A riboswitch-regulated antisense RNA in *Listeria monocytogenes*

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Riboswitches are ligand-binding elements located in 5′ untranslated regions of messenger RNAs, which regulate expression of downstream genes. In *Listeria monocytogenes*, a vitamin B_{12}-binding (B_{12}) riboswitch was identified, not upstream of a gene but downstream, and antisense to the adjacent gene, *pocR*, suggesting it might regulate *pocR* in a nonclassical manner. In *Salmonella enterica*, PocR is a transcription factor that is activated by 1,2-propanediol, and subsequently activates expression of the *pdu* genes. The *pdu* genes mediate propanediol catabolism and are implicated in pathogenesis. As enzymes involved in propanediol catabolism require B_{12} as a cofactor, we hypothesized that the *Listeria* B_{12} riboswitch might be involved in *pocR* regulation. Here we demonstrate that the B_{12} riboswitch is transcribed as part of a noncoding antisense RNA, herein named AspocR. In the presence of B_{12}, the riboswitch induces transcriptional termination, causing *aspocR* to be transcribed as a short transcript. In contrast, in the absence of B_{12}, *aspocR* is transcribed as a long antisense RNA, which inhibits *pocR* expression. Regulation by AspocR ensures that *pocR*, and consequently the *pdu* genes, are maximally expressed only when both propanediol and B_{12} are present. Strikingly, AspocR can inhibit *pocR* expression *in trans*, suggesting it acts through a direct interaction with *pocR* mRNA. Together, this study demonstrates how *pocR* and the *pdu* genes can be regulated by B_{12} in bacteria and extends the classical definition of riboswitches from elements governing solely the expression of mRNAs to a wider role in controlling transcription of noncoding RNAs.

asRNA | cobalamin | infection

The Gram-positive bacterium *Listeria monocytogenes* is a food borne pathogen which causes gastroenteritis as well as meningitis and encephalitis in immunocompromised individuals and abortions in pregnant women (1). The ability of the bacterium to invade diverse tissues while proliferating intracellularly in phagocytic and nonphagocytic cells has led to its adoption as a model organism in both infection biology and cell biology (2, 3). More recently, *L. monocytogenes* has also become a focus of studies of RNA-based regulation. Multiple transcriptomics studies in *L. monocytogenes* have now identified 134 putative small *trans*-acting RNAs (sRNAs), 86 antisense RNAs (asRNAs), and 42 *cis*-acting riboswitches (4).

In Gram-positive bacteria, the majority of *cis*-acting riboswitches affect transcription termination, although some affect translation, Rho-mediated termination, and mRNA cleavage (5–7). Transcribed as part of the 5′ UTR of mRNAs, transcription termination riboswitches adopt structured conformations able to bind specific ligands. Ligand-binding induces formation of a terminator in an elongating mRNA, causing RNA polymerase to cease transcription. As such, transcription termination riboswitches are positioned upstream of ORFs and regulate elongation of transcription into the downstream genes. Intriguingly, in *L. monocytogenes* we previously noticed one such B_{12} riboswitch positioned downstream of the *lmo1149* gene and in the opposite and convergent orientation to the next adjacent gene, *pocR* (*lmo1150*) (8). As this riboswitch lacked a downstream ORF, we hypothesized it might play a nonclassical role. Three previous studies supported the hypothesis that the riboswitch might be transcribed as part of, and regulate transcription of, an asRNA. In the first study, a transcript encompassing the riboswitch, Rli39, was detected by a tiling array analysis (Fig. 1*A*) (8). In the second study, a noncoding RNA (ncRNA), RliH, was identified by strand-specific Northern blot, antisense to the 5′ end of *pocR* (Fig. 1*A*) (9). Finally, in the third study, whole-genome differential RNA-seq analysis identified a transcription start site (TSS) for the Rli39 transcript, but no TSS was detected for RliH (10). These findings suggested that Rli39 and RliH might represent two parts of a long riboswitch-regulated asRNA.

The rationale for a B_{12}-regulated asRNA opposite to *pocR* became clearer upon a survey of the literature regarding the role of PocR. In *Salmonella enterica*, PocR is a transcription factor that, in the presence of propanediol, activates transcription of the *pdu* and *cob* genes, which mediate propanediol catabolism and vitamin B_{12} biosynthesis, respectively (11). Propanediol is a molecule produced in the intestine by commensal bacteria as a byproduct of the fermentation of rhamnose and fucose, and propanediol catabolism requires a B_{12}-dependent diol dehydratase encoded by the *pduCDE* genes (12). We hypothesized that in *L. monocytogenes* a B_{12}-regulated asRNA opposite *pocR* might fine tune the expression of *pocR* in response to B_{12}. This could inhibit PocR-mediated activation of the B_{12}-dependent *pdu* genes when propanediol is present but B_{12} has yet to be synthesized. At present, however, it is unknown how *pocR* or the *pdu* genes are regulated in response to B_{12} in any bacterium.

