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J R Mellin, Mikael Koutero, Daniel Dar, Marie-Anne Nahori, Rotem Sorek, et al.. Riboswitches. Sequestration of a two-component response regulator by a riboswitch-regulated noncoding RNA.. Science, 2014, 345 (6199), pp.940-3. 10.1126/science.1255083 . pasteur-01120664

HAL Id: pasteur-01120664

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Submitted on 24 Apr 2015

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Sequestration of a two-component response regulator by a riboswitch-regulated non-coding RNA

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1 **Abstract:**

2 Riboswitches are ligand-binding elements contained within the 5' untranslated
3 regions of bacterial transcripts, which generally regulate expression of downstream
4 open reading frames. Here we show that in *Listeria monocytogenes*, a Vitamin B₁₂-
5 binding (B₁₂) riboswitch controls expression of a non-coding regulatory RNA, Rli55.
6 Rli55 in turn controls expression of the *eut* genes, which enable ethanolamine
7 utilization and require B₁₂ as a cofactor. Defects in ethanolamine utilization, or in its
8 regulation by Rli55, significantly attenuate *Listeria* virulence in mice. Rli55 functions
9 by sequestering the two-component response regulator, EutV via a EutV binding-site
10 contained within the RNA. Thus Rli55 is a riboswitch-regulated member of the small
11 group of regulatory RNAs that function by sequestering a protein and reveals a
12 unique mechanism of signal integration in bacterial gene regulation.

1 Ethanolamine is an abundant molecule in the vertebrate intestine (1, 2), and
2 genes of the ethanolamine utilization pathway (*eut*) are widely conserved in
3 pathogenic bacteria (3-5). This includes the Gram positive intracellular human
4 pathogen *Listeria monocytogenes*, in which *eut* expression has been shown to be up-
5 regulated in the intestine during infection of mice (6), suggesting that ethanolamine is
6 important for *Listeria* pathogenesis. In *Enterococcus faecalis* *eut* expression is
7 activated in response to ethanolamine by a two-component response regulator,
8 EutVW (7, 8). Ethanolamine utilization requires B₁₂ as a cofactor (9), and we noted
9 the presence of a B₁₂-binding riboswitch located upstream of the first gene in the *eut*
10 locus of *L. monocytogenes* (Fig. 1A) (10), suggesting *eut* expression might also be
11 regulated in response to B₁₂ availability.

12 To investigate a role for B₁₂, we examined expression of the *eut* locus in
13 response to B₁₂ and ethanolamine by RNA-seq (Fig. 1B) and qRT-PCR (fig. S1). We
14 observed expression of the *eutVW* genes under all conditions, albeit at low levels,
15 suggesting that the cell maintains a pool of EutVW to sense and respond to
16 ethanolamine. In contrast, higher-level expression of *eutVW* and expression of other
17 *eut* genes requires both B₁₂ and ethanolamine (Fig. 1B). These data indicated B₁₂ is
18 required to activate *eut* expression and suggested that the B₁₂ riboswitch does not
19 prevent transcription of the *eut* locus in the presence of B₁₂, as might be expected for
20 a classical riboswitch (11).

21 To clarify if the riboswitch has a role in the B₁₂-dependent regulation of *eut*
22 expression we examined transcription of the riboswitch locus (Fig. 1C) in response to
23 B₁₂ and ethanolamine. We were unable to detect any long transcript, which might
24 extend into the downstream *eut* locus. However, we did detect a ~450nt transcript,
25 Rli55 (10, 12) in the absence of B₁₂ (Fig. 1D), and a smaller (~200nt) transcript which
26 accumulated in the presence of B₁₂ (Fig. 1D). This result suggested the riboswitch
27 mediates transcription termination of the ~450nt Rli55 transcript in response to B₁₂,
28 and any remaining long transcript is rapidly degraded (fig. S2A). A strain with a

1 deletion in the B₁₂ riboswitch (Δ ribo), constitutively expressed the long Rli55
2 transcript under all conditions (Fig. 1E, fig. S2B), confirming B₁₂-dependent regulation
3 by the riboswitch. Conversely, neither the long or short Rli55 transcripts were
4 detected in a strain (Δ rli55) in which the entire *rli55* locus was deleted (Fig. 1E, fig.
5 S2B). Thus the B₁₂ riboswitch determines whether Rli55 is expressed as a long or
6 short transcript in response to B₁₂.

7 We reasoned Rli55 might act as a regulatory RNA controlling expression of
8 the *eut* locus. To test this hypothesis we examined expression of *eut* genes by qRT-
9 PCR in the wild-type, Δ ribo and Δ rli55 strains. In the wild-type strain *eut* expression
10 was elevated only when both ethanolamine and B₁₂ were present in the media (Fig
11 1F, fig S3A&B), whereas in the Δ ribo strain, which constitutively expresses the long
12 Rli55, *eut* genes were never expressed under any conditions. In contrast, in the
13 Δ rli55 strain, expression of the *eut* genes was high in the presence of ethanolamine
14 alone in addition to ethanolamine and B₁₂ together. Together, these data support a
15 model in which Rli55 prevents the expression of the *eut* locus in the absence of B₁₂,
16 ensuring the *eut* genes are expressed only in the presence of both ethanolamine
17 (substrate) and B₁₂ (cofactor) (fig. S3C).

18 We tested whether defects in ethanolamine utilization, or its regulation,
19 impacted *L. monocytogenes* virulence, by examining the wild-type, Δ ribo and Δ rli55
20 strains in a mouse intravenous infection model. We also tested a mutant lacking the
21 *eutB* gene encoding an ethanolamine lyase subunit, rendering it unable to catabolize
22 ethanolamine. The Δ ribo and Δ eutB strains both had significantly reduced bacterial
23 loads at 24 hours post infection compared to the wild-type strain, and these
24 differences increased at 48 hrs and 72 hrs post infection (~10 – 50-fold) (Fig. 2 A -
25 F). In contrast, the Δ rli55 strain, in which *eut* expression is not inhibited, was present
26 in comparable amounts to the wild-type strain in the spleen and liver. Thus defects in
27 ethanolamine utilization or activation of *eut* expression significantly attenuate *L.*
28 *monocytogenes* virulence.

1 In *E. faecalis*, ethanolamine is sensed by the sensor-kinase EutW, which
2 subsequently phosphorylates the response regulator, EutV (7). Phosphorylated EutV
3 in turn binds ANTAR elements (AmiR and NasR Transcriptional Antiterminator
4 Regulator) in the 5'UTRs of actively transcribed *eut* mRNAs, which prevents the
5 formation of a transcription terminator and consequently activates *eut* expression
6 (13). In *L. monocytogenes* ANTAR sites were identified upstream of the *eutA* and
7 *eutV* genes and a third site was identified in the *rli55* locus upstream of the *eutG*
8 gene and downstream of the B₁₂ riboswitch (13). We also identified a second ANTAR
9 site in the *rli55* locus (Fig. 3A), suggesting that one or both of these ANTAR elements
10 could be transcribed as part of the 3' end of Rli55 RNA. Indeed, our RNA-seq data
11 showed that, in the absence of B₁₂, high levels of Rli55 are transcribed as a long
12 transcript encompassing the first ANTAR element (Fig. 3A). In contrast, *rli55*
13 transcription terminates abruptly after the riboswitch in the presence of B₁₂, indicating
14 the riboswitch determines if Rli55 is transcribed with or without an ANTAR element.
15 This notion was supported by the detection of putative Rli55 orthologues in *E.*
16 *faecalis* and *Streptococcus sanguinis* (fig. S4). In the latter, the riboswitch, in
17 conjunction with a single ANTAR element, has undergone an inversion relative to the
18 adjacent *eutG* gene, suggesting the riboswitch and the first ANTAR element are
19 functionally linked (fig. S4B), and that this is also the case in *L. monocytogenes*.

20 To test if the ANTAR elements are involved in Rli55-mediated regulation, we
21 complemented the $\Delta rli55$ strain with chromosomally integrated *rli55* alleles carrying
22 mutations in the ANTAR elements and examined which ones restored Rli55-
23 mediated regulation of *eut* expression (Fig 3B&C, fig. S5). A strain with an empty
24 construct ($\Delta rli55::\text{Empty}$) could not prevent expression of the *eut* genes in
25 ethanolamine alone (as in the parental $\Delta rli55$ strain), whereas a strain with a wild-
26 type copy of *rli55* ($\Delta rli55::rli55$) fully restored Rli55-mediated inhibition. However, a
27 strain with a deletion in the riboswitch ($\Delta rli55::\Delta\text{ribo}$) inhibited *eut* expression in all
28 conditions, as the riboswitch can no longer terminate *rli55* transcription in response to

1 B₁₂. In a strain $\Delta rli55::rli55\Delta M1$, wherein four uridine residues in the first ANTAR site
2 were mutated to adenines (Fig. 3D, $\Delta M1$), inhibition of *eut* expression by Rli55 was
3 abolished in the presence of ethanolamine alone. In contrast, in strain
4 $\Delta rli55::rli55\Delta M1/M2$, where compensatory mutations were made to the opposite side
5 of the ANTAR stem-loop (Fig. 3D, $\Delta M2$), wild-type regulation of *eut* expression was
6 restored. Mutation of the six nucleotides in the stem-loop of the second ANTAR
7 element (Fig. 3D, $\Delta M3$, $\Delta rli55::rli55\Delta M3$) had no significant effect on Rli55-mediated
8 regulation. Thus the first ANTAR element is necessary and sufficient for Rli55-
9 mediated regulation.

