Supporting Information

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SI Materials and Methods

Constructions of Plasmids and Strains. All cloning followed standard molecular biology protocols. Mutants were constructed using the pMAD shuttle plasmid (1). For construction of pMAD-based plasmids for generation of deletion mutants, 500- to 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 using the first two PCR products as template to generate a upstream–downstream (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 overnight at 16 °C with T4 DNA ligase (New England Biolabs; M0202S). A total of 2 μL of each ligation was transformed into chemically competent *Escherichia coli* Top10 (Invitrogen; C404003) cells according to the manufacturer's instructions. Transformants were screened by PCR for the presence of the UD insert and purified plasmid was isolated from positive colonies by miniprep using QIAprep spin columns (Qiagen; 27104). Electrocompetent *Listeria monocytogenes* strains were transformed with each respective plasmid and mutagenesis carried out as described previously (1). All mutants were confirmed by DNA sequencing. For construction of ΔP+ribo::Ø and ΔP+ribo::*aspocR* strains, constructs were cloned into the XmaI/SalI sites of the pAD vector (2) derived previously from the pPL2 vector (3). Plasmids were transformed into electrocompetent ΔP+ribo bacteria and integrants were confirmed by PCR.

RNA Isolation. A total of 25 mL cultures of *L. monocytogenes* was grown in LB alone or LB supplemented with 50 mM 1,2 propanediol (Sigma; 398039) and/or 20 nm vitamin B_{12} (Sigma; C0884) to an OD_{600} of 0.3–0.5. Cultures were subsequently pelleted in a Sigma 4K15 swing bucket centrifuge at 2862 × *g* for 20 min in 50-mL Falcon tubes. Pellets were resuspended in 1 mL 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 s, speed 6.5 followed by an additional 30 s, speed setting 6.5). Subsequently tubes were centrifuged for 5 min at 8,000 × *g*, at 4 °C in a tabletop centrifuge to separate beads from lysates. The lysate was drawn off and transferred to a 2-mL Eppendorf tube. RNA isolation proceeded according to the manufacturer's instructions. Briefly, 200 μL chloroform (Carlo Erba Reagents; 438601) was added to the lysate, shaken and incubated for 10 min at room temperature, followed by centrifugation for 15 min at 13,000 × *g*, at 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 min. RNA was pelleted by centrifuging for 10 min at 13,000 × *g*, at 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. Northern blots were carried out according to the Northernmax-Gly kit protocol (Ambion; AM1946). DNA probes were generated by PCR. RNA probes were generated using the T7 Maxiscript kit (Ambion; AM1314). Probes were radioactively labeled with α-[^{32}P]ATP or α-^{32}P-UTP (Perkin-Elmer; BLU007 × 500UC), respectively. PCR templates were amplified from genomic DNA. For each sample, 10–20 μg of RNA was incubated with 1:2 volumes of 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% (wt/vol) denaturing agarose gels (Ambion; 9040) and electrophoresed in Northernmax denaturing gel buffer (Ambion; AM8676) at 60 mV for ∼2 h. RNA was transferred to Nytran membranes (Sigma; Z670286) by passive downward transfer with Northernmax transfer buffer (Ambion; AM8672). Membranes were photographed under UV light to capture ethidium bromide staining of ribosomal RNA bands as loading controls. The positions of RNA size markers (Ambion; AM7150) were marked on the membranes with a scalpel. RNA was cross-linked to membranes by exposure to UV light for 5 min and membranes were prehybridized in Ultrahyb buffer (Ambion; AM8670) for 30 min before probes were added and incubated overnight. For Northern blots against the AspocR transcript, hybridizations were carried out at 68 °C. All other hybridizations were carried out at 60 °C. The following morning, membranes were washed twice for 5 min in 2× SSC, 0.1% (vol/vol) SDS at room temperature with constant agitation, followed by two washes in 0.1× SSC, 0.1% (vol/vol) SDS at the respective hybridization temperature with constant agitation. Membranes were wrapped in cellophane and subsequently used to expose films (GE Healthcare; 28906844).