In this study we show that a B_{12} riboswitch in *L. monocytogenes* regulates a *cis*-encoded asRNA opposite to *pocR*. This transcript, named AspocR, encompasses the previously identified Rli39 and RliH transcripts. Binding of B_{12} to the riboswitch leads to premature termination of *aspocR* transcription, whereas a longer AspocR transcript is produced in the absence of B_{12}. Expression of the long form of AspocR inhibits *pocR* expression and consequently the activation of the *pdu* genes, when B_{12} is unavailable. As propanediol catabolism is thought to be important for the pathogenesis of many intestinal pathogens, our study has implications for both *L. monocytogenes* and enterobacterial pathogenesis.

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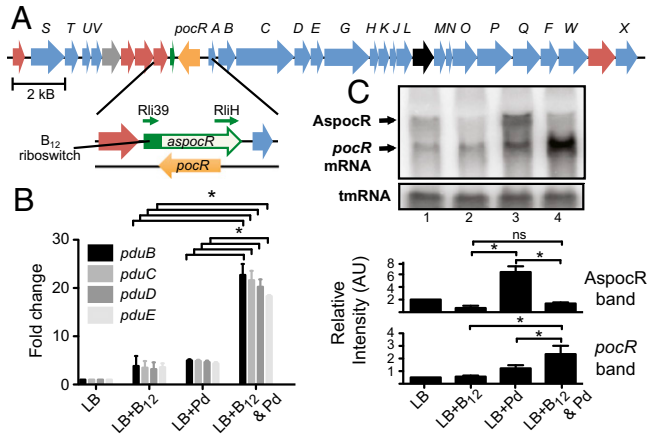

Fig. 1. Regulation of the *pdu* locus by B_{12} and propanediol. (*A*) Schematic representation of the *L. monocytogenes* propanediol utilization (*pdu*) locus: *pocR* gene (orange), *pdu* genes (blue), B_{12} biosynthesis genes (red), unknown gene (gray), ethanolamine utilization gene (black), and B_{12} riboswitch (green). Enlargement shows the *aspocR/pocR* locus. Green arrows denote the Rli39 and RliH transcripts. (*B*) Expression levels of the *pduBCDE* genes evaluated by qRT-PCR. RNA was isolated from bacteria grown to an OD_{600} of 0.3–0.5 in LB, LB+B_{12} (20 nM), LB+Pd (50 mM), or LB+both. Results represent three biological replicates. Error bars show SE. (*C*) Northern blot of the *pocR* and AspocR transcripts. Blots were probed with a radioactively labeled PCR probe spanning the *aspocR/pocR* locus. Band intensities were calculated from four experiments. Units are arbitrary. Statistically significant differences ($P < 0.05$) are marked with asterisks. RNA was isolated from bacteria grown as described above. tmRNA was used as a loading control.

Results

Maximal Expression of the *pdu* Genes Requires both B_{12} and Propanediol. In *L. monocytogenes*, the *pdu* genes are located in two cassettes of 8 and 20 genes, which surround the *pocR* gene (Fig. 1*A*). Additionally, a B_{12} riboswitch lies 3′ of *pocR*, but positioned in the antisense orientation (Fig. 1*A*). Little is known about the regulation of *pocR* or the *pdu* genes in response to either propanediol or B_{12}. We thus investigated the regulation of *pdu* genes in response to the presence of propanediol or B_{12} by quantitative reverse transcriptase PCR (qRT-PCR). To this end we first evaluated the transcript levels of four *pdu* genes encoding an accessory protein of propanediol catabolic reactions (*pduB*), and the three subunits of the B_{12}-dependent diol dehydratase (*pduCDE*). When either propanediol or B_{12} alone was present in the medium, expression of the *pduB*, *pduC*, *pduD*, and *pduE* genes increased, albeit mildly (~5-fold), compared with the control condition (LB). In contrast, activation of all four genes was significantly higher (~25-fold) when both propanediol and B_{12} were present together (Fig. 1*B*), indicating that maximal expression requires the presence of both propanediol and B_{12}.

