Multiple posttranscriptional regulatory mechanisms partner to control ethanolamine utilization in *Enterococcus faecalis*

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Ethanolamine, a product of the breakdown of phosphatidylethanolamine from cell membranes, is abundant in the human intestinal tract and in processed foods. Effective utilization of ethanolamine as a carbon and nitrogen source may provide a survival advantage to bacteria that inhabit the gastrointestinal tract and may influence the virulence of pathogens. In this work, we describe a unique series of posttranscriptional regulatory strategies that influence expression of ethanolamine utilization genes (*eut*) in *Enterococcus*, *Clostridium*, and *Listeria* species. One of these mechanisms requires an unusual 2-component regulatory system. Regulation involves specific sensing of ethanolamine by a sensor histidine kinase (EutW), resulting in autophosphorylation and subsequent phosphoryl transfer to a response regulator (EutV) containing a RNA-binding domain. Our data suggests that EutV is likely to affect downstream gene expression by interacting with conserved transcription termination signals located within the *eut* locus. Breakdown of ethanolamine requires adenosylcobalamin (AdoCbl) as a cofactor, and, intriguingly, we also identify an intercistronic AdoCbl riboswitch that has a predicted structure different from previously established AdoCbl riboswitches. We demonstrate that association of AdoCbl to this riboswitch prevents formation of an intrinsic transcription terminator element located within the intercistronic region. Together, these results suggest an intricate and carefully coordinated interplay of multiple regulatory strategies for control of ethanolamine utilization genes. Gene expression appears to be directed by overlapping posttranscriptional regulatory mechanisms, each responding to a particular metabolic signal, conceptually akin to regulation by multiple DNA-binding transcription factors.

riboswitch | 2-component system

Enterococcus faecalis is a significant pathogen in the hospital environment; however, its most common lifestyle is that of a commensal in the gastrointestinal (GI) tract of mammals. In susceptible patients, this commensat relationship can serve as a source of infection (1). Growth under hostile conditions such as the GI tract requires metabolic flexibility, and enterococci can catabolize various energy sources including ethanolamine (2). Ethanolamine is a product of the catabolism of phosphatidylethanolamine, an abundant phospholipid in both mammalian and bacterial membranes (3, 4). Both the host diet and cells within the intestine (bacterial and epithelial) are thought to provide a rich ethanolamine source (5). Correspondingly, several bacterial species found within the GI tract encode the machinery necessary for ethanolamine catabolism, including, but not limited to, *E. faecalis*, *Streptococcus sanguinis*, *Escherichia coli*, and *Salmonella*, *Listeria*, and *Clostridium* species.

For *Listeria monocytogenes*, the genes of the ethanolamine utilization (*eut*) locus exhibit increased expression inside the host cell, and loss of one of the key enzymes, EutB, causes a defect in intracellular growth (6). In *Salmonella typhimurium*, expression of the *eut* operon is affected by a global regulator of invasion genes (CsrA) (7). In *E. faecalis*, expression of genes in the *eut* locus is also influenced by the Fsr global virulence regulatory system (8), and a transposon insertion in this region attenuated killing of *Caenorhabditis elegans* (9). These data suggest that the ability to use ethanolamine may affect virulence. However, despite a possible role for the *eut* locus in bacterial pathogenesis, studies of the mechanisms for genetic regulation of these genes are just beginning (10).

Ethanolamine utilization has been best studied in *S. typhimurium* (11). The catabolism of ethanolamine occurs in a multiprotein complex called the carboxysome, and results in the production of the metabolically useful compound acetyl-CoA (12). The process highly depends on bioavailability of adenosylcobalamin (AdoCbl), because the central enzymatic step requires AdoCbl as a cofactor (13). Expression of these genes in *Salmonella* is positively regulated by a DNA-binding transcription factor, EutR, encoded within the *eut* operon and active in the presence of AdoCbl and ethanolamine (5, 14). *E. faecalis* lacks the EutR regulator, suggesting the existence of alternative regulatory mechanisms for control of ethanolamine catabolism (10).

