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Cumate-Inducible Gene Expression System for Sphingomonads and Other *Alphaproteobacteria*

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Tunable promoters represent a pivotal genetic tool for a wide range of applications. Here we present such a system for sphingomonads, a phylogenetically diverse group of bacteria that have gained much interest for their potential in bioremediation and their use in industry and for which no dedicated inducible gene expression system has been described so far. A strong, constitutive synthetic promoter was first identified through a genetic screen and subsequently combined with the repressor and the operator sites of the *Pseudomonas putida* F1 *cym*/*cmt* system. The resulting promoter, termed P_{Q5}, responds rapidly to the inducer cumate and shows a maximal induction ratio of 2 to 3 orders of magnitude in the different sphingomonads tested. Moreover, it was also functional in other *Alphaproteobacteria*, such as the model organisms *Caulobacter crescentus*, *Paracoccus denitrificans*, and *Methylobacterium extorquens*. In the noninduced state, expression from P_{Q5} is low enough to allow gene depletion analysis, as demonstrated with the essential gene *phyP* of *Sphingomonas* sp. strain Fr1. A set of P_{Q5}-based plasmids has been constructed allowing fusions to affinity tags or fluorescent proteins.

Regulated gene expression systems are a powerful tool to study physiology, allowing, for example, dosage-effect studies, conditional expression of toxic alleles, and depletion analysis of essential genes; accordingly, they are well developed for model organisms (1–7) but are missing for many non-model organisms. Most systems rely on a transcriptional repressor that tightly binds to operator sites in the promoter region of target genes in the absence of an inducer, thereby preventing transcription; when an inducer is present, it allosterically binds to and inactivates the transcriptional regulator, leading to derepression of promoters. Despite this simple concept, identification of inducible promoters, control elements, and inducing conditions in a particular organism is not always a trivial task. On the other hand, it is often difficult to exploit a particular system for use in organisms other than the original host because of the need for dedicated transporters for the inducer or a different promoter specificity of the RNA polymerase holoenzyme or the requirement of a coactivator for full promoter activity (8). Some of these obstacles can be circumvented by engineering artificial inducible promoters by placing a constitutive minimal promoter known to be active in a particular organism with operator sequences and the repressor of a heterologous system (2, 9, 10). We here describe such a system for sphingomonads, a phylogenetically diverse group of environmentally abundant bacteria comprising the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* (11). Members of this group are well known for their unusual ability to degrade a wide range of compounds, including pesticides, herbicides, xenobiotics, and many other aromatics. Due to this property, they are prospective candidates for bioremediation; in addition, several strains are used in industrial applications, and others have been described as potential candidates for biocontrol (12–15). Despite this interest in many different aspects of their physiology, no dedicated inducible gene expression system for sphingomonads has existed so far. Here, by combining the operator sequences (CuO) and the cumate-responsive CymR repressor from the *Pseudomonas putida* F1 *cym*/*cmt* system (16, 17) with an engineered minimal housekeeping promoter, P_{syn2}, derived from *Sphingomonas* sp. strain Fr1 (henceforth *Sphingomonas* Fr1), we have developed a highly inducible and rapidly responding conditional gene expression system that shows low basal expression without induction (i.e., is tight). We demonstrate its functionality in a number of different sphingomonads as well as in more distantly related *Alphaproteobacteria* not belonging to the *Sphingomonadales*. Due to the modular design of the system, core promoter sequences can be readily exchanged, opening the possibility to adapt the system to other organisms as well.

MATERIALS AND METHODS

Strains, growth conditions, and plasmid delivery. *Escherichia coli* TOP10 (Invitrogen), "*ccdB* survival" (Invitrogen), and "*dam*$^-$ *dcm*$^-$" (NEB; catalog no. C2925I) were used for cloning. *Sphingomonas* Fr1 wild-type (JVZ857) and Δ*phyR* Δ*phyP*::*loxP* mutant (JVZ1357) strains were described previously (18). Other *Sphingomonas*, *Sphingobium*, and *Novosphingobium* strains used in this work were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Methylobacterium extorquens* AM1 *rif* (19), *Caulobacter crescentus* CB15 (20), and *Agrobacterium tumefaciens* C58 (21) were laboratory stocks. *Paracoccus denitrificans* JVZ2585 is a spontaneous rifampin-resistant derivative of *P. denitrificans* DSM 413 (DSMZ) obtained by selection on LB-Lennox containing 50 μg rifampin/ml; it carries the A1610T mutation in "cluster I" of *rpoB*, resulting in amino acid change Q537L (equivalent to Q513L in *E. coli*), which likely accounts for rifampin resistance (22). *E. coli* strains were routinely grown on LB-Lennox at 37°C. Other strains were grown at 28°C on solidified medium (1.5% agar) or as 20-ml cultures in 100-ml baffled flasks (with shaking at 160 rpm) in LB-Lennox (*Sphingomonas* Fr1, *P. denitrificans*, and *A. tumefaciens*), PYE (0.2% peptone, 0.1% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot H_2O$; *C. crescentus*), Nutrient broth (Fluka Analytical, catalog no. 17181) (all other sphingomonads), or min-