The B_{12} Riboswitch Regulates a Long Transcript. The demonstration that the *pduBCDE* genes are regulated in response to propanediol and B_{12} together is consistent with the hypothesis that the B_{12} riboswitch regulates an asRNA opposite to *pocR*. The asRNA could in turn regulate *pocR*, and subsequently *pdu* gene expression, in response to B_{12}. To analyze whether such an asRNA is produced, we examined transcription from both strands of the *pocR* locus by Northern blot using a double-stranded DNA probe to identify the putative antisense RNA and the *pocR* transcript simultaneously (Fig. 1*C*). When bacteria were cultured in LB medium alone, both the *pocR* transcript (~1,000 nt) and a putative antisense RNA (~1,400 nt, herein named AspocR) were detected at similar levels (Fig. 1*C*, lane 1). When B_{12} was added to the medium, the level of AspocR decreased (Fig. 1*C*, lane 2), supporting the hypothesis that transcription of *aspocR* is controlled by a B_{12} riboswitch. Surprisingly, the level of *pocR* transcript remained relatively unchanged despite the reduction in AspocR levels, suggesting the cell maintains a basal level of *pocR* transcript even in the presence of low levels of AspocR, although the mechanism of this regulation is unknown. In the presence of propanediol, however, we observed a significant increase in the levels of both AspocR as well as *pocR* mRNA (Fig. 1*C*, lane 3). This increase is likely due to propanediol-dependent activation by PocR itself. PocR has been shown to autoregulate its expression in *S. enterica*, and both *aspocR* and *pocR* contain two nearly identically spaced sites in their promoters, which closely resemble the *S. enterica* consensus PocR binding site (Fig. S1*A*) (8, 13, 14). In contrast, in the presence of both propanediol and B_{12} we observed an even greater increase in *pocR* mRNA, but the AspocR transcript was no longer detected (Fig. 1*C*, lane 4), in agreement with our original hypothesis. We also examined expression of both *pocR* and *aspocR* by Northern blot using single-stranded RNA probes to detect each transcript individually (Fig. S1 *B* and *C*). Using a probe complementary to the riboswitch we detected a short transcript (Fig. S1*B*, thin arrow), corresponding to the previously reported Rli39 transcript under all conditions (8). However, we also detected a long AspocR transcript when bacteria were grown in presence of propanediol alone (Fig. S1*A*, thick arrow), and this transcript was detected in LB alone, if blots were overexposed. Expression of *pocR* increased in the presence of propanediol, but was most strongly activated in the presence of both B_{12} and propanediol. Together, these results suggest that, in the presence of B_{12}, the riboswitch causes *aspocR* to be transcribed as a short truncated transcript, whereas in the absence of B_{12}, *aspocR* is transcribed as a long transcript, which could act as an asRNA to inhibit *pocR* expression.

Expression of *pocR* Is Regulated by B_{12} via AspocR. Our data fit well with a model whereby the B_{12} riboswitch controls expression of an asRNA, which can inhibit *pocR* expression when B_{12} levels are low (Fig. 2*A*). To determine if the long transcript acts as a *cis*-encoded asRNA regulating *pocR*, we constructed two mutant strains. In the first mutant (Δribo), we deleted a 229-nt region encompassing the B_{12} riboswitch, while leaving intact the predicted promoter upstream of the riboswitch (Fig. 2*B*). We expected that the Δribo mutant would be unable to terminate *aspocR* transcription, even in the presence of B_{12}. In a second mutant (ΔP+ribo), a 263-nt region encompassing both the riboswitch and the −10 promoter region was deleted (Fig. 2*B*). We expected that, as it lacked a promoter, the ΔP+ribo mutant would never express the AspocR transcript. Both mutants grew similarly to the wild-type strain under all conditions (Fig. 2*C*), and RNA was isolated from all strains grown in LB medium alone, LB plus propanediol or B_{12}, or LB plus both in combination. Strand-specific Northern blotting was carried out to analyze levels of the long AspocR transcript and *pocR* mRNA. In the wild-type strain, low levels of AspocR were produced when bacteria were grown in LB medium (Fig. 2*D*, lane 1), and *aspocR* expression increased when bacteria were grown in the presence of propanediol (Fig. 2*D*, lane 7). However, when only B_{12}, or B_{12} in combination with propanediol was present in the medium, no AspocR was detected (Fig. 2*D*, lanes 4 and 10). In agreement with the expected expression patterns of the mutants, the Δribo mutant showed a constitutive expression of *aspocR*, and *aspocR* expression increased when bacteria were grown in the presence of propanediol (Fig. 2*D*, lanes 2, 5, 8, and 11). The absence of the riboswitch results in the observed band migrating slightly faster than that of the wild-type strain and likely accounts for the higher levels of AspocR observed in the Δribo mutant as the riboswitch also leads to the production of truncated transcripts in the wild-type strain. In contrast, no AspocR transcript was

