Supplemental Material

The iron-sensing aconitase B binds its own mRNA to prevent sRNA-induced mRNA cleavage

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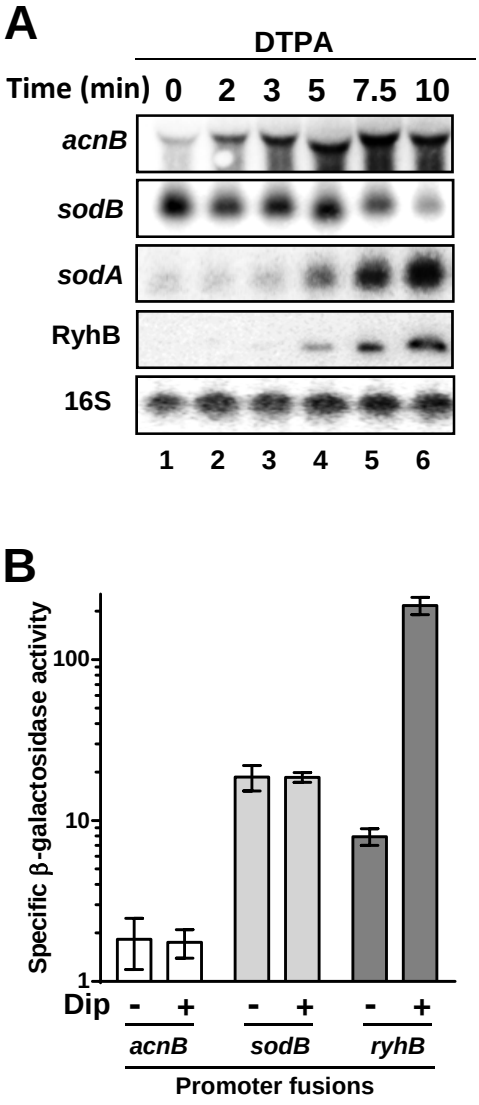

Figure S1. Effects of iron chelation on *acnB* expression. (A) Northern blot of total RNA hybridized with *acnB*, *sodB*, *sodA*, RyhB and 16S probes. RNA extraction was performed after addition of the iron chelator diethylene triamine pentaacetic acid (DTPA, final concentration of 100 µM) at time 0 to induce RyhB expression in EM1055 cells. Time course was performed at indicated time, at an OD_{600} of 0.5. *sodB* mRNA was used as control for RyhB sRNA negative target and *sodA* mRNA as Fur protein negative target. 16S rRNA was used as a loading control. (B) Effect of Fe starvation on β-galactosidase activity of *acnB*, *sodB* and *ryhB* promoter transcriptional *lacZ* fusions in Δ*ryhB* strains (EM1238). Fe chelator Dip (200 µM) was added or not at an OD_{600} of 0.1 and specific activity was measured in triplicate. Transcriptional *sodB-lacZ* fusion was used as a negative control and transcriptional *ryhB-lacZ* fusion as a positive control for Fe-dependent promoter regulation.

