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A novel pair of inducible expression vectors for use in *Methylobacterium extorquens*

Lon M Chubiz¹, Jessica Purswani¹,², Sean Michael Carroll¹ and Christopher J Marx¹,³*

**Abstract**

**Background:** Due to the ever increasing use of diverse microbial taxa in basic research and industrial settings, there is a growing need for genetic tools to alter the physiology of these organisms. In particular, there is a dearth of inducible expression systems available for bacteria outside commonly used γ-proteobacteria, such as *Escherichia coli* or *Pseudomonas* species. To this end, we have sought to develop a pair of inducible expression vectors for use in the α-proteobacterium *Methylobacterium extorquens*, a model methylotroph.

**Findings:** We found that the Pᵣ promoter from rhizobial phage 16-3 was active in *M. extorquens* and engineered the promoter to be inducible by either p-isopropyl benzoate (cumate) or anhydrotetracycline. These hybrid promoters, Pᵣ/cmtO and Pᵣ/tetO, were found to have high levels of expression in *M. extorquens* with a regulatory range of 10-fold and 30-fold, respectively. Compared to an existing cumate-inducible (10-fold range), high-level expression system for *M. extorquens*, Pᵣ/cmtO and Pᵣ/tetO have 33% of the maximal activity but were able to repress gene expression 3 and 8-fold greater, respectively. Both promoters were observed to exhibit homogeneous, titratable activation dynamics rather than on-off, switch-like behavior. The utility of these promoters was further demonstrated by complementing loss of function of *ftfL* - essential for growth on methanol - where we show Pᵣ/tetO is capable of not only fully complementing function but also producing a conditional null phenotype. These promoters have been incorporated into a broad-host-range backbone allowing for potential use in a variety of bacterial hosts.

**Conclusions:** We have developed two novel expression systems for use in *M. extorquens*. The expression range of these vectors should allow for increased ability to explore cellular physiology in *M. extorquens*. Further, the Pᵣ/tetO promoter is capable of producing conditional null phenotypes, previously unattainable in *M. extorquens*. As both expression systems rely on the use of membrane permeable inducers, we suspect these expression vectors will be useful for ectopic gene expression in numerous proteobacteria.

**Background**

As the amount of bacterial genome sequencing information continues to grow, the need for broad-host-range, extensible genetic tools will become increasingly ubiquitous. In particular, the capacity for heterologous gene expression in diverse microbial taxa will be of paramount importance for numerous research goals, as well as industrial and synthetic biological applications. To this end, we explored the use of two well-characterized transcriptional repressors (TetR and CymR) in conjunction with a phage-derived promoter (Pᵣ from phage 16-3) to produce a novel of set inducible expression vectors for use in the facultative methylotroph *Methylobacterium extorquens*.

Methylotrophic bacteria are a ubiquitous group of microorganisms defined by their capacity to utilize reduced single-carbon (C₁) compounds as a sole source of energy and biomass. The facultatively methylotrophic, α-proteobacterium *Methylobacterium extorquens* has been a model organism in the study of C₁ metabolism for over 50 years. In the last decade, due in part to the development of a repertoire of genetic tools [1-4] *Methylobacterium* species have become increasingly useful in the study of horizontally transferred metabolic pathways [5-7] and microbial evolution [8-10]. Furthermore, in the past few years genome sequences have become available...
for eight representatives within *Methylobacterium* [11,12]. While considerable progress has been made for genetic manipulation of *M. extorquens*, an area that remains underrepresented by comparison is the development of regulated expression systems.

To date, only one regulated expression system has been demonstrated to be functional in *M. extorquens*. Choi and coworkers constructed an inducible expression system utilizing the cumate responsive transcriptional repressor, CymR, from *Pseudomonas putida* F1 and the strong P~maaF~ promoter that drives the expression of methanol dehydrogenase in *M. extorquens* [13]. This hybrid system has been modified and utilized to test the fitness consequences of gene expression levels of different formaldehyde oxidizing enzymes in *Methylobacterium* [14,15]. While functional, this promoter-operator pairs are extremely “leaky”, wherein the basal level of expression in non-inducing conditions is quite high [14]. This limitation makes heterologous gene expression exceedingly difficult, and hampers the exploration of conditionally null phenotypes.