We describe a unique partnership of multiple posttranscriptional regulatory elements for control of ethanolamine utilization in *E. faecalis.* One involves a 2-component regulatory system with RNA-binding activity, and the other comprises metabolite-sensing RNA elements, or riboswitches. We postulate that both regulatory systems exert control by influencing the stability of a series of intrinsic transcriptional terminators, as modeled in Fig. 1. Two-component regulatory systems typically include a histidine kinase that autophosphorylates on sensing a specific signal and subsequently transfers the phosphoryl group to a dedicated response regulator. Response regulators typically include a domain for phosphorylation at a conserved aspartate residue and a second domain required for eliciting the regulatory response. The latter most often occurs through protein-DNA or protein–protein interactions (15). However, ≈0.9% of identified eubacterial response regulators (16) contain an ANTAR (AmiR and NasR transcriptional antiterminator regulators) domain (17). The *eut*-associated response regulator shares this arrangement (10). Two proteins containing ANTAR domains have previously been shown to interact with target mRNA transcripts to prevent formation of a transcription termination signal (16–20). We present evidence that the *eut*-associated

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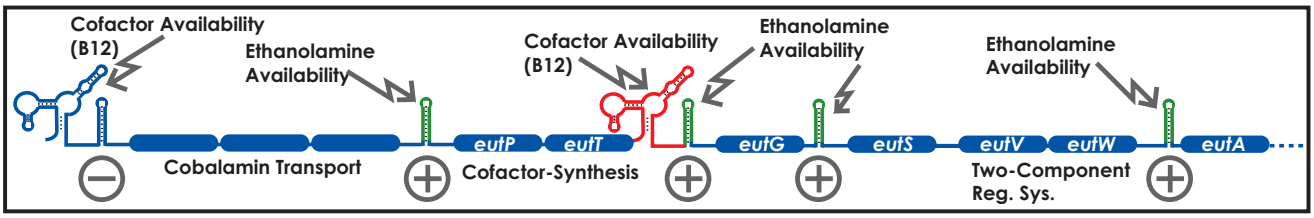

Fig. 1. Model for posttranscriptional control of gene expression in the *eut* locus of *E. faecalis*. A subset of genes present in the *eut* locus of *E. faecalis* are shown (blue boxes). A classical AdoCbl-sensing riboswitch (blue) is schematically represented upstream of genes predicted to encode for cobalamine transport proteins. A novel, AdoCbl-sensing riboswitch subclass described herein is indicated (red) within the intergenic region upstream of *eutG*. Conserved hairpins predicted to be transcriptional terminators are shown as green stem-loops. The stability of these terminators is postulated to be affected by the presence of ethanolamine, via the EutWV two-component system, or adenosylcobalamin, via a riboswitch. Posttranscriptional mechanisms predicted to increase downstream expression in response to their metabolic stimulus are indicated by a plus sign, whereas those predicted to decrease expression are indicated with a minus.

2-component regulatory system also interfaces with RNA-mediated genetic control strategies. Intriguingly, this system is also retained by other species (e.g., *Listeria* and *Clostridium*) that are capable of ethanolamine catabolism, suggesting a common post-transcriptional regulatory circuitry.

In addition to regulation by a 2-component system at the RNA level, we also demonstrate regulation by at least 1 metabolite-sensing riboswitch. Riboswitches are *cis*-acting regulatory RNA elements located in 5′ untranslated regions (5′-UTR) or intercistronic portions of target mRNAs (21). They consist of 2 domains: a conserved aptamer domain that interacts directly with a specific metabolite, and a variable domain involved in modulating gene expression. Riboswitches responsive to various metabolites have been previously identified. However, the coordinated involvement of protein factors in concert with riboswitch-based mechanisms has not been reported.

Together, these data suggest that multiple posttranscriptional regulatory mechanisms are coordinated for regulation of the genes within the *eut* loci of certain bacterial species. Also, the experiments shown provide support for the hypothesis that bacterial operons can be controlled by multiple posttranscriptional regulatory pathways, each responding to a different input signal, akin to regulation by multiple independent DNA-binding transcription factors.

Results and Discussion

The *E. faecalis* Genome Contains a Locus Encoding Genes for Ethanolamine Utilization. Previously, we found that a transposon mutant containing a disruption in one of the ethanolamine utilization genes caused an attenuated phenotype in a *C. elegans* infection model (9). The gene was originally designated a *pduJ* homolog (22). However, closer analysis reveals that it is more likely a *eutK* homolog. We performed BLAST analysis [supporting information (SI) Table S1] of the genes surrounding the transposon insertion, and found many homologs of the *S. typhimurium eut* genes (11), as did another group in Del Papa and Perego (10). The carboxysome structural genes and essential enzymatic genes have largely been maintained, albeit with some differences. Of particular interest was the absence in *E. faecalis* of *eutR*, which encodes for a DNA-binding transcription regulator, and the presence of a 2-component regulatory system originally named HK17 and RR17 (Table S1; see ref. 23). Here, we rename them eutW (histidine kinase), and eutV (response regulator), to conform to nomenclature standards. This observation suggested that EutW/EutV in *E. faecalis* might replace the regulatory role of EutR in *Salmonella*.