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imal medium (23) with methanol (*M. extorquens*). When appropriate, concentrations (in μg/ml) of antibiotics for *E. coli*, sphingomonads, and *M. extorquens* were used as follows: tetracycline, 10; kanamycin, 50; carbenicillin, 50; streptomycin, 100; chloramphenicol, 34. For other strains, tetracycline to maintain plasmids was added to the media in the following concentrations (in μg/ml): 2 (*C. crescentus*); 3 (*A. tumefaciens*); and 1 (*P. denitrificans*).

Plasmids were delivered into sphingomonads by conjugal transfer or electroporation as described previously (24). For *A. tumefaciens* and *C. crescentus*, electroporation was performed on a GenePulser Xcell system (Bio-Rad) using the standard preset *E. coli* program; for *M. extorquens*, electroporation was performed as described before (24). For *P. denitrificans*, plasmids were delivered by conjugal transfer from *E. coli* S17-1λ*pir* (25) with mating on minimal medium without a carbon source as previously described (24). Mating mixtures were plated on LB-Lennox containing tetracycline (10 μg/ml) and rifampin (50 μg/ml) for plasmid selection and *E. coli* counterselection, respectively.

Measurement of promoter activity. Cumate (4-isopropylbenzoic acid) was purchased from Sigma-Aldrich (catalog no. 269402-5G) and dissolved in 100% ethanol to result in a 100 mM stock solution. Dilutions were prepared in ethanol to give a 1,000-fold stock. For "no cumate" controls, an equal volume of ethanol was added to cultures.

For measuring the steady-state activity of P_{Q5} in *Sphingomonas* Fr1, precultures of the wild-type strain harboring pQF-lacZ were grown for 8 to 10 h to an optical density at 600 nm (OD_{600}) of 1.0, diluted into fresh medium at an OD_{600} of 0.0005 to 0.001 with different cumate concentrations (see Results and Discussion), and grown for 20 to 24 h. For measuring induction kinetics in *Sphingomonas* Fr1, cultures were grown to the exponential phase (OD_{600} of 0.5 to 1.5) and cumate was added to reach a final concentration of 50 μM. For depletion analysis, cultures were grown in LB containing 50 μM cumate to the exponential phase (OD_{600} of 1 to 2), washed 5 times with 20 ml of LB, and resuspended in 20 ml of LB (OD_{600} of 0.025) with (50 μM) or without cumate. Cultures were grown for 20 to 24 h before measuring *nepR*p-*lacZ*$^+$ activity.

Measurements of steady-state activity of P_{Q5} in other sphingomonads were performed as described for *Sphingomonas* Fr1 using 25 μM cumate. For *A. tumefaciens* and *P. denitrificans*, cultures inoculated from a single colony were grown overnight (16 to 20 h) with (25 μM) or without cumate. For *C. crescentus*, cultures in the exponential phase (OD_{600} of 0.6 to 0.8) were split in two and induced with 50 μM cumate or subjected to mock treatment with ethanol for 4 h before measurement of P_{Q5} activity. For *M. extorquens*, the wild-type strain harboring the *lux* reporter plasmid (pQ5-lux or pQ2148-lux) was grown to the exponential phase (OD_{600} of 0.3 to 0.6) and P_{Q5} or P_{Q2148} was induced by adding 90 μl of culture to wells containing 10 μl of a 10-fold aqueous stock solution of cumate at different concentrations. Measurements were started immediately (<2 min after induction) and followed for approximately 3 h in 2-min intervals.

β-Galactosidase activity was determined using the chromogenic substrate 2-nitrophenyl-β-D-galactopyranoside (ONPG; Fluka) according to the method of Miller (26). For *M. extorquens*, luciferase activity was measured in 96-well black/white isoplates (PerkinElmer) on a Victor^3 multilabel plate reader (PerkinElmer) essentially as described previously (27) except that the measured values were corrected for the initial OD_{600}.

