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Bestowing Inducibility on the Cloned Methanol Dehydrogenase Promoter (P_{mxaF}) of *Methylobacterium extorquens* by Applying Regulatory Elements of *Pseudomonas putida* F1▽

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P_{mxaF} is a strong methanol-inducible promoter in *Methylobacterium extorquens*. When this promoter is cloned in expression vectors and used to drive heterologous gene expression, methanol inducibility is either greatly reduced or entirely lost. In order to bestow inducibility upon the cloned P_{mxaF} promoter in expression vectors, we adopted combinational methods (regulatory elements of the *Pseudomonas putida* F1 *cym* and *cmt* operons and Tn*7* transposon system) to control reporter gene expression at the transcriptional level in *M. extorquens*. An operator fragment (26 nucleotides) of the *cmt* operon was inserted downstream of the cloned P_{mxaF} promoter in the broad-host-range expression vector (pCHOI3). The repressor gene (*cymR*) located upstream of the *cym* operon in *P. putida* F1 was amplified by PCR. To avoid cellular toxicity for *M. extorquens* caused by the overexpression of CymR, single and/or double copies of *cymR* were integrated into the chromosome of *M. extorquens* using the mini-Tn*7* transposon system. Cultures containing the chromosomally integrated *cymR* gene were subsequently transformed with pCHOI3 containing modified P_{mxaF} (i.e., P_{mxaF} plus operator). In this construct, inducibility is afforded by cumate (*p*-isopropylbenzoate). In this report, we describe the inducible and tightly regulated expression of heterologous genes (*bgl* [for β-galactosidase], *est* [for esterase], and *gfp* [for green fluorescent protein]) in *M. extorquens*. This is the first documented example of an inducible/regulated heterologous gene expression system in *M. extorquens*.

Methylotrophic bacteria are a diverse group of microorganisms with the ability to utilize single-carbon (C_1) substrates more reduced than carbon dioxide as their sole source of carbon and energy. Among the methylotrophs, members of the genus *Methylobacterium* have been described as being ubiquitous, participating in a myriad of favorable interactions with nature (24, 26, 27). Furthermore, *Methylobacterium* spp. naturally produce several substances of commercial importance, including poly-β-hydroxybutyrate (3, 4), vitamin B_{12} (28), pyrroloquinoline quinone (1, 10), and carotenoids (29). Over the past decade, *Methylobacterium extorquens* AM1 has been extensively studied and characterized both genetically and physiologically (6, 18, 20). The wealth and depth of understanding of *M. extorquens* and closely related strains suggest the potential of *M. extorquens* as a source of industrially pertinent natural products and recombinant proteins. The salient aspects for this potential have been described elsewhere (2, 8, 9), and they include (i) simple and inexpensive cultivation requirements, (ii) optimized high-cell-density fermentation protocols, (iii) available genome sequence for *M. extorquens* AM1, and (iv) availability of suitable genetic tools for *M. extorquens* (8, 13, 19, 21, 22). Application of these tools has made it possible to overexpress a variety of recombinant proteins in the range of 3 to 6 g/liter under high-cell-density growth conditions (8, 9, 13). However, inducible/regulated expression of recombinant genes in *M. extorquens* or in any other methylotroph, to our knowledge, has not yet been attained. *M. extorquens* possesses native methanol-inducible promoters, notably promoters which are located upstream of genes that encode methanol dehydrogenase and other proteins required for its activity and enzymes required for the synthesis of the methanol dehydrogenase prosthetic group, pyrroloquinoline quinone. Of these, the promoter P_{mxaF} has been thoroughly scrutinized both biochemically and in expression studies (8, 9, 19, 21, 30). In its native form in the chromosome, this strong promoter is methanol inducible. However, when this promoter is cloned in expression vectors, it acts essentially in a constitutive mode. The mechanism by which P_{mxaF} is regulated in the chromosome is still not fully known. Therefore, the reason the recombinant P_{mxaF} reverts from inducible to constitutive remains speculative. Expression of the green fluorescent protein (GFP)-encoding gene (*gfp*) under the control of cloned P_{mxaF} in the plasmid pCM110 was observed even after the culture was grown repeatedly on succinate as the sole source of carbon (unpublished results). This fact makes P_{mxaF} unsuitable in applications where regulated expression is paramount, such as in expression of recombinant proteins potentially toxic to the host or in metabolic flux and pathway engineering applications where the effect of expression of specific genes on metabolism is required. Several other heterologous inducible promoters, such as P_{lac}, λP_L, and P_R, have been tested with *M. extorquens*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference(s) or source
M. extorquens		
ATCC 55366	Wild type	ATCC
Transformants		
CymR1	Modified host strain containing single copy of *cymR* expression cassette in the chromosome	This study
CymR2	Modified host strain containing double copy of *cymR* expression cassette in the chromosome	This study
GFP	*M. extorquens* (CymR2) containing pCUM-gfp	This study
BGL	*M. extorquens* (CymR2) containing pCUM-bgl	This study
EST	*M. extorquens* (CymR2) containing pCUM-est	This study
Pseudomonas putida F1	Source of *cymR* gene in the *cym* operon	11, 12
E. coli strains		
Top10	Strain for cloning and propagating plasmid DNA	Invitrogen, Inc.
S17-1/λ (*pir*)	Host strain for pUX-BF13	17
Plasmids		
pBK-miniTn7-ΩSm2	pUC19-based delivery plasmid for a miniTn7-Km transposon; Km^r Sm^r	17
pBRI-tet	pUC19-based delivery plasmid for a mini-Tn7 transposon; Tet^r	This study
pBRI80	pUC19-based delivery plasmid for a mini-Tn7 transposon containing P_{mxaF} and RBS; Tet^r	This study
pBRI-cymR1	pBRI80 plasmid containing one copy of *cymR* expression cassette	This study
pBRI-cymR2	pBRI80 plasmid containing two copies of *cymR* expression cassettes	This study
pUX-BF13	R6K replicon-based helper plasmid	17
pCR2.1-TOPO	PCR cloning vector	Invitrogen, Inc.
pCR-cymR	pCR2.1-TOPO plasmid containing *cymR*	This study
PCR-MDHOP	pCR2.1-TOPO plasmid containing P_{mxaF}+operator	This study
pCR-bgl	pCR2.1-TOPO plasmid containing *bgl*	This study
pCR-est	pCR2.1-TOPO plasmid containing *estI*	This study
pCR-gfp	pCR2.1-TOPO plasmid containing *gfp*	This study
pCM110	Wide-host-range cloning vector containing P_{mxaF}; Tet^r	19
pCHOI3	Wide-host-range cloning vector containing P_{mxaF}; Km^r	This study
pCUM50	Newly constructed regulative expression vector for *M. extorquens* (CymR2)	This study
pCUM-bgl	pCUM50 plasmid containing β-galactosidase expression cassette	This study
pCUM-est	pCUM50 plasmid containing esterase expression cassette	This study
pCUM-gfp	pCUM50 plasmid containing GFP expression cassette	This study
pUC19	Multipurpose cloning vector	Invitrogen, Inc.
pCESTa	*estI* gene source	9
pEBIG4	*bgl* gene source	15
pQBI63	*gfp* gene source	Qbiogene, Inc.