Building on these previous findings, we have employed an additional transcriptional repressor, TetR, from the transposon Tn10. As the foundational member of the TetR-family of DNA binding proteins [16], to whom CymR is also a member, TetR has been extensively studied yielding much data on ligand binding, DNA binding kinetics, and operator site specificity [17]. In the absence of inducer, TetR and CymR bind tightly to their respective operator sites (see Figure 1), thereby inhibiting transcriptional initiation by RNA polymerase. Upon binding of ligands such as tetracycline or anhydrotetracycline (a high-affinity ligand) in the case of TetR, or cumate (*p*-isopropyl benzoate) with CymR, the affinity of TetR and CymR for their respective operator sites is nearly abolished, allowing for transcription initiation to proceed. Exploiting these characteristics, numerous studies have modified existing expression systems to behave in a dose-dependent manner. In fact, TetR and related transcriptional repressors have found use in numerous synthetic biology applications in bacteria, archaea, and eukaryotes [13,18-23].

Here we describe the construction of two IncP-based, inducible expression vectors for use in *M. extorquens*, and possibly numerous other proteobacteria with minor modification. The novelty of these vectors lies in their use of two separate transcriptional repressors, TetR and CymR, along with a strong promoter from the rhizobial phage 16-3. We demonstrate the utility of these vectors by showing that i) induction is dose-dependent, ii) induction is continuous through time, and iii) the regulatory range of both systems exceeds those currently available for *M. extorquens*. Collectively, these results supply researchers investigating *M. extorquens*, and likely numerous other proteobacteria, with two alternative systems to express genes in traditional and synthetic biology applications.

**Findings**

**Promoter design and rationale**

During the process of selecting an appropriate promoter, we desired that the promoter i) be sufficiently active in *M. extorquens* and ii) not be subject to regulation by native transcription factors. Based on these two criteria, a natural source for such a promoter was from bacteriophage. Many bacteriophage promoters have a wide host range and often have strong, constitutive activity in the absence of their transcriptional control mechanisms. However, numerous well characterized coliphage-derived promoters such as λ*P*~L~, λ*P*~R~, T5*P*~N25~, T7*P*~A1~ are weakly active or inactive in *M. extorquens* [13]. To this end, we looked to other bacteriophage promoters that have been shown to be active in α-proteobacteria. Based on this metric, we explored the use of promoters from the control region of the rhizobial phage 16-3 (*P*~L~ and *P*~R~). Phage 16-3 has been extensively examined with physiological and biochemical studies in both its host, the α-proteobacterium *Sinorhizobium melloti*, and *Escherichia coli* [24,25], suggesting that *P*~L~ and *P*~R~ may be functional in a variety of hosts. Additionally, the only transcriptional regulator known to interact with *P*~L~ and *P*~R~ is the 16-3 C repressor [25].

In a set of exploratory experiments, we found that *P*~R~ was active in *M. extorquens* (data not shown). As we desired to construct inducible systems, we focused attention to engineering *P*~R~ derivatives containing operator sites for the CymR and TetR regulators (Figure 1). The resulting hybrid promoters, *P*~R~/*cmtO~ and *P*~R~/*tetO~, were found to produce the widest regulatory range without interfering with *P*~R~ promoter activity. Interestingly, we found that placing the operators, specifically *tetO*, throughout other regions of the promoter resulted in either loss of promoter repression or activity (data not shown). This was a somewhat surprising result given the flexibility of many other phage-derived systems to be manipulated with multiple repressor and activator operator sites [18,26]. Collectively, these findings allowed us to engineer two inducible promoters with similar maximal activity (Figure 2).

