Supporting Information

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SI Methods

Bacterial Strains, Growth Media, and Chemicals. All bacterial strains, plasmids, and oligonucleotides used for this research are listed in Table S2. Culture media was purchased from Difco, and chemicals were purchased from Sigma, unless otherwise noted. *Escherichia coli* strains were grown in LB medium supplemented with erythromycin (erm) 200 μg/mL, or ampicillin (amp) 100 μg/mL, or kanamycin (kan) 100 μg/mL when appropriate. *Enterococcus faecalis* strains were cultured in brain heart infusion (BHI) medium or BHI with 40% horse serum (BHIS), and supplemented with erm 50 μg/mL and rifampin (rif) 100 μg/mL when appropriate.

Strain Construction. An in-frame markerless double deletion of *eutV* and *eutW* histidine kinase was made by using P-*pheS** counterselection system (1). Sequence flanking the 5′ end of *eutV* and the 3′ end of *eutW* were amplified by PCR by using primers AB60, AB61 and AB62, AB63, respectively. These 2 products were introduced into the pGEM-T easy vector (Invitrogen) and fused together by using the primer-introduced BamHI sites. The resulting fragment was released from pGEM-T easy by using the NcoI and PstI sites. The released fusion product was ligated into the vector pCJK47 (also digested at NcoI and PstI), resulting in pAR1; pAR1 was electroporated into CK111 cells, and the counterselection protocol was carried out as previously described to create strain AR1 (1). The resulting deletion of *eutVW* was confirmed by PCR and sequencing.

The plasmid pKAF6 was used as the parent plasmid to construct transcriptional *lacZ* fusions used for ß-galactosidase assays. The rhamnose promoter was excised from the vector pCJK96 by cutting at the BamHI and BglII restriction sites and then ligating the compatible cohesive ends to make vector pCJK96–2. The *lacZ* gene from pCJK47 was amplified by using primers ODG136 and ODG137. Both the amplified fragment and pCJK96–2 were digested at PstI and SphI, and then ligated together to create pKAF6.

To construct pKAF11, first, the region extending from the *eutP* 5′ UTR to the beginning of the *eutG* coding region was amplified by using the primers KF102 and KF146. The resulting fragment was introduced into pKAF6 by using the SalI and BamHI restriction enzyme sites. We constructed pKAF12 and pKAF13 by using the method described for pKAF11 with the following changes: pKAF12 contains the *eutP* 5′ UTR amplified with KF102 and KF147; and pKAF13 contains the *eutG* 5′ UTR amplified by using primers KF103 and KF146.

To produce N-terminal His_6-tagged EutW and EutV, the genes were amplified by using ODG141 and ODG142, and KF152 and KF153, respectively. The resulting fragments were digested at the primer-encoded BamHI and XhoI restriction sites, and ligated into pET-28a(+) (Novagen) digested with the same enzymes. The resulting plasmids, pDAG90 and pKAF10, were confirmed by sequencing and transformed into *E. coli* strain BL21(DE3) (Novagen) to create strains DAGE240 and KAFE2.

ß-Galactosidase Assays. Strains KAF9, 10, and 11 were cultured overnight in BHI broth containing 50 μg/mL erm. Overnight cultures were diluted 1:50 in Fresh BHIB or BHIS. Cultures were grown at 37 °C for 4 h, and cells were collected by centrifugation. Cells were resuspended in 1:10 Z buffer (Z buffer, 60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$/5 mM β-mercaptoethanol, pH 7.0). The OD_{600} of each sample was measured by using a microplate reader. Cells were lysed by vortexing 5 min with 0.1 mm Zirconia/Silica beads (Biospec Products). Cell lysates were cleared by centrifugation. Cleared lysates were incubated with *o*-nitrophenyl β-D-galactoside (ONPG), and incubated until yellow color developed. Data were analyzed as previously described (2).

