Jungle Express is a versatile repressor system for tight transcriptional control

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Tightly regulated promoters are essential for numerous biological applications, where strong inducibility, portability, and scalability are desirable. Current systems are often incompatible with large-scale fermentations due to high inducer costs and strict media requirements. Here, we describe the bottom-up engineering of ‘Jungle Express’, an expression system that enables efficient gene regulation in diverse proteobacteria. This system is guided by EilR, a multidrug-binding repressor with high affinity to its optimized operator and cationic dyes that act as powerful inducers at negligible costs. In E. coli, the engineered promoters exhibit minimal basal transcription and are inducible over four orders of magnitude by 1 µM crystal violet, reaching expression levels exceeding those of the strongest current bacterial systems. Further, we provide molecular insights into specific interactions of EilR with its operator and with two inducers. The versatility of Jungle Express opens the way for tightly controlled and efficient gene expression that is not restricted to host organism, substrate, or scale.
**ARTICLE**

**Bacterial** have evolved diverse mechanisms to sense and adapt to changes in the environment. Typically, these responses are mediated at the transcriptional level by allosteric transcription factors, DNA-binding proteins that establish specific contacts with a chemical signal in the ligand-binding domain. The interaction triggers a conformational change that transduces the signal to the DNA-binding domain; this in turn changes its affinity to a specific operator within the promoter region, which results in differential gene expression. The decoupling of regulatory modules from their native context provides a means to gain external control over transcription of target genes by the addition of their inducing effector molecules.

Inducible promoters are indispensable tools in biological research, including the study of gene function and cellular regulation, and the engineering of strains with new capabilities. Tight transcriptional control is essential, since basal expression levels can obscure the interpretation of phenotypic studies and cause detrimental off-target reactions in biotechnology projects. Inducible systems are also vital for biotechnological applications, including high-level gene expression for the production of enzymes and therapeutic proteins, and the regulation of metabolic pathways for the biosynthesis of pharmaceuticals, fragrances, nutraceuticals, chemical building blocks, and biofuels. While strong promoters are often required in such applications, gene overexpression also causes a general metabolic burden on cells, and heterologous expression is often toxic to the host organism. These stresses result in reduced growth rates and increase the risk of plasmid loss and escape mutants when using constitutive or leaky promoters, ultimately leading to poor productivities. Such detrimental effects can be minimized by using a tightly repressible induction system that completely decouples growth from production, which allows for the establishment of a fast-growing, healthy culture prior to expression of target genes during the production phase.

There is a long history of resourcing regulatory parts from nature and refactoring them as inducible gene expression systems. Major developments that employed transcriptional machineries from phages are now routinely used for high-level gene expression. The output of induction systems has been varied by combining operator sites with phage promoters and by selecting suitable operator/promoter hybrids out of randomized libraries. The increasing knowledge of transcription factors in combination with progress in DNA sequencing and synthesis has promoted the mining of genomic databases and the screening for ligands to discover novel regulatory systems for applications. Despite the sizeable knowledgebase of transcription factors, their cognate operator sites and corresponding ligands, only a few induction mechanisms, including the LacI-IPTG, the TetR-aTc, and AraC-arabinose systems, are routinely used in practice. Depending on the application, these systems often exhibit limitations, such as high basal expression, narrow host range, or specific growth requirements. Media components might interfere with conventional inducible systems, resulting in loss of regulation and poor performance. For example, the presence of galactose, arabinose, or rhamnose in plant biomass-based fermentations reduces the regulatory effect of common promoters induced by these monosaccharides. While glucose concomitantly prevents full activation due to carbon catabolite repression. Such considerations become critical when scaling up from culture tube to large bioreactors, where high efficiency and low operating costs are essential. In particular, the high costs of current inducers usually preclude their use in industrialized fermentations. Consequently, suboptimal constitutive promoters are often standard practice, at the expense of high productivities that are particularly required for generating low-cost products.

Alternative induction strategies include the use of promoters that are activated by cell density-dependent signals, by starving cells of an essential nutrient, or by dynamic pathway regulation controlled by intermediates. Although these systems do not depend on the addition of inducing compounds, the timing and level of expression are generally difficult to control, and in many instances reduce metabolic activity and require host engineering or stringent media compositions. In contrast, an ideal inducible system would exhibit minimal basal activity, display strong and uniform expression levels upon induction, operate at low costs, and not be influenced by the host metabolism, media components, and other inducible systems.

Here, we describe the development and the applicability of a bacterial broad-host expression system that is inducible and displays minimal basal transcription. We previously identified EiIR as a regulatory component of a multidrug efflux system from *Enterobacter lignolyticus*, a bacterium isolated from the soil of a Puerto Rican rainforest because of its ability to catalyze lignin components. In its native context, EiIR regulates expression of EilA, an inner membrane transporter that confers tolerance to imidazolium-based ionic liquids, reagents that enhance the microbial conversion of lignocellulosic biomass to chemicals. The ability of EiIR to respond to these reagents provides a substrate-responsive, auto-regulated tolerance system that maintains its functionality in a biofuel-producing *E. coli* strain. In this work, we generated an operator with increased affinity for EiIR by comparing conserved sequences across multiple bacterial genomes. We then combined this operator with *E. coli*-phage immediate-early promoters that are recognized by the host RNA polymerase. By using an EiIR-regulated promoter probe, we identified several cationic dyes that act as efficient low-cost inducers. These molecules bind to EiIR with high affinities, capable of releasing the repressor from its operator at nM to µM concentrations in *E. coli* and three distantly related proteobacteria. Using data from X-ray crystallography, we present insights on EiIR interaction with its operator and identify contacts with two inducers, crystal violet (CV) and malachite green (MG). Alluding to the source of EilR as a regulatory component of a multidrug efflux system from *E. lignolyticus* and the engineering of strains with new capabilities, Tight transcriptional control is essential, since basal expression levels can obscure the interpretation of phenotypic studies and cause detrimental off-target reactions in biengineering projects.