**Activation of *P*~R~/*cmtO~ and *P*~R~/*tetO~ is dose-dependent**

A desirable property for regulated expression systems is for levels of gene expression from the promoter to be proportional to the concentration of inducer. In order to explore the range of induction of *P*~R~/*cmtO~ and *P*~R~/*tetO~, the promoters along with their respective regulatory proteins were introduced onto broad-host-range plasmids (*IncP* compatibility group) to create the expression vectors pLC290 and pLC291 (Figure 1). Since previous studies
have demonstrated mCherry to be a sensitive measure of gene expression in *M. extorquens* [14], we decided to use mCherry fluorescence as a metric of promoter activity. We placed the red-fluorescent protein variant mCherry under the control of each promoter in pLC290 and pLC291 and introduced the resulting vectors (pJP18T and pJP22T) into *M. extorquens*. To induce expression from P_{R/cmtO} and P_{R/tetO}, we supplied varied concentrations of cumate (Q) and anhydrotetracycline (aTc), respectively, to *M. extorquens* cultures.

In general, both promoters were found to be responsive to concentrations of Q and aTc that were in agreement with previous studies in *M. extorquens* or other organisms [13,18,27]. The P_{R/cmtO} promoter was observed to respond to a range of 0.1 to 5 μg/ml (0.6 to 30 μM) of Q and the P_{R/tetO} promoter from 0.1 to 25 ng/ml (0.2 nM to 50 nM) aTc. Interestingly, the induction profile of P_{R/cmtO} increased in a log-linear fashion over the entire concentration range, whereas P_{R/tetO} was observed to have a much more concave profile. In terms of regulatory range, P_{R/cmtO} and P_{R/tetO} were observed to have 10-fold and 30-fold induction, respectively, with both promoters having the same maximum absolute levels of expression (Figure 2). Importantly, the basal level of expression from P_{R/cmtO} was found to be approximately 3-fold higher than that of P_{R/tetO}. Taken together, these data suggest that while P_{R/cmtO} may be more tunable, P_{R/tetO} serves as a superior expression system for
genes requiring tight repression, such as cytotoxic proteins. Also, we found that there was minimal cross-talk between the CymR and TetR ligand specificity or promoter binding indicating these systems would work independent of one another (pJP18T: 4.6 Uninduced/4.2 with aTc; pJP22T: 1.0 Uninduced/1.1 with Q; Grown in succinate).

Comparing the levels of gene expression and regulatory range of $P_{R/cmtO}$ and $P_{R/tetO}$ to the cumate inducible $P_{mxaF}$ promoter previously reported [13,14], we found that in M. extorquens these promoters achieve 33% of the maximal activity of $P_{mxaF}$ (the strongest known Methylobacterium promoter) and provide a greater degree of repression. Specifically, a cumate-inducible $P_{mxaF}$ mCherry expression vector, pHC115m, yielded relative fluorescence values of 15.6 ± 1.5 (uninduced) to 157.1 ± 3.7 (induced). While this 10-fold regulatory range was similar to $P_{R/cmtO}$, the minimal and maximal expression from $P_{R/cmtO}$ were both 3-fold lower. By comparison, $P_{R/tetO}$ with a 30-fold regulatory range, was able to repress expression 8-fold lower than the $P_{mxaF}$ system with only a 3-fold difference in maximum expression. Collectively, these results demonstrate that both $P_{R/cmtO}$ and $P_{R/tetO}$ provide improvement over previously explored systems. However, we do note that $P_{mxaF}$ may remain a superior promoter in cases when high-level protein over-expression is desired.

Importantly, these hybrid promoters allow for more relevant exploration of cellular physiology as their expression levels and ranges fall well within or above native promoters in M. extorquens.

**Maximal activation of $P_{R/cmtO}$ and $P_{R/tetO}$ is substrate dependent**

An issue with many expression systems designed with host-derived promoters is the possibility of interactions with native transcription factors. Specifically, the $P_{mxaF}$ promoter is known to be more highly active in cells grown on methanol as opposed to succinate [1,28]. To explore this possibility, with respect to $P_{R/cmtO}$ and $P_{R/tetO}$, we cultured M. extorquens harboring pJP18T and pJP22T in media with either methanol or succinate as the sole carbon source (Table 1). We found that succinate grown cells possessed a nearly 2-fold increase in maximal gene expression, compared to methanol grown cells; effectively, the opposite behavior seen with $P_{mxaF}$.