In Vitro Transcription/Translation. PCR fragments spanning upstream of the *pocR* promoter and upstream of the B_{12} riboswitch promoter were amplified from lysates of wild-type, Δribo, or ΔP+ribo strains and subcloned into the pJET1.2/blunt cloning vector (Thermo Scientific). Plasmid constructs were sequenced and digested to cut out the subcloned PCR fragments, which were then gel purified. A total of 900 ng of each purified DNA fragment were used as templates for the in vitro transcription/translation reaction using *E. coli* S30 Extract System for Linear Templates (Promega; L1030). Reaction set-ups were according to the manufacturer with the addition of 1 μM vitamin B_{12} (Sigma; C0884) and 50 mM 1,2-propandiol (Sigma; 398039). Reactions were incubated for 2 h at 37 °C. Samples were put on ice and the reactions were precipitated by adding four volumes of acetone on ice for 15 min before centrifuging 13,400 × *g*, 5 min at 4 °C. Pellets were dried in a speedvac for 10 min and thereafter resuspended in 30 μL 1× protein sample buffer. Loaded material was evaluated by Coomassie staining to ensure approximately equal amounts of material were loaded in each well.

***pocR* mRNA Stability Experiment.** Wild-type, Δribo, or ΔP+ribo strains were grown in LB supplemented with 52 mM 1,2 propanediol (Sigma; 398039) and 20 nm vitamin B_{12} (Sigma; C0884) at 30 °C until OD_{600} ∼0.4. Rifampicin was added at a concentration of 250 μg/mL and 25 mL of cultures were withdrawn at timepoints 0, 2, 4, 8, and 16 min after rifampicin addition and added to tubes containing 0.2 volumes stop solution (5% phenol in 96% ethanol). Tubes were immediately frozen in liquid nitrogen. For RNA isolation tubes were thawed on ice and centrifuged 2 min, 11,200 × *g* at 4 °C. Pellets were resuspended in 400 μL resuspension solution [10% (wt/vol) glucose, 12.5 mM Tris pH 7.6, 5 mM EDTA] and 60 μL fresh 0.5 M EDTA. Suspensions were transferred to bead beater tubes containing ∼0.4-g glass beads (0.1 mm; BioSpec Products) and bacteria were lysed in a FastPrep apparatus (45 s, speed 6). After centrifugation

(5 min, 16,000 × g, 4 °C), supernatants were added to tubes containing 1 mL Trireagent (Ambion) and incubated for 5 min, at room temperature (RT). A total of 100 μL of chloroform/isoamylalcohol (24:1) was added. Samples were vortexed and centrifuged for 5 min, 16,000 × g at 4 °C. The upper aqueous phase was subjected to two additional chloroform/isoamylalcohol extractions and thereafter precipitated with 0.7 volumes isopropanol. RNA was pelleted by centrifugation (20 min, 16,000 × g, 4 °C) and pellets were dissolved in 180 μL of water. RNA was DNase treated (Roche) for 45 min at 37 °C before addition of 200 μL of phenol/chloroform/isoamylalcohol (1:24:1, pH 6.6). Centrifuged samples (5 min, 16,000 × g, 4 °C) were chloroform/isoamylalcohol (1:24) extracted and precipitated with 2.5 volumes 99.5% ethanol and 10% 3M NaAC (pH 4.5). Pellets were resuspended in water and RNA concentration was determined using a Nanodrop (Nanodrop ND-1000 Spectrophotometer).

SDS/PAGE and Western Blot. Samples were denatured at 95 °C for 5 min and separated on a 12% (vol/vol) SDS/PAGE before being transferred to a Hybond-P membrane (Amersham) using semi-wet transfer. The membrane was blocked in 5% (wt/vol) powder milk in TBS-T for 2 h at room temperature. Primary antibody, α-PocR (dilution 1:2,000) was incubated overnight at 4 °C. The membrane was washed 6 × 5 min in TBS-T at room temperature. Secondary antibody, α-rabbit-HRP (Agrisera), was incubated for 1 h at RT (1:8,000 dilution) in TBS-T before washing the membrane as described above. The membrane was developed using ECL2 prime Western Blot Detection (Pierce) according to the manufacturer's instructions and images were analyzed using a CCD camera (LAS 4000; Fujifilm). α-PocR sera was obtained from rabbits immunized with two peptides CIQTEETDWKNDKDL and CFRKEFDVTPKGYRETFK conjugated to KLH (Storkbio) and affinity purified on an EAH Sepharose 4B column (GE Healthcare) coupled with 2 mg of each peptide.