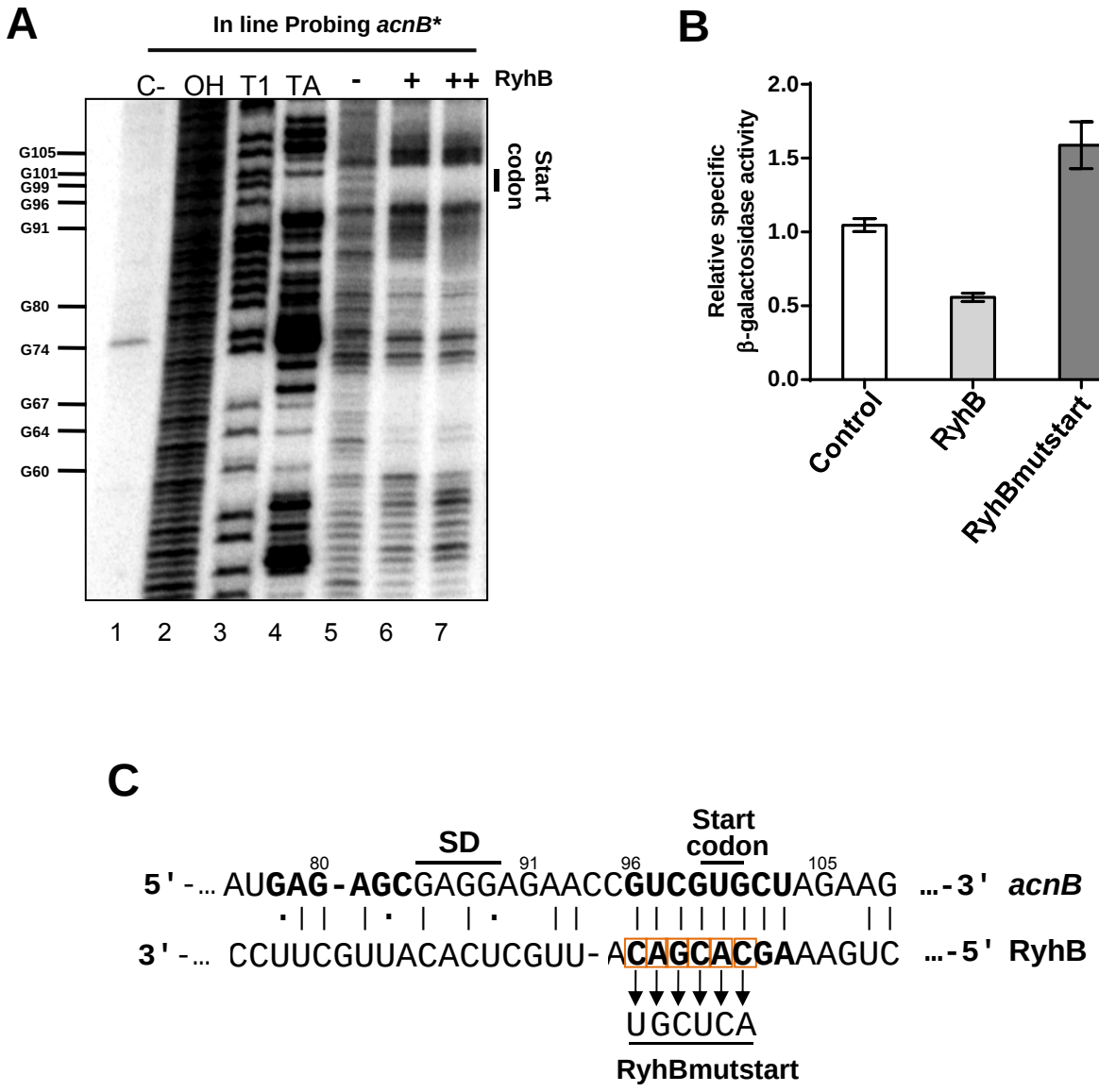

Figure S2. RyhB sRNA pairs to the translational initiation region of *acnB* mRNA. (A) *In vitro* pairing between sRNA RyhB and 5'end-radiolabelled *acnB* mRNA. (Lane 1) Control of *acnB* mRNA transcript alone. In-line probing reaction where *acnB* 5'UTR RNA transcript was incubated with RyhB sRNA in rates of 1:0, 1:10 and 1:20 (Lanes 5-7). (Lane 2) NaOH ladder. (Lane 3) RNase T1 ladder. (Lanes 4) RNase TA ladder. (B) The translational fusion $AcnB_{177}$-LacZ is specifically repressed by RyhB when induced from pBAD-*ryhB*. The sRNA RyhB is induced by Ara (0.01%) at an OD_{600} of 0.1 and specific β-galactosidase activity has been measured 2h later. Expression of the RyhBmutstart (shown in (C)) from the pBAD-*ryhB*mutstart construct does not repress translation of $AcnB_{177}$-LacZ translational fusion. This absence of repression suggests that the 6 nt-long mutation in RyhBmutstart prevents pairing between RyhB and *acnB* mRNA. The empty vector pNM12 has been used as a control. Specific activity was measured in triplicate and standard deviation are presented on graph. (C) *In sillico* pairing prediction of RyhB sRNA on *acnB* mRNA on its translational initiation region by TargetRNA (57).