We suspect that this disparity in maximal expression may be due to an external factor, such as different plasmid copy numbers, between methanol and succinate growth. Previously reported XylE and β-galactosidase promoter probe vectors used in M. extorquens, such as pCM130 and pCM132 (plasmids with the same backbone as pLC290 and pLC291), exhibit between 2 and

| Table 1 Growth substrate dependence on $P_{R/cmtO}$ and $P_{R/tetO}$ activation |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Plasmid | Promoter | Methanol | Succinate |
| | | Uninduced | Induced | Uninduced | Induced |
| pJP18T | $P_{R/cmtO}$ | 5.32 ± 0.64 | 28.94 ± 2.14 | 5.79 ± 0.44 | 61.93 ± 2.42 |
| pJP22T | $P_{R/tetO}$ | 1.46 ± 0.27 | 33.13 ± 2.63 | 1.95 ± 0.62 | 54.67 ± 5.60 |

Gene expression as measured by mCherry fluorescence from M. extorquens cells harboring pJP18T or pJP22T. Cells were grown in succinate or methanol medium in the presence or absence of Q (5 μg/ml) or aTc (25 ng/ml). Values are relative fluorescence (arbitrary units) and reported error is the 95% confidence interval (N = 4).
3-fold increases in background activity during succinate versus methanol growth [1]. As pCM130 and pCM132 possess no promoter sequences upstream of their reporter genes, the only likely variation that might exist is in plasmid copy number. Comparing these findings to our own, where \( P_{R/cmtO} \) and \( P_{R/tetO} \) contain no host-related transcription factor binding sites, we see similar fold changes in maximal expression suggesting that a similar mechanism may be affecting these expression systems. Taken together, these data indicate that single-copy or chromosomally integrated systems be used in situations where uniform expression is desired across substrates.

**Induction of \( P_{R/cmtO} \) and \( P_{R/tetO} \) is continuous**

A problematic feature of many expression systems, particularly those associated with metabolic pathways, is that gene expression can exhibit phenotypic heterogeneity throughout the population of cells, such as an on-off, switch-like behavior [29-31]. To explore this possibility, we grew *M. extorquens* strains bearing the mCherry expression vectors pJP18T and pJP22T to mid-log phase, induced cultures with either Q or aTc, and measured the time course of individual-cell fluorescence by flow cytometry. We found that over 8 hours of induction the induced populations activated transcription in a uniform, continuous manner (Figure 3). Though we did observe residual uninduced cells, we suspect this may be due to debris introduced by our cell fixing method or possibly cells losing mCherry due to costly over-expression. These data demonstrate the utility of the \( P_{R/cmtO} \) and \( P_{R/tetO} \) expression systems in studying aspects of cellular physiology requiring uniform gene expression.

![Figure 3 Single-cell dynamics of \( P_{R/cmtO} \) and \( P_{R/tetO} \) activation](image-url)

Histograms of relative fluorescence values for pJP18T (A) and pJP22T (B) harboring *M. extorquens* PA1 as determined by single-cell flow cytometry. Cultures were grown to mid-log phase and induced with 5 \( \mu \)g/ml Q (A) or 25 ng/ml aTc (B). At times 0, 2, 4, 6, 8, and 24 hrs, cells were harvested and fixed in carbon-free Hypho medium supplemented with 100 mg/ml streptomycin. The 8 and 24 hr time points have nearly overlapping fluorescence distributions. Fluorescence units are presented as arbitrary units (A.U.) and normalized as described in Materials and Methods.

**Complementation and conditional null phenotypes using \( P_{R/tetO} \) constructs**

To examine the utility of these vectors for studying *M. extorquens* physiology, we complemented a gene encoding a key enzyme in methanol metabolism using the \( P_{R/tetO} \)-based plasmid pLC291. We chose to use utilize \( P_{R/tetO} \) due to the tight induction properties we have observed using an mCherry reporter (Figure 2 and Table 1). The product of \( ftfL \) (formate-tetrahydrofolate ligase) is required for the assimilation of formate into biomass during one-carbon metabolism [32]. A disruption in \( ftfL \) results in a methanol minus growth phenotype. By complementing a \( ftfL \) knockouts using \( ftfL \)-expressing vectors under the control of \( P_{R/tetO} \), in the presence of aTc, we found that we could fully restore growth on methanol (Figure 4). Importantly, in the absence of aTc, we observed that we were able to produce a complete null phenotype for \( ftfL \) (Figure 4). These results demonstrate the utility of \( P_{R/tetO} \) to study *M. extorquens* physiology and generate conditional null mutants regulated by aTc.