ATCC 55366; however, gene expression under the control of these promoters was leaky and weak (unpublished results). To develop a regulated and inducible expression system, we adapted the regulatory element of *Pseudomonas putida* F1. *P. putida* F1 degrades *p*-cymene (*p*-isopropyltoluene) through *p*-cumate (*p*-isopropylbenzoate) to isobutyrate, pyruvate, and acetyl coenzyme A (11, 12). The genes encoding the enzymes required for this degradation are grouped in two distinct operons, called *cym* and *cmt*. The *cym* operon encodes the conversion of *p*-cymene to *p*-cumate. Located downstream of the *cym* operon is the *cmt* operon, which encodes the catabolism of *p*-cumate. A regulatory protein known as CymR, encoded upstream of the *cym* operon, has been shown to bind to specific operator-promoter regions in both operons and controls expression of both operons. Induction is afforded by *p*-cumate, the end product of the first operon, but not by *p*-cymene (11, 12).

We decided to apply the regulatory elements, the *cymR* gene and the operator fragment of the *cmt* operon from *P. putida* F1, to *M. extorquens* ATCC 33566 in the hope of bestowing inducibility and regulation on the existing constitutive expression vectors for *Methylobacterium* strains. If successful, we would obtain an inducible expression system able to operate during growth of *Methylobacterium* on a preferred growth substrate, methanol, while controlling the expression of heterologous genes from the strong P_{mxaF} promoter using the nontoxic and inexpensive inducer cumate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *M. extorquens* (ATCC 55366) was used for the preparation of the heterologous gene expression host. *Escherichia coli* strain Top10 was used for cloning and propagation of recombinant plasmid DNA, and S17-1 λ *pir* (*recA thi pro hsdR*$^-$ M^+ RP4:2-Tc:Mu:Km Tn*7*, λ *pir* lysogen; Sm^r Tp^r) was used for propagation of the helper plasmid (17). *E. coli* was cultured in Luria-Bertani broth at 37°C. *M. extorquens* was grown in CHOI medium, as previously described (9), and 1% (vol/vol) methanol was used as the sole carbon source. Media were solidified by 1.8% agar (Difco) when appropriate. Antibiotics were used for *E. coli* and *M. extorquens* at the following concentrations (in μg/ml): ampicillin, 100; kanamycin (Km), 40; tetracycline (Tc), 35.