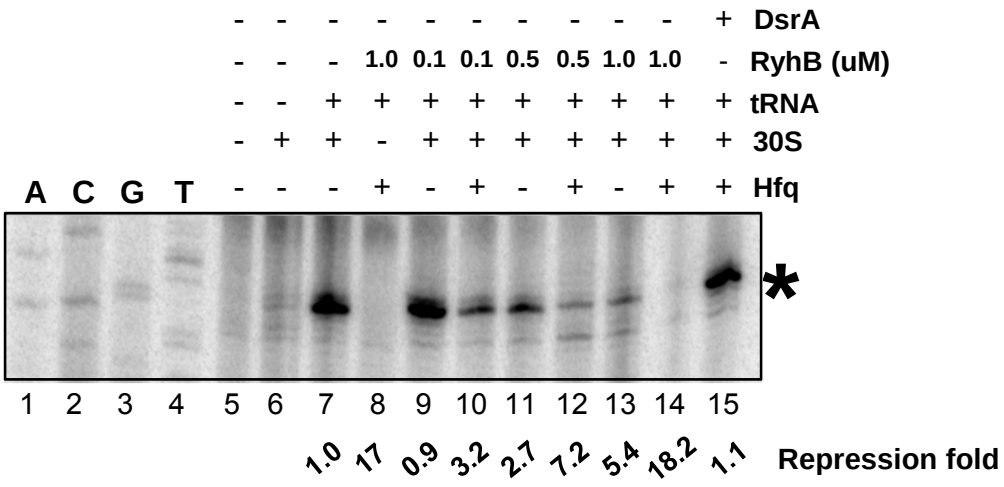

Figure S3. Toeprinting assays suggesting that RyhB sRNA and Hfq prevented 30S complex formation on *acnB* mRNA. Unlabeled *acnB* was incubated in the presence of increasing amount of RyhB (lanes 8 to 14) in the presence (lanes 8, 10, 12, 14) or absence (lanes 7, 9, 11, 13) of Hfq (0.5 µM). Ratio of *acnB*:Hfq:RyhB were 1:2.5:0.1 (lane 10), 1:2.5:0.5 (lane 12) and 1:2.5:1.0 (lane 14). ACGT refers to sequencing ladder generated with the same oligonucleotide (EM1484) that was used for toeprinting analysis. Toeprinting reverse transcription was performed with γ-P^{32} 5'-end labelled oligonucleotide. Densitometry analysis of fold of repression relative to toeprint control (lane 7) is shown. DsrA sRNA was used as a negative control of translational repression of *acnB* mRNA (lane 15).