The EutWV 2-Component System Is Necessary for High-Level Expression of the *eut* Locus Under Inducing Conditions. Previous work found that EutV contains a domain consistent with the ANTAR family of proteins (10). The latter consists of a unique class of proteins that have RNA-binding antiterminator function (17). Two response regulators containing this domain have been characterized, AmiR and NasR (18–20). These proteins associate directly with the 5′ UTR of the nascent transcript to prevent formation of an intrinsic terminator. RNA-binding activity of AmiR is activated when it becomes disassociated from its negative regulator AmiC in the presence of free amidates (24). In contrast, RNA-binding activity of NasR is stimulated by direct binding of nitrates to NasR (19). The *E. faecalis* EutV protein differs from AmiR and NasR in that it contains a classic N-terminal receiver domain, including a conserved phosphoaccepting aspartic acid residue (10).

Because the possible effects of the EutWV 2-component system on *eut* gene expression has never been examined, we constructed 3 translational fusions to *lacZ* (Fig. 2*A*). The region encompassing *eutPTG* was fused to *lacZ* to generate *eutPTG-lacZ*. The intergenic regions upstream of *eutP* and *eutG* were also fused to *lacZ* for *eutP-lacZ* and *eutG-lacZ*, respectively. These constructs were transformed into *E. faecalis* strain OG1RF and assayed in brain heart infusion (BHI) medium. Under these conditions, *eutPTG-lacZ* and *eutP-lacZ* exhibited a similar level of expression, whereas no expression was observed for *eutG-lacZ* or a vector-only control (Fig.

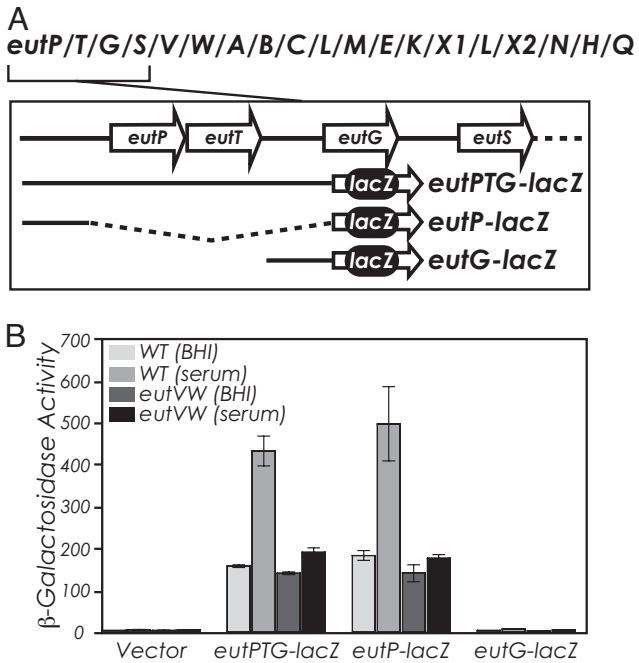

Fig. 2. The 2-component system, EutWV, encoded within the *eut* locus is required for induced expression. (*A*) Organization of the *eut* locus in *E. faecalis* with the inset showing the translational fusions made to *lacZ* in an *E. faecalis* shuttle vector. For the predicted functions of the genes, see Table S1. (*B*) β-galactosidase activity levels observed in wild-type or *eutVW E. faecalis* strains containing the vectors with the *lacZ* fusions shown in *A*.