Recombinant EutV and EutW Preparation. Strains KAFE2 and DAGE240 were cultured in LB medium, and expression induced at A_{260} = 0.8 with 0.25 mM IPTG at 18 °C for 18 h. The 3-g cell pellets were resuspended in buffer A [25 mM Hepes, pH 7.0/500 mM NaCl/2 mM β-mercaptoethanol (β-ME)]. After cell disruption and centrifugation, the supernatant was passed over 5 mL of Cobalt TALON resin (Clontech) followed by 20 column volume (CV) washes of buffer A and 15 CV of buffer A + 8 mM imidazole. Protein was eluted with 2 CV of 100 mM imidazole in buffer A followed by 2 CV of 200 mM imidazole in buffer A, and finally with 2 CV of 400 mM imidazole in buffer A. The stepwise gradient was used to prevent precipitation (at >100 and >100 μg/mL for EutW and EutV, respectively) of proteins during purification. Eluted protein was loaded onto a G25 desalting column (Amersham). Purity of EutV and EutW was >90%, as judged by 10% SDS/PAGE followed by Coomassie-staining.

Phosphorylation Assay. EutW and/or EutV were incubated in the presence of 2 mM $MgCl_2$, 1 mM ATP (doped with γ-^{32}P ATP), and specified concentrations of ethanolamine for 30 min at 25 °C. The samples were resolved by 10% SDS/PAGE on 2 gels, one stained with Coomassie Brilliant blue and the other visualized by using a PhosphorImager (Amersham).

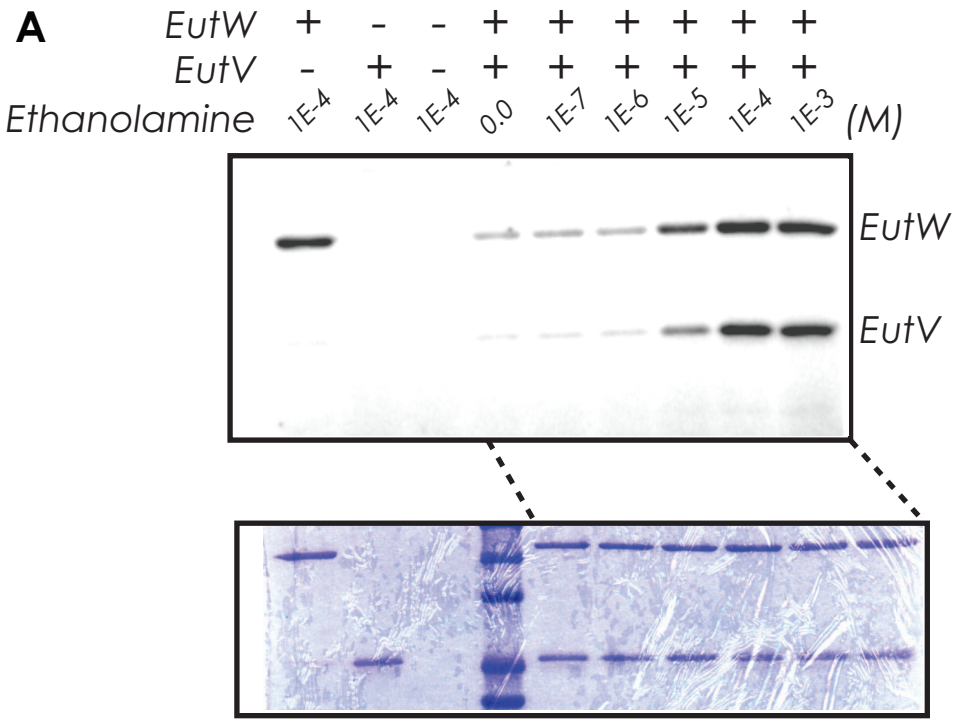
Electrophoretic Mobility Shift Assay. To remove the hexahistidine-tag, EutV was incubated with Thrombin (at 5U/mg EutV; Calbiochem). To generate phosphorylated EutV, cleaved EutV was incubated for 30 min at 25 °C with an equimolar amount of EutW, in the presence of 1 mM ethanolamine and 5 mM ATP. The sample was loaded on fresh Qiagen Ni^{2+} NTA resin to separate EutV (flowthrough) from EutW (fractions) and concentrated by using Amicon filtration units. The 5′ region of *eutP* was synthesized in vitro with oligos AR025 and AR026, and radiolabeled with γ-^{32}P ATP; 10 fmol of RNA was incubated with specified concentrations of unphosphorylated or phosphorylated EutV in the presence of 2.5 mM $MgCl_2$ for 60 min at 25 °C, and resolved by nondenaturing gel electrophoresis in a 4% TBMG gel [Tris-borate, 2 mM $MgCl_2$, 5% (vol/vol) glycerol; 37.5:1 acrylamide:bis].