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**Results**

**Palindromic consensus operator increases affinity to EiIR.** EiIR belongs to the TetR family of transcription factors, which commonly regulate divergently transcribed adjacent genes. Using an electrophoretic mobility shift assay (EMSA) to test the affinity of purified EiIR to each of the two native DNA sequences and to the consensus operator, we identified a 24-bp consensus motif embedded in both the *eilA* and *eilA* promoter regions (Fig. 1a). By searching for motifs in regions located between *eilR* homologs and divergently aligned *eilA* homologs in gamma-proteobacteria, we identified a 24-bp consensus motif, consisting of two conserved, inverted 11-bp sequences separated by two base pairs that is represented twice in these intergenic regions. In *E. lignolyticus*, this palindromic motif is located 56–79 bp (eilO1) and 20–43 bp (eilO2) upstream of *eilA*, with *eilO2* embedded in both the *eilR* and *eilA* promoter regions (Fig. 1a).

Using an electrophoretic mobility shift assay (EMSA) to test the affinity of purified EiIR to each of the two native DNA sequences and to the consensus operator, we confirmed their function as EiIR binding sites. In particular, we observed that *eilO2* binds with high affinity to EiIR, exceeding that of the native *E. lignolyticus* operators (Fig. 1b).

**Cationic dyes release EiIR from its operator.** To identify potential EiIR ligands, we created a reporter plasmid containing
a constitutively driven eilR, and a truncated eilOc flanked by randomized promoter hexamers (−35 and −10 sites) upstream of the gene encoding the red fluorescent protein (RFP). E. coli carrying this randomized promoter library was then screened in the presence of the known EilR effector 2-ethyl-1-methylimidazolium chloride27 to isolate promoter PEilO1t, which showed the highest RFP expression level in response to this effector (Supplementary Fig. 1).

Next, the P_EilO1t-carrying reporter strain was exposed to three other known substrates of the cognate multidrug efflux pump EilA 27, all being hydrophobic ammonium cations. The long-chained cetylpyridinium chloride and the bivalent cation methyl viologen caused only minimal de-repression at sublethal concentrations (Supplementary Fig. 2). In contrast, the acridine dye proflavine induced the reporter to a higher RFP expression level at a concentration ~10⁴ fold lower than that required for maximally achievable induction by 2-ethyl-1-methylimidazolium chloride. Given the sensitive response of EilR to µM levels of proflavine, we expanded the screen to other readily available hydrophobic cationic dyes, some of which are known to interact with the multidrug-binding repressors QacR31 and RamR32.

**Fig. 1** EilR repressor-regulated promoter engineering.  

**a** Alignment of intergenic regions that separate the genes encoding efflux pumps and their cognate repressors in gamma-proteobacteria homologous to the E. lignolyticus eilAR locus. All intergenic regions contain two repressor-binding sites eilO1 and eilO2, from which the 24-bp consensus operator (eilOc) motif emerges. The asterisks indicate the base pairs conserved in all operators. Predicted promoter hexamers are shown for the repressor genes (−35r, −10r) and for the efflux pumps (−35e, −10e). Each colored box represents a nucleotide: A (green); T (magenta); G (yellow); C (blue).

**b** Electrophoretic mobility shift binding assays of purified EilR with the full-length native E. lignolyticus operators (eilO1, eilO2), the full consensus operator (eilOc), half consensus operator (½ eilOc) and random DNA. Molar ratios of the 21.6 kDa EilR monomer and duplex DNA are indicated.

**c** A library of randomized E. coli consensus promoter boxes fused with a truncated consensus operator generated the biosensor P_EilO1t into which an additional full-length eilOc was placed at the transcriptional start site to yield PEilO2t. Immediate-early coliphage-promoters PE20 (PJExD) and PH207 (PJExH1, PJExH2) from phage T5, PL (PJExL) from phage lambda, and PA1 (PJExA1, PJExA2) from phage T7, were reorganized by placing a truncated eilOc into the spacer region, partially overlapping the −35 or the −10 hexamers, followed by addition of a full-length consensus operator at the transcriptional start site. An arrow indicates the transcriptional start site. Colors of the nucleotides belonging to the eilOc-operator are highlighted and the promoter −35 and −10 hexamers are boxed.
We found that several members of the acridine, phenothiazine, phenazine, and xanthene families induced the EilR-regulated reporter in the nM to low µM range (Fig. 2). Each of the identified effectors triggered a distinct promoter response. For example, increasing the concentration of acridine orange or of pyronin Y resulted in a relatively gradual induction. In contrast, crystal violet (CV) rapidly induced transcription, indicating a strong positive cooperativity. Since CV also displayed the highest potency (EC50 of 120 nM) and efficacy of all compounds tested (Fig. 2), we chose this purple triarylmethane dye as standard inducer in further experiments.

Engineered promoters are inducible in diverse proteobacteria. The high affinity of EilR for its consensus operator, as well as for CV motivated us to develop an EilR-regulated bacterial expression system. We chose a set of immediate-early promoters from E. coli phages, namely P20 from phage T5, P1 from phage T7, and PL from phage lambda, all of which are recognized by the host RNA-polymerase. Analogously to PeilO1, we first placed the truncated eilO1 operator site into the 17-bp spacer region between and partly overlapping the −35 and −10 transcription motifs (Fig. 1c).

Insertion of a full-length second consensus operator at the transcriptional start site not only enhanced repression, but also elevated RFP levels in the induced state (Supplementary Fig. 3). The higher protein level is a likely consequence of increased transcript abundance, since the palindromic full-length operator has the potential to stabilize mRNA by forming a strong 5’-terminal stem-loop. In the absence of inducer, basal activity of these EilR-regulated promoters fell below that of the three routinely used inducible systems PBAD, P tet, and Ptrc (Fig. 3). Both in complex and defined media with glucose as carbon source, most PJEx promoters exhibited approximately 10-fold stronger repression than that of the tightly regulated P tet and the arabinose-responsive, glucose-repressible PBAD promoter. While basal RFP expression was barely detectable in the repressed state, CV induced PJExD in a population-uniform manner (Fig. 3c), resulting in more than a 104-fold dynamic range in both media tested.