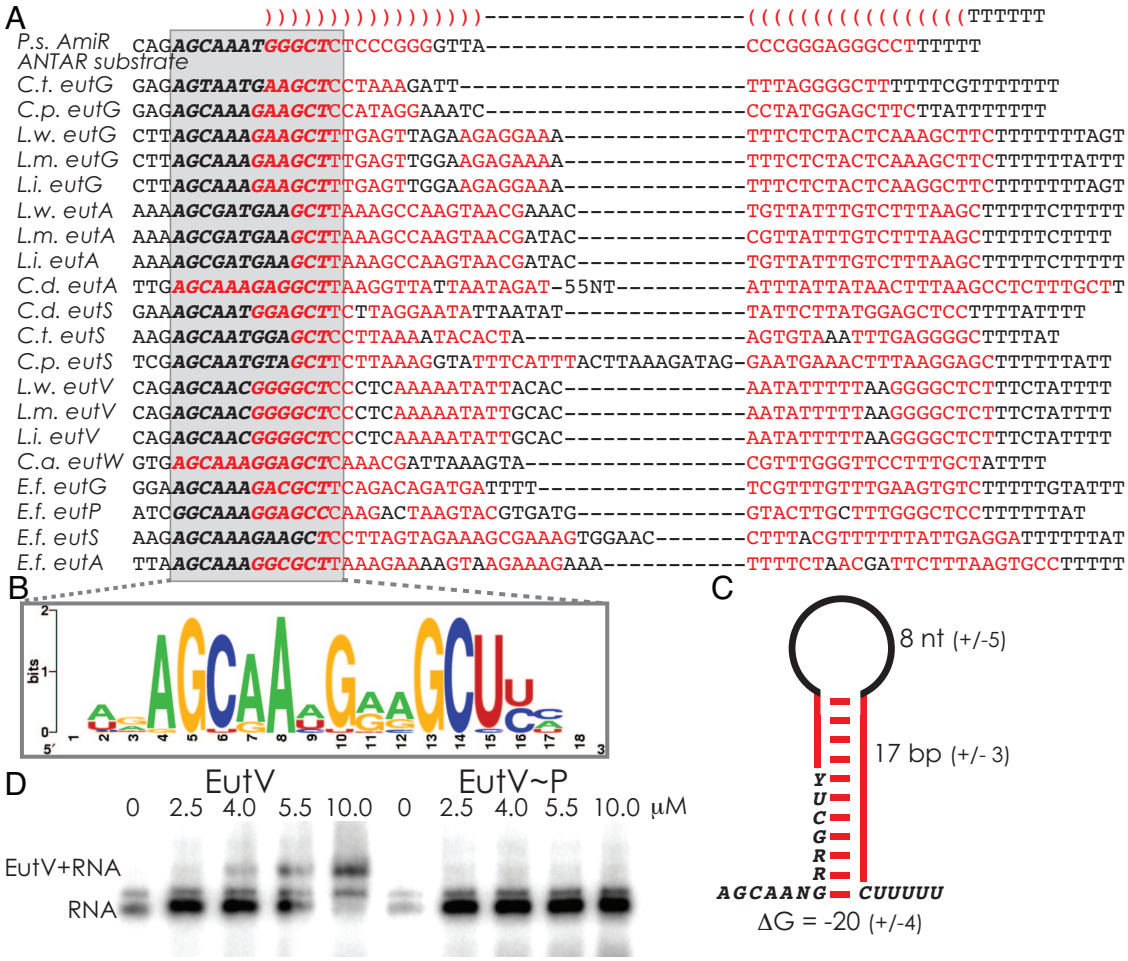

Fig. 3. A putative ANTAR recognition motif. (*A*) Comparative sequence alignment of portions of the intergenic regions upstream of *eutG*, *eutP*, *eutS*, *eutV*, and *eutA* from *Enterococcus*, *Listeria*, and *Clostridium* species. A potential base-paired region is shown (red, helical residues) with a conserved primary sequence motif (gray box), which likely constitutes an ANTAR recognition motif. (*B*) Weblogo representation (38) of the conserved sequence motif. Size of the letter is indicative of frequency of occurrence. (*C*) A consensus secondary structure model for the base-paired stem and putative ANTAR recognition motif. (*D*) An electrophoretic mobility shift assay using γ-^{32}P-radiolabeled RNA that encompasses the *eutP* 5′ UTR demonstrates an interaction of the RNA with unphosphorylated (*Left*), but not phosphorylated (*Right*) EutV.

2*B*). Together, these data suggest that there is likely to be a promoter-active region upstream of *eutP*, but not *eutG*.

Previous studies have found that many *E. faecalis* genes involved in virulence are expressed in serum (25, 26). Because of the possible connections of the operon to pathogenesis, we examined gene expression in BHI medium containing 40% serum. The medium induced expression of the *eutPTG-lacZ* and *eutP-lacZ* reporter fusions ≈3-fold above growth in plain BHI (Fig. 2*B*). To determine whether the EutWV 2-component system affected basal and/or induced expression, the *lacZ* fusions were assayed for strains containing a deletion of *eutVW*. Serum-dependent induction of *eutPTG-lacZ* and *eutP-lacZ* expression was abrogated in the *eutVW* background. Instead, low-levels of β-galactosidase activity were maintained under these conditions. The identical results for the *eutPTG-lacZ* and *eutP-lacZ* fusions suggested that a EutWV regulatory determinant(s) is likely located upstream of *eutP*.

Discovery of a Repeated and Conserved RNA Element Within the *eut* Locus. Previous work and our own experiments showed that ethanolamine activates autophosphorylation of EutW and phosphorylation of EutV (Fig. S1; ref. 10). Because EutV contains a putative ANTAR output domain, we postulated that phosphoryl transfer between EutW and EutV might be coupled with RNA-binding activity. Also, the observation that the EutWV 2-component regulatory system is conserved between *E. faecalis* and *Listeria* and *Clostridium* species suggested that a common RNA substrate motif could exist.