**Conclusions**

To date, only a handful of expression systems exist for bacterial models outside *E. coli* and other closely related \( \gamma \)-proteobacteria. In an effort to expand the genetic toolkit available to researchers working with *M. extorquens*, and presumably other proteobacteria, we have constructed a set of two inducible expression vectors that utilize the CymR and TetR (cumate and tetracycline repressors) in conjunction with the strong \( P_{R} \) promoter from phage 16-3. The pLC290 and pLC291 vectors were found to provide uniform, high-level expression in *M.
E. coli strains were cultured in Luria-Bertani broth as [10], with succinate at 5 mM or methanol at 20 mM. Minimal medium as described by Chou and coworkers supplemented with kanamycin at 50 μg/ml or ampicillin described by Miller [35] or nutrient broth. Media was used for all bacterial strains, except Methylobacterium extorquens PA1 strain CM2730 (ΔcelABCD) [34] or nutrient broth. All bacterial strains used in this work are derivatives of Escherichia coli NEB10β (New England Biolabs), E. coli LC100 (F− rpsL-1 ilvG attλ::[spcR lacO1 tetR]) [33], Methyllobacterium extorquens PA1 strain CM2730 (ΔcelABCD) [34] or M. extorquens AM1. Growth of all strains, except E. coli, was performed in modified ‘Hypho’ minimal medium as described by Chou and coworkers [10], with succinate at 5 mM or methanol at 20 mM. E. coli strains were cultured in Luria-Bertani broth as described by Miller [35] or nutrient broth. Media was supplemented with kanamycin at 50 μg/ml or ampicillin at 100 μg/ml to select for the presence of all plasmids. Inducers anhydrotetracycline (aTc) and cumate-KOH (Q) were supplied at 25 ng/ml or 5 μg/ml from aqueous stocks, respectively, unless otherwise indicated. Growth and gene expression experiments were performed at 30°C using an automated growth system described by Delaney and coworkers [34,36].

Plasmid and strain construction
Promoter designs were initially constructed and subsequently mutated in a pBluescript(SK-) (Stratagene) backbone. Synthetic oligonucleotides CAACAACCTTATACC ATGGCTCTACAAAAAGGCGAACATGGTACCATGACGACTCATACAA and GTCCGTTCGTAAATCTCATTICTCT CAACATCATATTGTTGTAACGCCAGGGTTTTCCC containing the sequence for a 91 nt region encoding the P_{R/\text{tetO}} promoter from the rhizobial phase 16-3. The oligonucleotides were annealed to form a 91 bp dsDNA fragment, followed by PCR amplification with primers ATAGGGCCCAACAATCCATACATTCCACATTCAACG and ATAGGTACCGTCCGTGTTACACTCTATA CAC to introduce PspOMI and KpnI restriction sites. The resulting fragment was digested with PspOMI and KpnI and cloned into the respective sites in pBluescript(SK-) to form pLC265. TetR and CymR operator sites (tetO and cmtO), were introduced at the distal end of P_{R/\text{tetO}} in pLC265 using enzymatic inverse PCR (EI-PCR) [37] using primers ATACGTCCTCTCCTCCATCAGTAGAGAGTTTGTAGATTGTAAACGAGGAC, ATAC GTCTCAGGGAGCTCAGATGACCATTGTTTGCC, ATACGTCCTCAACAAAAAGGAGAAAATCTGCTGTGTTTTG GTGACCCAATGGCCCATTAAG, and ATACGTCCTCA TTGGTTTACAACTCTACACTCAATTGTGTGTG followed by BsmBI digestion and ligation to generate plasmids pLC271 (P_{R/\text{tetO}} containing) and pLC277 (P_{R/cmtO} containing).