We investigated whether the EilR-mediated promoter PJEx1 interferes with these three inducible systems and their cognate inducer molecules arabinose, anhydrotetracycline, and IPTG, respectively (Supplementary Fig. 4). The absence of crosstalk demonstrates that EilR-based promoters are suitable for orthogonal gene regulation.

**Fig. 2** Cationic dyes induce EilR-regulated promoters. **a** Response of P_{JExD} to the inducing dyes as measured by single-cell RFP fluorescence of E. coli containing the low-copy plasmid pTR_sJExD-rfp. **b** Values for EC50, amplitudes (ymax), and Hill-coefficients are used to characterize the response of P_{JExD} to cationic dyes. Colored circles in the chemical structures indicate N (blue), S (yellow), and O (red). Photographs show the corresponding inducing dyes in concentrated aqueous solutions. Values and error bars in **a** represent the means and standard deviation of biological triplicate measurements. Response curves (R² > 0.99) and numerical values in **b** were obtained by non-linear regression analysis in a 4-parameter variable slope model using average values of fluorescence measurements from three independently grown cultures.
To test the host range of our system, we introduced the unmodified cassette comprising eilR and the suite of P_{Ex} promoters into three non-enteric model proteobacteria: the metabolically versatile soil bacterium *Pseudomonas putida* KT2440; the N2-fixing plant symbiont *Sinorhizobium meliloti* Rm1021; and the aquatic oligotroph *Caulobacter crescentus* NA1000. In each of these hosts, EilR maintained its repressing capability and CV induced all of the examined promoters (Fig. 4). In these three bacteria, most EilR-regulated promoters displayed lower basal activities and higher expression maxima than those of the TetR-dependent phage promoter P_{LtetO-1}.

In the gamma-proteobacteria *P. putida* and *E. coli*, P_{ExD} exhibited the lowest basal activity and very high levels of expression in its induced state. Likewise, in the phylogenetically more distant *S. meliloti* and *C. crescentus*, this promoter exhibited relatively low basal expression and high activity when induced. However, in these two alpha-proteobacteria, P_{ExH1} and P_{ExH2} maintained tighter repression that resulted in larger dynamic ranges, even though they did not achieve expression maxima as high as that of P_{ExD}.

**Fig. 3** RFP expression from EilR-regulated promoters in *E. coli*. Single-cell fluorescence of *E. coli* expressing RFP from EilR-regulated and three common inducible promoters after growth in either defined media with 0.2% glucose (a) or LB media (b). P_{Ex} promoters were induced with 1µM CV, P_{tet} with 400 nM anhydrotetracycline, P_{BAD} with 13 mM arabinose and P_{Trc} with 1mM IPTG. c Flow cytometry measurements show the distribution of single-cell fluorescence of stationary phase populations expressing RFP from P_{ExD} after growth without (blue) or with increasing concentrations of CV (purple). The hatched histogram represents auto-fluorescence of cells lacking rfp. *E. coli* DH10B was used in a–c, with rfp expressed from medium copy plasmids (p15A ori). Values and error bars in a and b represent the means and standard deviation of biological triplicate measurements after subtracting background fluorescence emitted by *E. coli* lacking rfp.
levels that are toxic in the presence, and unlike in conventional conditions or induced by 1 µM CV caused normally in the absence or presence of high levels of sucrose. We observed that induction by 1 µM CV caused sacB expression at levels that are toxic in the presence, and unlike in conventional cultures expressing RFP under the control of EilR-regulated PJEx promoters, either in their repressed state, or induced by 1 µM CV. The TetR-regulated PJEx-A1 promoter, induced with 100 nM anhydrotetracycline, was included for comparison.

Under repressed conditions, the transformed E. coli bacteria due to its conditional toxicity in the presence of sucrose. We demonstrated tight repressability of PJEx promoters, we first placed the toxic sacB gene under control of PJEx-A1 and PJEx-D at medium and low copy-numbers in E. coli. This B. subtilis gene, encoding levansucrase, together with its upstream regulatory region are used as a counterselection marker in Gram-negative bacteria due to its conditional toxicity in the presence of sucrose. Under repressed conditions, the transformed E. coli grew normally in the absence or presence of high levels of sucrose. We observed that induction by 1 µM CV caused sacB expression at levels that are toxic in the presence, and unlike in conventional sacb-counterselection cassettes, also in the absence of sucrose (Fig. 5a).

Next, we showed that the PJEx system allows tight gene regulation in E. coli and S. meliloti. We placed the cell cycle regulator gene pleC under the control of PJEx-H1 in an S. meliloti mutant lacking this essential factor. PJEx-H1-mediated pleC repression caused a complete block of viability, while induction with 1 µM CV established normal growth (Fig. 5b). The ability to control the phenotype by tight gene regulation makes Jungle Express a useful instrument for physiological studies, bioengineering projects, and the expression of toxic proteins.

|                | PJEx-A1 | PJEx-A2 | PJEx-L | PJEx-D | PJEx-H1 | PJEx-H2 | PJEx1 | PJLxID | Vector |
|----------------|---------|---------|--------|--------|---------|---------|-------|--------|--------|
| **P. putida**  |         |         |        |        |         |         |       |        |        |
| Uninduced     | 42 (1)  | 79 (13) | 43 (2) | 41 (1) | 45 (1)  | 43 (1)  | 56 (3) | 67 (2) | 42 (1) |
| Induced       | 3088 (34) | 3408 (98) | 2098 (123) | 3866 (4) | 2005 (13) | 522 (19) | 4342 (350) | 1218 (31) | 92 (5) |
| **C. crescentus** |         |         |        |        |         |         |       |        |        |
| Uninduced     | 40 (7)  | 36 (8)  | 44 (5) | 38 (13) | 22 (3)  | 22 (3)  | 59 (9) | 50 (4) | 17 (3) |
| Induced       | 288 (34) | 253 (21) | 208 (19) | 326 (17) | 158 (8) | 177 (27) | 248 (37) | 233 (10) | 28 (2) |
| **S. meliloti** |         |         |        |        |         |         |       |        |        |
| Uninduced     | 46 (2)  | 84 (2)  | 64 (5) | 35 (2) | 14 (1)  | 19 (1)  | 119 (3) | 41 (2) | 13 (1) |
| Induced       | 6977 (323) | 7089 (249) | 2459 (116) | 5299 (380) | 1401 (75) | 384 (22) | 4273 (152) | 3498 (271) | 78 (3) |