SDS-PAGE and Western blots. Aliquots (1 ml) were taken from the same cultures used to measure *nepR*p-*lacZ*$^+$ activity, cells were pelleted by centrifugation (6,000 × *g*, 5 min) and resuspended to an OD_{600} of 10 in SDS-PAGE sample buffer (28), and 5-μl samples were resolved on SDS-PAGE gels (12.5%) followed by transfer to nitrocellulose membranes. Western blotting was performed with primary mouse monoclonal anti-FLAG M2 antibodies (Invitrogen) and secondary goat anti-mouse horseradish peroxidase-coupled IgG antibodies (Bio-Rad) and ECL Western blotting detection reagents (GE Healthcare).

Plasmid construction. Standard molecular biology protocols were followed (29). PCR was done with Phusion DNA polymerase (Thermo Scientific) in GC buffer containing 10% dimethyl sulfoxide (DMSO), and oligonucleotides were purchased from Microsynth (Balgach, Switzerland). Restriction enzymes were from Thermo Scientific, and T4 DNA ligase was from New England BioLabs. For oligonucleotide annealing, the two complementary oligonucleotides (10 μM each) were mixed in 100 μl of oligonucleotide annealing buffer (8 mM Tris-HCl [pH 7.5], 40 mM NaCl) in a microcentrifuge tube; the tube was placed in 1 liter of boiling water, which was then allowed to cool to room temperature for several hours. Relevant plasmids are listed in Table 1 and oligonucleotides in Table S1 in the supplemental material.

P_{Q5}-based plasmids. pQF was assembled in multiple steps, mostly from synthetic DNA (Invitrogen and MWG Operon Eurofins) and short double-stranded DNA (dsDNA) (generated by oligonucleotide annealing), and details are outlined in the supplemental material. All other P_{Q5}-based plasmids were derivatives of pQF, except pQF-lacZ, from which pQF itself was derived (see the supplemental material). The multiple-cloning site (MCS) of pQF is flanked by sequences encoding triple-FLAG (3×FLAG) tags. Derivatives of pQF were obtained by exchanging the N-terminal 3×FLAG tag for other affinity tags or fluorescent proteins while maintaining the reading frame of the original MCS and the C-terminal 3×FLAG in all constructs. The N-terminal tags in the different plasmids are as follows: pQF, 3×FLAG tag; pQH, hemagglutinin (HA) tag; pQM, *myc* tag; pQG, enhanced green fluorescent protein (EGFP); pQY, "super" yellow fluorescent protein 2 (SYFP2); pQR, mCherry; pQC, enhanced cyan fluorescent protein (ECFP). Their construction is detailed in the following explanation. pQH was generated by replacing the HindIII-VspI fragment of pQF by a fragment generated by annealing oligonucleotides oJVZ1088 and oJVZ1089. Similarly, pQM was generated by replacing the HindIII-VspI fragment of pQF with a fragment generated by annealing oligonucleotides oJVZ1090 and oJVZ1091. pQF derivatives for C-terminal fusions to fluorescence proteins were constructed by replacing the HindIII-VspI fragment of pQF with PCR fragments encoding fluorescence proteins amplified with oligonucleotides oJVZ1107 and oJVZ1108. PCR templates for genes encoding fluorescent proteins were as follows: pBBRBB-egfp (30) (Addgene plasmid 32549) for EGFP; pLM-sYFP2 (18) for SYFP2; pMP4516 (31) for ECFP; and pHC119 (a gift from David H. H. Chou) for mCherry. Destination vectors based on P_{Q5} compatible with Gateway cloning (Invitrogen) were obtained by PCR amplification of the Gateway cassette (including *att* sites, *ccdB*, and *cat*) from pDEST-565 (Addgene plasmid 11520) with oligonucleotides oJVZ740 and oJVZ739, digestion with XbaI and Acc65I, and ligation into the same sites of pQF and its derivatives. Destination plasmids were given the parent's name followed by a "D" (e.g., pQHD was derived from pQH by insertion of the Gateway cassette and allows fusion to the N-terminal HA tag). The pQH derivative pQH2 contains the pBBR1 origin of replication (oriV) and was constructed by PCR amplification of a fragment containing *rep*, *mob*, and oriT from pBBR1MCS5 (32) using oligonucleotides oJVZ1436 and oJVZ1437, digestion with PscI and Eco147I, and cloning between the same sites of pQH.