To test this hypothesis, we manually examined intergenic regions larger than 75 nt in the *eut* locus for bacteria containing EutWV homologs and searched for common features. We identified putative stem-loop elements closely resembling intrinsic transcription terminator hairpins (Figs. 1 and 3). Interestingly, these putative terminator elements were found to share a common 13-nt sequence (AGCAANGRRGCUY) overlapping the 5′-proximal portion (Fig. 3 *B* and *C*). Comparative sequence alignments revealed that the primary sequence of the predicted terminator stem-loops varied significantly, whereas the 13-nt sequence exhibited a high degree of conservation.

Remarkably, the portion of the 5′ UTR of the amidase operon that is required for association with AmiR shared the consensus pattern for the *eut*-associated terminator motif (Fig. 3*A*). The fact that sequence and structural motifs in the *eut* loci closely resemble an established RNA substrate of an ANTAR regulatory protein suggests strongly that they are likely EutWV regulatory targets. We identified the conserved element primarily upstream of a subset of the *eutG*, *eutS*, *eutA*, and *eutV* genes for each of the relevant species (Figs. 1 and 3*A*). It could be clearly identified upstream of *eutP*, *eutG*, *eutS*, and *eutA* in *E. faecalis*. These observations suggest

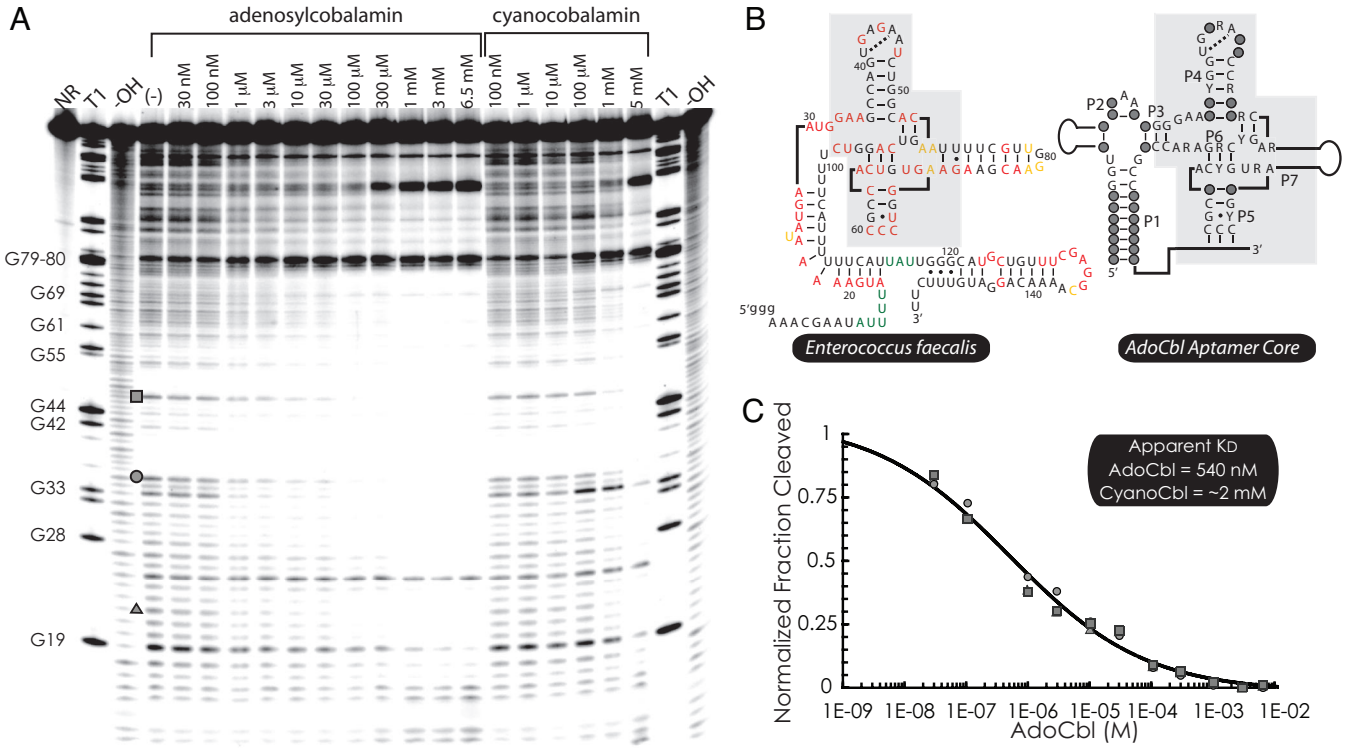

Fig. 4. AdoCbl induces structural changes within the *eutG* 5′ UTR. (*A*) Representative in-line probing data for the *E. faecalis eutG* riboswitch region with increasing concentrations of AdoCbl and cyanocobalamin. Controls include nonreacted RNA (NR), partial digestion by RNase T1 (cleavage at G residues) and partial alkaline digestion (cleavage at all residues). Shaded symbols mark bands used for quantitative analyses. (*B*) Proposed secondary structure model for the *E. faecalis eutT-G* riboswitch showing nucleotides that are more constrained (red), less constrained (green), or unaffected (yellow) on addition of AdoCbl. Also shown is the consensus secondary structure model of the classical AdoCbl-sensing riboswitch. The gray box highlights the conserved core region (32). (*C*) Normalized fraction cleaved plotted against AdoCbl concentration is shown for the residues indicated in *A*. The gray line results from a 4-parameter logistic fit of the data. The results of the nonlinear regression analysis suggest a binding affinity of 540 nM for AdoCbl, and >2 mM for cyanocobalamin.

multiple points of posttranscriptional regulatory control by EutWV within the *eut* loci. Interestingly, recent data has suggested that a region encompassing the *eutS* upstream sequence was induced in the presence of ethanolamine (10). Our work suggests that the region upstream of *eutP* is required for activation by EutWV (Fig. 2). Both observations agree well with identification of the terminator element upstream of the *eutS* and *eutP* genes, and are consistent with a regulatory role. Because our analysis was limited to *eut* operons, it is possible that the conserved sequence element may be present elsewhere in the genome as part of a broader regulon.