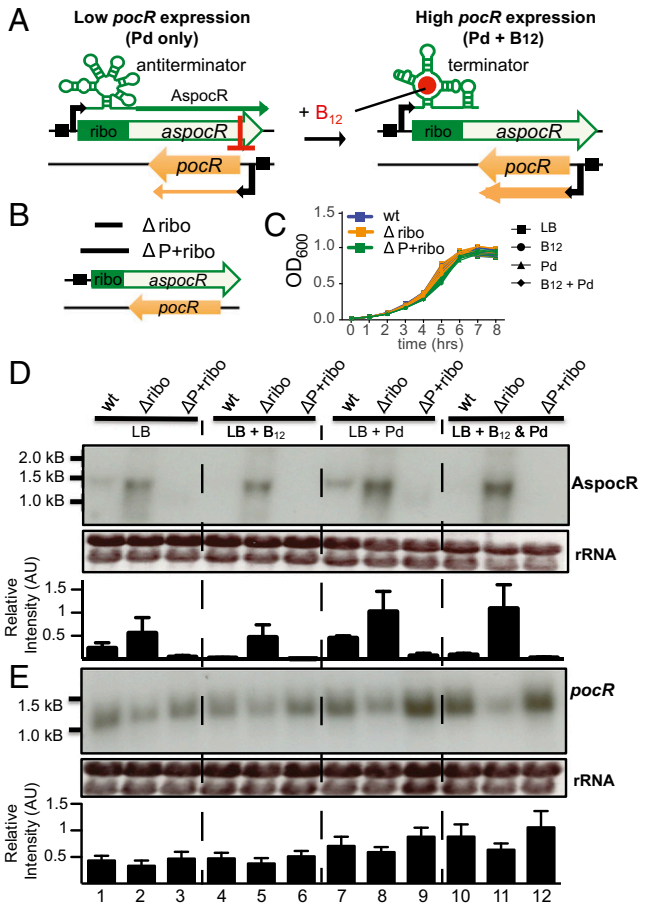

Fig. 2. Expression of *pocR* is regulated by B_{12} via AspocR. (*A*) Model showing proposed regulation of *pocR* by AspocR. (*B*) Schematic representation of the wild-type *aspocR/pocR* locus with the B_{12} riboswitch (green) and *aspocR* promoter (black box). Regions deleted in the Δribo and ΔP+ribo mutants are indicated by black bars. (*C*) Growth curves of the three strains under conditions indicated. Results represent three experiments. (*D* and *E*) Northern blots were carried out using single-stranded RNA probes to detect the long AspocR and *pocR* transcripts. Band intensities were quantified from three separate experiments. Units are arbitrary. RNA was isolated from bacteria grown to an OD_{600} of 0.3–0.5 in LB, LB+B_{12} (20 nM), LB+Pd (50 mM), or LB+both. Ethidium bromide staining of rRNA is shown as a loading control.

detected in the ΔP+ribo mutant under any conditions (Fig. 2*D*, lanes 3, 6, 9, and 12).

We also examined expression of *pocR* and found that, in all cases, *pocR* mRNA levels inversely correlated with levels of AspocR. In particular, in the Δribo mutant, which constitutively expresses *aspocR*, *pocR* mRNA levels were low under all conditions (Fig. 2*E*, lanes 2, 5, 8, and 11). In contrast, in the ΔP+ribo mutant, which never expresses *aspocR*, levels of *pocR* mRNA were elevated compared with the wild-type strain when bacteria were grown in the presence of propanediol alone (Fig. 2*E*, lane 9 vs. 7). This indicates that in the ΔP+ribo mutant, *pocR* was highly expressed when only propanediol was present, whereas in the wild-type strain, *pocR* mRNA levels were lower when bacteria were grown in the presence of propanediol alone and increased significantly only when bacteria were grown in the presence of both propanediol and B_{12}. Together our data imply a model wherein *pocR* expression is activated in the presence of propanediol, but is simultaneously inhibited by transcription of the AspocR asRNA. Only when B_{12} is also present will the riboswitch terminate transcription of the long AspocR transcript, allowing maximal *pocR* expression.

Effects of *aspocR* Expression on PocR-Regulated Genes. We identified putative PocR binding sites upstream of the *pduA* (Fig. 3*A*), *pduU*, and *pduS* genes (Fig. S2*A*) suggesting, as in *S. enterica*, the *pdu* genes are regulated by PocR. To determine if AspocR-mediated control of *pocR* expression affects regulation of the *pdu* genes, we evaluated the transcript levels of *pduBCDE*, *pduU*, and *pduS* genes by qRT-PCR in the wild type, Δribo, and ΔP+ribo mutants (Fig. 3 and Fig. S2). Bacteria were grown to midlog phase in LB medium in the presence of either propanediol or B_{12} alone or in combination, and RNA was extracted. When cells were grown in propanediol alone, the *pduBCDE* genes were strongly up-regulated in the ΔP+ribo mutant compared with the wild type or the Δribo mutant (Fig. 3). Conversely, the *pduBCDE* genes were minimally expressed in the Δribo mutant, which constitutively expresses *aspocR*, even in the presence of both propanediol and B_{12}. We observed the same pattern of expression for the *pduS* and *pduU* genes as for the *pduBCDE* genes (Fig. S2), however induction of these genes was lower than for the *pduBCDE* genes. These data suggest that the regulation of *pocR* via AspocR is the main switch controlling *pdu* gene expression in response to B_{12}.

As the vitamin B_{12} biosynthesis operon has been shown to be activated by PocR in *S. enterica* (11), we also examined expression of the *cblT* (*lmo1190*) and *cbiA* (*lmo1191*) genes. *cblT* and *cbiA* are the first two genes of the vitamin B_{12} biosynthesis operon in *L. monocytogenes* (Fig. S3*A*) and we identified a PocR-binding site upstream of *cblT* (Fig. S3*B*). *cblT* encodes a putative transporter of a B_{12} precursor and *cbiA* encodes a cobyrinic acid synthase involved in B_{12} synthesis. Interestingly, whereas *L. monocytogenes* appears to carry only the genes for anaerobic synthesis of B_{12} (15), both genes are nonetheless activated under aerobic conditions in response to propanediol. This activation is PocR dependent as levels of both *cblT* and *cbiA* remained low in