In-Line Probing Reactions. RNA for in vitro studies was prepared as described previously (3). The *E. faecalis eutT-G* intergenic region was amplified with primers JES025 (containing T7 promoter sequence) and JES023 (Table S2). RNAs were synthesized by T7 RNAP and were 5′-radiolabeled with [γ-^{32}P]ATP as described previously (3). In-line probing reactions contained ≈1 nM RNA, 50 mM Tris·HCl, pH 8.3/100 mM KCl/20 mM $MgCl_2$ and specified concentrations of AdoCbl or cyanocobalamin. RNA was incubated at 25 °C for ≈40 h, and products were resolved by 10% PAGE. All gels were quantified and analyzed as described previously (3).

Transcription Termination Assays. Template for the transcription termination assay included sequence from the *eutT-G* intergenic region, and were amplified with primers JES027 and ODG140.

JES027 contained a portion that annealed to the *eutT-G* region (bold letters), but also added the *B. subtilis mgtE* promoter region (indicated by italics). ODG140 annealed downstream of the putative transcription terminator. Synchronized in vitro transcription reactions were performed as described previously by using *E. coli* RNA polymerase (Epicentre) (3).

1. Kristich CJ, Chandler JR, Dunny GM (2007) Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of Enterococcus faecalis. *Plasmid* 57:131–144.
2. Miller JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
3. Dann CE, III, et al. (2007) Structure and mechanism of a metal-sensing regulatory RNA. *Cell* 130:878–892.
4. Barrick JE, Breaker RR (2007) The distributions, mechanisms, and structures of metabolite-binding riboswitches. *Genome Biol* 8:R239.
5. Murray BE, et al. (1993) Generation of restriction map of Enterococcus faecalis OG1 and investigation of growth requirements and regions encoding biosynthetic function. *J Bacteriol* 175:5216–5223.
6. Kristich CJ, Chandler JR, Dunny GM (2007) Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of Enterococcus faecalis. *Plasmid* 57:131–144.
7. Leenhouts K, et al. (1996) A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet MGG* 253:217–224.