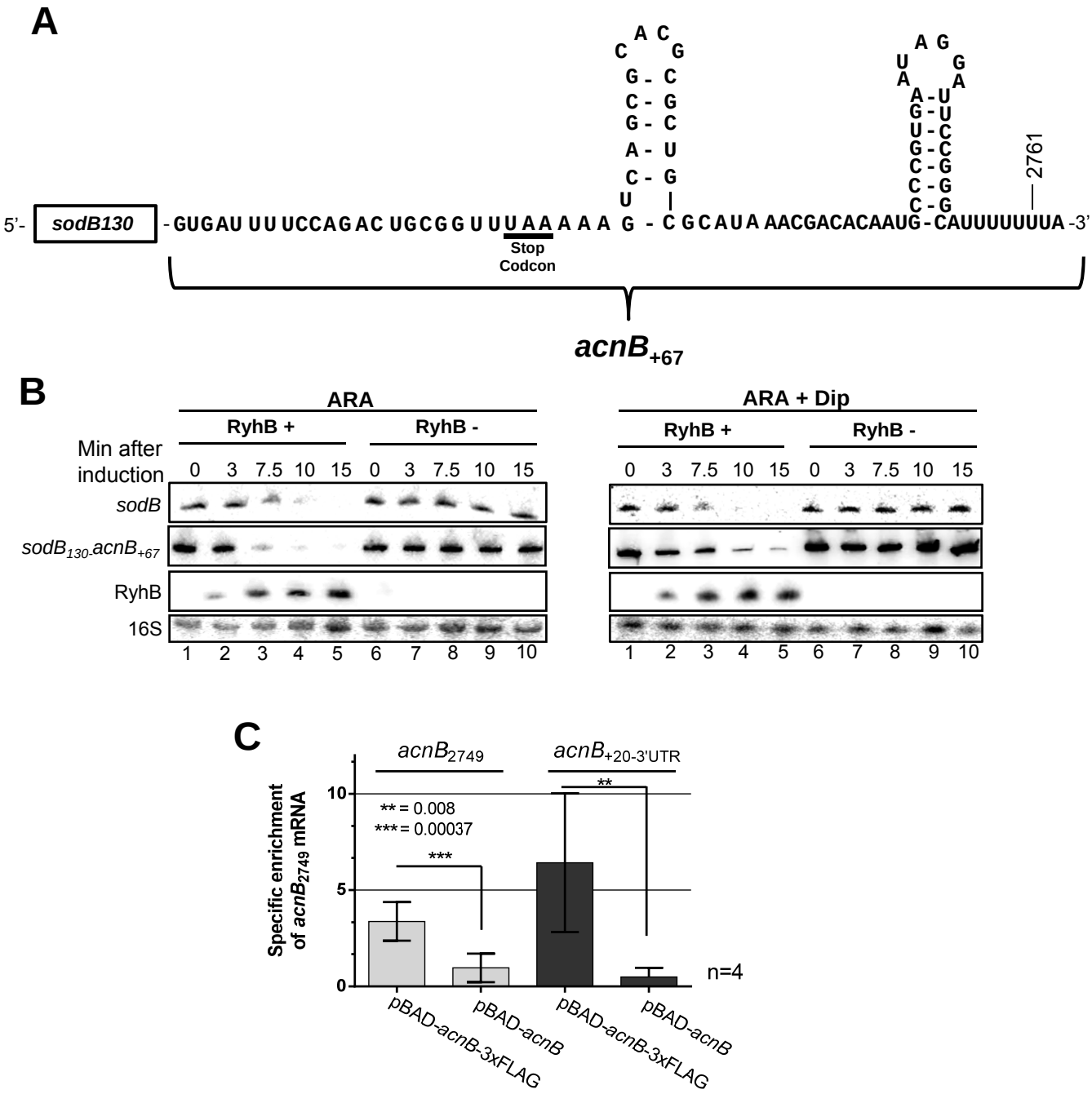

Figure S4

The *acnB* 3'UTR acts ectopically to stabilize a $sodB_{130}$-$acnB_{+67}$ chimeric fusion. (A) Schematic view of $sodB_{130}$-$acnB_{+67}$ construct in which *sodB* 5'UTR RNA (from nt 1 to nt 341) was fused to *acnB* from nt 2670 to nt 2761. (B) Northern blots of total RNA using an RNA probe specific to *sodB* showing the effect of RyhB expression on $sodB_{130}$-$acnB_{+67}$ construct. Left panel: arabinose (0.05% final) was added to induce expression of RyhB (pBAD-*ryhB*) or the control (pNM12) at an OD_{600} of 0.5. Right panel, same as left panel except the Fe chelator Dip (200 µM final) was added 10 min before RyhB induction. Expression of endogenous *sodB* mRNA and RyhB sRNA is also shown. 16S ribosomal is used as a control. (C) $AcnB_{3xFLAG}$ RNA-IP experiment of $acnB_{2749}$ construct was compared with $acnB_{2749-+20}$ construct. In both construct, *acnB* were expressed from endogenous promoter in Fe-poor media. Then, AcnB was expressed (JAB292) in cells with plasmids pBAD-$acnB_{3xFLAG}$ and pBAD-*acnB*. RNA-IP was performed at an OD_{600} of 0.5. qRT-PCR was performed for determination of $acnB_{2749}$ and $acnB_{2749-+20}$ specific enrichment by using 3'-end specific probes. Mean and s.d. of experiments are shown.

Suplementary Material and Methods

Strain and plasmids

Derivatives of *E. coli* MG1655 were used in all experiments. DH5α strain was used for routine cloning procedures. Construction of $acnB_{117}$ *–lacZ, sodB-lacZ* and *ryhB-lacZ* (EM487-EM488) transcriptional fusions was constructed as described in Mitarai *et al.* (1).

To construct the $AcnB_{2688}$-LacZ translational fusion, a PCR product obtained with oligonucleotides EM531 and EM549 was digested with *Bam*HI and ligated into *Bam*HI-digested pRS1551 (creating an in-frame translational fusion with *lacZ*). This fusion was then delivered as a single copy into the chromosome of different strains (see table S1) at the λ *attI* site, as described previously (2). Stable lysogens were screened for single insertion of recombinant λ by PCR (3).