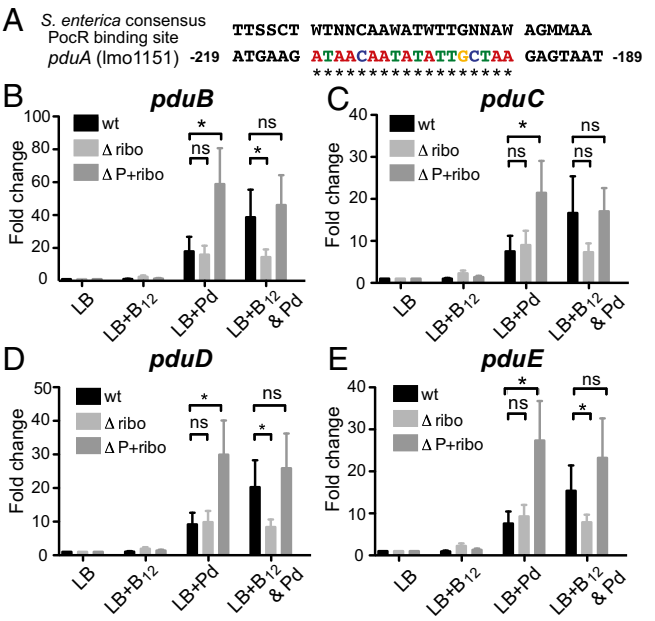

Fig. 3. Effects of *aspocR* expression on PocR-regulated genes. (*A*) Potential PocR-binding site upstream of the *pduA* gene is shown. Numbering denotes the position relative to the translation start site. Asterisks denote matches to the *S. enterica* consensus PocR-binding sequence. Levels of the (*B*) *pduB*, (*C*) *pduC*, (*D*) *pduD*, and (*E*) *pduE* genes were evaluated by qRT-PCR in the wild-type (WT), Δribo, or ΔP+ribo strains. Statistically significant differences ($P < 0.05$) are indicated by asterisks. RNA was isolated from bacteria grown to an OD_{600} of 0.3–0.5 in LB, LB+B_{12} (20 nM), LB+Pd (50 mM), or LB+both. Results represent the average of four biological replicates. Error bars show SE.

the Δribo mutant. We also noted that the intergenic region between *cblT* and *cbiA* contains a B_{12} riboswitch (8), and expression of *cbiA* was repressed in all strains in the presence of B_{12} (Fig S3 *C* and *D*). Thus, whereas expression of the B_{12} biosynthesis operon is activated by PocR in response to propanediol, repression of *cbiA* and potentially the downstream B_{12} biosynthesis genes is likely mediated by the B_{12} riboswitch between *cblT* and *cbiA* in response to B_{12}.

AspocR Inhibits *pocR* Expression *in Trans* and in Vitro. To date, only a few studies have examined how asRNAs mediate their effects on target genes (16, 17). Owing to their perfect complementarity with the sense-encoded gene, it has been proposed that asRNAs might act through a base-pairing mechanism. However, it has also been proposed that transcription of an asRNA might interfere with transcription of the sense transcript by affecting transcription elongation on the opposite strand. Such a transcriptional interference mechanism requires the asRNA to be transcribed from the same locus as the sense gene. To examine these two possible mechanisms of AspocR-mediated regulation, we tested whether AspocR could ectopically affect expression of *pocR*. A fragment containing the *aspocR*/*pocR* locus, but lacking the promoters for either gene, was cloned into the pAD vector, which stably integrates into the chromosomal $tRNA^{ARG}$ locus. A constitutive promoter was cloned in front of *aspocR*, and a Rho-independent terminator was incorporated at the end of *aspocR*. A control plasmid carrying only the promoter and terminator was also constructed. The plasmids were transformed into the ΔP+ribo background, which does not express *aspocR* to generate the ΔP+ribo::*aspocR* and ΔP+ribo::Ø strains. Bacteria were subsequently grown in LB medium alone, LB in the presence of either propanediol or B_{12} alone, or in combination, RNA was extracted, and Northern blotting was carried out against the long form of AspocR and the *pocR* transcript.

In the ΔP+ribo::Ø strain no AspocR transcript was detected under any condition (Fig. 4, lanes 1, 3, 5, and 7), whereas in the ΔP+ribo::*aspocR* strain, an AspocR transcript was detected under all conditions, indicating it was constitutively expressed (Fig. 4, lanes 2, 4, 6, and 8). In the ΔP+ribo::Ø strain, *pocR* transcript levels were unaffected by the presence of the control construct and showed the same pattern of induction as observed previously for the ΔP+ribo mutant. Namely, *pocR* mRNA levels were elevated in the presence of propanediol alone or propanediol plus B_{12}. In contrast, in the ΔP+ribo::*aspocR* strain, which constitutively expresses *aspocR*, levels of *pocR* transcript were significantly reduced under all conditions. This indicates that AspocR can act *in trans* and does not need to be transcribed from the same locus to inhibit expression of *pocR*. While this does not exclude the possibility that AspocR also mediates transcriptional interference when transcribed from its native locus, it suggests that the main effects of AspocR on *pocR* expression are mediated by base pairing to *pocR* mRNA.