***lacZ* reporter constructs.** The *lacZ* transcriptional reporter plasmid pAK501 was constructed in several steps that involved cloning of a putative transcriptional terminator, a "low-background" multiple-cloning site (MCS), and the reporter gene *lacZ* in plasmid pCM62 (33) followed by exchange of the antibiotic resistance cassette and the origin of replication. Individual steps are described in the following explanation. First, TERM193, a fragment encompassing tandem copies of a putative bidirectional transcriptional terminator encoded in the *Sphingomonas* Fr1 genome, was amplified with oligonucleotide pair oJVZ745 and oJVZ746 from pTOPO-TERM193 (see Fig. S1 in the supplemental material) and cloned into pCM62 via PscI and HindIII sites, generating plasmid pAK127. Next, *lacZ* was amplified from *E. coli* BTH101 (Euromedex) using oligonucleotide pair oJVZ1082 and oJVZ1083 and cloned between the EcoRI and Acc65I sites of pAK127, resulting in the *lacZ* reporter plas-

TABLE 1 Plasmids used in the study

Plasmid category and name	Description/relevant features[a]	Source or reference
Basic cloning plasmids		
pAK200	pBBR oriV; Km^r	18
pAK206	pBBR oriV; Gm^r	18
pAK200Cm	pAK200 derivative; Cm^r	This study
pBBR1MCS5	pBBR oriV; Gm^r	32
pCM62	IncP oriV; ColE1 oriV; RP4 oriT; Tc^r	33
pME6010	pVS1 oriV; p15A oriV; Tc^r	34
Standard P_{Q5}-based plasmids		
pQF	pCM62 with *cymR**, P_{Q5}, and MCS for N- and C-terminal fusions to 3×FLAG tag; Tc^r	This study
pQH	pQF derivative for C-terminal fusion to 3×FLAG and N-terminal fusion to HA tag; Tc^r	This study
pQM	pQF derivative for C-terminal fusion to 3×FLAG and N-terminal fusion to myc tag; Tc^r	This study
pQG	pQF derivative for C-terminal fusion to 3×FLAG and N-terminal fusion to EGFP; Tc^r	This study
pQY	pQF derivative for C-terminal fusion to 3×FLAG and N-terminal fusion to SYFP2; Tc^r	This study
pQR	pQF derivative for C-terminal fusion to 3×FLAG and N-terminal fusion to mCherry; Tc^r	This study
pQC	pQF derivative for C-terminal fusion to 3×FLAG and N-terminal fusion to ECFP; Tc^r	This study
pQH2	pQH derivative with pBBR oriV; Tc^r	This study
pQFD	pQF derivative with Gateway cassette; contains *ccdB*; Cm^r Tc^r	This study
pQHD	pQH derivative with Gateway cassette; contains *ccdB*; Cm^r Tc^r	This study
pQMD	pQM derivative with Gateway cassette; contains *ccdB*; Cm^r Tc^r	This study
pQGD	pQG derivative with Gateway cassette; contains *ccdB*; Cm^r Tc^r	This study
pQYD	pQY derivative with Gateway cassette; contains *ccdB*; Cm^r Tcr	This study
pQRD	pQR derivative with Gateway cassette; contains *ccdB*; Cm^r Tc^r	This study
Reporter plasmids		
pTOPO-TERM193	pCR2.1-TOPO with TERM193; Km^r	This study
pAK127	pCM62 with TERM193; Tc^r	This study
pAK127lacZ	Promoter-probe plasmid (intermediate construct); pAK127 with *E. coli lacZ*; Tc^r	This study
pAK127lacZ(MCS)	Promoter-probe plasmid (intermediate construct); pAK127lacZ with new MCS; Tc^r	This study
pAK200Cm-lacZ	Promoter-probe plasmid (intermediate construct); pAK200Cm with TERM193-MCS-*lacZ* fragment of pAK127lacZ(MCS); Cm^r	This study
pAK501	Promoter-probe plasmid; pME6010 with TERM193-MCS-*lacZ-cat* fragment of pAK200Cm-lacZ; Cm^r	This study
pAK501-nepRp	*nepR* promoter (from −42 to −2) in pAK501; Cm^r	This study
pAK128	Promoter-probe plasmid; pAK127 with SYFP2; Tc^r	This study
pQF-lacZ	pCM62-based reporter plasmid with *cymR** and P_{Q5}-*lacZ*$^+$ fusion; Tc^r	This study
pLM05	Promoter-probe plasmid carrying *luxCDABE* from *Photorhabdus luminescens*; Km^r	27
pQ5-lux	pLM05 with *cymR** and P_{Q5}-*luxCDABE* fusion; Km^r	This study
pQ2148	pQH derivative in which P_{syn2} is replaced by the META1_2148 promoter from *M. extorquens* AM1; Tc^r	This study
pQ2148-lacZ	Reporter plasmid; pQ2148 with *lacZ*; Tc^r	This study
pQ2148-lux	pLM05 with *cymR** and P_{Q2148}-*luxCDABE* fusion; Km^r	This study
Other plasmids		
pQH-PhyP	pQH derivative encoding PhyP-3×FLAG fusion; Tc^r	This study
pAK200-QPhyP	pAK200 derivative with PQ5 driving PhyP-3×FLAG expression; Km^r	This study
pCM62-PhyR	pCM62 harboring *phyR* under the control of its native promoter and RBS; Tc^r	18
pCM62-PhyR(D194A)	pCM62 harboring *phyR*(D194A) under the control of its native promoter and RBS; Tc^r	18