Construction of expression hosts. The *M. extorquens* expression hosts (CymR1 and CymR2) were constructed by insertion of the *cymR* gene of *P. putida* F1 using the mini-Tn*7* integration system with the mini-Tn*7* vector pBRI80 with a helper plasmid (12, 17). Primers used in the *cymR* amplification were designed on the basis of the nucleotide sequence of the *P. putida* F1 *cym* operon (11, 12). In order to achieve tightly regulated induction, two copies of the *cymR* gene were integrated into the *M. extorquens* chromosome. Briefly, the mini-Tn*7* base vector pBRI80 was constructed as follows: a 1,955-bp PstI fragment containing *tetA* and *tetR* was amplified from pCM110 (19) by PCR using the primers Tet-F-Pst (5′-GCTGCAGTCAATCGTCACCCTTTCTCGGTC-3′) (PstI site is underlined) and Tet-R-Pst (5′-GCTGCAGTCAGCGATCGGCTCGTTGCCCTG-3′) (PstI site is underlined). This fragment was then replaced with a kanamycin-resistant-protein-encoding gene in pBK-miniTn7-ΩSm2 to form pBRI-tet.

The P_{mxaF} promoter plus the ribosomal binding site (P_{mxaF}-RBS) was ampli-

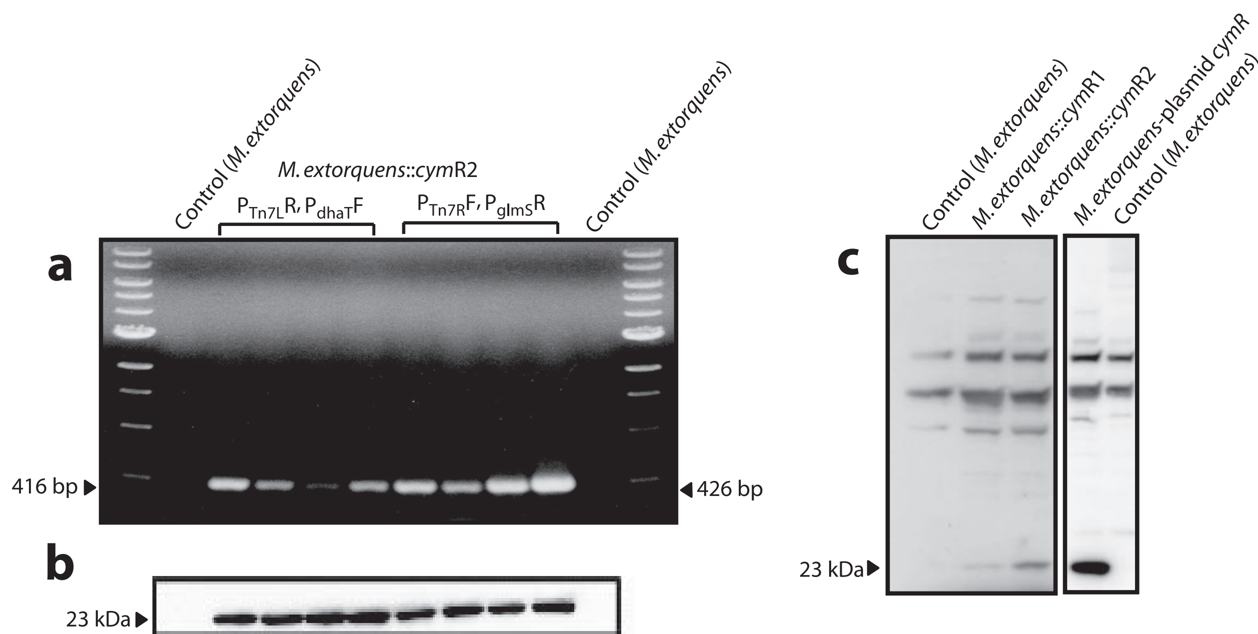

FIG. 1. Analysis of intracellular polypeptides from recombinant strains of *M. extorquens*::*cymR* expressing repressor protein. (a) Colony PCR profiles. The physical presence of the integrated *cymR* gene in the chromosome of *M. extorquens* was tested in eight randomly selected *M. extorquens* (CymR2) clones by colony PCR utilizing the primers described in Materials and Methods. The position and size of the expected PCR products are marked. $P_{Tn7L}R$, $P_{dhaT}F$, $P_{Tn7R}F$, and $P_{glmS}R$ represent the primers used to generate PCR products. (b) The presence of the repressor protein in eight randomly selected clones (see panel a) was confirmed by Western blot analysis. Arrows indicate the position and the molecular size (23 kDa) of the repressor protein. (c) Western blot analysis of repressor protein expressed by native *M. extorquens*, a single-copy *cymR* integrant, a double-copy *cymR* integrant, or plasmid (pCM110)-expressed *cymR*, respectively.

fied from pCESTc using the primers MDH-F-Pst (5′-GGCTGCAGGTTGACGACAACGGTGCGATG-3′) (PstI site is underlined) and MDH-R-Mlu (5′-CCGACGCGTATGTATATCTCCTTCTTAAAG-3′) (MluI site is underlined). The PCR fragment containing P_{mxaF}-RBS was cloned into pBRI-tet, which was partially digested with PstI/MluI to delete the Sm^r/Sp^r cassette to generate pBRI80.