**Fig. 4** PJEx-mediated transcription in non-enteric proteobacteria. a Fluorescence of P. putida KT2440, S. (Ensifer) meliloti Rm1021 and C. crescentus NA1000 cultures expressing RFP under the control of EilR-regulated PJEx promoters, either in their repressed state, or induced by 1 µM CV. The TetR-regulated PJEx-A1 promoter, induced with 100 nM anhydrotetracycline, was included for comparison. b Fluorescence of cultures expressing RFP from three PJEx promoters induced by increasing CV concentrations. Data represent the averages of plate reader measurements from two (P. putida) or three (S. meliloti, C. crescentus) independently grown stationary phase cultures, with the standard deviation indicated by error bars (curves) or in parentheses (table). Fluorescence values are in arbitrary units and cannot be compared directly across different hosts because different instruments were used for measurements.

**Fig. 5** Tight regulation enables control of toxic and essential genes in E. coli and S. meliloti. a Ten-fold serial dilutions of E. coli expressing the toxic gene sacB from PJEx-A1 and PJEx-D located either on low (l) or medium (m) copy-number plasmids, grown under repressed conditions or induced by 1 µM CV. A strain lacking sacB (c) was used as control. Induction caused sacB expression at levels that were toxic in the absence of sucrose or in the presence of high sucrose concentrations. Note that unlike in sacB counterselection plasmids, the PJEx plasmids do not include the native B. subtilis 5′ region, which contains a terminator element that is neutralized by a sucrose-inducible antiterminator. b Ten-fold serial dilutions of either S. meliloti wild type (WT) or a ΔpleC mutant with a plasmid-borne sole copy of the essential cell cycle regulator gene pleC driven by PJEx-H1. Strains were plated under repressed conditions or induced by 1 µM CV.
**Fig. 6** Protein overexpression from the EilR-mediated P_{IELD} promoter in *E. coli*. a Production of the -1.15 kDa tetrameric β-galactosidase protein LacZ expressed in *E. coli* BL21(DE3) from either the CV-inducible P_{IELD} or the IPTG-inducible P_{T7} on medium-copy plasmids (pSC101 ori). Soluble (S) and insoluble (I) fractions were separated prior to loading the SDS gel. b Production of the -25 kDa RFP protein expressed from either the CV-inducible P_{IELD} or the IPTG-inducible P_{T7} on low-copy plasmids (pSC101 ori). Apart from the promoter/regulator cassette, the plasmids were identical. Crude cell extracts for SDS-PAGE were taken 4 h after induction and growth at 37 °C in LB-medium (a), or at the point of induction at early logarithmic phase (t0) and 24 h after induction and growth at 30 °C in terrific broth (b). c Concentrations and costs of chemicals used to fully induce existing expression systems and EilR-regulated promoters in a 1000-L *E. coli* fermentation, based on extrapolation of lab-scale experiments.

Jungle Express provides high protein levels at low cost. We applied Jungle Express as tool for protein production in an *E. coli* expression strain, and compared its expression capability with that of P_{T7}, a strong promoter routinely used for high-level expression. Inducing P_{IELD} with 1 µM CV generated soluble and active β-galactosidase (Fig. 6a) at approximately three higher levels than when expressed from the common, IPTG-inducible P_{T7} (see Methods). Similarly, induction of P_{IELD} with nM concentrations of CV enabled high production of the RFP protein at low gene copy numbers (Fig. 6b, Supplementary Fig. 5), with yields exceeding those of P_{T7}. While the T7-system displayed a high level of RFP expression in uninduced cells, no visible accumulation of the target protein was observed in repressed P_{IELD} cultures.

While several of the EilR-inducing dyes, notably methylene blue and malachite green, inhibited growth of *E. coli* at high concentrations, CV, acridine orange, and pyronin had only minimal effects on growth rate at concentrations required for full induction (Supplementary Fig. 6). Minor mutagenic activities have been reported in different organisms for some of the dyes at higher concentrations; however, the mutagenic effect of the optimal inducer CV at fully inducing levels (0.5 to 1 µM CV) was negligible in the hosts tested, *E. coli* and *S. melliloti* (Supplementary Table 1). These results demonstrate that the engineered promoters achieve high-level gene expression when induced by low concentrations of CV, a low-cost compound (Fig. 6c, Supplementary Table 2) that is stable in growth medium (Supplementary Fig. 7) and has marginal perturbation to the host.

Mechanisms of operator recognition and ligand binding. To characterize the mechanism of this system at the molecular level, we carried out structural and mutagenesis experiments. Using X-ray crystallographic analysis (Supplementary Table 3), we determined the structure of the repressor in complex with the consensus operator DNA, and found that EilR binds as a homodimer (Fig. 7a). Amino acid residues 1–192 of each monomer form 9 α-helices arranged in two domains: the N-terminal DNA-binding domain consists of residues 1–52, forming three α-helices, and the C-terminal ligand-binding domain comprises residues 53–192, forming six α-helices. The C-terminal domain, like homologous domains in other members of the TetR family of transcription factors, is also responsible for dimerization. EMSA experiments confirmed that EilR functions as a single homodimer (Fig. 1b), similarly to TetR. In contrast, QacR, another repressor of the TetR family, binds to its target DNA through two homodimers. Similarly, the TetR family repressor RamR binds CV, but superpositioning the EilR and RamR structures in complex with CV indicated that the location of the CV binding site and the surrounding protein structure is significantly different (Supplementary Fig. 8).