[a] Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

mid pAK127lacZ. Because pAK127lacZ had a high level of background *lacZ* activity despite the presence of terminators, the MCS of pAK127lacZ was replaced by another MCS generated by cloning annealed oligonucleotides (oJVZ1086 and oJVZ1087) between the HindIII and Acc65I sites of pAK127lacZ, giving pAK127lacZ(MCS). This plasmid showed an approximately 10-fold-reduced background compared to its parent. To construct pAK501, the "*lacZ* reporter cassette" of pAK127lacZ(MCS) comprising TERM193-MCS-*lacZ* was combined in several steps with a different broad-host-range oriV (pSV1) and a different antibiotic-resistant marker (*cat*). To this end, pAK200Cm was first constructed by PCR amplification of *cat* from pBAD33 (1) using oligonucleotide pair oJVZ1103 and oJVZ1104 and cloning the BamHI-BcuI-digested PCR product between the BglII and BcuI sites of pAK200 (18). pAK200Cm-lacZ was then constructed by cloning a NcoI-NheI-digested PCR product containing the *lacZ* reporter cassette (TERM193-MCS-*lacZ*) from pAK127lacZ(MCS) amplified with oligonucleotides oJVZ1105 and oJVZ1106 into the PscI and NheI sites of pAK200Cm. Finally, pAK501 was generated by ligating a Eco147I-BglII-digested fragment amplified by PCR with oligonucleotides oJVZ1102 and oJVZ1103 containing TERM193, the MCS, *lacZ*, and *cat* from pAK200Cm-lacZ with the 5.1-kb fragment of pME6010 (34) released by Eco147I-BamHI digestion (pME6010 was purified from the *E. coli* "*dam*$^-$ *dcm*$^-$" strain since Eco147I cleavage is methylation impaired in the context of pME6010). Plasmid pAK501 carries the broad-host-range pSV1 origin of replication and is thus compatible with other commonly used origins of replication, such as IncP and pBBR1.

Plasmid pAK501-nepRp reporting general stress response (GSR) activity was generated by cloning annealed oligonucleotides oJVZ1291 and oJVZ1292 harboring the mapped *Sphingomonas* Fr1 *nepR* promoter (18) between the HindIII and BamHI sites of pAK501.

***syfp2* reporter constructs.** The *syfp2* reporter plasmid pAK128 used for screening for strong promoters was constructed by PCR amplifcation of *syfp2* from pLM-sYFP2 (18) with oligonucleotides oJVZ743 and oJVZ744 and cloning into pAK127 (see above) via Acc65I and EcoRI sites. Construction of the *rpmB*p mutant promoter library is described in a separate section (see below).

***luxCDABE* reporter constructs.** To construct pQ5-lux, a fragment containing P_{Q5} and *cymR** was subcloned from pQF-lacZ into the luciferase reporter plasmid pLM05 (27) using restriction sites NotI and Acc65I. pQ2148-lux was constructed in several steps. First, P_{syn2} of plasmid pQH was replaced by a fragment containing the META1_2148 promoter generated by oligonucleotide annealing of oJVZ1434 and oJVZ1435 using HpaI and BsrGI restriction sites, giving plasmid pQ2148. Then, as an intermediary step, a XbaI-EcoRI fragment of pAK127lacZ(MCS) containing *lacZ* was inserted between the SpeI and EcoRI sites of pQ2148, generating pQ2148-lacZ. Finally, a fragment containing *cymR** and P_{Q2148} was subcloned from pQ2148-lacZ into pLM05 using NotI and Acc65I sites, giving pQ2148-lux.