10 The long form of Rli55 containing an ANTAR element might bind and
11 sequester EutV, preventing it from activating expression of the *eut* genes in the
12 presence of ethanolamine but absence of B₁₂. When sufficient levels of B₁₂
13 accumulate, B₁₂ would bind the riboswitch, producing truncated Rli55 transcripts,
14 which would lack an ANTAR element and be unable to sequester EutV. To examine
15 this hypothesis, we constructed a strain with an additional copy of the *eutV* gene
16 carrying a 2XFLAG-tag (EutV^{FLAG}) and first showed that expression of EutV^{FLAG}
17 protein is regulated identically to the native *eutV* gene in response to ethanolamine
18 and B₁₂ (fig. S6A). We also constructed a strain with an additional *eutV* gene lacking
19 a FLAG tag (EutV^{NOFLAG}). Anti-FLAG immunoprecipitations with cell lysates from
20 these two strains (fig. S6B&C) followed by RNA-seq analysis (Fig. 3E&F) showed
21 that Rli55 is enriched by co-immunoprecipitation with EutV^{FLAG} primarily when
22 bacteria are grown in the presence of ethanolamine alone, while we saw no
23 enrichment in a parallel immunoprecipitation with the EutV^{NOFLAG} strain (Fig. 3E). In
24 contrast, the ANTAR element upstream of the *eutV* gene (Fig. 3F) is enriched by co-
25 immunoprecipitation of lysates from EutV^{FLAG} bacteria, but not EutV^{NOFLAG} bacteria,
26 grown in the presence of ethanolamine and B₁₂ together but not from lysates of
27 bacteria grown in ethanolamine alone. To a lesser extent the ANTAR containing
28 region upstream of *eutA* and the entire *eutA-Q* locus is enriched under the latter

1 condition (fig. S7). These data support a model in which the majority of EutV is bound
2 and sequestered by Rli55 in the presence of ethanolamine alone. Conversely, in the
3 presence of ethanolamine and B₁₂, the riboswitch produces short truncated Rli55
4 transcripts, which cannot bind EutV, allowing EutV to bind *eut* mRNAs and activate
5 *eut* expression (Fig. 3G).

6 This riboregulatory mechanism coordinates expression of the ethanolamine
7 utilization (*eut*) locus with the availability of B₁₂, the essential cofactor for
8 ethanolamine catabolism. Our results highlight a so-far undescribed role for
9 ethanolamine utilization in infection. Previously ethanolamine utilization has been
10 shown to be important after oral infection (3, 4, 14), however, the contribution of
11 ethanolamine utilization to *L. monocytogenes* pathogenesis in an intravenous mouse
12 infection model suggests that ethanolamine utilization is important outside of the
13 intestine and possibly in the intracellular environment. This study also extends the
14 role of riboswitches in the regulation of non-coding RNAs (15, 16). Finally, our data
15 show that Rli55 represents a new member of the small family of regulatory RNAs that
16 function by sequestering a protein, which also includes the 6S and CsrB/C RNAs (17)
17 and highlights a unique means of signal integration in bacterial gene regulation.

1 **Figure 1. Control of Ethanolamine utilization (*eut*) genes by a B₁₂-riboswitch**
2 **regulated ncRNA. (A)** The *eut* locus. Green arrow denotes B₁₂ riboswitch. Vertical
3 arrows denote positions of ANTAR elements (13) **(B)** RNA-seq coverage of *eut*
4 operon. **(C)** Regions deleted in the $\Delta rli55$ and $\Delta ribo$ strains are indicated by solid
5 black lines. Northern blot of the *rli55* transcript in the **(D)** wild-type strain with probe
6 indicated by thick dashed line in schematic or **(E)** wild-type (wt), $\Delta ribo$ or $\Delta rli55$
7 strains with probe indicated by thin dashed line in schematic. Ethidium bromide
8 staining of rRNA is shown as a control. Quantitation of bands is shown below each
9 blot. Expression of the **(F)** *eutV*, *eutA* & *eutT* genes was evaluated by qRT-PCR in
10 the indicated strains. Values represent means +/- SEM, n = 3, * = p<0.05, ns = not
11 significant.

12

13 **Figure 2. Ethanolamine utilization and pathogenesis.** BalbC mice were injected
14 intravenously with ~ 4,500 CFU of the indicated strain. Mice were sacrificed at **(A &**
15 **B)** 24 hr, **(C & D)** 48 hr or **(E & F)** 72 hr and spleens and livers removed to assess
16 bacterial load per organ. Results represent two independent experiments with 3-4
17 mice per group in each experiment. * = p<0.05, ns = not significant.

18

19 **Figure 3. Rli55 sequesters the two-component response regulator EutV. (A)**
20 RNA-seq coverage in reads per million (RPM) of the *rli55* locus from bacteria grown
21 in as indicated. **(B & C)** qRT-PCR of *eutV* & *eutA* genes. vitamin B₁₂ (B₁₂),
22 ethanolamine (Ea). Values represent means +/- SEM, n=3. All differences were
23 significantly different (* = p<0.05) from the $\Delta rli55::$ Empty strain in the Ea condition,
24 except where indicated (ns). All differences were not significantly different from the
25 $\Delta rli55::$ Empty strain in the B₁₂ + Ea condition, except where indicated **(D)** Secondary
26 structure of the ANTAR elements encoded in the boxed region of Fig. 3A. Mutations
27 are shown in orange. **(E & F)** Histogram shows RNA-Seq coverage in reads/million
28 (RPM) of the *rli55* and *eutV* loci with RNA isolated by co-immunoprecipitation of cell

1 lysates from either EutV^{FLAG} or EutV^{NOFLAG} cultures grown in the presence of
2 ethanolamine (Ea) or ethanolamine and B₁₂ together. **(G)** Proposed model of Rli55-
3 mediated regulation of EutV in the presence of ethanolamine (Ea) alone or
4 ethanolamine + B₁₂.

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5 **Acknowledgments:**

6 We are grateful to Wade Winkler, Danielle Garsin, Nicole King and Joseph Pederson
7 for helpful and insightful discussions, and Yanjie Chao and Jörg Vogel for assistance
8 with the CLIP-Seq protocol. This work was supported by grants to PC, ERC
9 Advanced grant (233348), ANR (BACNET 09-BLAN-0024-02), ANR Investissement
10 d'Avenir Programme (10-LABX-62), Fondation Le Roch and Fondation Jeantet. PC is
11 a International Senior Research Scholar of the Howard Hughes Medical Institute.

12

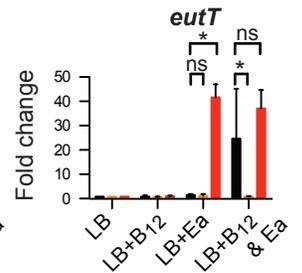
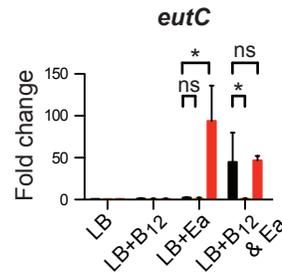
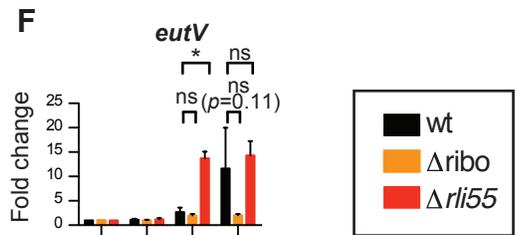
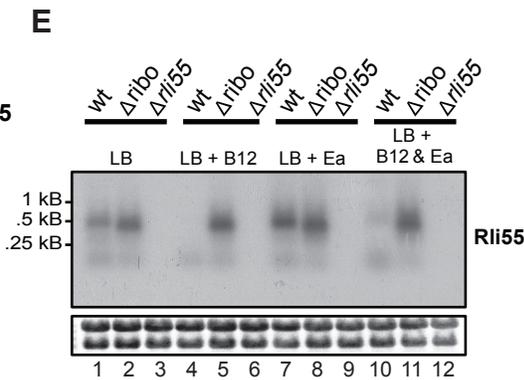
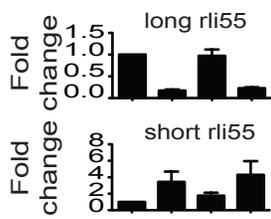
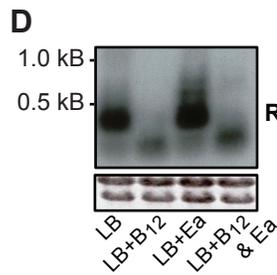
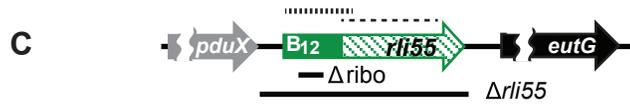
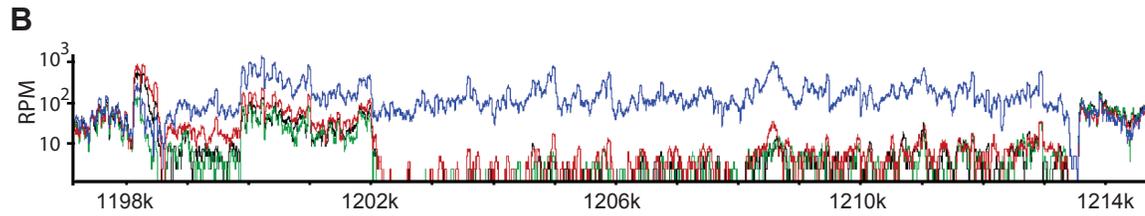
13 **Supplementary Materials:**