The DNA-binding domain of EilR contains a helix-turn-helix (HTH) motif, a common structure among DNA-binding proteins. The HTH structure consists of two approximately perpendicular α-helices (α-helix2 and α-helix3) connected by a short turn. While EilR establishes non-specific DNA contacts through hydrogen bonding with the DNA sugar-phosphate backbone and a salt bridge with a phosphate group, three residues are responsible for the specific binding of EilR to its DNA operator (Fig. 7a, b): Residues Arg32 and His47 in the HTH domain establish direct contacts with two nucleotide backbone and a salt bridge with a phosphate group, three residues are responsible for the specific binding of EilR to its DNA operator (Fig. 7a, b): Residues Arg32 and His47 in the HTH domain establish direct contacts with two nucleotide bases in the *eilO* major groove. Unlike other characterized members of the TetR family, EilR also specifically interacts with a base located in the minor groove, established by residue Tyr3. To confirm the mechanism for specific EilR-eilO interaction observed by crystallographic analysis, we compared operator binding strength of wild type EilR with mutants containing Ala substitutions for amino acids Arg32, His47, and Tyr3 using EMSA binding assays (Supplementary Fig. 9). Arg32 and His47 substitutions completely impaired operator binding, while the decreased operator affinity of the Tyr3 mutant indicates that this interaction in the minor groove is important for binding specificity.

In a similar manner to the EilR-operator complex, we used crystallography to investigate the EilR structure in complex with each of two cationic triarylmethane ligands, malachite green (MG) and the most potent inducer CV (Fig. 2). Structural analysis revealed the binding of two ligand molecules per EilR dimer. This is similar to the TetR repressor in complex with tetracycline, but different from QacR, which binds only one ligand per dimer. The two cationic triarylmethane ligands...
with a propeller-like geometry\textsuperscript{16} bind within the negatively charged core of the C-terminal domain (Fig. 7c). The ligand-binding site of EilR contains several Glu and Asp residues (Fig. 7d), a distinctive feature this repressor shares with other transcription factors that recognize small cationic molecules\textsuperscript{45,47}.

To demonstrate the specificity of these negatively charged residues for ligand binding, we created Ala substitutions of EilR and examined their ability to de-repress RFP expression via PJExD in the presence of MG and CV (Supplementary Fig. 10). While the Glu90 and Asp175 mutants induced RFP to a similar extent as wild-type EilR, the absence of Asp163 caused a dramatic decrease in expression, suggesting that Asp163 is required for EilR to recognize the positive charge of these inducers.

Both CV and MG established hydrophobic interactions with the EilR by binding 14 residues from all six α-helices present in the C-terminal domain within van der Waals contact distance,
but CV established five additional van der Waals contacts via the extra dimethyl amino group (Fig. 7d, Supplementary Fig. 10), explaining the higher potency of CV compared to MG.

Comparison of EilR in its induced and DNA-bound forms showed a significant conformational change around the ligand-binding sites (Supplementary Fig. 11). A small difference in the positions of the DNA-binding domains of the EilR dimer was observed between the induced and DNA-bound states. Such a shift suggests a possible cascade event for the signal to be transferred to the DNA-binding domain, as has been previously been postulated for the TetR-repressor48.

Discussion
This work describes the development of an inducible broad-host expression system from scratch, taking an approach that can serve as a guideline for designing regulatable prokaryotic promoters. Out of the inexhaustible resource found in microbes, we made use of the multidrug efflux regulator EilR from a rainforest bacterium as the key component of this system. This repressor not only recognizes the major groove of its operator DNA like other members of the TetR-family, but it also establishes additional interactions with a base in the minor groove that result in increased DNA affinity and specificity. Phylogenetic analysis provided the second component required for tight regulation, a consensus operator with a higher affinity for EilR compared to that of the two native repressor-binding sites. Using a promoter-probe, we identified the third set of players, a range of inducing cationic dyes, of which the EilR-ligand CV acts with an EC50 at nM concentrations. As a triarylmethane cation, CV is attracted by the negatively charged core of EilR, with Asp163 specifically required for tight binding, and its hydrophobic character increases affinity to the repressor via multiple van der Waals contacts. These additional contacts enhance the conformational changes within the EilR-dimer, which explains the positive cooperativity responsible for the high Hill coefficient in the concentration-response curve of CV.

Combining the fully symmetrical operator with immediate-early E. coli-phage promoters generated Jungle Express, an orthogonal and highly reproducible bacterial expression system. Out of the vast resource for genetic parts available, we engineered a functional and highly versatile, low nutritional requirements, and high stress-tolerant E. coli strain with a metabolic and biochemical platform3,5. While Jungle Express was designed for functionality of robust and stable strains with reduced cellular burden and single chromosomal copy, therefore permitting the development for tight binding, and its hydrophobic character increases affinity to the repressor via multiple van der Waals contacts. These additional contacts enhance the conformational changes within the EilR-dimer, which explains the positive cooperativity responsible for the high Hill coefficient in the concentration-response curve of CV.

Phylogenetic analysis and design of a consensus operator. The intergenic regions of eilR and eilA homologs in gamma-proteobacteria were extracted using pre-computed gene trees available at the OrthoDB platform3,5. To improve the specificity of motif reconstruction, we filtered out intergenic regions with more than 90% of sequence similarity using Jalview31, which resulted in a set of non-redundant intergenic regions from the following bacteria: Enterobacter lignolyticus; Citrobacter koseri; Citrobacter rodentium; Salmonella enterica paratyphi; Salmonella enterica serovar Typhi; Klebsiella pneumoniae; Klebsiella oxytoca; Salmonella enterica serovar Enterobacteriaceae sp. 63B; Pantoea ananatis; LMG 20103; Acinetobacter sp. ADP1; Acinetobacter baumannii. Intergenic regions from these organisms were used to identify putative EilR binding site motifs by MEME32. The MEME algorithm was applied with default parameters, restricting the motifs types to palindromes only and searching any number of site repetition on the same strand. The motif with the lowest E-value was considered as a putative eil-operator.