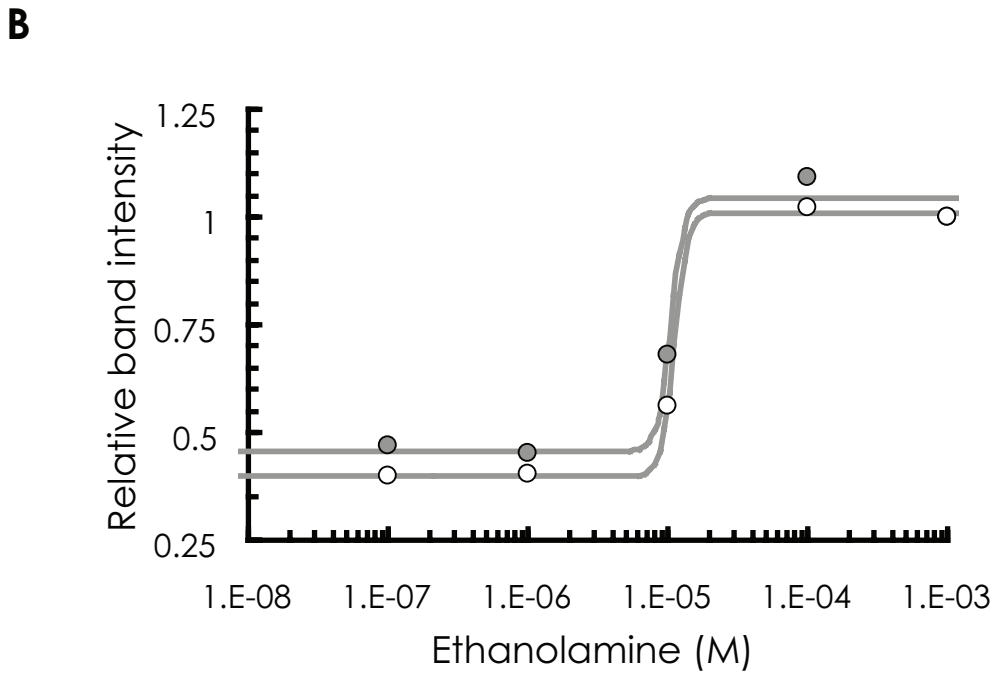


Fig. S1. Ethanolamine induces phosphoryl transfer for the EutWV 2-component system. (*A*) Autoradiogram of phosphorylation assays by using [γ-^{32}P]ATP (*Upper*) shows ethanolamine-responsive autophosphorylation of EutW and phosphoryl transfer to EutV. An identical gel (*Lower*) stained with Coomassie Brilliant Blue shows equal loading of protein samples. The protein markers correspond to 50-, 37-, 25-, and 20-kDa proteins. (*B*) The relative band intensity shown in Fig. 2 is plotted against ethanolamine concentration for EutW (filled circles) and EutV (open circles).