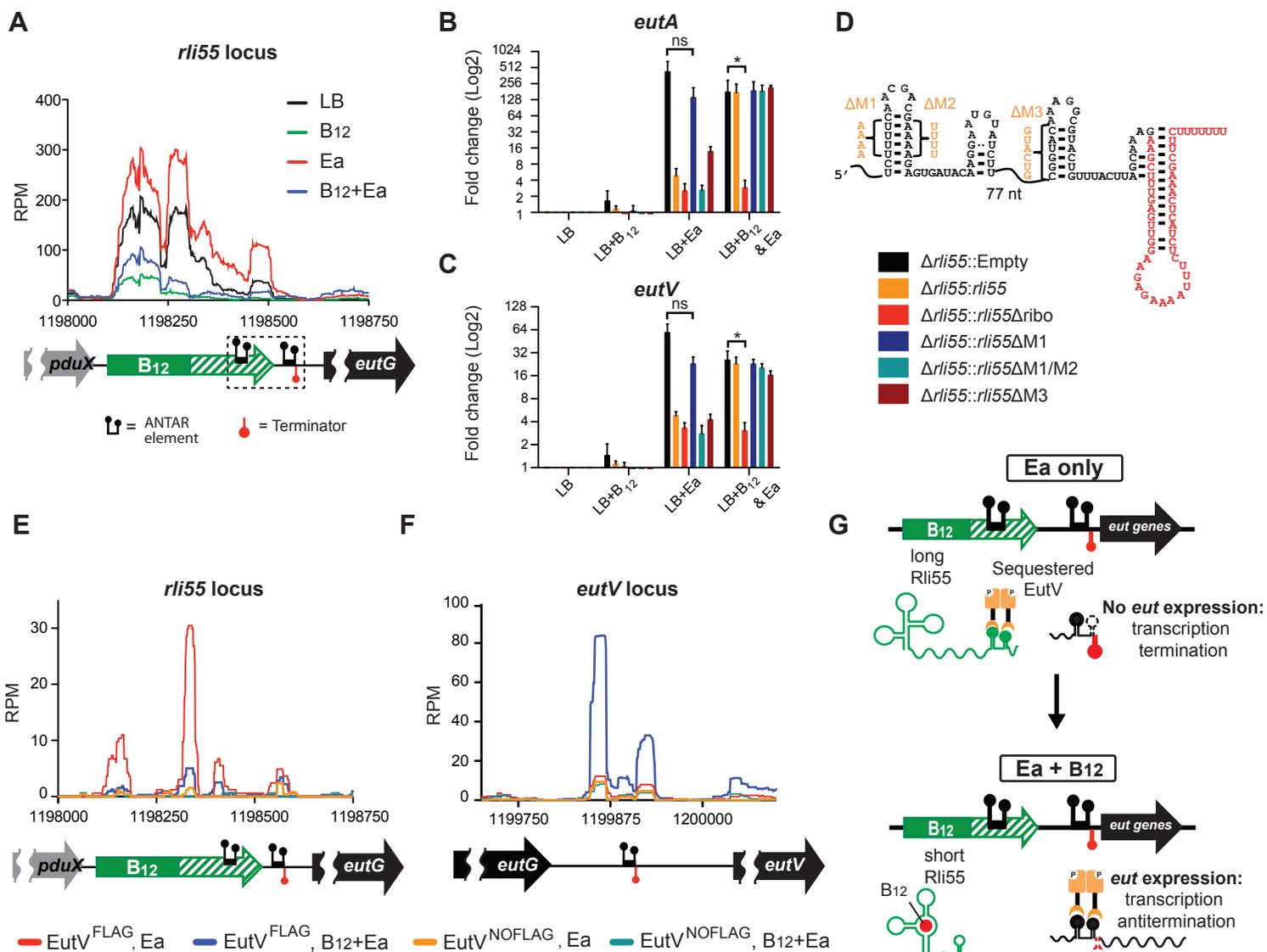
14 Materials and Methods

15 Figures S1-S7

16 Tables S1-S2

A ethanolamine utilization (*eut*) operon







Supplementary Materials for

Sequestration of a two-component response regulator by a riboswitch-regulated non-coding RNA

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Materials and Methods
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Materials and Methods

Bacterial Strains, Plasmids, Primers and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table S1. Primers used in this study are listed in Table S2. For all northern blot and qRT-PCR experiments *Listeria monocytogenes* and *Enterococcus faecalis* were grown in LB broth with shaking at 200 rpm in Erlenmeyer flasks at 37° C. *Streptococcus sanguinis* was grown in BHI broth. LB and BHI were supplemented with 10 mM ethanolamine (Sigma, 98136) or 20 nM Vitamin B₁₂ (Sigma, C0884), as indicated. *E. coli* was grown in LB broth. For all RNA isolations bacteria were inoculated to OD₆₀₀ 0.02 and grown to and OD₆₀₀ of 0.3 – 0.5.

Constructions of Plasmids and Strains

All cloning followed standard molecular biology protocols. For construction of pMAD-based plasmids for generation of mutants, regions of 500 – 700 nt regions of complementarity both upstream and downstream of a targeted region were PCR amplified with Phusion High fidelity polymerase and reagents (Finnzymes, F-553) using genomic DNA as a template. Upstream and downstream regions were joined with a second overlapping extension PCR reaction using the first two PCR products as template to generate a “UD” PCR product. UD PCR products were subsequently purified with QIAquick PCR purification columns (Qiagen, 28104), digested with the appropriate restriction enzyme (New England Biolabs), purified again as before, and ligated into digested pMAD plasmid at overnight at 16° C with T4 DNA ligase (New England Biolabs, M0202S). 2 µl of each ligation were transformed into chemically competent *E. coli* Top10 (Invitrogen, C404003) cells according to the manufacturers instructions. Transformants were screened by PCR for the presence of the appropriate insert and purified plasmid was isolated from positive colonies by miniprep using QIAprep spin columns (Qiagen, 27104). Electrocompetent *L. monocytogenes* strains were transformed with each respective plasmid and grown at 30° C for two days on BHI X-Gal plates. Blue colonies were selected and grown in BHI at 30° C for 6 hours. Cultures were subsequently diluted 1:1000 in fresh BHI and grown overnight at 42° C. Culture were serially diluted and plated onto BHI X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (80 µg/ml), Erythromycin (5 µg/ml) plates. Blue, resistant colonies have the plasmid integrated into the chromosome, as the pMAD plasmid cannot replicate at high temperature. Colonies with the plasmid integrated into the chromosome were subsequently inoculated into fresh BHI and grown at 30° C the subsequent day for 6 hrs and then shifted to 42° C for three hours. Cultures were again serially diluted and plated onto BHI, X-Gal plates without selection. White colonies were screened by PCR, for loss of the plasmid, which can occur through recombination with the repeated upstream or downstream regions, to either regenerate the wild-type chromosome or a deletion mutant. All mutants were confirmed by DNA sequencing. For complementation plasmids,

genomic DNA was used to amplify a fragment containing the *rli55* gene. Subsequent mutations were introduced by site-directed mutagenesis using primers containing mismatches at the desired bases. Briefly, two fragments containing overlapping regions with the desired mutation were produced and then joined by a subsequent splice overlap extension PCR, digestion, and ligation into the pJM2 vector. For the construction of the EutV^{FLAG} strain, a synthetic gene containing a C-terminal FLAG tag was ordered from IDT DNA, and used as a template for amplification and subsequent digestion and cloning into the pJM2 vector.