Plasmid construction for E. coli assays. All plasmids are listed in Supplementary Table 4. To construct the Padox sensor strain, the eilR gene was PCR amplified from a fosmid containing E. lignolyticus genomic DNA that confers ionic liquid tolerance27. The eilR gene was then cloned after the weak constitutive promoter pEraft17 into pFAB0888 (provided by Vivek Mutalik), containing genes encoding kanamycin resistance and a monomeric red fluorescence protein (RFP) as reporter12. The resulting plasmid, pFAB-eilR was then used as template to generate the library of randomized ~10 and ~35 regions upstream of rfp. Primers were designed in a way to fit a truncated consensus eil-operator into a 17-bp spacer region between the ~35 and ~10 sites (see Supplementary Fig. 1). To create the described promoter library, pFAB-eilR was PCR amplified with the primer set: eilO-pFAB_random_for and eilO-pFAB_random_rev (Supplementary Table 5), digested with Dpn1 (Thermo-Fisher), phosphorylated (using 100 ng PCR product) with polynucleotide kinase (Thermo-Fisher) and self-ligated with 10 μg DNA ligase (Thermo-Fisher) at 16 °C for ca. 24 h. A single microtiter purification product was transformed into chemically competent E. coli DH10B containing plasmid pBbS5c-eilA enabling C5Cm-Tolerance via the IPTG-inducible eilA gene. Transformed cells were plated on 200 × 200 mm LB agar plates supplemented with kanamycin (50 μg L−1) and chloramphenicol (12.5 μg L−1) and incubated at 37 °C overnight. One hundred and thirty six colonies were transferred separately into 96-deep-well microtiter plates and grown in a 96-well microtiter plate reader in EZ-Rich media containing 0.2% glucose and 10 μM IPTG either without or with 300 mM [C2C1im]Cl. To identify variants that respond to [C2C1im]Cl, RFP fluorescence of cells was measured in a Tecan E20pro plate reader. Promoter PPadox, located on the resulting plasmid pFABeilO1t, is the variant with the highest dynamic range.

After removal of the BglII-site upstream of PPadox, the region spanning from eilR to the transcriptional start downstream of PPadox was transferred from pFAB-eilR between the AatII and the EcoRI sites of a BigBlue plasmid backbone53 (p15A ori, Kan6) containing the rfp gene and its RBS by isothermal DNA assembly, following the manufacturer’s instructions (New England Biolabs), which resulted in plasmid pTR_EilO1t.

To construct the PEx-promoter suite, phage promoters PPE1, PPE2, and PPE3, with truncated eilO operators in their 17-bp spacer regions were ordered as gBlocks (IDT), with the flanking regions containing at least 40 bp identity with ends of the PCR amplified modified version of pFAB-eilR gBlocks were cloned into the linearized vector backbone by isothermal DNA assembly. The resulting plasmids were PCR-amplified with primers that each contained half an operator at the transcriptional start (Supplementary Table 5). PCR products were self-ligated to obtain promoters with two eilO operators. PPE1 was generated by taking the same approach, using pTR_EilO1t as template plasmid. All assemblies were transferred into E. coli DH10B, and the promoter region and rfp sequence-verified.

To engineer sacB-plasmids, the sacB cassette was PCR amplified from pKW1 (sacB counterselection suicide plasmid, gift from Kelly Wetmore) to replace the rfp gene on pTR_sExA1-rfp, pTR_sExA2-rfp, pTR_sExA3-rfp, and pTR_sExD-rfp via Golden Gate cloning, while the RBS on these plasmids was maintained. The resulting plasmids pTR_sExA1-sacB, pTR_sExA2-sacB, pTR_sExA3-sacB, and pTR_sExD-sacB were transformed into E. coli DH10B and sequence-verified.
To generate lacZ plasmids, the lacZ gene was PCR-amplified from E. coli MG1655 genomic DNA to replace the rfp gene on pTR_sJExD-rfp and pBbA7k-rfp via Golden Gate cloning, while the RBS on these plasmids was maintained. Plasmids were transformed into E. coli DH10B and sequence-verified.

**E. coli fluorescence measurements.** Cells were induced for RFP expression as indicated and measured after growing at 37 °C to stationary phase, unless otherwise described. Microplate measurements were performed in a BioTek Synergy 4 reader for absorbance at 600 nm and fluorescence (575 nm excitation, 620 nm emission).

**Flow cytometry.** Single-cell fluorescence and population homogeneity were measured in stationary phase E. coli cultures expressing RFP after a 1:20 dilution in PBS buffer. An LSRII Fortessa (BD, CA, USA) instrument, equipped with a yellow-green laser (561 nm excitation) was used to detect mRFP fluorescence during dynamic range measurements shown in Fig. 3a, b. For each sample, 50,000 events were measured with the following settings: 637 V, SSC-H (side scatter): 279 V, PE-Texas Red-H: 450 V (mRFP detection). A Guava easyCyte (Millipore) flow cytometer was used for generating the histogram in Fig. 3c. For each sample, 5000 events were counted by forward and side scatter acquisition, and the cellular accumulation of RFP was measured by fluorescence intensity. Data acquisition was performed using InCyte software version 2.2 (Millipore).

**EiR-regulated promoters in other bacteria.** Maps of plasmids constructed for assays in P. putida, S. meliloti and C. crescentus are shown in Supplementary Fig. 12. The broad-host-range vector pJC543 was assembled using In-Fusion HD Cloning Kit (Clontech), by inserting BglII and SpeI digested pZS4Int111 into pZE21-MCS1 at the BglII site. Specifically, tetR was amplified using primers DVA00311 (5′-AGGAGTCTCTGCTTTCGATCAATCAGTTAAGAGCCCATCTGCTAATTAGTG-3′ and DVA00312 (5′-AGGGATCTCTGCTTTCGATCAATCAGTTAAGAGCCCATCTGCTAATTAGTG-3′) and DVA00313 (5′-GGGTGCCGGAAGGATCAAGATCTATGTCTAGATTAGATAAAAGTAAAGTGA-3′) and DVA00314 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′) and DVA00315 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′) and DVA00316 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′). The two PCR products were combined with the two fragments of pZE21-MCS1 resulting from digestion with BglII and SpeI. The assembled pJC543 plasmid, which contains the PLtetO-1 promoter and encodes its cognate regulator TetR, can be used to transform Rm1021 but not MG1655 genomic DNA to replace the complementing plasmid pJC476, resulting in strain JOE5635. The resulting PCR product was combined with the appropriate E. coli IS528 and the divergently transcribed T1 transcriptional terminator and generating pJC548. Expression of RFP from this plasmid can be induced with anhydrotetracycline.