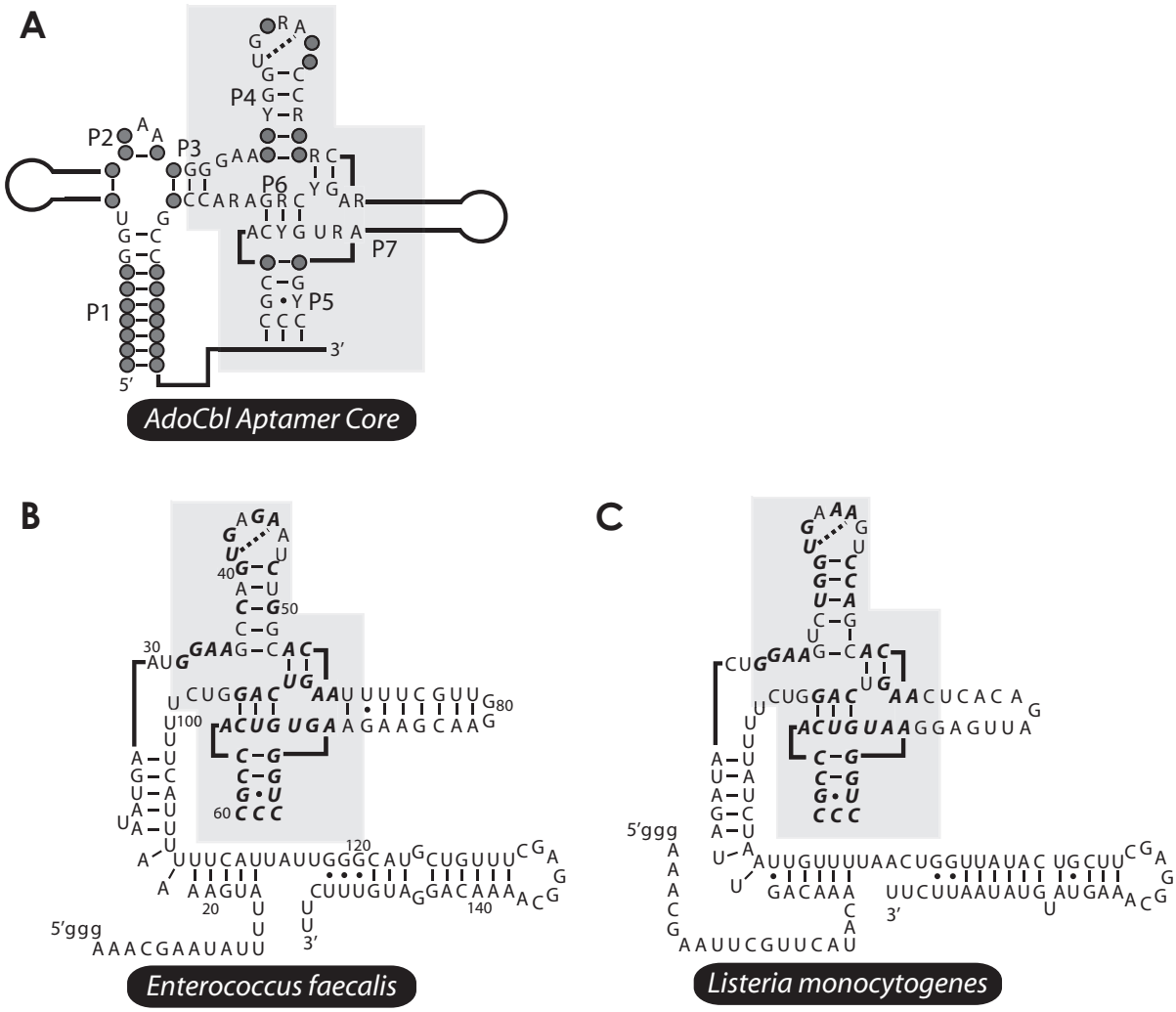


Fig. S2. Comparison of the classical AdoCbl-sensing riboswitch (4) with those (*A*) found in the *eutT-G* region of *E. faecalis* (*B*) and *Listeria monocytogenes* (*C*). The conserved, core regions are highlighted by the gray boxes and bold, italicized letters. Despite significant differences in primary sequence, a secondary structure model of the *L. monocytogenes eutT-G* putative riboswitch closely resembles that of *E. faecalis*.

Table S1. The *eut* genes in *E. faecalis* compared with *Salmonella*

Gene conservation	Gene name	*E*	Function
—	*eutB*	5 e-145	EA ammonia-lyase
—	*eutH*	5 e-98	permease
—	*eutA*	2 e-75	EAL reactivase
—	*eutG*	2 e-66	alcohol dehydrogenase
Highly Conserved	*eutL*	3 e-62	metabolosome
—	*eutC*	7 e-53	EA ammonia lyase
—	*eutE*	6 e-47	aldehyde dehydrogenase
—	*eutS*	2 e-21	carboxysome
—	*eutM*	2 e-20	carboxysome
—	*eutQ*	7 e-18	unknown
—	*eutP*	4 e-17	unknown
—	*eutT*	7 e-13	corrinoid adenosyltransferase
Conserved	*eutN*	1 e-06	carboxysome
—	*eutK*	0.002	carboxysome
—	*eutD*	N/A	phosphotransacetylase
Salmonella only	*eutJ*	N/A	chaperone
—	*eutR*	N/A	transcription activator
—	*eutW*	N/A	histidine kinase
Enterococcus only	*eutV*	N/A	response regulator
—	unknown 1	N/A	unknown
—	unknown 2	N/A	unknown

N/A, not applicable.