RNA isolation

For all RNA isolations, 25 ml cultures of *L. monocytogenes* were grown to an OD₆₀₀ of 0.3 – 0.5 and subsequently pelleted by centrifugation at 4000 rpm for 20 mins in 50 ml Falcon tubes with swing bucket rotor. Pellets were resuspended in 1ml Tri Reagent (Sigma, 93289), transferred to 2 ml Lysing Matrix Tubes (MP Biomedicals, 6911) and mechanically lysed by bead beating in a FastPrep apparatus (45 secs, speed 6.5 followed by an additional 30 secs, speed 6.5). Subsequently tubes were centrifuged for 5 min at 8000 X G, 4° C in a tabletop centrifuge to separate beads from lysates. Lysates were drawn off and transferred to 2 ml Eppendorf tubes. RNA isolation proceeded according to the manufacturers instructions. Briefly, 200 µl Chloroform (Carlo Erba Reagents, 438601) was added to the lysate, shaken and incubated for 10 minutes at room temperature followed by centrifugation for 15 min at 13,000 X G, 4° C. The upper aqueous phase was removed and transferred to a new 1.5 ml Eppendorf tube and RNA was precipitated by the addition of 500 µl Isopropanol and incubation at room temperature for 5-10 mins. RNA was pelleted by centrifuging for 10 mins at 13,000 X G, 4° C. The supernatant was discarded and the pellet was washed twice with 75% ethanol. RNA pellets were resuspended in 50 µl water.

Northern Blots

RNA probes radioactively labeled with α-32P-UTP (Perkin-Elmer, BLU007X500UC) were generated using the T7 Maxiscript kit (Ambion, AM1314) and PCR templates were amplified from genomic DNA using primers listed in Table S2. For each sample, 10 – 20 µg of RNA was incubated 1:2 by volume with Formaldehyde loading dye (Ambion, 8552) supplemented with 20 µg/ml ethidium bromide, and incubated at 65° C for 15 min to denature RNA secondary structures. Samples were loaded onto 1% agarose gels (Ambion, 9040) and electrophoresed in Northernmax Denaturing Gel Buffer (Ambion, AM8676) at 60 mV for approximately 2 hrs. RNA was transferred to Nytran membranes (Sigma, Z670286) by passive downward transfer with 20X SSC. Membranes were photographed under ultraviolet light to capture ethidium bromide staining of ribosomal RNA bands for loading controls. The positions of RNA size markers (Ambion, AM7150 or a in-house generated set) were marked with a scalpel. RNA was crosslinked to membranes by exposure to UV light for five minutes and membranes were prehybridized in Ultrahyb buffer (Ambion, AM8670) for 30 mins

before probes were added an incubated overnight. Hybridizations were carried out at 60 - 68° C. The following morning, membranes were washed twice for 5 mins in 2X SSC, 0.1% SDS at room temperature with constant agitation, followed by two washes in in 0.1X SSC, 0.1% SDS at the respective hybridization temperature with constant agitation. Membranes were wrapped in cellophane and subsequently used to expose films (GE Healthcare, 28906844). Quantitation of blots represent mean values of three biological replicate experiments +/- SD, normalized to loading controls.

Determination of RNA half-life

Strains were grown to an OD₆₀₀ of 0.3 – 0.5 in either ethanolamine alone (0.01 mM) or ethanolamine (0.01 mM) and B₁₂ (20 nM). Cells were subsequently treated with rifampicin (500 µg/ml final concentration) and Nalidixic acid (40 µg/ml final concentration), dissolved in DMSO, to halt further transcription and DNA synthesis. Aliquots were removed time 0 (just before treatment) or after treatment at 2 min, 5 min, 9 min, 16 min, 24 min and 33 min. Cells were harvested by centrifugation in a tabletop centrifuge at 13,000 rpm for 1 min. Pellets were resuspended in 1 ml Trizol, flash frozen in liquid nitrogen and subsequently RNA was isolated as described above. For northern blots, 5 µg of Total RNA was run on 1% agarose gels, and northern blots were carried out as described above with either the T7riboprobe or the T716S RNA probes made by T7 RNA transcription as described above. Blots were imaged with a Typhoon FLA9000 phosphoimager and quantified with the MultGauge V3.0 software suite. Values represent mean values of three biological replicate experiments +/- SD, normalized to 16S loading controls.

Quantitative Real Time PCR

For each sample, 10 µg of RNA was treated with Turbo DNase (Ambion, AM2238) for 1 hr at 37 C in a 20 µl reaction. RNA was subsequently purified by ammonium acetate precipitation, washed once with 75% ethanol and resuspended in water. cDNA was synthesized from 1 µg of RNA using the iScript cDNA Synthesis kit (Bio-Rad, 170-8890) according to the manufacturer's instructions and reactions were subsequently diluted with 180 µl of water. qRT-PCR reactions were prepared in 10 µl volumes on 384 well plates (ABI, 4309849). Reactions were prepared as follows: 2 µl of diluted cDNA, 2 µl water, 5 µl SYBR Green master mix (ABI, 4309155), .5 µM of gene specific oligonucleotides, water to 10 µl total volume. Oligonucleotides were designed using a primer design program available at <http://eu.idtdna.com/Scitools/Applications/Primerquest/>, and are listed in Table S2. Primer efficiencies were evaluated by generating a standard curve with serial dilutions and shown to be between 90%-110% in all cases. Reactions were carried out and quantified on an ABI Prism 7900HT machine with SDS2.4 software. PCR conditions were as follows: 95° C for 10 min followed by 40 cycles of 95° C 15 secs and 60° C 15 secs. Specificity of the amplified product and primer dimer formation was verified for each primer set by the presence of only a single peak in a dissociation step

carried out after each run. The absence of contaminating DNA was verified with control samples for each RNA sample for which no prior reverse transcription reaction was carried out. Expression levels of all genes were normalized to the *L. monocytogenes* *rpoB* gene, and fold changes were calculated using the $\Delta\Delta CT$ method. All samples were evaluated in triplicate and at least three independent experiments were carried out for each gene evaluated. For statistical evaluation, all values were log₂ converted and evaluated by paired t-test. Differences were considered statistically significant when p values were < 0.05.

Intravenous mouse infections

BalbC mice were injected intravenously with 5000 CFU +/- 1800 CFU of the indicated strain. Inoculums were verified by plating of serially diluted aliquots onto BHI plates before and after injection followed by counting of colony forming units after overnight growth at 37° C. Mice were sacrificed at subsequent timepoints and spleens and livers removed, homogenized and serially diluted. Dilutions were plated onto BHI plates and grown overnight at 37° C. Colonies were counted to assess bacterial load per organ. Two independent experiments were carried out with 4 mice per group in each experiment. (Note: two data points were removed due to contamination of the plates, and one mice injected with the Δ ribo strain died within 24 hr after injection, likely due to a misplaced injection, and was not included in the analysis). Statistically significant differences were evaluated by the Mann-Whitney test, and differences were considered statistically significant when p values were < 0.05.

Whole transcriptome RNA-Sequencing

10 μ g DNase I treated RNA was depleted of ribosomal RNA using MicrobeExpress (Life Technologies, AM1905). 1 μ g RNA was fragmented at 72° C for 2min using a fragmentation kit (Ambion, AM8740) and cleaned using AMPure XP magnetic beads (Agencourt, A63880). cDNA was generated by reverse transcription (RT) with random hexamers (Thermo Scientific, SO142) using the Affinity Script enzyme (Agilent, 930107-51) according to the manufacturer's instructions at a final volume of 20 μ l. Second strand synthesis was performed in by adding the following to the completed RT reaction: 8 μ l NEB2 10X buffer, 3 μ l dNTPs 10mM, 3 μ l dATP 10mM, 24 U of E. coli DNA polymerase I, 200 U RNase H and 400 U of T4 DNA ligase (New England BioLabs, M0202S). Final volume was adjusted to 80 μ l using DNase free water and the reaction was incubated at 16° C for 2h. dsDNA was cleaned using magnetic beads and then subjected to A-base addition and adapter ligation with adapters carrying the Illumina sequences as described (18). The resulting library was amplified with 16 cycles of PCR using the Kappa-HiFi enzyme (KAPA Biosystems, KK2601). Libraries were sequenced on the Hiseq2500 and reads were mapped to the corresponding reference genome (NC_003210) using Novoalign (Novocraft V3.02.02). Data analysis was performed using in-house Perl scripts.