To construct the $acnB_{2749}$*–lacZ* transcriptional fusion, a PCR product was generated with oligonucleotides EM531 and EM1557 and then digested with *Bam*H1 and ligated into *Bam*H1-digested pFRΔ vector. To generate mutated versions of $acnB_{2749}$*–lacZ*, a PCR reaction was performed using $acnB_{2749}$ PCR product as the DNA template with the following oligonucleotides: $acnB_{2749\text{-Mstem}}$ (EM531-EM1956), $acnB_{2749\text{-stem}}$ (EM531-EM2004) and $acnB_{\text{CC-stop-GCC}}$ (EM531-EM1821). For $acnB_{+20\text{-3'UTR}}$ construct, a two-step PCR strategy was used. While the first PCR was performed with oligonucleotides EM531-EM1958, the second step was performed by using oligonucleotides EM531-EM1959. The $acnB_{+20\text{-GCC}}$ construct was obtained by performing a PCR reaction with oligonucleotides EM531-EM2057 followed by another amplification reaction with oligonucleotides EM531 and EM2058. The resulting PCR product was then digested with *Bam*HI and ligated into *Bam*HI-digested pFRΔ. The cloning strategy used to generate plasmids pBAD33-$acnB_{2749}$, pBAD33-$acnB_{2749\text{Mstem}}$, pBAD33-$acnB_{2749\text{-stem}}$ and pBAD33-$acnB_{+20\text{-3'UTR}}$ was the same as described above for pFRΔ cloning except that the vector used for ligation was *Bam*H1-digested pBAD33. Those plasmids were transformed into appropriate strains before performing experiments.

To construct $sodB_{130}$-$acnB_{3'}$, a three-step PCR strategy was performed. A first PCR product ($sodB_{130}$) was obtained by using oligonucleotides EM423-EM424 and *E. coli* genomic DNA. Then a second PCR was performed with oligonucleotides EM423-EM1957 on the previous $sodB_{130}$ PCR product as template. A third PCR product was obtained by amplifying the previous PCR product with EM423 and EM1939. Finally, the PCR product was digested with *Eco*R1 and *Bam*HI and then ligated into *Eco*RI/*Bam*H1-digested pFRΔ. The resulting transcriptional fusion was delivered as a single copy into the chromosome as described previously.

The plasmid pBAD24-*acnB* was generated by PCR reactions using oligonucleotides EM1695-EM1258 to produce a promoter-less *acnB* fragment. The resulting PCR product was digested with *Bam*H1/*Pst*1 and ligated into *Bam*H1/*Pst*1-digested pBAD24. To construct the pBAD24-*acnB-3x-FLAG* plasmid, the following

oligonucleotides (EM1689-EM1747 and EM1695-EM549) were used to generate two PCR products. Following this, both products were mixed and amplified with oligonucleotides EM1695-EM1748 to generate *acnB-3x-FLAG*. Then, the final PCR was digested with *Bam*H1 and ligated into *Bam*H1-digested pBAD24.

Plasmid pET21b-*acnB-FLAG* was produced by a two-step PCR reaction to generate a promoter-less *acnB* fragment using oligonucleotides EM1259-EM1260, which then served as template for a second PCR reaction with oligonucleotides EM1259-EM1261. The final PCR product was digested with *Nde*1/*Xho*1 and ligated into pET21b.

Plasmid pGD3 is a pBAD33 derivative (4) that has been mutagenized to insert a *Xho*I site at the -6 to -1 region of the +1 transcription start site (see (5) for details). pGD3-*ryhB* was generated by a PCR reaction producing a promoter-less *ryhB* DNA fragment with a *Xho*I site upstream and an *Eco*RI site downstream (oligonucleotides EM1572-EM455). The PCR product was digested with *Xho*I and *Eco*RI and ligated into *Xho*I/*Eco*RI-digested pGD3.