Table S2. Strains, plasmids and oligos used in this study

	Description	Source
Strains		
E. faecalis		
OG1RF	Wild-type strain, Fa^R, Rf^R	Ref. 5
KAFF9	OG1RF with plasmid pKAF11, Fa^R, Rf^R, Em^R	This work
KAFF10	OG1RF with plasmid pKAF12, Fa^R, Rf^R, Em^R	This work
KAFF11	OG1RF with plasmid pKAF13, Fa^R, Rf^R, Em^R	This work
KAFF12	Δ*eutV/eutW* with plasmid pKAF11, Fa^R, Rf^R, Em^R	This work
KAFF14	Δ*eutV/eutW* with plasmid pKAF12, Fa^R, Rf^R, Em^R	This work
KAFF15	Δ*eutV/eutW* with plasmid pKAF13, Fa^R, Rf^R, Em^R	This work
AR1	CK111 with plasmid pAR1, Em^R, Kn^R	This work
CK111	Conjugative donor strain containing a chromosomal *repA* allele for plasmid maintenance, Sp^R	Ref. 6
AR2	Δ*eutV/eutW* Fa^R, Rf^R	This work
E. coli		
EC1000	*E. coli* strain used for manipulation of *repA* dependent plasmids, Kn^R	Ref. 7
DAGE240	BL21(DE3) containing pDAG90, Kn^R	This work
KAFE2	BL21(DE3) containing pKAF10, Kn^R	This work
Plasmids		
pCJK47	Plasmid used for the construction of in-frame deletion mutants by P-*pheS** counterselection	Ref. 6
pAR1	pCJK47 + *eutV* upstream:*eutW* downstream fragment	This work
pKAF6	pCJK96–2 with *lacZ* from pCJK47	This work
pKAF11	pKAF6 + *eutP* 5′ UTR to *eutG* 5′ UTR	This work
pKAF12	pKAF6 + *eutP* 5′ UTR	This work
pKAF13	pKAF6 + *eutG* 5′ UTR	This work
pCJK96	Parent plasmid for construction of pCJK96–2	Ref. 6
pCJK96–2	pCJK96 with the rhamnose promoter excised	This work
pDAG90	pET-28a(+) (Novagen) containing *eutW*	This work
pKAF10	pET-28a(+) (Novagen) containing *eutV*	This work
Primers		
AB60	5′TTTCAGATGCGTTGTGC 3′	This work
AB61	5′ CGGGATCCTTTCTTCACGCCC 3′	This work
AB62	5′ CGGGATTCAGCAAAGGCGCT 3′	This work
AB63	5′ CCGATGTCCCCATATTT 3′	This work
ODG136	5′AACTGCAGCACCTTCGTTTTCAGACTTT 3′	This work
ODG137	5′CGGGATCCGGGCCGTCGTTTTACAAGTT3′	This work
KF102	5′AAAGTCGACGATATCCATTTAGCGTTAACGCTAAG3′	This work
KF146	5′AAAGGATCCCCATAGTTCGGTAGGGAAATGAATTGT3′	This work
KF147	5′AAAGGATCCCACCGATAGCTCCCATTAAAATGATTCGTTT3′	This work
KF103	5′AAAGTCGACGCAGAACGCATCATTGTTCG3′	This work
ODG141	5′AAAAAAGGATCCAAACGATTAGAGCAATTATGTCAC3′	This work
ODG142	5′AAAAAACTCGAGTCAATGAACAACATCACTGGTGTTC3′	This work
KF152	5′GGATCCGATGGACGAATTGTAATAGTCGATGA3′	This work
KF153	5′CTCGAGTTATTCATCATCCATTACAATCAATTCTGC3′	This work
AR025	5′TAATACGACTCACTATAGGGACAAGTTTTTTGGAAACAGG3′	This work
AR026	5′ CCCATTAAAATGATTCGTTTCATCG3′	This work
JES025	5′TAATACGACTCACTATAGGGAAACGAATATTTTATGAAAAATATG3′	This work
JES023	5′TTAAGAAACATCCTGTTTTGCC3′	This work
JES027	5′TTACGAATTCAGTTGACATAGCTTGTTTTTTTCTGTAATCTCAGTGTGT**CAAACGAATATTTTATGAAAAATATGAATGG**3′	This work
ODG140	5′ GCGCCTTCTCCTACCCATAGTTCG3′	This work