CLIP-Seq (Cross-linking followed by immunoprecipitation and RNA-Seq)

I. Immunoprecipitation

The CLIP-seq protocol was adapted from (19). Strains carrying either a FLAG-tagged or untagged copy of the *eutV* gene in the *tRNA^{ARG}* locus were cultured overnight in LB media. Overnight cultures were inoculated into 500 ml of fresh LB media in 2L Erlenmeyer shake flasks to an OD₆₀₀ of 0.02 and grown to OD₆₀₀ 0.3-0.5. Cultures were centrifuged in a Sorval ultracentrifuge (RC26 Plus) at 8000 rpm for 20 mins, resuspended in 20 ml of fresh media, spread in 12 cm X 12 cm dished to a thickness of ~2mm and exposed to 0.8 J of 254 nm UV light in an Uvitec CL508S UV Crosslinker. Cultures were subsequently centrifuged again at 4000 rpm in a swing bucket centrifuge (Sigma, 4K15) for 20 mins. Pellets were resuspended in lysis buffer (20mM Tris pH8.0, 1mM MgCl₂, 150mM KCl, supplemented with 1 Complete protease inhibitor tab (Roche, 11697498001), 1 phosphoSTOP phosphatase inhibitor tab (Roche, 04906837001) and 0.2 U/ml RNasin (Promega, N251B per 30 ml) and lysed by 7 cycles of mechanical agitation in with Lysing Matrix B glass beads (MP Biomedicals, 6911-100) in a FastPrep apparatus (speed 6.5, 45 secs followed by 1 min on ice). Lysates were centrifuged in a tabletop centrifuge for 2 mins at 13,000 rpm to clarify lysates. 100 µl of lysate was removed and used for total RNA isolation. The remaining lysate was added to 40 µl of α-FLAG M2 affinity gel (Sigma, A2220) pre-equilibrated with two 200 µl washes in lysis buffer and incubated at 4°C for 4 hrs with agitation. Beads were subsequently washed 4 times for 15 mins in lysis buffer followed by elution with three washes of 80 µl 200 µg/ml 3X FLAG peptide (Sigma, F4799). RNA was subsequently isolated as described above with Tri Reagent (Sigma, 93289) according to the manufacturer's instructions, with the addition of glycogen (Ambion, AM9510) to the precipitation step to a concentration of 0.05 µg/µl.

II. RNA-Seq

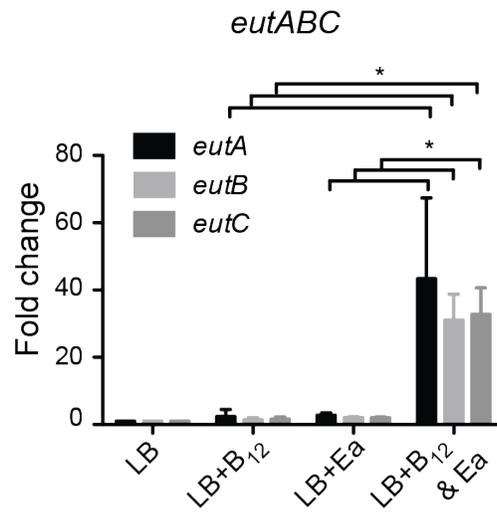
All column purifications were performed with RNA Clean & Concentrator-5, Zymo Research (Proteogene, R1015) following manufacturer's recommendations. 400-600 ng of RNA was fragmented 5 mins (Ambion, AM8740) and purified. RNA was subsequently treated with 2U of Antarctic Phosphatase (NEB, M0289S) for 30 mins at 37°C and the enzyme was heat inactivated by heating for 5 mins at 65°C. RNA fragments were 5' monophosphorylated with 20 U of T4 polynucleotide kinase (NEB, M0201S) and 1.667 mM ATP for 1 hour at 37°C before a column purification. Libraries were constructed using NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 1) (NEB, E7300S), adjusting adapter concentrations as suggested in manufacturer's recommendations. Library were subsequently purified with AMPure XP beads (Agencourt, A63880) and checked for quality with Bioanalyzer DNA High Sensitivity Chips (Agilent, 5065-4626). Sequencing was performed on a MiSeq (Illumina) in a multiplexed single read using a MiSeq Reagent Kit v3 (Illumina, MS-102-3001). Raw sequence files (fastq) were cleaned of 3' Adapter sequences (AGATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNATCTCGTATG CCGTCTTCTGCTTG), and low quality (cutoff 28) and short sequence (15nt) reads were removed using AdapterRemoval v1.5.2 (Lindgreen *et. al*, 2012)

software. Reads were aligned against the reference genome (NC_003210) with bowtie v1.0.0 (Langmead *et al.*, 2009) using default parameters, counted with HTSeq v0.6.0 and normalized to reads per million.

SDS-PAGE and Western blot

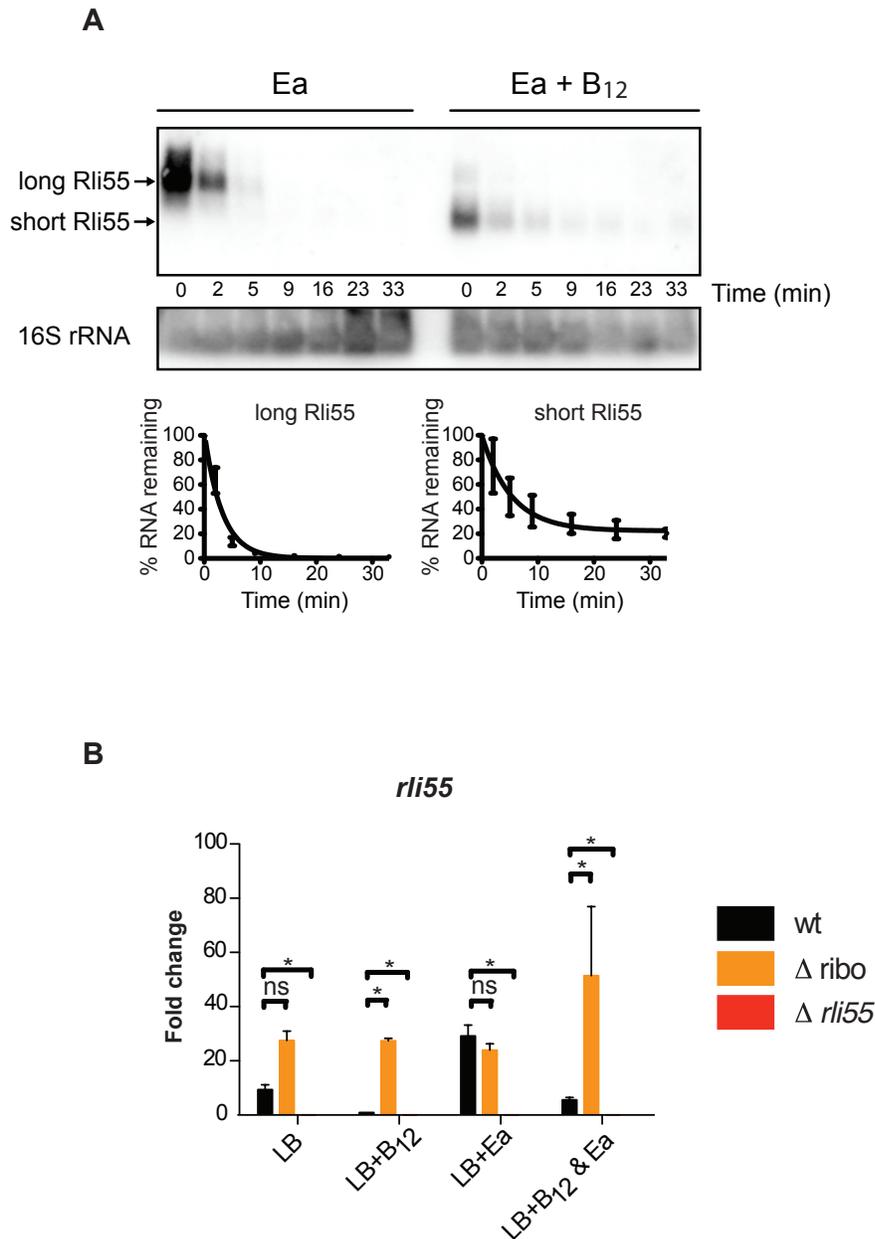
Samples were denatured at 95°C for 5 minutes and separated on a 12% SDS-PAGE before being transferred with a iBlot apparatus (Invitrogen, IB1001) using the P6 protocol for 6 mins according to the manufacturer's instructions. The membrane was blocked in 5% powder milk in TBS-Tween for two hours at room temperature. Primary antibody, α -FLAG (Sigma, F7425) (dilution 1:1000) was incubated overnight at 4° C. Membranes were washed 4 X 10 minutes in TBS-Tween at room temperature. Secondary antibody, α -rabbit-HRP (Agrisera) was incubated for 1 hour at RT (1:10000 dilution) in TBS-Tween before washing the membrane as described above. The membrane was developed using ECL2 prime Western Blot Detection (Pierce, 11884584) according to the manufacturer's instructions and subsequently exposed (LAS 4000, Fujifilm).