The first series of plasmids (pCM66-pCM73) containing EiR-regulated promoters (EiR promoter: Ptau-pleC, Ptau-dbl, Ptau-oriT-traf) were constructed by inserting each promoter and the divergently transcribed tetR gene was PCR-amplified using primers DVA00309 (5′-CTCCAGTTAAGAGCCCATCTGCTAATTAGTG-3′ and DVA00310 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′) and DVA00313 (5′-AGGGATCTCTGCTTTCGATCAATCAGTTAAGAGCCCATCTGCTAATTAGTG-3′ and DVA00314 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′) and DVA00315 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′) and DVA00316 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′) and DVA00317 (5′-GGGGCGGTTTCATGACGCTTGGATGTGGATGAAGAGGACGCG-3′). The two PCR products were combined with the two fragments of pZE21-MCS1 resulting from digestion with BglII and SpeI. The assembled pJC543 plasmid, which contains the PLtetO-1 promoter and encodes its cognate regulator TetR, can be used to transform Rm1021 but not MG1655 genomic DNA to replace the complementing plasmid pJC476, resulting in strain JOE5635. The resulting PCR product was combined with the appropriate E. coli IS528 and the divergently transcribed T1 transcriptional terminator and generating pJC548. Expression of RFP from this plasmid can be induced with anhydrotetracycline.

**Spotted assays.** E. coli DH10B containing pTR_sJExD-sacB, pTR_sJExD-rfp, pTR_sJExD-sacB or the non-sacB control plasmid pBbA105 were grown overnight in LB/Kan (50 µg/mL) and diluted to an OD600nm = 1 for 10-fold serial dilutions. Three microliter were spotted on LB/Kan (50 µg/mL) supplement with 8% sucrose and/or 1 µM CV, and colonies from OD600nm = 10-2 to 10-10 dilutions were photographed after 20 h growth at 37 °C.

**S. meliloti plasmid gene under the control of Ptau-pleC.** pJC681 was introduced into strain JOE3608 [Δ[pLacCΔ-C76][pC746], S. meliloti] by triparental mating, selecting for neomycin resistance in the presence of 100 mM taurine and 1 µM CV, to replace the complementing plasmid pJC476, resulting in strain JOE5635. Stationary-phase cultures of JOE3635, JOE3608 (Δ[pC746][pPnR]) were used to induce CV and measured as in Fig. 6d. All other cultures were induced with 10 mM taurine. β-Galactosidase (LacZ) activity measurement was performed with the Pierce β-Galactosidase Assay Kit (ThermoFisher Scientific). Cells were grown in 5 mL LB + Kan (50 µg/mL) in glass culture tubes. One sample of each strain was stored at –20 °C as un-induced sample. The other cultures were induced with the indicated amount of CV or IPTG and grown at 30 °C for 24 h. Samples were then centrifuged, re-suspended in 50 µL of 1× SDS-PAGE sample buffer (SB) and heated for 6 min in the microwave. Samples (1 µL) and a Novex sharp prestained protein standard were loaded onto 8–16% gels.

**β-Galactosidase (LacZ) activity measurement.** Activity of β-galactosidase was measured in the soluble fraction of the lysates used for the SDS-PAGE analysis, following an established spectrophotometric assay using ONPG (ortho-nitrophenyl-β-D-galactopyranoside) as the substrate (for the detailed method please see the Supplementary Methods). Proteins were separated in 7.5% polyacrylamide gels and visualized by using 0.25% (w/v) of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as substrate. After a color reaction at 37 °C for 2–3 h, the gels were scanned in the Synergy 4 plate reader.
Mixtures were incubated for 15 s at room temperature, prior to stopping the reaction with 600 μL 1 M Na2CO3. Absorbance was read on a spectrophotometer at 420 nm. Nucleotide compositions were 0.189 (P2 IPTG), 0.586 (P2 IPTG), and 0.021 (sample without lyase).

**Purification of the EiIR protein.** EiIR-His6 was expressed in *E. coli* harboring a PET-derived expression plasmid, pLan-eiIR, with an IPTG-inducible T5 promoter and a TEV protease-cleavable his6-tag. Cells were grown to stationary phase overnight, and diluted 1:100 in 500 mL Terrific Broth (TB) supplemented with 2 mM MgSO4 for cultivation in 2-L, non baffled flasks. These cultures were grown at 43 °C shaking at 200 rpm until the OD600 was ~1.3, then the temperature was lowered to 20 °C. IPTG was added to 0.5 mM, and the cultures continued for 3 days. For crystalllography, selenomethionine (Se-Met) labeled protein was produced using the method described by Studier and colleagues. At this time, the cells were harvested and the pellets stored at -80 °C. Expression levels were estimated using SDS-PAGE. Protein purification was begun by thawing the paste and re-suspending it in 50 mM Tris buffer, pH 8.0, containing 600 mM NaCl, 50 mM Na-glutamate, 50 mM arginine-β-Cl, 10 mM MgCl2, and 0.5 mM diethiothreitol (high salt buffer, HSB). The re-suspended cells were lysed using the Emulifex® C homogenizer. The lysate was clarified by centrifugation at 40,000 x g for 40 min at 4 °C. The clarified lysate was loaded onto a 5 mL His-Trap column and fractionated using an AKTA FPLC. The column was washed with HSB to establish an OD280 baseline prior to applying 100 mL (20 column volume) of a gradient of 2-99% of 1 M imidazole in HSB. To remove both imidazole and the His6-tag, the fractions containing EiIR-His6 were pooled and TEV protease was added at a 1:100 molar ratio. The pooled fraction was dialyzed against 1 L of HSB overnight at 4 °C. Cleavage was monitored by SDS-PAGE analysis of an aliquot. The dialysate was passed through a 1 mL His-Trap column to capture TEV and remaining His-tagged EiIR. EiIR lacking the His6 tag was collected in the flow through.