To further examine the mechanism of AspocR mediated regulation, we measured PocR expression using an in vitro transcription/translation system. In this system we first amplified fragments corresponding to the *aspocR*/*pocR* locus from the wild-type, Δribo, or ΔP+ribo strains and cloned them into plasmids. Plasmids were linearized and used as templates for in vitro transcription/translation experiments. When wild-type or Δribo templates were used in transcription/translation reactions, we observed low levels of PocR protein (Fig. 4*C*), probably due to constitutive AspocR expression from these templates. This conclusion was supported by the findings that a B_{12} riboswitch was unable to mediate transcription termination in the presence of B_{12} in another in vitro transcription system (18). In contrast, reactions with the ΔP+ribo template, which lacks the *aspocR* promoter, showed significant PocR expression in the presence of propanediol and B12 together (Fig. 4*C*), or propanediol (Fig. 4*C*) and B_{12} alone (Fig. S4), presumably due to the absence of AspocR-mediated inhibition.

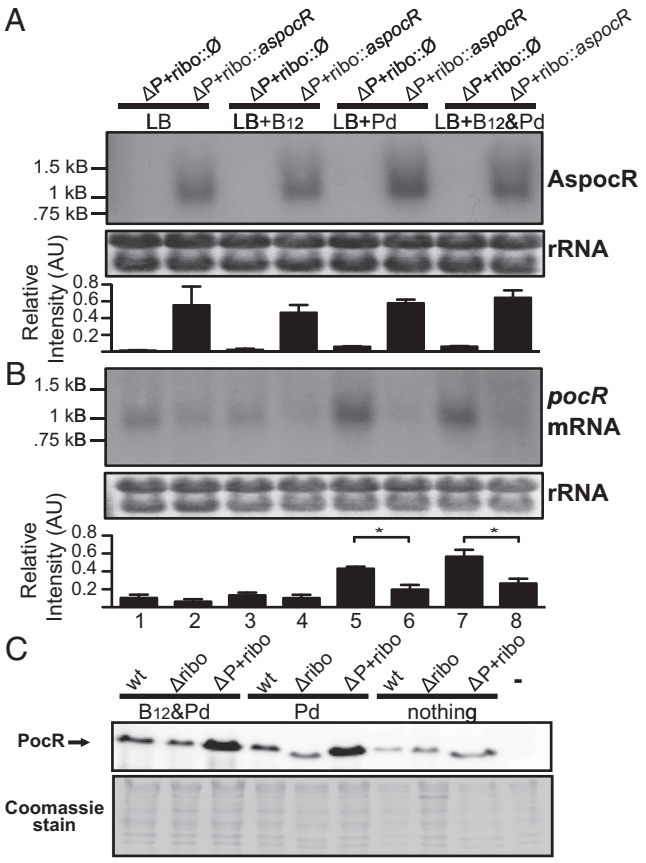

Fig. 4. AspocR represses *pocR* expression *in trans* and in vitro. Strains were constructed in the ΔP+ribo background with the *aspocR* gene preceded by a constitutive promoter and followed by a Rho-independent terminator (ΔP+ribo::*aspocR*) or with a constitutive promoter and terminator but lacking the *aspocR* gene (ΔP+ribo::Ø). RNA was isolated from bacteria grown to an OD_{600} of 0.3–0.5 in LB, LB+B_{12} (20 nM), LB+Pd (50 mM), or LB+both. Northern blots were carried out using single-stranded RNA probes to detect the (*A*) long AspocR and (*B*) *pocR* transcripts. Statistically significant differences ($P < 0.05$) are indicated by asterisks. Ethidium bromide staining of rRNA is shown as a loading control. Positions of molecular weight markers are indicated. Band intensities were quantified from three separate experiments. Units are arbitrary. (*C*) Western blot showing PocR protein levels from in vitro transcription/translation experiments. Fragments corresponding to the *aspocR*/*pocR* locus from the wild-type, Δribo, or ΔP+ribo strains were used as templates for reactions in the presence of Pd+B_{12}, Pd, or nothing. A control reaction with Pd+B_{12} but no template is also shown (–). Equal loading was verified by Coomassie staining (shown below).

Finally, we performed experiments to examine the stability of *pocR* mRNA in the presence or absence of AspocR. Wild-type, Δribo, and ΔP+ribo strains were cultured in LB medium in the presence of both propanediol and B_{12} to an OD_{600} of ~0.4 before rifampicin was added to the medium to stop transcription. RNA was extracted just before rifampicin treatment, and at various times thereafter, and Northern blotting was carried out to determine the levels of *pocR* mRNA. Consistent with our previous observations, we observed a significant reduction in the amount of *pocR* mRNA at time 0 in the Δribo mutant compared with the wild type or ΔP+ribo mutants (Fig. S5*A*, lanes 1, 6, and 11), indicating that the steady-state level of *pocR* transcript is indeed inhibited in the presence of AspocR. After rifampicin treatment, we observed a slight decrease in the half-life of *pocR* mRNA in the ΔP+ribo mutant; however, these differences were

not significant (Fig. S5*B*) and suggested AspocR does not promote the degradation of *pocR* mRNA.