Figure S1



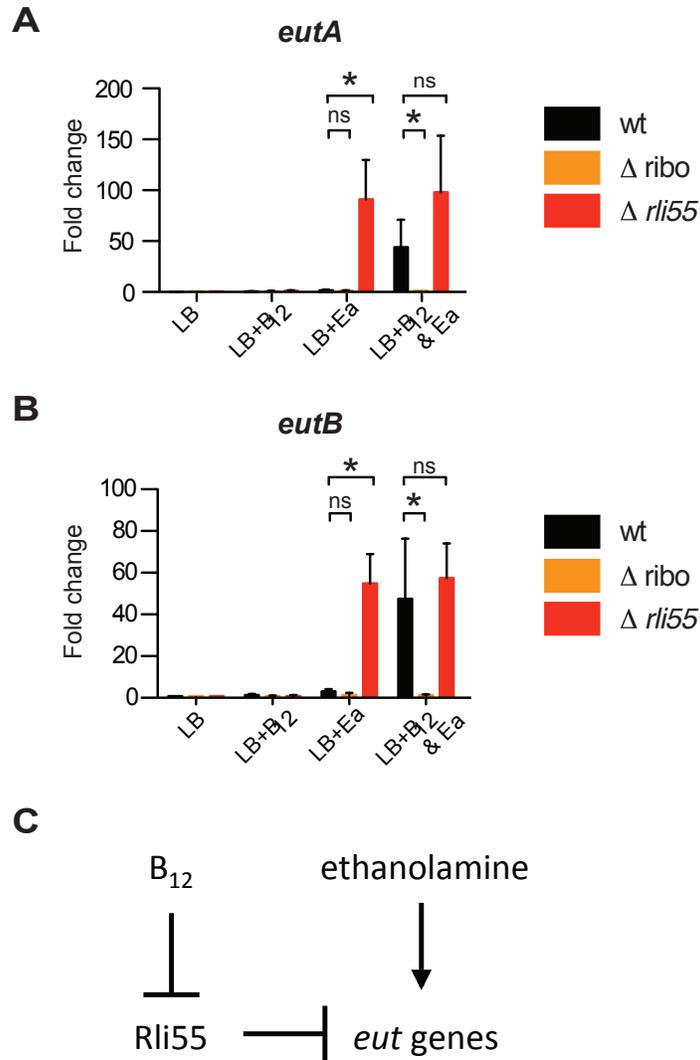
***eutABC* genes are regulated by both B₁₂ and ethanolamine.** Expression levels of the *eutABC* genes were evaluated by qRT-PCR. RNA was isolated from bacteria grown in LB, LB + 20 nM B₁₂ (LB + B₁₂), LB plus 10 μM ethanolamine (LB + Ea) or LB plus or LB + both (LB + B₁₂ & Ea) to an OD₆₀₀ between 0.3 and 0.5. Results represent the average of three biological replicates. Values depict means +/- SEM, * = p<0.05

Figure S2



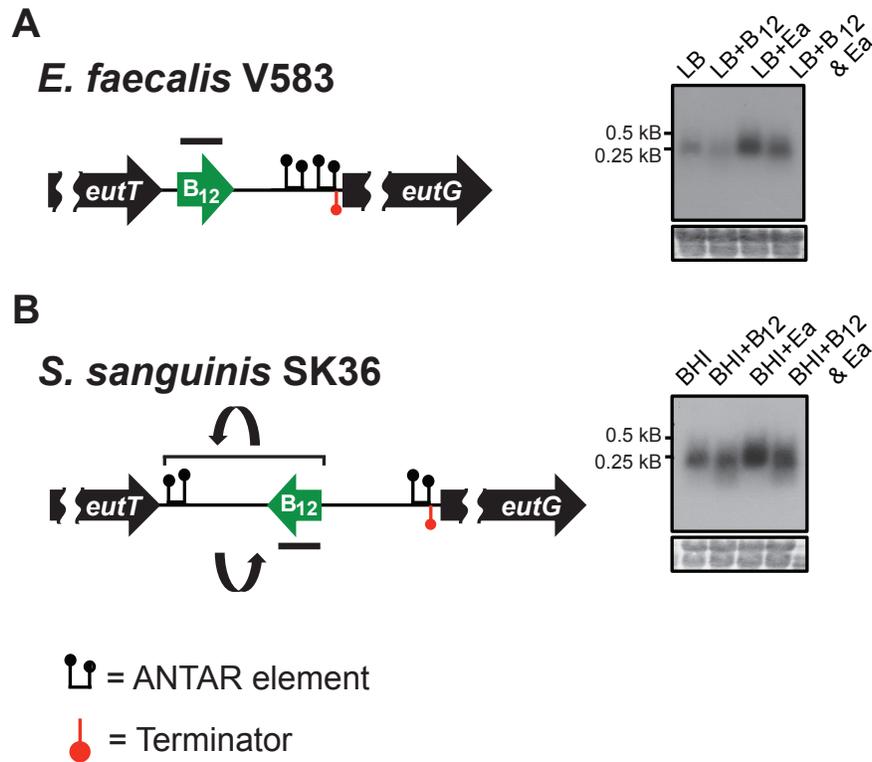
Rli55 stability and regulation. (A) northern blot showing expression of long and short Rli55 transcripts. RNA was isolated at the indicated timepoints after addition of Rifampicin and Naladixic acid to cultures. Blots were probed with a radioactively labeled, single-stranded RNA probe complimentary to the B₁₂ riboswitch. Northern blotting of 16S rRNA is shown as a loading control. Graphs show decay of RNA overtime. Value represent means +/- SD, n = 3 **(B)** Expression of *rli55* was evaluated by qRT-PCR. RNA was isolated from bacteria grown in LB, LB + 20 nM B₁₂ (LB + B₁₂), LB + 10 μM ethanolamine (LB + Ea) or both (LB + B₁₂ & Ea) to an OD₆₀₀ between 0.3 and 0.5. Results represent the average of three biological replicates. Error bars show standard error. Values represent means +/- SEM, n = 3, * = p<0.05, ns = not significant.

Figure S3



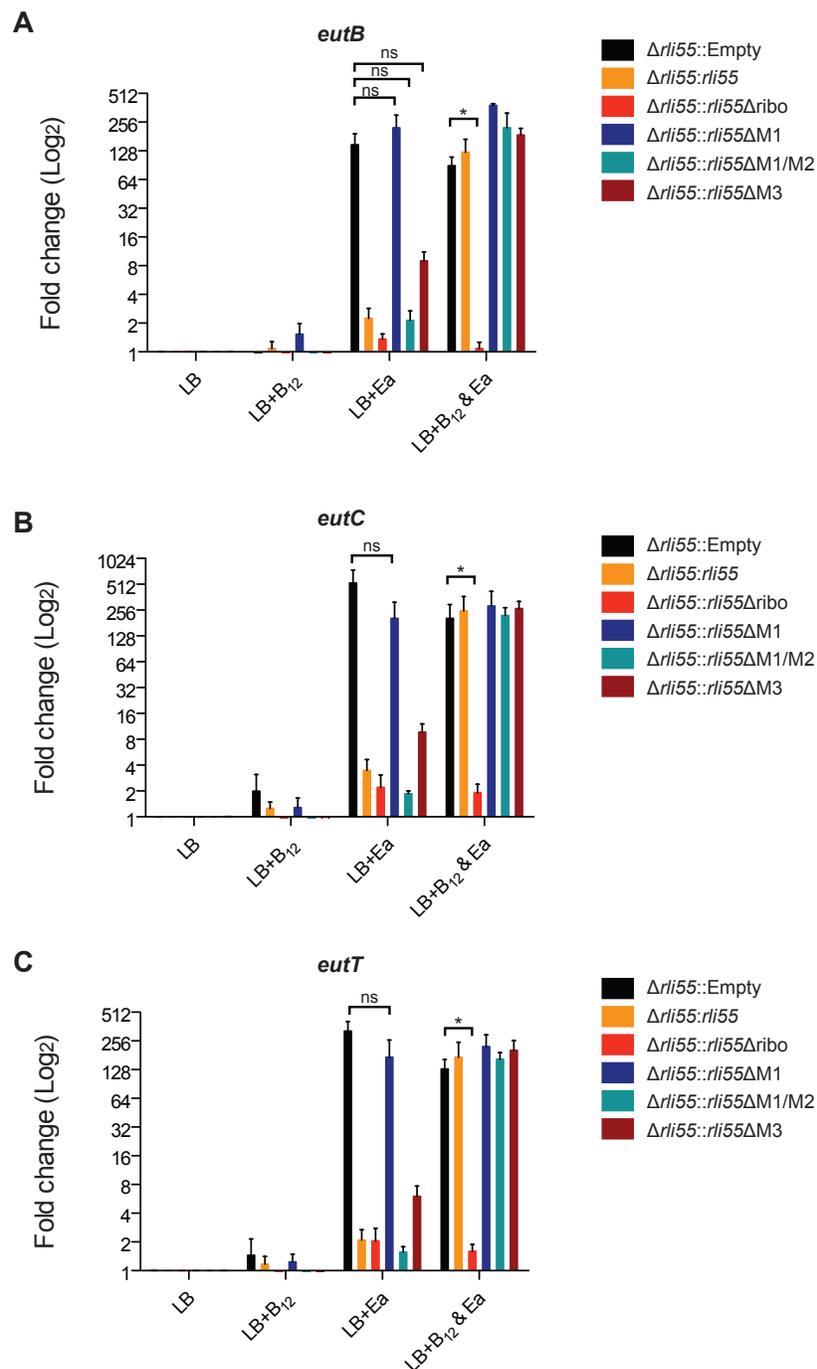
Rli55 regulates *eut* gene expression. Levels of the (A) *eutB* & (B) *eutC* genes were evaluated by qRT-PCR in the wild-type (wt), Δ ribo or Δ *rli55* strains. RNA was isolated from bacteria grown in LB, LB + 20 nM B₁₂ (LB + B₁₂), LB + 10 μ M ethanolamine (LB + Ea) or both (LB + B₁₂ & Ea) to an OD₆₀₀ between 0.3 and 0.5. Results represent the average of three biological replicates and error bars show standard error. Values represent means \pm SEM, Asterisks denote statistically significant differences ($p < 0.05$), ns=not significant. (D) Model of the regulatory interactions between B₁₂, ethanolamine, Rli55 and *eut* gene expression.