To generate pBRI-cymR1, *cymR* was amplified from chromosomal DNA of *P. putida* F1 using the primers CYM-F-Afl (5′-GCTTAAGAAGATGGTGATCATGAGTCCAAAGAGAAGAAC3′) (AflII site is underlined) and CYM-R-Not (5′-CAGCGGCCGCCTAGCGCTTGAATTTCGCGTACCGCTCTCGCG-3′) (NotI site is underlined). A 612-bp AflII-NotI fragment from pCR-cymR was ligated into the AflII-NotI site of pBRI80 to form pBRI-cymR1. To obtain pBRI-cymR2, containing two copies of *cymR* expression cassettes, a second copy of *cymR* was amplified from pBRI-cymR using the primers MDH-CYM-F-Not (5′-CAGCGGCCGCGTTGACGACAACGGTGCGATGGGTC-3′) (NotI site is underlined) and CYM-R-Apa (5′-CAGGGCCCCTAGCGCTTGAATTTCGCGTACCGCTCTCGCG-3′) (ApaI site is underlined). The amplified fragment containing P_{mxaF}-RBS-*cymR* was then ligated into the NotI-ApaI site of pBRI-cymR1 to generate pBRI-cymR2.

The genotypes of the *cymR*-integrated host strains were confirmed by Southern hybridization using the 612-bp *cymR* fragment as a probe. Since we have identified the specific Tn*7* insertion site (*att*Tn*7*) for *M. extorquens* in a previous study (8), the integration of the target gene into the chromosome of *M. extorquens* was also determined by colony PCR using designed primers which include common Tn*7* primers ($P_{Tn7R}F$, 5′-ATTAGCTTACGACGCTACACCC-3′; $P_{Tn7L}R$, 5′-CACAGCATAACTGGACTGATTTC-3′) and gene-specific primers ($P_{dhaT}F$, 5′-CATCGCGATTGTCGATTCGG-3′; and $P_{glmS}R$, 5′-CTGAAGGAAATCAGCTACATC-3′). The *cymR*-positive strains were finally confirmed by Western blotting. Then, the electrocompetent cells were prepared using *cymR*-positive *M. extorquens*, essentially as described previously (9, 13).

Inducible expression vector construction. Manipulations and sequencing of DNA were carried out using standard procedures. The operator sequence of the *cmt* operon from *P. putida* F1 was introduced downstream of the methanol dehydrogenase promoter, P_{mxaF}, by PCR. The pCUM50 regulative expression vector was obtained in several steps: first, the P_{mxaF} plus synthetic operator sequence (P_{mxaF}+operator) was amplified by PCR from pCM110 using primers MDH-F-PST (5′-GCTGCAGGTCGACTCTAGATCACCTCCTTAAGC-3′) (the PstI site is underlined) and MDH-CUM-R (5′-CGAATTCATAATACAAACAGACCAGATTGTCTGTTTGTTGCCCTTAGGATCCGCGGTATC-3′) (the EcoRI site is underlined). The 403-bp PCR fragment containing P_{mxaF}+operator was cloned into pCR2.1 to create pCR-MDHOP. Next, the kanamycin resistance gene of a 1,218-bp PstI fragment from pBK-miniTn7-ΩSm2 was cloned into the PstI site of pCM110, and then *tetA* and *tetR* were removed by restriction with BclI and self-ligation yielded pCHOI3. A 403-bp PstI-EcoRI fragment from pCR-MDHOP was then ligated between the PstI and EcoRI sites of pCHOI3; this replaced P_{mxaF} with P_{mxaF}+operator to form the cumate-inducible expression vector pCUM50.

To test heterologous protein production using this cumate switch system, we obtained an XbaI-ClaI fragment containing the *gfp* gene from pQBI63 and cloned it into SpeI-ClaI sites of pCUM50 to generate pCUM-gfp. The 2,100-bp fragment carrying the lactase gene (*bgl*) from *Bifidobacterium infantis* was amplified from pEBIG4 (15) using primers BGL-F-Nhe (5′-CGCTAGCGAACATAGAGCGTTCAAGTGGC-3′) (the NheI site is underlined) and BGL-R-Cla (5′-CATCGATTTACAGCTTGACGACGAGTACGCCG-3′) (the ClaI site is underlined). For the amplification of the esterase gene (1,800 bp; *estI*) of *Lactobacillus casei*, pCESTa (9) was used as a template with primers EST-F-Nhe (5′-GGCTAGCGATCAATCTAAAACAAATC-3′) (the NheI site is underlined) and EST-R-Cla (5′-CATCGATTTATTTATTTGTAATACCGTCTGC-3′) (the ClaI site is underlined). These NheI-ClaI fragments of *bgl* and *est* were then replaced with a *gfp* gene in pCUM-gfp to form pCUM-bgl and pCUM-est, respectively. The different proteins tested in the pCUM system were cloned via SpeI and ClaI (pCUM-gfp) or via NheI and ClaI (pCUM-bgl and pCUM-est).