Other plasmids. pQH-PhyP was constructed by amplifying *phyP* with primers oJVZ534 and oJVZ1093, digestion with HindIII and Acc65I, and ligation into pQH digested with the same enzymes; in this construct, PhyP is fused to a 3×FLAG tag at its C terminus and the expression of the PhyP-3×FLAG fusion is driven by P_{Q5} and the natural *phyP* ribosome binding site (RBS). pAK200-QPhyP was derived by subcloning a PscI-EcoRI fragment of pQH-PhyP into pAK200 digested with the same enzymes. This plasmid carries the pBBR oriV and is thus compatible with the pAK501 reporter plasmid (pSV1 oriV) and pCM62-based plasmids (IncP oriV). pCM62-PhyR and pCM62-PhyR(D194A) have been described previously (18).

***In silico* analysis of putative promoters of housekeeping genes.** A permanent draft of the *Sphingomonas* Fr1 genome (genome name "*Sphingomonas melonis* FR1") is available through the Integrated Microbial Genomes (IMG) system (35) on the DOE Joint Genome Institute website (http://img.jgi.doe.gov/cgi-bin/w/main.cgi). Upstream regions of putative housekeeping genes (see Table S2 in the supplemental material) were subjected to a motif search by MEME (36) with the following parameters: distribution of motif occurrences, 0 or 1 per sequence; number of different motifs, 15; minimum number of sites, 5; maximum number of sites, 30; minimum motif width, 20; maximum motif width, 50; searching, "given strand only." Putative *rpmB* promoter sequences of sphingomonads were retrieved from the IMG website and aligned using MultAlin (37) using symbol comparison table "DNA" and default parameters.

Screening for and identification of P_{syn2}. A 119-bp region upstream of the *rpmB* (Sphme2DRAFT_0682) open reading frame with randomized nucleotides in the putative −10 and −35 promoter regions was generated by oligonucleotide annealing and extension with Phusion DNA polymerase using oligonucleotides PL28pNTGACN_s and PL28pTANNNGC_as. Products were purified, digested with HindIII and BamHI, cloned into the same sites of pAK128 (a *syfp2* reporter plasmid), and transformed into *E. coli* TOP10. Plasmids were purified from a pool of approximately 10,000 colonies to generate the *rpmB* promoter library. This library was subsequently transformed into *Sphingomonas* Fr1, and approximately 20,000 colonies were screened for high fluorescence using an IVIS Spectrum system (Caliper LifeSciences). A total of 14 colonies showing high fluorescence were picked, and the *rpmB* promoter regions were amplified by colony PCR and sequenced (Microsynth, Switzerland).

Nucleotide sequence accession numbers. Sequences of pAK501 and pQF have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers KF536587 and KF536588, respectively.

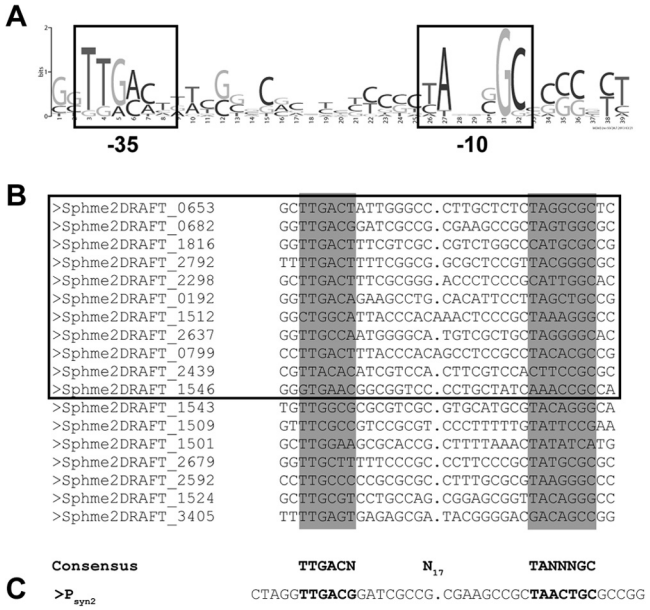

FIG 1 (A) Weblogo output of the motif found by MEME in 11 of 23 query sequences. (B) Alignment of the 11 sequences found by MEME harboring the putative promoter motif (boxed) and additional putative promoters that were aligned manually after reinspection of the promoter regions of the remaining housekeeping genes. Note that the linker length had been adjusted manually by inserting a 1-bp gap in most promoters to better align the −35 and −10 boxes of two promoters. Labels refer to the gene locus tags of the permanent genome draft (http://img.jgi.doe.gov/cgi-bin/w/main.cgi). (C) Sequence of P_{syn2} corresponding to positions −43 to +1 in the original *rpmB* (Sphme2DRAFT_0682) promoter. Note that 1 bp had been inserted in the linker region to align it to the sequences shown in panel B.