Quantitative Real-Time PCR. For each sample, 10 μg of RNA was treated with DNaseI (Ambion; DNase-Free AM1906) for 1 h 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), 0.5 μM of gene-specific oligonucleotides, and water to 10 μL total volume. Oligonucleotides were designed using a primer design program available at http://eu.idtdna.com/Scitools/Applications/Primerquest/. Primer efficiencies were evaluated by generating a standard curve with serial dilutions and shown to be between 90% and 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 s and 60 °C 15 s. 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 disassociation 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 ΔΔCT method. All samples were evaluated in triplicate and at least three independent experiments were carried out for each gene evaluated.

***In Silico* Analysis of Antisense Riboswitches.** Riboswitch and ORF annotations were culled from the RNA families (Rfam) database (4) and National Center for Biotechnology Information Refseq databases. We carried out analysis on the following riboswitch families (Rfam name in parentheses, if different): vitamin B_{12} (cobalamin, Ado_Cbl_riboswitch), Cyclic-di-GMP (GEMM_RNA_motif), S-adenosylmethionine (SAM), thiamine pyrophosphate (TPP), fluoride (crcB), T-box, Mg^{2+} (ykoK), flavin mononucleotide (FMN), lysine, purine, Pre-queosine (PreQ1), pyrimidine (PyrR), S-adenosylhomocysteine (SAH, SAH_riboswitch), and tetrahydrofolate (THF). A Java-based algorithm was used to cross-reference the two annotations and identify instance of riboswitches oriented antisense to the adjacent ORF.

Densitometry and Statistical Analysis. For analysis of Northern blot relative band intensities, bands were quantified by densitometry using ImageJ software. Intensity values were normalized to either tmRNA or rRNA bands. Units are arbitrary. For analysis of statistically significant differences in Northern blots and qRT-PCRs, values were log2 transformed and evaluated by paired t test using Graphpad Prism software.

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3. Lauer P, Chow MYN, Loessner MJ, Portnoy DA, Calendar R (2002) Construction, characterization, and use of two Listeria monocytogenes site-specific phage integration vectors. *J Bacteriol* 184(15):4177–4186.
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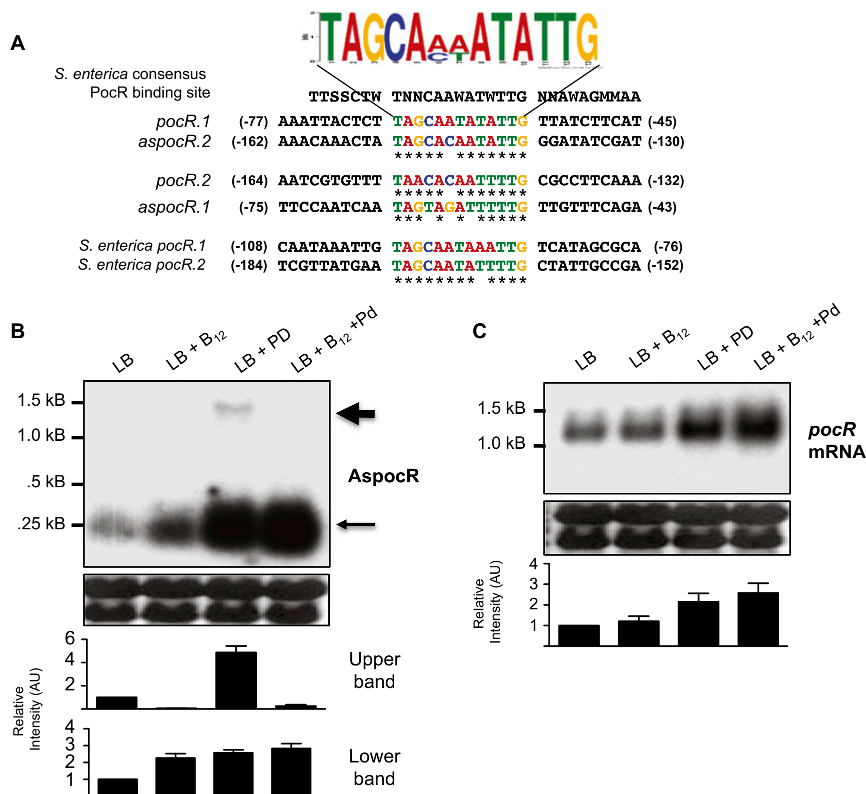