Detection of gene expression. Detection of GFP was carried out by fluorescence microscopy, and quantification was done using a microplate spectrofluorometer (SPECTRAFluor Plus; TECAN) under excitation and emission wavelengths of 485 and 508 nm, respectively. The concentration of GFP was calculated based on a linear relationship between concentration and fluorescence units determined using solutions of purified GFP (Qbiogene). The biomass was determined by cell dry weight measurement of the samples (Moisture Analyzer MA 30; Sartorius).

Esterase activity was determined by a spectrophotometric method using *para*-nitrophenyl caprylate as a substrate. The rate of hydrolysis of *para*-nitrophenyl

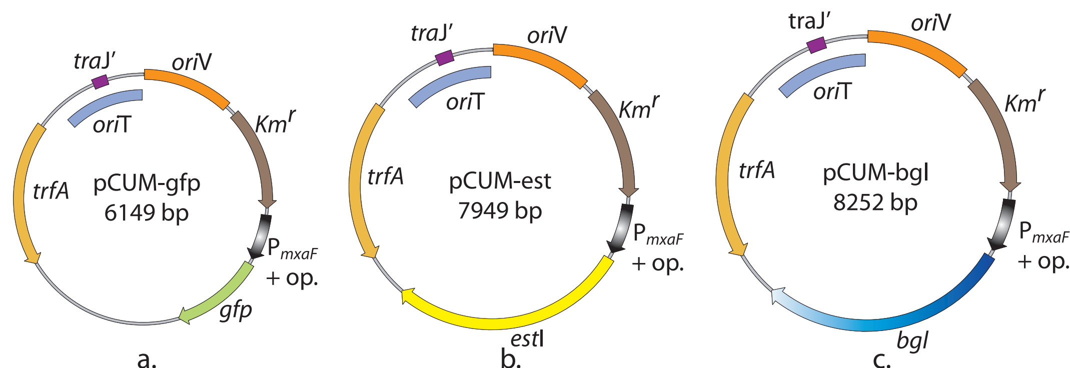

FIG. 2. Maps of recombinant plasmids containing the required regulatory elements. Abbreviations: *traJ′*, mutated conjugal transfer gene that is missing the final 85 of 123 amino acids; *oriT*, origin of transfer; *oriV*, vegetative origin; *trfA*, replication initiator gene; *Km^r*, kanamycin resistance gene; P_{mxaF} + op., methanol dehydrogenase combined with operator sequence from *cmt* operon of *P. putida* F1; *gfp*, green fluorescent protein-encoding gene; *estI*, esterase-encoding gene; and *bgl*, β-galactosidase-encoding gene.

caprylate at 37°C was measured in 50 mM sodium phosphate buffer (pH 7.0) according to the method described previously (9, 16). One unit of activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per min under the given assay conditions. The β-galactosidase activity was measured with *o*-nitrophenol-β-D-galactopyranoside as a substrate, and one unit of activity was defined as the amount of enzyme that liberated 1 μmol of *o*-nitrophenol per min (25). The protein concentration was estimated by the method of Bradford (5) using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Western blotting. Integrative expression of the repressor protein (CymR) was determined by Western blotting using a standard protocol. CymR was detected with rabbit anti-bCymR no. 422 antibody (0.1 g ml^{-1}; in-house antibody generated by our group) and a goat anti-rabbit immunoglobulin G (heavy plus light chains) horseradish peroxidase conjugate (0.1 μg ml^{-1}; catalog no. 31460; Pierce, West Grove, PA). Cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer.

RESULTS AND DISCUSSION

The construction of the cumate-regulated/inducible system in *M. extorquens* required the engineering both of the chromosome and of the cloned P_{mxaF} promoter, described hereafter.