The simplest interpretation of these data are that ethanolamine triggers phosphorylation of EutV, which in turn activates association with the *eut*-associated terminator motif, stimulating antitermination and synthesis of the ethanolamine catabolism genes. To test this hypothesis, EutW and EutV were cloned into expression vectors that added N-terminal hexahistidine tags, and the proteins were overproduced in *E. coli* and purified from cell lysates by using affinity chromatography. After verifying that autophosphorylation of EutW and phosphorylation EutV occurs in an ethanolamine-dependent manner (Fig. S1; ref. 10), we purified phosphorylated and unphosphorylated protein. Preliminary tests of RNA-binding activity suggest that, indeed, there is a physical relationship between EutV and the *eut*-associated RNA motif (Fig. 3*D*). However, in contrast to our expectations, unphosphorylated EutV may exhibit improved affinity for the RNA substrate, rather than phosphorylated EutV. Also, preliminary tests of RNA-binding activity of EutW suggest that it also may exhibit RNA-binding activity. Therefore, the molecular details of the relationship between EutV and EutW and the conserved RNA element require further elucidation.

AdoCbl-Sensing Riboswitches Are Found Within the *eut* Locus. In *Salmonella*, expression of ethanolamine catabolism genes is controlled by the EutR transcription factor, which responds to both ethanolamine and AdoCbl to drive transcription. Sensitivity to AdoCbl is logical, because it is required for the main enzymatic step in ethanolamine catabolism (13). Therefore, it would not be unexpected for other microorganisms to regulate ethanolamine catabolism in a manner sensitive to the presence of AdoCbl. Indeed, growth on ethanolamine as the sole carbon source in *E. faecalis* requires AdoCbl (10). Sensitivity of the EutWV 2-component regulatory system to ethanolamine, as published previously (10) and here (Fig. S1), suggests a basis for ethanolamine-responsive regulation of the *eut* locus. However, our studies also reveal a regulatory mechanism for sensing AdoCbl.

Because riboswitches that sense AdoCbl have previously been described, we manually examined intergenic regions within the *eut* locus for evidence of secondary structure and primary sequence conservation. These regions were also analyzed by using the RNA motif search program, Ribex (27). These efforts resulted in the identification of a putative regulatory RNA located between *eutT* and *eutG* (Figs. 1 and 3*B*). Notably, this RNA element exhibited partial similarity to an adenosylcobalmin-sensing riboswitch class described previously (Fig. 3*B*; refs. 14, 28–31). However, only a portion of the canonical AdoCbl-sensing riboswitch could be initially recognized, suggesting that the *E. faecalis eutT-G* RNA element may be significantly different. Indeed, this putative RNA element was not identified by prior bioinformatics-based searches for AdoCbl-sensing riboswitches (28, 29, 31). Closer inspection of the *eutT-G* intergenic region yielded 2 key observations. First, a sequence motif closely resembling the *E. faecalis eutT-G* element