The subsequent broad-host-range vectors were constructed using the expression vector pHC115 [14] as a template. A DNA region encoding Tn10 tetR was PCR amplified from LC100 using primers ATAGCTGAGGGAGAGGGGCGATGATGTTGATTGAC ATACGTCCTCAACAAAAAGGAGAAAATCTGCTGTGTTTTG GTGACCCAATGGCCCATTAAG, and ATACGTCCTCA TTGGTTTACAACTCTACACTCAATTGTGTGTG followed by BsmBI digestion and ligation to generate plasmids pLC271 (P_{R/\text{tetO}} containing) and pLC277 (P_{R/cmtO} containing).
The Kpn subcloning a pCM157 [3] into forming the Cre-recombinase expression plasmid curves, M. extorquens were performed as follows. For dose-dependent response assays to measure levels of fluorescence-based expression assays. Assays were conducted using pJP18T and pJP22T were created by subcloning a KpnI and EcoRI digestion product containing mCherry from pHCI15m [14] into the corresponding sites in pLC290 and pLC291, respectively. The vectors pLC290 (GenBank Accession KC296704) and pLC291 (GenBank Accession KC296705) are publicly available from the non-profit organization AddGene (http://www.addgene.org/Christopher.Marx).

Unmarked ftfL knockouts were generated by transforming the Crecombinase expression plasmid pCM157 [3] into M. extorquens AM1 derivatives CM216K.1 [39] generating strain CM2336 (ΔftfL::loxP). The ftfL operon was then deleted by subcloning a KpnI and EcoRI digestion product of a pHCI15-based ftfL plasmid (SMC unpublished) into the corresponding sites of pLC291, creating plasmids pSCP5. The vector, pSCP5, was introduced into CM2336 via triparental mating using the helper plasmid pRK2073 [40,41], to produce strains CM4103 (ΔftfL::loxP/pSCP5). Complementation was performed by inoculation of succinate grown CM4103 into methanol minimal medium containing 0 μg/ml or 20 μg/ml aTc.

Fluorescence-based expression assays

Assays to measure levels of mCherry protein expression were performed as follows. For dose-dependent response curves, M. extorquens strains harboring pJP18T or pJP22T were grown to saturation in 10 ml of Hypho-succinate medium. These cultures were then diluted 1:200 in fresh medium, followed by 630 μl aliquots being dispensed to clear, flat-bottom, 48-well microtiter plates (Costar). Cultures were grown for 4 hrs on a plate shaking tower (Caliper) at 150 rpm in a 30°C humidified room. After 4 hrs of growth, 10 μl of fresh medium containing Q or aTc was added to supply Q and aTc at desired concentrations. Cultures were allowed to continue growth for an additional 24 hrs prior to fluorescence (excitation 587 nm/emission 610 nm) and optical density (600 nm) measurements made using a Tecan Safire2 plate reader. Relative fluorescence values reported are:

Relative fluorescence (A.U.) = \frac{RFU}{OD_{600}} \times 10^{-3}

Dynamic expression assays were conducted under similar conditions as above with the following exceptions. Cells (200 μl of culture) were harvested after induction at 0, 2, 4, 6, 8, and 24 hrs. Culture samples were pelleted by centrifugation (6,000 x g) and resuspended in an equal volume of cold Hypho medium without succinate and supplemented with 100 mg/ml streptomycin to inhibit mCherry translation. Fixed cells were kept on ice prior to fluorescence measurements made using a BD LSR II Flow Cytometer. Flow cytometry data were then analyzed using the BioConductor flowCore package in R [42]. Reported fluorescence values for flow cytometry are raw values from the BD LSR II and were not correlated to those of the Tecan Safire2.

Competing interests

The authors (LMC, JP, SMC and CJM) declare no competing interests with respect to the findings in this article.

Authors’ contributions

LMC and CJM were responsible for the conceptions and design of the study. LMC, JP, and SMC conducted all vector and culture growth and fluorescence measurement experiments. LMC, SMC, and CJM drafted the manuscript. All authors read and approved the final manuscript.

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