RNA secondary structure probing

In line probing was performed as described previously (6). γ-5'-^{32}P-end labelled $acnB_{177}$ RNA (final concentration of 0.1 µM) was incubated with 1 µM or 2 µM RyhB RNA. The $acnB_{177}$ and RyhB RNA were produced using T7 RNA polymerase from a PCR product (see Table S3 for oligonucleotides). Ribonucleases T1 (0.1 U) (Ambion) and TA (2.5 U) (Jena Biosciences) were used for 5 min at 37^{o}C in sequencing buffer (Ambion). Alkaline ladder was generated in alkaline buffer (Ambion) for 5 min at 90^{o}C.

Toeprinting assays

Toeprinting analysis and 30S purification has been performed as described previously (5,7). Purification of Hfq protein was performed as described previously (8).

Table S1

List of all the strains and plasmids used in this study.

Strain Number	**Relevant Markers**	**Reference/Source**
EM1055	MG1655 Δ*lacX*74	(9)
EM1238	EM1055 Δ*ryhB*::*cat*	(9)
JF133	EM1055 *rne131 zce*-726::Tn*10*, *ryhB*::*cat*	(10)
EM1451	EM1055 Δ*ara*714 *leu*$^{+}$	(11)
EM1455	EM1055 Δ*ara*714 *leu*$^{+}$ *ryhB*::*cat*	EM1451 + P1vir EM1238
DY330	W3110 Δ*lacU169 gal490* ΔcI857 Δ (*cro-bioA*)	(12)
GD363	DY330 + *ryhB*::*tet* cassette	(5)
KP1135	EM1055 Δ*ara*714 *leu*$^{+}$ *ryhB*::*tet*	EM1451 + P1vir GD363
JF222	EM1055 *ryhB*::*cat* λ$acnB_{117}$'-*lacZ*	EM1238 + λpFRΔ-$acnB_{117}$'-lacZ

JAB017	EM1055 λ*ryhB*'-*lacZ*	EM1055 + λpFRΔ-*ryhB*'-lacZ
KP519	EM1055 $sodB_{130}$'-*lacZ ryhB*::*cat*	(10)
JAB242	EM1055 $sodB_{130}$-$acnB_{+67}$	EM1055 + λpFRΔ-$sodB_{130}$-$acnB_{3'}$-*lacZ*
JAB243	EM1055 *ryhB*::*cat* $sodB_{130}$-$acnB_{+67}$	EM1238 + λpFRΔ-$sodB_{130}$-$acnB_{3'}$-*lacZ*
JAB246	KP1135 Δ*ara*714 *leu*$^+$ *ryhB*::*tet* $sodB_{130}$-$acnB_{+67}$	KP1135 + λpFRΔ-$sodB_{130}$-$acnB_{3'}$-*lacZ*
GD432	JW0014-5 (*acnB*736::*kan*)	(Keio collection, Yale)
JAB154	EM1055 Δ*ara*714 *leu*$^+$ *acnB*736::*kan*	EM1451 + P1vir GD432
JAB212	EM1055 Δ*ara*714 *leu*$^+$ *ryhB*::*tet acnB*736::*kan*	JAB154 + P1vir GD363
JAB284	EM1055 *acnB*736::*kan*	EM1055 + P1vir GD432
JAB292	EM1055 *ryhB*::*tet acnB*736::*kan*	JAB284 + P1vir GD363
JAB153	EM1055 Δ*ara*714 *leu*$^+$ *ryhB*::*cat acnB*736::*kan*	EM1455 + P1vir GD432
JAB051	EM1055 Δ*ara*714 *leu*$^+$ *ryhB*::*cat* λAcnB$_{2688}$'-'LacZ	EM1055 + λpRS1551 AcnB$_{2688}$'-'LacZ
JAB068	EM1055 *ryhB*::*cat* λ AcnB$_{2688}$'-'LacZ	EM1238 + λpRS1551-AcnB$_{2688}$'-'LacZ
KP760	EM1055 Δ*ara*714 *leu*$^+$ *ryhB*::*cat* AcnB$_{2688}$'-'LacZ	EM1455 + λpRS1551-AcnB$_{2688}$'-'LacZ
Plasmids	**Description**	**Reference/Sources**
pBAD24		(4)
pNM12	pBAD24 derivative	(13)
pBAD-*ryhB*	pNM12 + RyhB (arabinose-inducible promoter)	(14)
pBAD-*ryhB*mutstart	pNM12 + RyhBmutstart (arabinose-inducible promoter)	This study
pGD3	pBAD33 derivative	(5)
pGD3-*ryhB*	pGD3 + RyhB (arabinose-inducible promoter)	This study
pBAD24-*acnB*-*3xFlag*	pBAD24 + *acnB* + 3xFlag tag (arabinose inducible promoter)	This study
pBAD24-*acnB*	pBAD24 + *acnB* (arabinose inducible promoter)	This study
pRS1551	Plasmid for construction of translational fusions	(2)
pFRΔ	pRS1553 derivative (for transcriptional fusions)	(15)
pFRΔ-$acnB_{2688}$	pFRΔ + $acnB_{2688}$	This study
pFRΔ-$acnB_{2749}$	pFRΔ + $acnB_{2749}$	This study
pFRΔ-$acnB_{2749\text{-}Mstem}$	pFRΔ + $acnB_{2749\text{-}Mstem}$	This study
pFRΔ-$acnB_{2749\text{-}stem}$	pFRΔ + $acnB_{2749\text{-}stem}$	This study
pFRΔ-$acnB_{CC\text{-}stop\text{-}GCC}$	pFRΔ + $acnB_{CC\text{-}stop\text{-}GCC}$	This study
pFRΔ-$acnB_{+20\text{-}3'UTR}$	pFRΔ + $acnB_{+20\text{-}3'UTR}$	This study
pFRΔ-$acnB_{+20\text{-}GCC}$	pFRΔ + $acnB_{+20\text{-}GCC}$	This study
pFRΔ-$sodB_{130}$-$acnB_{3'}$	pFRΔ + $sodB_{130}$-$acnB_{3'}$	This study
pBAD33		(4)
pBAD33-	pBAD33 + $acnB_{2749}$ (arabinose inducible promoter)	This study

