sopD2* 5'UTR	Same as pKP-83-2 but carries a single nt mutation in the SgrS interaction sequence.	pSC101*, Cm^R	This study
*sopD**::gfp*	pKP90-1	*sopD* 5'UTR	Same as pKP-67-2 but carries a single nt mutation in the SgrS interaction sequence.	pSC101*, Cm^R	This study
	pKD-4		Template for KmR mutant construction	oriRγ, Amp^R	(6)
	pKD-46	ParaB-γ-β-exo	Temperature-sensitive *lambda red* recombinase expression plasmid	oriR101, Amp^R	(6)
	pCP20		Temperature-sensitive FLP recombinase expression plasmid	oriR101, Amp^R and Cm^R	(6)
	pSUB11		Template for construction of 3xFLAG-tag sequence linked to a KmR cassette	R6KoriV, Amp^R	(9)

Table S4: DNA and RNA oligonucleotides used in this study.

Sequences are given in 5' → 3' direction; 5'P denotes a 5' monophosphate.

Name	Sequence	Used for
pLacOD	GTGCTCAGTATCTTGTTATCCG	sRNA cloning
pLacOB	CGCACTGACCGAATTCATTAA	sRNA cloning
pZE-XbaI	TCGTTTTATTTGATGCCTCTAGA	sRNA cloning
pZE-A	GTGCCACCTGACGTCTAAGA	sRNA cloning, sequencing oligo
pZE-T1	CGGCGGATTTGTCCTACT	templates Puresystem analysis
JVO-0298	GTTTTTTCTAGACCTGCGTGACCACAGAA	sRNA cloning
JVO-0900	GGAGAAACAGTAGAGAGTTGC	sRNA cloning
JVO-0901	TTTTTTCTAGATTAAATCAGAACGCAGA	sRNA cloning
JVO-0916	5'P~GATGAAGCAAGAGGAAGAG	sRNA cloning
JVO-0979	GTTTTTTTTTTAATACGACTCACTATAGGGAGGTATCTGCTGGCGGGT	SgrS riboprobe template
JVO-1366	CACCAATACTCAGTCACACATGATG	Northern Blot probe SgrS
JVO-1888	GTTTTTGCTAGCAAGCCGACTTTCATTAAGC	*sopD*::*gfp* reporter plasmid
JVO-1890	TCCGGCAGGCAGCCGGATTTTAAATTGGTTATATTACTGACCATATGAATATCCTCCTTAG	*sopD*::3XFLAG construction
JVO-2190	CATTGGTAAAGATGGGTGCAGTCGTAATATATTACTGACAGACTACAAAGACCATGACGG	*sopD*::3XFLAG construction
JVO-2418	5´P-CGGAACTGATGCAGTG	sRNA cloning
JVO-2428	GTTTTATGCATGTCGTTCAAATTCTCTCCTG	*sopD*::*gfp* reporter plasmid
JVO-2547	GTGACTCAGTATTGGTGTAGG	sRNA mutagenesis
JVO-2548	CAATACTGAGTCACACATGATG	sRNA mutagenesis
JVO-2553	ATGCGAGTCACTTTAAGCT	sRNA mutagenesis
JVO-2554	GACTCGCATAATATTTTCCT	sRNA mutagenesis
JVO-2573	ATAATAAAATTGCTGAGTTTTTCATCGGAGTTCCCCTTTTGTGTAGGCTGGAGCTGCTTC	Deletion of *Salmonella sgrR*
JVO-2574	TGTAGTGATTTTGTTGCCGGGCAGCAATCAAGAGCTGGCGGGTCCATATGAATATCCTCCTTAG	Deletion of *Salmonella sgrR*
JVO-2583	5'P~GTCGTTCAAATTCTCTCCTG	*sopD riboprobe template*
JVO-2594	GTTTTTTTTAATACGACTCACTATAGGATGAAGCAAGAGGAAGAG	template SgrS T7 RNA
JVO-2595	AAGCCAGCAGATATTATCTG	template SgrS T7 RNA
JVO-2596	GTTTTTTTTAATACGACTCACTATAGGTCGTTCAAATTCTCTCCTG	*sopD*::*gfp in vitro* transcript
JVO-2833	GTTTTTTATGCATAAATAGAGTGTGGTTTTAATCAA	*sopD2*::*gfp* reporter plasmid
JVO-2834	GTTTTTGCTAGCTAGCCGTGAATGATTAATTTC	*sopD2*::*gfp* reporter plasmid
JVO-2910	CCAGTCACGTTAAGTTTTGG	*sopD2*::*gfp* mutagenesis
JVO-2911	TAACGTGACTGGCATAATAACTC	*sopD2*::*gfp* mutagenesis
JVO-2984	CCAGTTACTTTAAGCTTCGGT	*sopD*::*gfp* mutagenesis
JVO-2985	AGTAACTGGCATAATATTTTCCT	*sopD*::*gfp* mutagenesis
JVO-2996	GTTTTTTTTTTAATACGACTCACTATAGGGAGGGATCCTGGACTTTATCCCAAC	*sopD riboprobe template*
JVO-3062	GTTTTTTTTAATACGACTCACTATAGGAAATAGAGTGTGGTTTTAATCAA	*sopD2*::*gfp in vitro* transcript
JVO-3147	GTTCTGATTGAGATATTTTGCC	sRNA mutagenesis
JVO-3148	CAATCAGAACTGGCGCATA	sRNA mutagenesis
JVO-3617	ATTTATAAGTGAAAAGTCGAGTTGTCGCAATATGCTTATAGACTACAAAGACCATGACGG	*sopD2*::3XFLAG construction
JVO-3618	TTACGCCATAAAAAGCGTACAAAAAAGGCTCCATATCAGTCCATATGAATATCCTCCTTAG	*sopD2*::3XFLAG construction
JVO-4032	ATGTGTCCAAAGAACGTTATGCGCTGTCCAGCGCCTGGCGGTGTAGGCTGGAGCTGCTTC	Deletion of *Salmonella sopD2*
JVO-4033	GAACAGCAGCAGGACGTTATTACTCGTACCTTCACGCAGACCATATGAATATCCTCCTTAG	Deletion of *Salmonella sopD2*
JVO-5218	GTGTAACTGAGTATTGGTGTAGG	sRNA mutagenesis
JVO-5219	TCAGTTACACATGATGTCTGC	sRNA mutagenesis
JVO-5496	CCAGTGACTTTAAGCTTCGGT	sRNA mutagenesis
JVO-5497	AGTCACTGGCATAATATTTTCCT	sRNA mutagenesis
JVO-5581	5'P~GTATTGGTGTAGGCGATAGC	sRNA mutagenesis
JVO-5582	CAGTCACACATGATGTCTGC	sRNA mutagenesis
JVO-5583	5'P~TGAGCATTGGTGTAGGCG	sRNA mutagenesis
JVO-5584	GTCACACATGATGTCTGCAATT	sRNA mutagenesis
	RNA oligonucleotides	
SgrS	GUGUGACUGAGUAU	Puresystem analysis

Supplementary references

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