RESULTS AND DISCUSSION

Screening for a strong housekeeping promoter. Since high gene expression at full induction for the inducible gene expression system was desired, we first sought to identify strong housekeeping promoters in *Sphingomonas* Fr1. This promoter should constitute a minimal (or core) promoter sequence in order to minimize the risk of additional factors influencing promoter activity. Regions of 99 to 120 bp upstream of 23 putative *Sphingomonas* Fr1 housekeeping genes (see Table S2 in the supplemental material) were subjected to a motif search using the MEME suite (36). The conserved motif TTGACN-N_{17}-TANNNGC resembling the σ^{70}-dependent promoter consensus of *E. coli* (TTGACA-N_{17}-TATAAT) was identified in 11 of 23 putative promoter regions by MEME (Fig. 1A), and 7 additional putative promoters from the original set could be manually aligned to this consensus (Fig. 1B). We focused in the following on one of those promoters, *rpmB*p, which drives expression of the gene encoding ribosomal protein L28 (Sphme2DRAFT_0682).

Alignment of the putative *rpmB*p regions of different sphingomonads showed that the presumed promoter motif is the most conserved part in the *rpmB* upstream region with the putative −35 box TTGACN consensus sequence and the −10 box TAN NNGC consensus sequence (see Fig. S3 in the supplemental material), suggesting that the identified motif is the true promoter of *rpmB*. However, the wild-type *rpmB* promoter was rather weak when tested as a transcriptional fusion to *E. coli lacZ* (data not shown). We reasoned that changing nonconserved nucleotides in

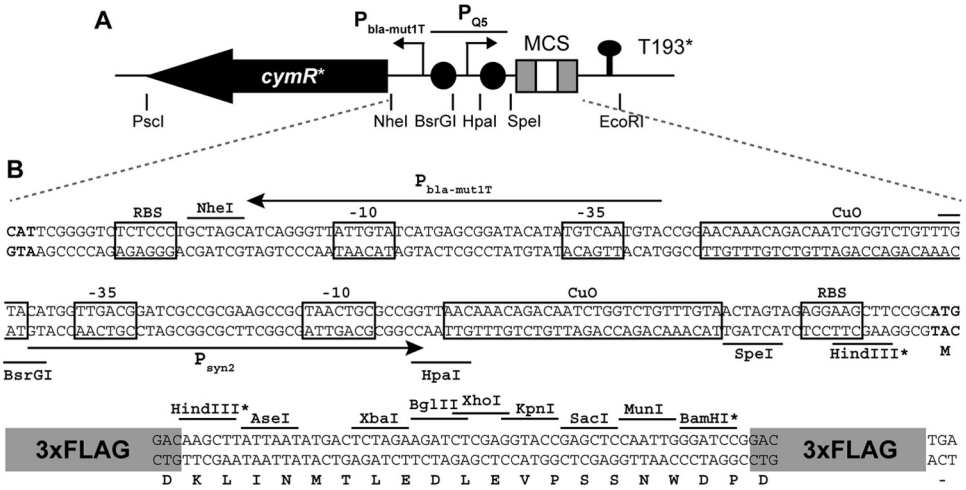

FIG 2 (A) Organization of *cymR**, P_{Q5}, and the multiple-cloning site (MCS) on plasmid pQF (not to scale). Restriction sites flanking important features are indicated. T193* denotes a putative transcriptional terminator derived from *Sphingomonas* Fr1 (see the supplemental material). Gray boxes in the MCS indicate 3×FLAG tags and black circles CuO sites. (B) Nucleotide sequence of the P_{Q5} promoter region and the MCS. *cymR** and MCS start codons are in bold. Ribosome binding sites (RBS), operator sequences (CuO), and −35 and −10 promoter elements are boxed. Core promoter sequences of P_{Q5} (P_{syn2}) and the promoter driving *cymR** expression ($P_{bla\text{-}mut1T}$) are indicated by arrows. Translation of the MCS is indicated below the nucleotide sequence. All restriction sites shown are unique in pQF, except those marked with an asterisk. The 3×FLAG tag (peptide sequence DYKDHDGDYKDHDIDYKDDDDK) is shown schematically.