acnB$_{2749}$		
pBAD33-*acnB*$_{2749\text{-Mstem}}$	pBAD33 + *acnB*$_{2749\text{-Mstem}}$ (arabinose inducible promoter)	This study
pBAD33-*acnB*$_{2749\text{-stem}}$	pBAD33 + *acnB*$_{2749\text{stem}}$ (arabinose inducible promoter)	This study
pBAD33-*acnB*$_{+20\text{-}3'\text{UTR}}$	pBAD33 + *acnB*$_{+20\text{-}3'\text{UTR}}$ (arabinose inducible promoter)	This study

88

89 **Table S2**

90 List of all the oligonucleotides used in this study.

Oligo number	**Sequence 5'-3'**
EM88	TGTAATACGACTCACTATAGGGCGATCAGGAAGACCCTCGC
EM89	AAAAGCCAGCACCCGGCTGGC
EM125	TGTAATACGACTCACTATAGGTGCGGTACTGGGCATTTACCC
EM140	TAATACGACTCACTATAGGGAGAGCATCACCAGGTCAGCCGAG
EM141	GATGAAGTGCGTGCTGGTGGC
EM188	TAATACGACTCACTATAGGGAGACCAGGCAGTTCCAGTAGAAAG
EM189	GCTAAAGATGCTCTGGCACCG
EM247	GTAACTTCCCGAACCGTCTG
EM293	TAATACGACTCACTATAGGGAGACGCTTTACGCCCAGTAATTCC
EM294	CTCCTACGGGAGGCAGCAGT
EM305	TAATACGACTCACTATAGGGAGAGCTTTGAAGTTATCAACGGAGC
EM306	TCAGACCTACGTAAACAACGC
EM411	ACATCCGTACTCTTCGTGCG
EM412	TGTAATACGACTCACTATAGGAGTCCAGGTTTTAGTTTCGCC
EM423	CAGTTGAATTCGGCGTGTATGTCGGCAACGG
EM424	GCTAGGGATCCTCGATGGTTTCCGCAGAAATG
EM455	CAGGCTGAAAATCTTCTCTCATC
EM487	CAGTTGAATTCCCGTGTTTCTGCGTGGCG
EM488	GCTAGGGATCCGCGAGACAATAATAATCATTC
EM531	GCTAGGGATCCTAGACCATCCTTAACGATTCAG
EM549	GCTAGGGATCCGCAGGTCTGGAAAATCACCC
EM635	TGTAATACGACTCACTATAGAACACATCAGATTTCCTGGTG
EM636	AAATCCCGACCCTGAGGG
EM1258	CCAATGCATTGGTTCTGCAGTTAAACCGCAGTCTGGAAAATCAC
EM1259	GGAATTCCATATG TGCGGTACTGGGCATTTACCC
EM1260	AACCGCAGTCTGGAAAATCACC
EM1261	CCGCTCGAGTTATTTATCGTCATCGTCTTTGTAGTC AACCGCAGTCTGGAAAATCACC
EM1484	GGTTTGCATCCAGGGGTTTGG
EM1557	GCTAGGGATCCGAATCTCATTCACCGGGCATTG
EM1572	GGGGCTCGAGGCGATCAGGAAGACCCTCGC
EM1573	GAATCTCATTCACCGGGCATTG
EM1578	TGTAATACGACTCACTATAGGAGTACCAGACCTACGTGGCG
EM1596	GAAATAAAAAAATGC CCG GGA ATC
EM1689	GACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGACTA CAAAGATGACGACGATAAATAGTAAGTGTAGGCTGGAGCTGCT
EM1695	GCTAGGGATCCTGCGGTACTGGGCATTTACCC