Antisense Oriented Riboswitches in Other Bacteria. As *pocR* and the *pdu* genes are widely conserved in enteropathogens, we examined the *pocR* genes of other bacteria for the presence of an associated antisense B_{12} riboswitch. This analysis identified B_{12} riboswitches antisense to the *pocR* genes in the closely related species of *Listeria welshimeri*, *Listeria innocua*, *Listeria seeligeri*, and *Listeria ivanovii* as well as other strains of *L. monocytogenes* (Dataset S1), suggesting that *pocR* expression in these bacteria is likely governed by orthologs of *aspocR*. We also noted a B_{12} riboswitch adjacent to the *pocR* gene in other species including *S. enterica*, *Citrobacter koseri*, *Escherichia coli*, *Shigella* spp., and *Yersinia enterocolitica* (Dataset S1); however, these B_{12} riboswitches were not in an antisense orientation to *pocR*. Thus, regulation of *pocR* by a B_{12} riboswitch-regulated asRNA appears limited to the *Listeria* genus.

Riboswitch-regulated asRNAs are so far limited to the example of the AspocR transcript described here and to another asRNA opposite the *ubiG* operon in *Clostridium acetobutylicum*, which is regulated by an S-adenosylmethionine (SAM) riboswitch (19). We were interested in examining whether riboswitch-regulated asRNAs might have been overlooked in bacteria. We designed an algorithm to identify antisense oriented riboswitches in 834 bacterial genomes using gene annotations derived from National Center for Biotechnology Information Refseq and riboswitch annotations from the RNA families (Rfam) database. We examined 15 classes of ligand-binding riboswitches. In total we identified 144 occurrences of riboswitches antisense to the adjacent downstream open-reading frame (Table 1), including the SAM riboswitch previously shown to regulate an asRNA in *C. acetobutylicum*. Surprisingly, antisense riboswitches were mostly represented in the B_{12}, S-adenosylmethionine (SAM), cyclic-di-GMP, Mg^{2+} and flouride-binding riboswitch families (2.1–9.1% antisense riboswitches/total), and very few or no antisense riboswitches were identified in the other riboswitch classes examined (detailed in Dataset S2). The reasons accounting for these differences are unknown. However, the presence of antisense-oriented riboswitches in a range of bacterial species suggests that riboswitch-regulated noncoding RNAs likely represent an overlooked mechanism bacteria use to regulate gene expression.

Table 1. Antisense-oriented riboswitches

Riboswitch type	# antisense*	% (total analyzed)
Vitamin B_{12}	45 (27)	3.8 (1,171)
SAM	18 (9)	2.1 (844)
Cyclic-di-GMP	16 (3)	4.5 (355)
T-box	15 (11)	0.7 (2,300)
Mg^{2+}	15 (12)	9.1 (165)
TPP	10 (4)	0.7 (1,403)
FMN	9 (7)	1.6 (557)
Fluoride	6 (1)	2.9 (207)
Glycine	5 (3)	0.8 (666)
Lysine	4 (2)	1.1 (369)
Purine	1 (1)	0.3 (380)
Pre-queosine	0	0.0 (113)
Pyrimidine	0	0.0 (253)
SAH	0	0.0 (85)
THF	0	0.0 (38)

FMN, flavin mononucleotide; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TPP, thiamine pyrophosphate. *Number of antisense riboswitches that partially overlap an adjacent ORF are presented in parentheses in this column.

Discussion

In this study, we identied a B_{12} riboswitch that is transcribed as part of, and regulates transcription of, a noncoding asRNA, AspocR. Regulation by AspocR ensures that *pocR*, and consequently genes of the propanediol utilization pathway, are maximally expressed only when the cofactor essential for propanediol catabolism, B_{12}, is present. Regulation of *pocR* by a riboswitch-regulated asRNA represents a unique mechanism for controlling expression of a transcriptional regulatory protein and our analysis of over 800 bacterial genomes suggests that AspocR represents an emerging family of riboswitch-regulated asRNAs.

Surprisingly, like rare cases of other long asRNAs (20), AspocR was able to act *in trans*, whereas the riboswitch-regulated asRNA from *C. acetobutylicum* was not (19). The ability of AspocR to act *in trans* suggests that it can diffuse and bind to the *pocR* mRNA leading to a number of outcomes. Studies have suggested that base pairing between an asRNA and an mRNA can lead to the degradation of the mRNA, interfere with translation initiation, or repress mRNA transcription through transcriptional attenuation (16). Our data suggest AspocR may work through the latter two mechanisms. Our in vitro transcription/translation experiments showed that significantly higher quantities of PocR protein were produced with a template lacking the *aspocR* promoter, suggesting that *aspocR* expression inhibits PocR translation, at least *in vitro*. Furthermore our in vivo experiments examining the effects of ectopic *aspocR* expression on *pocR* mRNA levels showed that the expression of *aspocR* results in lower steady-state levels of *pocR* transcript. However, the stability of the *pocR* mRNA itself was largely unaffected by *aspocR* expression, suggesting that AspocR may repress *pocR* expression at the level of transcription rather than by affecting *pocR* transcript stability. As only a few asRNAs have been studied in detail at the mechanistic level, AspocR, and asRNA-mediated regulation in general, thus deserve further study.