Fig. S1. Regulation of *aspocR* and *pocR* by PocR, B_{12} and propanediol. (*A*) The promoter regions of the *S. enterica pocR* gene, and the *L. monocytogenes pocR* and *aspocR* genes were compared using the MEME software package (1) to search for binding motifs. A conserved motif was identified matching the consensus PocR binding site previously determined in *Salmonella enterica* (2). Asterisks denote matches to the *S. enterica* consensus PocR-binding sequence. Numbers denote the distance from either the translation initiation site or the transcription initiation site (in parentheses). (*B* and *C*) Northern blots evaluating levels of the AspocR and PocR transcripts. RNA was isolated from bacteria cultured in LB, LB+B_{12} (20 nM), LB+Pd (50 mM), or LB+both and grown to an OD_{600} of 0.3–0.5. Blots were probed with single-stranded RNA probes complementary to the annotated B_{12} riboswitch or the *pocR* ORF, respectively. For the AspocR blot, the thin arrow denotes the truncated transcript (formerly annotated as Rli39) and the thick arrow denotes the long AspocR transcript. Quantitation of relative band intensities from three independent experiments is shown below each blot. Units are arbitrary. Ribosomal RNA is shown as a loading control. Error bars show SE.

1. Bailey TL, Elkan C (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* 2:28–36.
2. Chen P, Andersson DI, Roth JR (1994) The control region of the pdu/cob regulon in Salmonella typhimurium. *J Bacteriol* 176(17):5474–5482.

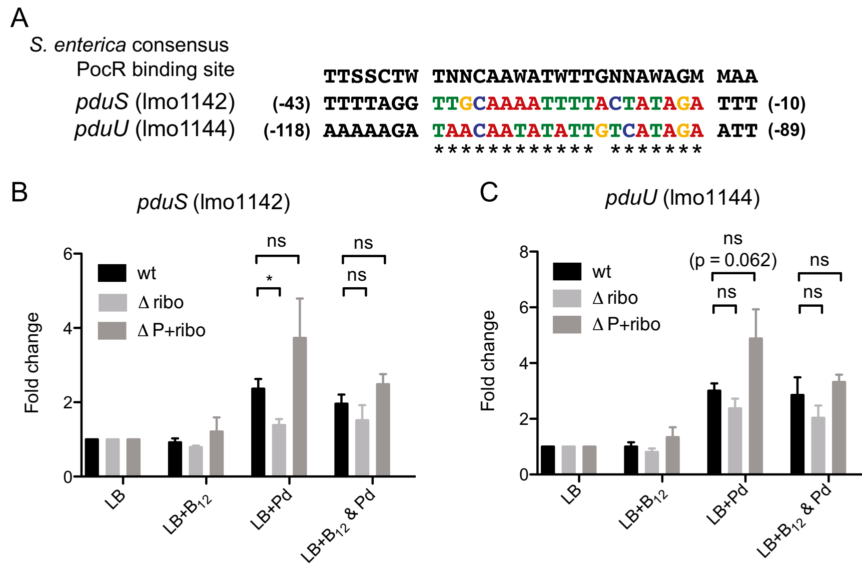

Fig. S2. Effects of AspocR expression on PocR-regulated genes. (*A*) PocR-binding sites identified upstream of the *pduS* and *pduU* genes are shown. Numbering denotes position relative to the transcription start sites of *pduS* and *pduU*, respectively. Asterisks denote matches to the *S. enterica* consensus PocR-binding sequence. (*B* and *C*) Levels of the *pduS* and *pduU* genes were evaluated by qRT-PCR in the wild-type (WT), Δribo, or ΔP+ribo strains. RNA was isolated from bacteria cultured in LB, LB+B_{12} (20 nM), LB+Pd (50 mM), or LB+both and grown to an OD_{600} of 0.3–0.5. Results represent the average of at least three biological replicates. Error bars show SE. Differences were not statistically significant (NS).