Figure S4



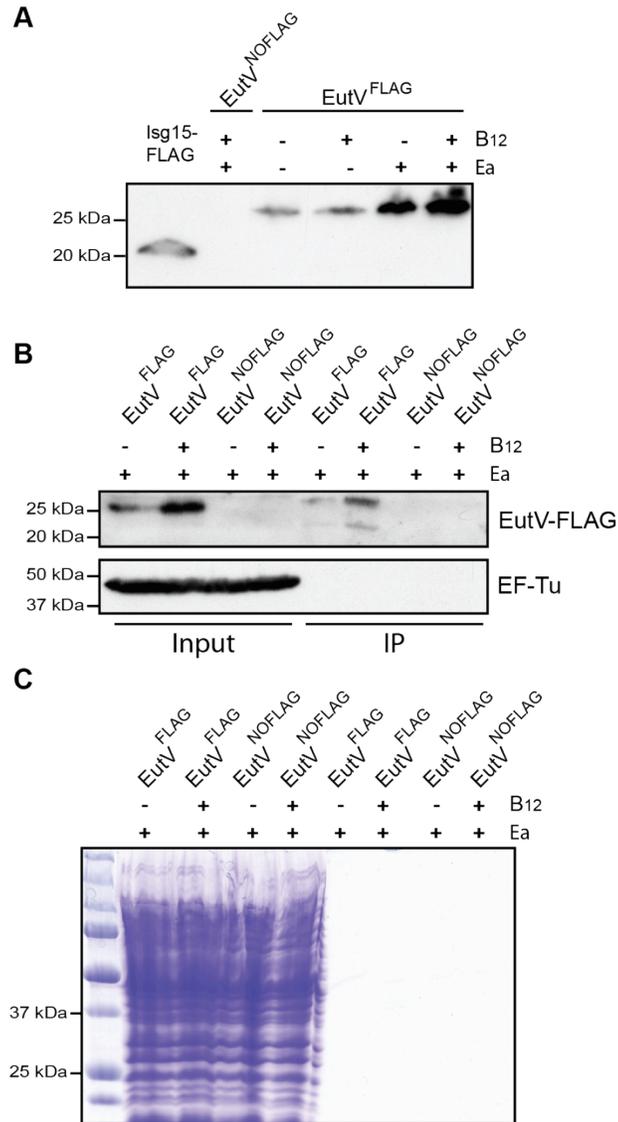
Putative Rli55 Orthologues. Schematics show the orthologous *rli55* loci of *E. faecalis* and *S. sanguinis*, including B₁₂ riboswitches (green arrows), ANTAR elements (black double lollipops) and terminators (red inverted lollipops). An inversion of the B₁₂ riboswitch and a single ANTAR element in *S. sanguinis* (area within brackets) is denoted by arced arrows. Northern blots show transcripts detected with single stranded RNA probes directed against the B₁₂ riboswitch from each locus (black solid bars) using RNA isolated from bacteria grown in either LB or BHI, + 20 nM B₁₂ (+ B₁₂), 10 μM ethanolamine (+ Ea) or both (+ B₁₂ & Ea) as indicated.

Figure S5



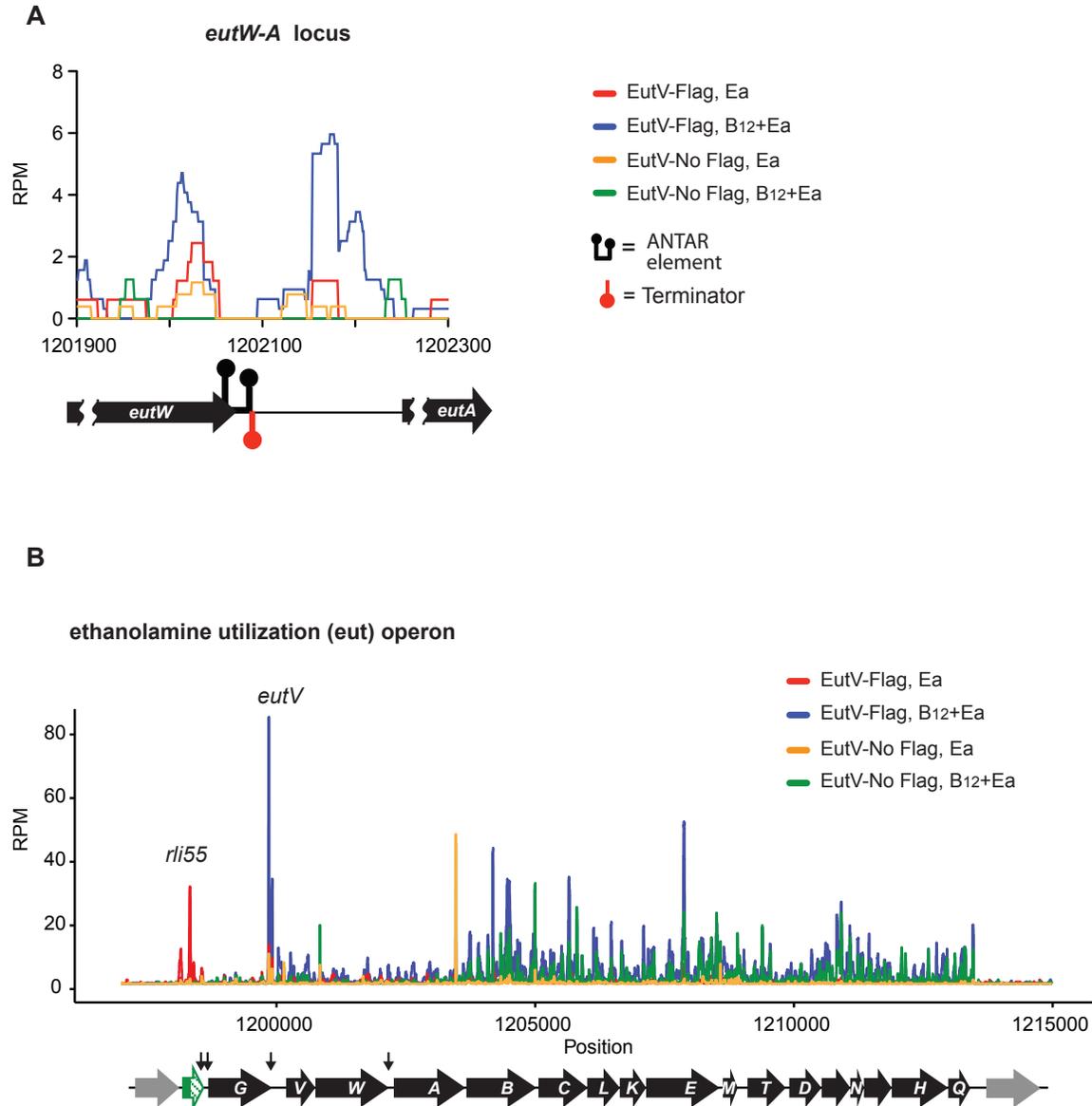
Complementation of Rli55-mediated regulation. Expression of the (A) *eutB*, (B) *eutC* & (C) *eutT* genes was evaluated by qRT-PCR. Graphs show the fold-change relative to the reference condition (LB) in log₂ units for the indicated strains grown in LB, LB + 20 nM B₁₂ (LB + B₁₂), LB + 10 μM ethanolamine (LB + Ea) or both (LB + B₁₂ & Ea). Values represent means +/- SEM, n = 3. Asterisks denote statistically significant differences (p<0.05), ns=not significant.