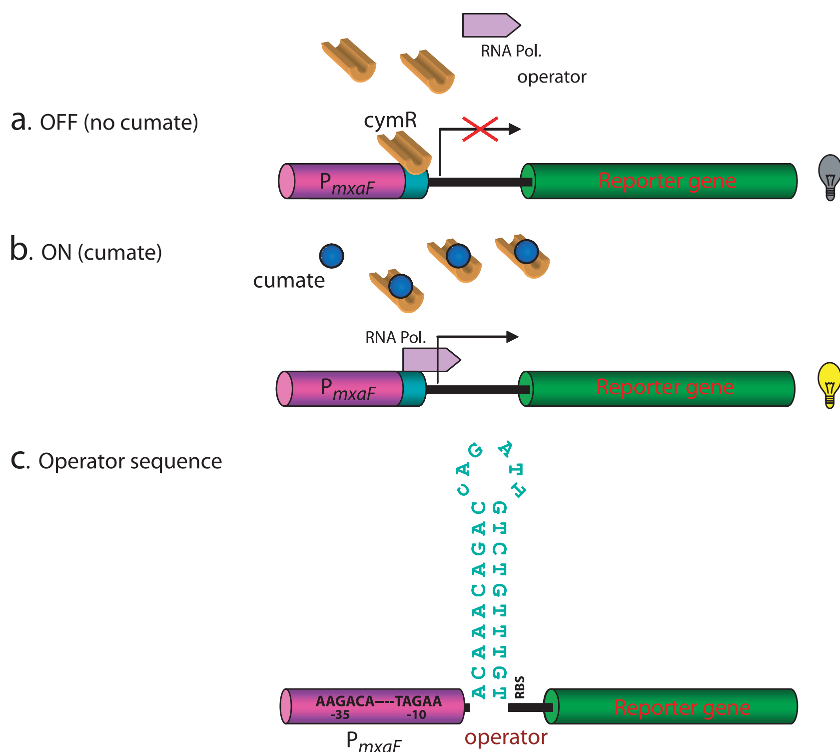

FIG. 3. A schematic diagram of the mechanism of action of the cumate-switchable expression system. (a) In the absence of the inducer, cumate, the repressor protein (CymR) is bound to the operator site upstream of the reporter gene or gene of interest and inhibits transcription. (b) The presence of cumate is necessary for transcription of the gene of interest. The addition of cumate rapidly alters the binding of the operator to the repressor CymR, thereby facilitating the formation of the CymR-cumate complex. The detachment of CymR from the operator site activates transcription of the downstream reporter gene. The CymR-cumate complex is unable to bind to the operator site. (c) Nucleotide sequence of the operator, which represents the putative binding site for the helix-turn-helix transcription factor (see Materials and Methods for details of the construction of the P_{mxaF}+operator fragment).

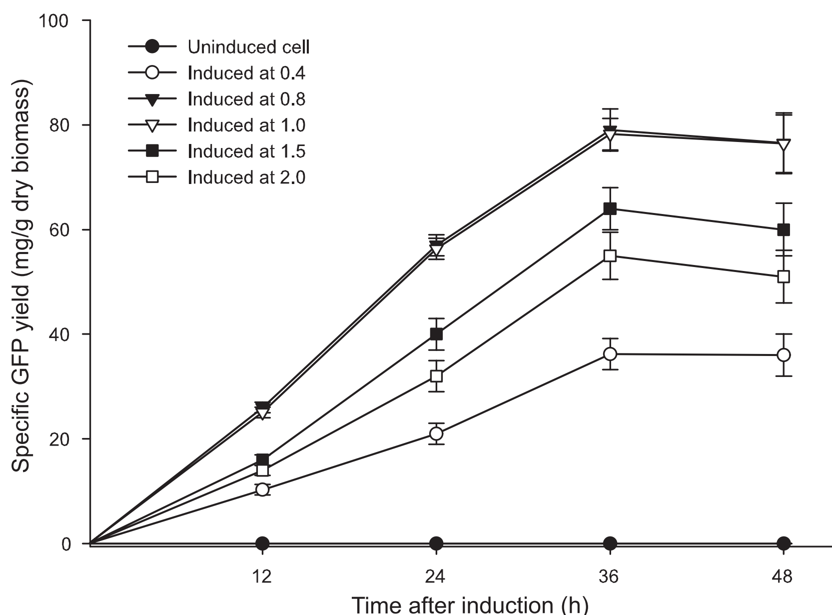

FIG. 4. Identification of the optimal time of induction. Maximum gene expression was obtained when the culture was induced with 15 μg/ml cumate at an OD_{600} of 0.8 to 1.0. Induction at an OD_{600} of 0.4, 1.5, and 2.0 resulted in reduced expression of the target gene.

The structural components required for an operational cumate-regulated expression system are the *cymR* gene, which encodes the CymR repressor protein, and the CymR binding DNA region fused to the 3′ end of a selected promoter. For the cumate-regulated system to work effectively in *M. extorquens*, the culture must be able to express and produce the functional repressor protein (CymR) constitutively. First, the 612-bp *cymR* gene from *P. putida* F1 was cloned into pCM110 under the control of the P_{mxaF} promoter. CymR expression was confirmed by Western blot analysis (Fig. 1c). However, the culture grew very poorly (data not shown), indicating a potential toxicity of CymR to the host. In order to reduce the level of CymR production and, by inference, its toxicity, an alternative to the plasmid-based *cymR* expression was adopted. In the absence of information on the minimal amount of CymR required for optimal repression of transcription, DNA fragments containing either one or two copies of the *cymR* gene were integrated into the chromosome of *M. extorquens*, generating the clones *M. extorquens* (CymR1) and *M. extorquens* (CymR2), respectively. Integration was achieved using the mini-Tn7 transposon system described elsewhere (7, 8). Insertion of mini-Tn7-P_{mxaF}-*cymR* into the unique Tn7 attachment site (*att*Tn7) of *M. extorquens* was verified by colony PCR (Fig. 1a). Expression was once again confirmed by Western blot analysis (Fig. 1b). Importantly, growth of the *cymR*-expressing clones was indistinguishable from that of wild-type *M. extorquens* (data not shown). To verify if the levels of CymR produced by the transformants were sufficient to repress transcription and thereby regulate expression, an expression vector possessing the engineered P_{mxaF} promoter had to be constructed. The expression vector (pCUM50) was constructed containing the operator sequence of the *cmt* operon from *P. putida* F1 downstream of the cloned homogeneous promoter, P_{mxaF}, in pCHOI3, a derivative of pCM110 where the tetracycline marker was exchanged for kanamycin (Table 1). Three different constructs containing a gene encoding GFP, esterase, or β-galactosidase were inserted into the multiple cloning site downstream of the operator, generating pCUM-gfp, pCUM-est, or pCUM-bgl, respectively (Fig. 2). Expression of GFP in the recombinant host *M. extorquens* (CymR2) transformed with the expression vector pCUM-gfp was not detected in the absence of the inducer, cumate. However, the recombinant host *M. extorquens* (CymR1) transformed with the expression vector pCUM-gfp grown in the absence of the inducer showed leaky expression, amounting to 15 mg GFP/g dry biomass. This leaky expression represents about 20% of the GFP yield of a fully cumate-induced culture (80 mg GFP/g dry biomass). Incomplete repression in *M. extorquens* (CymR1) hosts was assumed to be due to the insufficient CymR production (Fig. 1c). Hence, competent cells containing two *cymR* copies [*M. extorquens* (CymR2)] were used for further studies.