EM1744	GCTAGGGATCCTGCGGTACTGGGCATTTACCC
EM1747	CGCAGTCTGGAAAATCACCCGACTACAAAGACCATGACGGTG
EM1748	GCTAGGGATCCTTACTATTTATCGTCGTCATCTTTG
EM1767	CTCTACAAGTGCGGCCATTTGGTT
EM1821	GCTAGGGATCCGGCATTGTGTCGTTTATGCGCAGCGCGTGCGCTGACG GCTTAGGCCGCAGTCTGGAAAATCAC
EM1939	GCTAGGGATCCGTGATTTTCCAGACTGCGGTTTAAAAAGTCAGCGCAC GCGCTGCGCATAAACGACACAATGCCCGGTGAATGAGATTCCCGGGC ATTTTTTTATTTCGAATTCAGTCAG
EM1956	GCTAGGGATCCGAATCTCATTCACCGGGCATTGTGTCGTTTATGCGGT GCGCGTGCGCTGACTTTTTAAACCGCAG
EM1957	CCGCAGTCTGGAAAATCACCTCGATGGTTTCCGCAGAAATGTG
EM1958	GTGATTTTCCAGACTGCGGTTTAAAAAGCGUCGCUAAUUCUUGCCAG GTCAGCGCACGCGCTGCG
EM1959	GCTAGGGATCCGAAATAAAAAAATGCCCGGGAATC
EM2004	GCTAGGGATCCGAATCTCATTCACCGGGCATTGTGTCGTTTATGCGGT GCGCGTGCGCACACTTTTTAAACCGCAG
EM2057	CTGGCAAGAATTAGCGACGCGGCTTAGGCCGCAGTCTGGAAAATC
EM2058	GCTAGGGATCCGGAATCTCATTCACCGGGCATTGTGTCGTTTATGCGC AGCGCGTGCGCTGACCTGGCAAGAATTAGCGACGC

Table S3

A list of all the RNA transcripts produced by T7 RNA polymerase in vitro transcription. Indicated are the final product (and its purpose), the oligonucleotides used to generate the DNA template by PCR and the DNA template used for the PCR.

Final product	**Oligonucleotides**	**DNA template**
acnB (Northern probe)	EM140 - EM141	Genomic DNA
RyhB (Northern probe)	EM88 - EM89	Genomic DNA
sodB (Northern probe)	EM188 - EM189	Genomic DNA
sodA (Northern probe)	EM305 - EM306	Genomic DNA
hns (Northern probe)	EM411 - EM412	Genomic DNA
16S rRNA (Northern probe)	EM293 - EM294	Genomic DNA
$acnB_{177}$ (5'end) (PbAc probing/toeprinting)	EM125 – EM1767	Genomic DNA
acnB (3'end) (Footprint/ degradation assay)	EM1578 – EM1596	Genomic DNA or pFRΔ-$acnB_{\text{CC-stop-GCC}}$
RyhB (PbAc probing/toeprinting)	EM88-EM89	Genomic DNA
DsrA (PbAc probing/toeprinting)	EM635-EM636	Genomic DNA
acnB (qRT-PCR)	EM247-EM1573	Genomic DNA

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