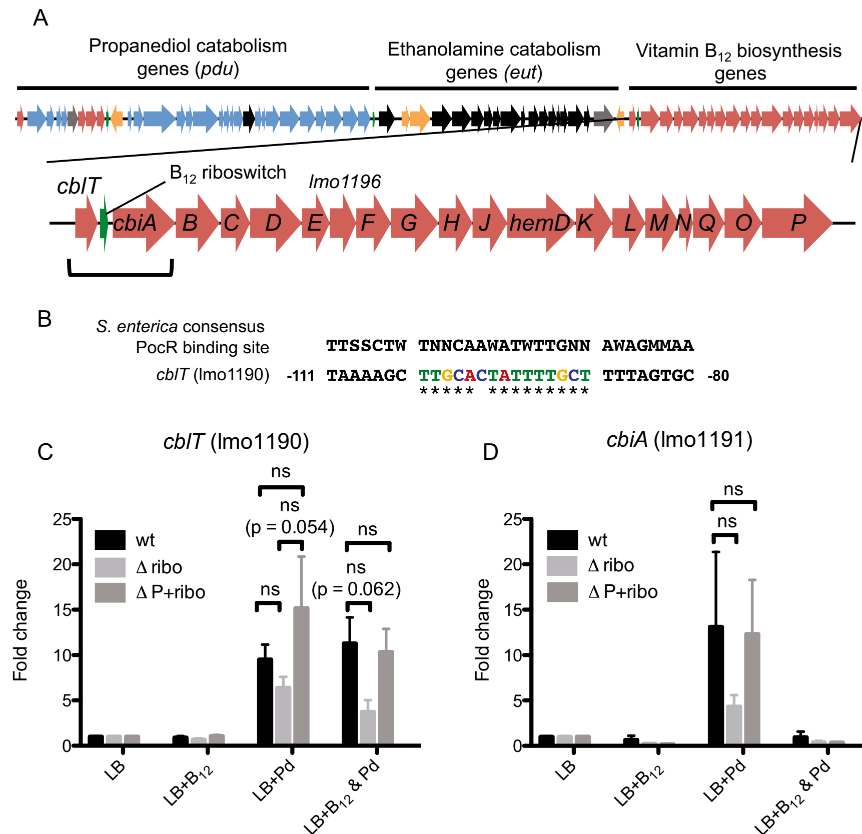

Fig. S3. Effects of AspocR expression on regulation of the vitamin B_{12} biosynthesis operon. (*A*) Schematic representation showing the genomic organization of the propanediol catabolic genes (blue), the ethanolamine catabolic genes (black), and the vitamin B_{12} biosynthesis genes (red). Putative regulatory genes are shown in orange. B_{12} binding riboswitches are denoted by green arrows and unknown genes are shown in gray. The *Inset* shows a close-up of only the B_{12} biosynthesis genes. (*B*) PocR-binding site identified upstream of the *cblT* gene. Asterisks denote matches to the *S. enterica* consensus PocR-binding sequence. (*C* and *D*). Levels of the *cblT* (*lmo1190*) and *cbiA* (*lmo1191*) genes were evaluated by qRT-PCR in the wild-type (WT), Δribo, or ΔP+ribo strains. RNA was isolated from bacteria cultured in LB, LB+B_{12} (20 nM), LB+Pd (50 mM), or LB+both and grown to an OD_{600} of 0.3–0.5. Results represent the average of three biological replicates. Error bars show SE.

Fig. S4. 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 amplified and cloned into plasmids. Plasmids were linearized and used as templates for reactions in the presence of B_{12}, or Pd+B_{12}.

Fig. S5. Effects of AspocR expression on *pocR* mRNA stability. (*A*) Northern blot showing *pocR* mRNA levels in wild-type, Δribo, or ΔP+ribo strains cultured in LB medium+B_{12} (20 nM) and Pd (52 mM) either before rifampicin treatment (time 0) or at various times after as indicated (in minutes). Blots were probed with α-^{32}P-UTP–labeled, single-stranded RNA probes complementary to the *pocR* ORF. A blot of the tmRNA is shown as a loading control. Lane numbers are indicated below lanes. (*B*) Band intensities were quantified by densitometry from three separate blots. Error bars show SE. The half-life of *pocR* mRNA in the indicated strain was determined by nonlinear regression analysis with Graphpad Prism software.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)
Dataset S4 (XLSX)