Interestingly, we noted that the AspocR transcript never completely represses *pocR* expression. Even in the Δribo mutant, which constitutively expresses *aspocR*, some *pocR* transcript remains detectable. AspocR seems to act as a rheostat, modulating *pocR* mRNA levels rather than acting as a complete inhibitor of *pocR* expression. This modulation makes sense in light of PocR's dual role in activating the expression of the propanediol catabolic genes and also the genes responsible for anaerobic, de novo synthesis of vitamin B_{12}. If AspocR strongly repressed *pocR* in the absence of B_{12}, PocR levels would likely be insufficient to activate expression of the B_{12} biosynthetic genes, locking the cell in a state where it would be unable to activate B_{12} synthesis. Instead, expression of *aspocR* only partially represses *pocR* at low B_{12} conditions. In the presence of propanediol, this level of PocR appears sufficient to initiate expression of the B_{12} biosynthesis genes, which under anaerobic conditions will produce B_{12} essential for propanediol catabolism, but it is insufficient to maximally activate expression of the *pdu* genes. Presumably, only when sufficient levels of B_{12} accumulate, either through uptake or de novo synthesis, does B_{12} bind the riboswitch, terminating transcription of *aspocR*, and leading to maximal expression of *pocR* and the *pdu* genes. Thus, AspocR seems to serve as a fine-tuning element, rather than an all-or-nothing switch.

To our knowledge, our study is unique in showing how *pocR* and the *pdu* genes are regulated by B_{12} in any bacterium. Regulation of propanediol catabolism has recently garnered attention as propanediol, along with the closely related molecule ethanolamine (which is also degraded in a B_{12}-dependent manner), are increasingly considered important nutrient sources for bacterial pathogens during infection (21). An elegant set of studies recently showed that, in the case of *S. enterica*, growth on ethanolamine in

the intestine depends upon the inflammatory immune response induced by the bacterium (22, 23). Inflammation leads to the recruitment of neutrophils to the intestine, whose generation of reactive oxygen species result in the production of an electron acceptor, tetrathionate. *S. enterica* can then use tetrathionate to anaerobically respire on ethanolamine and outgrow commensal bacteria. The tetrathionate reductase system also supports anaerobic respiration on propanediol in *S. enterica* (24), but *L. monocytogenes* lacks an obvious ortholog of tetrathionate reductase. Furthermore, we were unable to grow *L. monocytogenes* in minimal medium supplemented with propanediol and B_{12}, under aerobic, microaerobic, or anaerobic conditions, even when we included tetrathionate, nitrate, dimethyl sulfoxide, or fumarate as possible electron acceptors. Nonetheless, the observations that *L. monocytogenes* up-regulates the *pdu* and ethanolamine utilization (*eut*) genes during both intracellular growth in intestinal epithelial cells (25), as well as in the intestine of germ-free mice pretreated with lactobacilli (26), suggest that propanediol and ethanolamine both play a role in *L. monocytogenes* pathogenesis. We suspect that *L. monocytogenes* may require an electron acceptor specific to its in vivo niche to use propanediol, and it will be of considerable interest to determine how *L. monocytogenes* and other pathogens use these carbon, nitrogen and energy sources differently during infection.

Finally, our bioinformatics analysis suggests that riboswitch-regulated asRNAs are not uncommon in bacteria. Up to 15 different types of riboswitches have now been identified in prokaryotic genomes, and the position of many transcription termination riboswitches, either in an antisense orientation, or far from the adjacent ORF start, suggests they would be unable to regulate these ORFs in a classical manner. These "marooned" riboswitches are strong candidates to regulate asRNAs, and plausibly sRNAs, suggesting many riboswitch-regulated noncoding RNAs have been thus far overlooked.

Materials and Methods

For greater detail, see Datasets S3 and S4.

Bacterial Strains, Plasmids, Primers, and Growth Conditions. Strains and plasmids used are listed in Dataset S3. Primers used are listed in Dataset S4. *L. monocytogenes* and *E. coli* were grown in LB broth with shaking at 200 rpm at 30 °C and 37 °C, respectively. LB was supplemented with 50 mM 1,2 propanediol and/or 20 nm vitamin B_{12}. For all experiments, bacteria were grown to an OD_{600} of 0.3–0.5.

Northern Blots. Blots were carried out according to the Northernmax-Gly kit protocol. Blots were probed with double-stranded DNA probes or single-stranded RNA probes labeled with α-[^{32}P]ATP or α-^{32}P-UTP, respectively.

Quantitative Real-Time PCR. One microgram of DNaseI-treated total RNA was used for cDNA synthesis with an iScript cDNA Synthesis kit. qRT-PCR reactions were prepared with SYBR Green master mix. Reaction cycling and quantification was carried out in an ABI Prism 7900HT. Expression levels were normalized to the *rpoB* gene. Samples were evaluated in triplicate and results represent at least three independent experiments. Statistically significant differences were calculated using log2 transformed values evaluated by paired *t* test.

In Vitro Transcription/Translation and Western Blots. Fragments encompassing the *aspocR/pocR* locus of either wild type, ΔP+ribo, or ΔP+ribo strains were used as templates for in vitro transcription/translation reactions with *E. coli* S30 extracts. Reactions were precipitated, washed, resolubilized, and run on 12% (vol/vol) SDS/PAGE gels. Gels were transferred to Hybond-P membranes and blotted with affinity-purified α-PocR rabbit sera.

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