could also be identified within the *eut* operon of *Listeria* species, suggesting conservation of this element. Comparative sequence alignment of the *eutT-G* intergenic region for *Listeria* and *Enterococcus* species revealed many individual nucleotide differences between them (Fig. 4*B* and Fig. S2). However, many of the changes are such that base-pairing potential is maintained, in support of the postulated secondary structural arrangement. Also, the *eut* T–G element appeared to contain many of the structural and sequence features of the minimal core region (32) of AdoCbl-sensing riboswitches (Fig. 4*B* and Fig. S2), suggesting a functional relationship. However, the *eutT-G* RNA element contains important differences. Specifically, canonical AdoCbl riboswitches include a paired region (P1) formed from the 5′ and 3′ terminal nucleotides. In contrast, the *eutT-G* RNA element lacks both this and a neighboring helical element (P2). Also, the *eutT-G* RNA element appears to contain an extra base-paired region near the 3′ terminus that is absent from the canonical AdoCbl riboswitch. Based on these observations, we hypothesized that the *eutT-G* RNA element constitutes a subclass of the previously described AdoCbl riboswitch. Interestingly, although previous bioinformatics techniques were unable to identify the *eutT-G* element, they were able to identify an additional, canonical AdoCbl riboswitch (28, 31), immediately upstream of the *eut* locus, located in the promoter region of an operon predicted to encode for cobalamin transport (Fig. 1).

To test for riboswitch function, the *eutT-G* intergenic region was synthesized in vitro and subjected to in-line probing, a sequence-independent method of qualitatively assessing changes in RNA secondary structure (33, 34). In-line probing of the entire *eutT-G* intergenic region (>400 nt) indicated that only a portion of the overall region appeared to exhibit a significant degree of secondary structure content. Further truncations of the transcript narrowed this region to ≈200 nt that include the putative riboswitch. Then, in-line probing was used to investigate changes in the *eutT-G* riboswitch secondary structure arrangement on addition of varying concentrations of AdoCbl. Addition of AdoCbl resulted in decreased band intensity at many different RNA positions (Fig. 4*A*), suggesting that AdoCbl promoted stabilization of an array of secondary structure features that agree well with our model (Fig. 4*B*). Each of the positions exhibiting a change in band intensity responded similarly to AdoCbl, suggesting a concerted change in RNA structure. In total, these data suggest that AdoCbl induces a global conformational change for the *eutT-G* riboswitch with an apparent K_d of ≈540 nM (Fig. 4*C*), comparable with disassociation constants for the canonical AdoCbl riboswitch (30). In contrast, the apparent K_d for an analog, cyanocobalamin, was substantially poorer, demonstrating selectivity for AdoCbl.

Association of AdoCbl with the *eutT-G* Riboswitch Induces Transcription Antitermination. In general, riboswitches regulate gene expression by exerting control over transcription termination, translation initiation, or mRNA stability (21). Typically, riboswitches in Gram-positive bacteria control formation of an intrinsic transcription terminator in response to a metabolite. Consistent with this expectation, putative transcription terminator helices could be identified immediately downstream of both the canonical AdoCbl riboswitch and the *eutT-G* AdoCbl riboswitch subclass (Figs. 1 and 3*A*). Therefore, we investigated whether AdoCbl changes transcription termination in the *eutT-G* intergenic region.

Measurements of transcription in vitro for DNA templates encompassing the entire *eutT-G* region resulted in 2 primary transcripts (Fig. 5*A*), which correlated to the expected sizes of a runoff and a terminated transcript. The majority (85%) of the products corresponded to the terminated transcript. However, this fraction decreased on addition of AdoCbl, reaching a minimum of 65% termination. Half-maximal decrease in termination was achieved with 370 nM AdoCbl (Fig. 5*B*). These data reveal that association of AdoCbl with the *eutT-G* riboswitch inhibits termination, thus, promoting synthesis of the downstream genes. This