For crystalllographic studies, purified EiIR was dialyzed against 1 L of 50 mM Tris buffer, pH 8.0, containing 150 mM NaCl, 50 mM Na-glutamate, 50 mM Arginine-β-Cl, and 0.5 mM diethiothreitol, and concentrated to 10.5 mg mL⁻¹.

**Electrophoretic mobility shift assays (EMSA).** In the assay comparing EiIR affinity to various operator versions, the purified EiIR molecules and the duplexed oligonucleotides (Supplementary Table 6) were mixed in 30 mM Tris, pH 7.7, 100 mM NaCl, 25 mM arginine, 25 mM glutamine, 5 mM MgCl2 and left at room temperature for 1.5 h before their run in a 2% agarose gel in Tris-borate-EDTA buffer. A total of 231 pmoles of protein were used for the 2:1 EiIR-DNA, and 462 pmoles of protein for the 4:1 EiIR-DNA mixtures. The samples were run on a 2% agarose gel in TBE buffer stained with SYBR safe dye (Invitrogen), and imaged with an Alpha Innotech FluorChem® instrument. In the EiIR-mutant EMSA assay, EiIR versions were mixed with the duplexed oligonucleotide 5'-AAAAAGTTGGAACAGGTCGTTTCC′-3′ (εiO operator in bold letters) in 50 mM Tris, pH 8.0, 150 mM NaCl, 50 mM arginine-β-Cl, and 0.5 mM MgCl2, and 0.5 mM diethiothreitol, then dialyzed against 1 L of reservoir solution.

**Crystalization of EiIR in complex with εiO and inducers.** The final concentration of EiIR used for crystallization trials was 10 mg mL⁻¹. Oligonucleotides were synthesized at the 1 μmol scale and purified to remove small molecule impurities by commercial vendors, such as IDT/ DNA (Coralville, Iowa). The oligonucleotides were resuspended in 20 mM Tris- HCl pH 7.5 containing 10 mM MgCl2. Oligonucleotide pairs were annealed in equimolar ratios by heating at 85 °C for 10 min and gradual冷却 to room temperature. The EiIR-εiO complexes were formed by adding the duplexed oligonucleotide AAAAAAGTTGGA-CAGGTGGCAACTTCCC′-3′ (εiO operator in bold letters) to the protein solution in a 2:1 (protein:DNA) molar ratio. The EiIR apoenzyme and EiIR-εiO complexes were screened using the sparse matrix method43 with a Phoenix Robot (Art Robbins Instruments, Sunnyvale, CA) and the following crystallization screens: Berkeley Screen (Lawrence Berkeley National Laboratory, Berkeley, CA), Crystal Screen, SaltRx, PEG/ion, Index, and PEGRx (Hampton Research, Aliso Viejo, CA). Crystals of EiIR apoenzyme were formed in 0.2 M trisodium citrate, 20% (w/v) polyethylene glycol 3350 and 10% hexanediol. EiIR apoenzyme and EiIR-εiO complexes were obtained after 3 days by the sitting-drop vapor diffusion method with the drops consisting of a mixture of 0.2 μL of protein solution and 0.2 μL of reservoir solution.

**X-ray data collection and structure determination.** The crystals of EiIR-εiO, EiIR-MG, and EiIR-CV were placed in a reservoir solution containing 20% (v/v) glycerol, then flash-frozen in liquid nitrogen. X-ray data sets were collected at the Berkeley Center for Structural Biology beamlines 8.2.2 of the Advanced Light Source at Lawrence Berkeley National Laboratory (BLNL). The diffraction data were recorded using an ADSC-Q315r detector. The data sets were processed using the program HKL-2000.44 The EiIR-εiO complex structure was determined using selenomethionine-labeled protein by the single-wavelength anomalous dispersion method45 with the phenix.autosol46 and phenix.autosol46 programs. The EiIR-MG and EiIR-CV complex structures were determined by the molecular-replacement method with the program Phaser45 taking the EiIR structure from the EiIR-εiO complex as the search model. Structure refinement was performed by phenix.refine program48. Manual rebuilding using COOT18 and the addition of water molecules allowed for construction of the final model. Five percent of the data were randomly selected for cross validation. The final models of the EiIR-εiO, EiIR-MG, and EiIR-CV complexes showed an R factor of 19.4%/Rfree of 22.8%, R factor of 19.6%/Rfree of 24.0% and R factor of 20.4%/Rfree of 27.1%, respectively.

Data availability All plasmids listed in Supplementary Table 4 have been deposited in the Joint BioEnergy Institute Public Registry (https://publicregistry.jbei.org/folders/378) and are available for searching and reviewing the sequences and annotations. Sequences for the Enterobacter lignolyticus εiO gene, εiO promoter, and εiIR protein have the GeneBank accession number MH688001. The atomic coordinates and structural factors of EiIR-εiO, EiIR-MG, and EiIR-CV complexes have been deposited in the Protein Data Bank: 5V19, 5V1G, and 5VL1, respectively.

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Author contributions
T.L.R. conceived and designed the experiments for promoter development and inducer identification. V.K.M. and T.L.R. conceived the sensor plasmid. T.L.R. performed promoter experiments in *E. coli* except for protein overexpression. J.H.P. crystallized EidR and elucidated its structure. J.C.C. performed experiments in non-enteric hosts. P.N. performed phylogenetic analysis. A.D. performed EMSA experiments and protein overexpression, and A.D. and G.P.T. purified EidR for crystallization. B.A.S., P.D.A., N.J.H., S.W.S., and J.C. C. contributed reagents and research facility infrastructure, and M.P.T. coordinated research activities leading to this publication. T.L.R., M.P.T., J.C.C., J.H.P., and V.K.M. wrote the manuscript. P.D.A. and N.J.H. reviewed the manuscript, and M.P.T. prepared the manuscript for publication. All authors read and approved the final manuscript.

Additional information
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Competing interests: T.L.R. at LBNL has applied for a patent (application US20170002363) for the development and applications of the Jungle Express system. The remaining authors declare no competing interests.

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