M. extorquens (CymR2) transformed with the plasmids pCUM-gfp, pCUM-est, and pCUM-bgl grew well, and expression could not be detected in the absence of cumate. The addition of cumate to the culture reduces the binding capacity of the repressor protein (CymR) for the operator region, and transcriptional repression is thereby alleviated. Tight inducible expression of the reporter gene is thereby attained. The basic mechanism of this system is summarized in Fig. 3. The optimal inducer concentration for recombinant *M. extorquens* (CymR2) transformed with pCUM-gfp was determined in shake flask cultures grown to the mid-log phase (0.8 to 1.0 units at an optical density at 600 nm [OD_{600}]) at 30°C, followed by induction with cumate at different concentrations, ranging from 1 to 30 μg cumate/ml. GFP expression was detectable at 4 h postinduction at all concentrations tested. Cumate induction was shown to be very sensitive, since the addition of 1 μg cumate/ml resulted in GFP production representing approximately 47% of the GFP yield of a culture fully induced with 15 μg cumate/ml (data not shown). Growth inhibition was not ob-

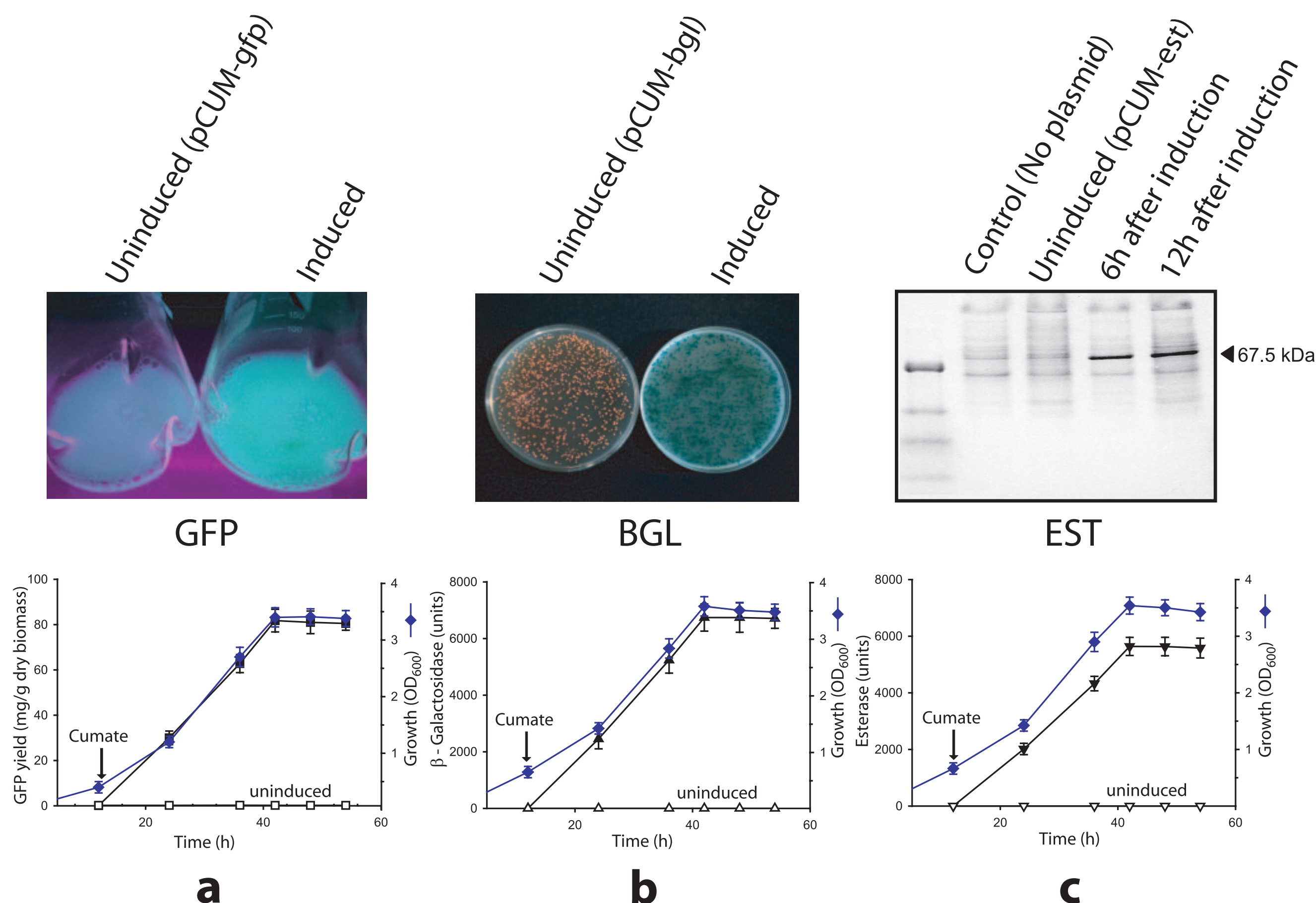

FIG. 5. Induction and production of recombinant proteins by the cumate-regulated expression system. The inducible expression system was validated with three different heterologous genes, encoding green fluorescent protein (a), β-galactosidase (b), and esterase (c). Without cumate induction, target proteins were not detected, as analyzed by UV for GFP, the chromogenic substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for β-galactosidase, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for esterase. The lower lane shows the recombinant protein production and bacterial growth profiles from each construct. The arrow indicates the point of induction with cumate (15 μg/ml). Protein yield or enzyme activities for induced cultures, closed symbols (■, ▲, and ▼); for uninduced cultures, open symbols (□, △, and ▽).

served with the cumate concentrations tested (1 to 30 μg/ml), indicating that cumate does not negatively affect cell metabolism at these concentrations in shake flask experiments, where typical biomass yields are expected to reach 1.5 to 1.7 g of cells (dry weight)/liter. The optimal cumate concentration for effective induction of GFP expression in shake flask growth experiments was 15 μg/ml (approximately 0.1 mM). Understandably, in high-cell-density fermentations, where biomass yields can reach over 50 g of cells (dry weight) per liter, higher concentrations of cumate may be required.