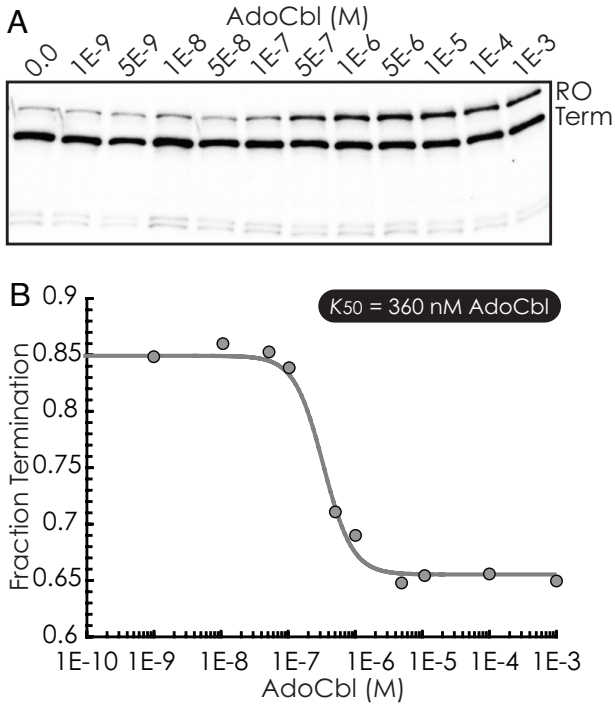

Fig. 5. AdoCbl induces antitermination in vitro. (*A*) In vitro transcription analysis of the *eutG* 5′ UTR with varying concentrations of AdoCbl. Each individual reaction resulted in transcripts corresponding to premature termination (*Lower*) and runoff transcription of the DNA template (*Upper*). (*B*) The fraction of transcripts resulting from premature transcription termination is shown plotted against [AdoCbl]. The half-maximal change in the fraction termination corresponded to 350 nM AdoCbl.

result is in contrast to previously characterized AdoCbl-sensing riboswitches, which, in concordance with their function in feedback inhibition of cobalamin biosynthesis or import, promote termination in response to AdoCbl (29, 31).

Interestingly, close inspection of the *eutT-G* terminator helix revealed that it contains the conserved sequence found in the other *eut* terminator elements that we have proposed as substrates for the EutWV 2-component signaling pathway (Fig. 3*A*). This observation raises the intriguing hypothesis that 2 separate posttranscriptional regulatory pathways, the AdoCbl riboswitch and EutWV-mediated antitermination, both exert regulatory control over the same intrinsic terminator helix (Fig. 1). It remains to be determined whether these posttranscriptional regulatory mechanisms function independently or in concert to influence terminator formation.

Conclusions

Many individual DNA-binding transcription factors that function as repressors or activators have been identified in bacteria. Also, it has been established that bacterial operons are oftentimes subjected to regulatory control by combinations of transcription factors (35). Thus, the use of multiple transcription factors, functioning either independently or via hierarchical relationships, allows for control of gene expression in response to combinatorial input signals. However, in the past decade it has become increasingly apparent that posttranscriptional regulatory circuitry is also fundamentally important for bacterial gene regulation. There are many different types of RNA-mediated regulatory mechanisms that have been described, including regulatory RNAs that function *in trans*, relative to their target transcripts, and those functioning in *cis*. Of the latter, almost all published examples are located in the 5′-untranslated region or within intergenic regions of polycistronic transcripts. Typically, one particular class of *cis*-acting regulatory RNA is

associated with a given operon, akin to regulation by a single, signal-responsive transcription factor. However, we have found that multiple posttranscriptional regulatory strategies converge for regulation of ethanolamine catabolism in *Enterococcus*, *Clostridium*, and *Listeria* species. The coupling of different RNA-mediated control mechanisms allows for coordinated regulation in response to 2 separate metabolic stimuli, ethanolamine and AdoCbl (Fig. 1). Therefore, these data suggest that bacterial operons may be subjected to overlapping and independent RNA-mediated regulatory circuitry, conceptually similar to the use of multiple transcription factors.

Materials and Methods

A brief overview is provided here. For greater detail, see *SI Methods* and Table S2.

β-Galactosidase Assays. OG1RF was the *E. faecalis* strain background used in our studies. Strains containing constructs described in Fig. 1*A* were cultured overnight in BHI medium. Overnight cultures were diluted 1:50 in fresh BHI medium or BHI with 40% horse serum (BHIS), and grown at 37 °C for 4 h, collected, and β-Galactosidase assays carried out as previously described (36).

EutV and EutW Biochemical Assays. Strains capable of producing N-terminal His_6-tagged EutW and EutV were created and induced. The cells were disrupted and the proteins affinity purified by use of a Cobalt TALON resin (Clontech). Purity of EutV and EutW was >90%, as judged by 10% SDS/PAGE followed by Coomassie-staining. To examine phosphorylation, EutW and/or EutV were incubated in buffer containing 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). To examine the ability of *eutV* to bind the untranslated 5′ region of *eutP*, this region was synthesized in vitro and radiolabeled with γ-^{32}P ATP. The 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-Line Probing Reactions and Transcription Termination Assays. The *E. faecalis eutT-G* intergenic region was amplified, and RNAs synthesized by T7 RNAP and 5′-radiolabeled with γ-^{32}P ATP as described previously (37). After incubation in reaction buffer at 25 °C for ≈40 h, the products were resolved by 10% PAGE. The same region was amplified for transcription termination assays and the reactions performed as described previously by using *E. coli* RNA polymerase (37).

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