Figure S6



Regulation and immunoprecipitation of EutV^{FLAG} (A) Western blot showing regulation of EutV^{FLAG} under the indicated growth conditions. A purified human Isg15^{FLAG} protein and a lysate of strain EutV^{NOFLAG}, carrying an additional untagged *eutV* gene grown in the presence of both B₁₂ and ethanolamine were included as controls. (B) Western blots of protein isolated from lysates of EutV^{FLAG} and EutV^{NOFLAG} strains grown either in ethanolamine (Ea) alone or ethanolamine and B₁₂ together either before (input) or after immunoprecipitations (IP) with α-FLAG affinity gel. Blots of the EF-Tu protein were evaluated as a control. (C) A Coomassie stained gel run in parallel shows the overall protein content of all lanes.

Figure S7



Enrichment of the *eut* operon by colP with EutV^{FLAG}. Histograms shows RNA-Seq coverage in reads per million (RPM) of the (A) *eutW* – *eutA* intergenic region and the (B) full *eut* locus with RNA isolated by co-immunoprecipitation of cell lysates from either EutV^{FLAG} or EutV^{NOFLAG} cultures grown in the presence of ethanolamine (Ea) or ethanolamine and B₁₂ together. Schematics below show each loci. Arrows in the second schematic indicate positions of ANTAR elements.

Table S1

Strains and plasmids used in this study

Strain, Plasmid or Oligonucleotide	Description	Reference
Strains		
<i>Listeria monocytogenes</i>		
EGDe (BUG1600)		Mackness GB <i>et al.</i> , 1964
Δ ribo (BUG3593)	Mutant containing a deletion of nucleotides 1198141-1198190 encompassing a portion of the B12 riboswitch at this position.	This study
Δ rli55 (BUG3594)	Mutant containing a deletion of nucleotides 1198066-1198566 encompassing the B12 riboswitch and downstream region.	This study
Δ rli55::Empty (BUG3550)	Strains with named plasmids integrated into the tRNA ^{ARG} locus	This study
Δ rli55::rli55 (BUG3551)	Strains with named plasmids integrated into the tRNA ^{ARG} locus	This study
Δ rli55:: Δ ribo (BUG3552)	Strains with named plasmids integrated into the tRNA ^{ARG} locus	This study
Δ rli55::rli55 Δ M1 (BUG3553)	Strains with named plasmids integrated into the tRNA ^{ARG} locus	This study
Δ rli55::rli55 Δ M1/M2 (BUG3554)	Strains with named plasmids integrated into the tRNA ^{ARG} locus	This study
Δ rli55::rli55 Δ M3 (BUG3555)	Strains with named plasmids integrated into the tRNA ^{ARG} locus	This study
EutV ^{FLAG} (BUG3559)	Strain carrying a 2X FLAG-tagged additional copy of the <i>eutV</i> gene in the tRNA ^{ARG} locus	This study
EutV ^{NOFLAG} (BUG3558)	Strain carrying an untagged additional copy of the <i>eutV</i> gene in the tRNA ^{ARG} locus	This study
<i>Streptococcus sanguinis</i> SK36		Xu P <i>et al.</i> , 2007
<i>Enterococcus faecalis</i> V583		
<i>E. coli</i> Top 10		F- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 araD139 Δ (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ -
Plasmids		
pMAD	shuttle vector used for creating plasmids for mutagenesis	Arnaud M <i>et al.</i> 2004
pMAD-ribo-UD (BUG3591)	plasmid used to create the Δ ribo mutant	This study
pMAD-rli55-UD (BUG3592)	plasmid used to create the Δ ribosRNA mutant	This study
pJM2 (BUG3544)	derived from pAD vector (Balestrino <i>et al.</i> 2010)	This study
pJM2- Δ rli55::rli55 (BUG3545)	pJM2 vector carrying wild-type <i>rli55</i> allele	This study
pJM2- Δ rli55:: Δ ribo (BUG3546)	pJM2 vector carrying <i>rli55</i> allele with Δ mutation	This study
pJM2- Δ rli55::rli55 Δ M1 (BUG3547)	pJM2 vector carrying <i>rli55</i> allele with Δ M1 ANTAR mutation	This study
pJM2- Δ rli55::rli55 Δ M1:M2 (BUG3548)	pJM2 vector carrying <i>rli55</i> allele with Δ M1 and Δ M2 ANTAR mutations	This study
pJM2- Δ rli55::rli55 Δ M3 (BUG3549)	pJM2 vector carrying <i>rli55</i> allele with Δ M3 ANTAR mutation	This study

Table S2

Name	Sequence
Oligonucleotides used to create mutants	
$\Delta rli55$ -F	GGTCTTTTATCTAATTGTTTTAACTG
$\Delta rli55$ -R	CAGTAAAACAATTAGATAAAAAGACCACCAGACTTCCAGTATCTAAC T
Δ ribo-Up-F	AATGACCCGGGTCCGAAGTCACTTTATCCGATAAA
Δ ribo-Up-R	AGAGAAATTTTCTCTTCCAACCTATTGCAAGAAGAATTTGCTTTACG AA
Δ ribo-Down-F	TGAGTTGGAAGAGAAAATTTCTCT
Δ ribo-Down-R	TCGAG GTCGAC TCTTTGTAAGCACGTTCTAAGTAT
Δ eutB-Up-F	AATGACCCGGGTGAATGTATCTCTTTTTCCGGAG
Δ eutB-Up-R	TCGCTTAGTTCCTCCCTTCCAGCTATTTATGTGGATCAGTTAAAG
Δ eutB-Down-F	CTTTAACTGATCCACATAAATAGCTGGAAGGGAGGAACTAAGCGA
Δ eutB-Down-R	TCGAGGTGACTCACCTACAAGTAAGCAAACCTAC
Oligonucleotides used to create T7 templates for Northern blot probes	
T7riboprobetemp-F	TTCATACAAACAGTTAGATACTG
T7riboprobetemp-F	ACGTTAATACGACTCACTATAGGGAGAATAAAAACGAAGCGGTGCAC A
T7 <i>rli55</i> downstream-F	TGCACCGCTTCGTTTTATGGT
T7 <i>rli55</i> downstream-R	ACGTTAATACGACTCACTATAGGGAGAAGCTTTGAGTAGAGAAATTT TCTCT
T7Efriboprobetemp-F	ACGTTAATACGACTCACTATAGGGAGA GAAACATCCTGTTTTGCC
T7Efriboprobetemp-R	CGAATATTTTATGAAAAATATGAATGGAA
T7Ssriboprobetemp-F	ACGTTAATACGACTCACTATAGGGAGAATTTAAGAATAAAGACCTGA CTTAA
T716S-F	ATGCCACGCCTTTGAAAGATG
T716S-R	ACGTTAATACGACTCACTATAGGGAGATGCACTCCAGTCTTCCAGT TT
T7Ssriboprobetemp-R	AATTGATTTTAGTACTTTTAAGGATA
Oligonucleotides used for qRT-PCR	
qRTrli55-F	ACAAATAAGCCCACTGTAAATG
qRTrli55-R	AAACCAAACACGTGGAAATG
qRTeutA-F	ATCTCAACGGGTGCGCTAAGTGAT
qRTeutA-R	AATTTCACTGTGTGTGAACCGGC
qRTeutB-F	ACTGGTTACCACGAAACAGCCACT
qRTeutB-R	ATGCATCTCCAGCACGGCTAGTTA
qRTeutC-F	AAATCGGTGAAATCACTGGCGCTG
qRTeutC-R	AACTGTACGACGAGCTTCTGGCAT
qRTeutT-F	GACGCATTTGCGTGGTAATCTGCT
qRTeutT-R	TGCAACGGAACATTGGGTTTCCAG
qRTeutV-F	TTATTTCAGAAGGGCTTGCTGGCG
qRTeutV-R	GTTTCTCGCCCTTTGGCAACTC
qRTrpoB-F	GCGAACATGCAACGTCAAGCAGTA
qRTrpoB-R	ATGTTTGGCAGTTACAGCAGCACC
Oligonucleotides used for $\Delta rli55$ complementation constructs	
<i>rli55</i> -F	AATGACCCGGGAGCAAATTCTTCTTGCAAGCGG
<i>rli55</i> -R	AATGAGTGCAGCAGATTACCTCCTAGTTTTTTTTAAAATA
<i>rli55</i> Δ M1-F	GGTGGGCAATGATCAAAAACAACGACGAAAAGA
<i>rli55</i> Δ M1-R	TCTTTTCGTCGTTGTTTTGATCATTGCCACC
<i>rli55</i> Δ M1:M2-F	TCAAAAACAACGACGTTTTGAGTGATACAAGG
<i>rli55</i> Δ M1:M2-R	CCTTGATCACTCAAAACGTCGTTGTTTTGA
<i>rli55</i> Δ M3-F	AAATAAAATATTTTAGTCATGAAAGGCGTACTGTTTACTT
<i>rli55</i> Δ M3-R	AAGTAAACAGTACGCCTTTCATGACTAAAATATTTTATTT