To determine the optimal time of induction for maximal protein production, *M. extorquens* (CymR2) cells stably transformed with pCUM-gfp were induced at different stages of growth (OD_{600} of 0.4, 0.6, 0.8, 1.0, and 1.2) in shake flask experiments with 15 μg cumate/ml. Cells induced at an OD_{600} of 0.8 to 1.0 generated maximal specific yields of GFP. At these induction points, the specific yield of the recombinant protein was consistently 20 to 50% greater than at other induction points (Fig. 4). However, it should be noted that stationary-phase cultures could still be induced, albeit resulting in reduced levels of expression (data not shown).

To further validate cumate inducibility, we expressed two other proteins (esterase and β-galactosidase) in the recombinant host strain *M. extorquens* (CymR2). These transformants were analyzed for induction profiles and expression levels and were compared to GFP-expressing cultures. All clones, cultured up to an OD_{600} of 0.8 to 1.0, did not produce detectable levels of recombinant proteins in the absence of cumate (Fig. 5). In all cases, at 4 h postinduction with 15 μg cumate/ml, expression of the recombinant proteins was detected. Production of GFP, esterase, and β-galactosidase remained active even after 24 h postinduction, with maximum expression at 30 h postinduction (Fig. 5a, b, and c). The recombinant protein expression was not detected in noninduced cells, and the expression occurred as long as there was cell growth. Using this system, 6.1 g GFP/liter and 5,637 ± 320 and 6,742 ± 480 U of recombinant esterase and β-galactosidase were obtained, respectively (Fig. 5a, b, and c).

The well-characterized promoters (T5, T7, and *lac*) utilized in *E. coli* expression vectors require isopropyl-β-D-thiogalactopyranoside for induction. Although isopropyl-β-D-thiogalactopyranoside is an effective inducer for *E. coli*, it is expensive and can elicit cellular toxicity at concentrations which are deemed optimal for expression of recombinant proteins (14, 23). The

innovative aspect of our inducible system is the fact that we have kept a powerful native promoter for heterologous gene expression, added an operator region downstream of the promoter, and integrated a repressor-encoding sequence in the chromosome which expresses sufficient repressor protein to repress expression of the target gene completely. Furthermore, cumate, a nontoxic organic and nonmetabolizable compound for *M. extorquens*, at low concentrations is capable of traversing the cell's outer membranes and alleviates repression effectively. Cumate did not cause any growth inhibition even at concentrations twofold higher than that required for optimal induction. This is the first documented description of a tightly regulated recombinant expression system in *M. extorquens*. Temporal expression of selected gene products and applications in metabolic flux and pathway engineering may now be possible with *M. extorquens*.

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