the −10 and −35 boxes (see above) could generate a stronger promoter and thus screened a library of mutant *rpmB*p promoters in which those nucleotides had been randomized for increased activity (see Materials and Methods). Among the 14 promoters identified in this screen (see Fig. S4 in the supplemental material), five were identical with the core promoter motif TTGACG-N_{17}-TAACTGC, and the corresponding 44-bp fragment ranging from position −43 to position +1 is referred to as P_{syn2} in the following (Fig. 1C).

Construction of a cumate-inducible promoter. In order to render P_{syn2} regulated, we combined it with the control elements of the *Pseudomonas putida* F1 *cym*/*cmt* system, i.e., the transcriptional repressor CymR and its corresponding operator sites (CuO) (17). This system was chosen because the inexpensive inducer cumate is membrane permeative, i.e., does not require any dedicated transporter, and *Sphingomonas* Fr1 apparently cannot metabolize it since its genome lacks homologs of cumate utilization genes. These features should make induction stable with minimal interference with physiology.

Because it is difficult to predict the behavior of a promoter when it is placed under the control of heterologous regulatory elements (38), several arrangements of P_{syn2} with CuO operator sequences and different promoters for *cymR* were tested (data not shown). In the final configuration shown in Fig. 2, the cumate-inducible promoter, referred to as P_{Q5}, consists of P_{syn2} flanked by two CuO operator sequences; expression of a high-GC-content codon-optimized gene for the CymR repressor (*cymR**) is driven divergently by a modified *bla* promoter ($P_{bla\text{-}mut1T}$) in which the −35 box has been changed to TTGACA and the −10 box to TAC AAT. This unit has been assembled together with a multiple cloning site flanked by sequences encoding triple FLAG (3×FLAG) tags into plasmid pCM62 (33), generating plasmid pQF. Details on construction of pQF are given in Fig. S2 and Materials and Methods in the supplemental material.

Characterization of P_{Q5}. To test whether *E. coli lacZ* could be used as a reporter gene for measuring cumate-dependent gene expression, we first determined the endogenous β-galactosidase activity of the *Sphingomonas* Fr1 wild type grown with or without cumate. Irrespective of the cumate concentration, no significant β-galactosidase activity (<5 Miller units) was observed, indicating that the strain had no endogenous β-galactosidase activity under the conditions tested and that *lacZ* is an appropriate reporter gene. Thus, to characterize P_{Q5}-dependent gene expression, a transcriptional fusion of P_{Q5} with *lacZ* (plasmid pQF-lacZ) was used and promoter activity was followed by measuring the β-galactosidase activity of *Sphingomonas* Fr1/pQF-lacZ cultures grown with different cumate concentrations. As seen from the dose-response curve in Fig. 3A, P_{Q5} steady-state activity was dependent on the inducer concentration and showed a maximal induction ratio of >250-fold at 25 and 50 μM cumate (see also Table 2). We noted that at cumate concentrations of >10 μM, growth was somewhat impaired. However, this was likely due to the high expression level of *lacZ* and not to the cumate concentration itself because *Sphingomonas* Fr1 without plasmid pQF-lacZ or carrying another gene, *phyP*, under P_{Q5} control (see below) did not show any growth defect even at 100 μM cumate. In time course experiments, addition of 50 μM cumate to an exponentially growing noninduced culture of the same strain (*Sphingomonas* Fr1/pQF-lacZ) led to a rapid and sustained increase in β-galactosidase activity that was apparent within 10 min after induction and peaked after 3 h (Fig. 3B). The differences in levels of absolute *lacZ* expression between the experiments whose results are shown in Fig. 3A and B were likely due to the different experimental setups (see Materials and Methods). In summary, these results demonstrate that P_{Q5} provides a rapidly responding and tunable system for gene expression in *Sphingomonas* Fr1.

P_{Q5} allows gene depletion analysis. One of the most informative applications of conditional gene expression systems is the study of essential genes by depletion analysis. To test whether this was possible with P_{Q5}, we chose the essential gene *phyP* of *Sphingomonas* Fr1 (18). *phyP* encodes a putative phosphatase of the response regulator PhyR that is a master regulator of the general stress response (GSR) in *Alphaproteobacteria* (39). *phyP* cannot be

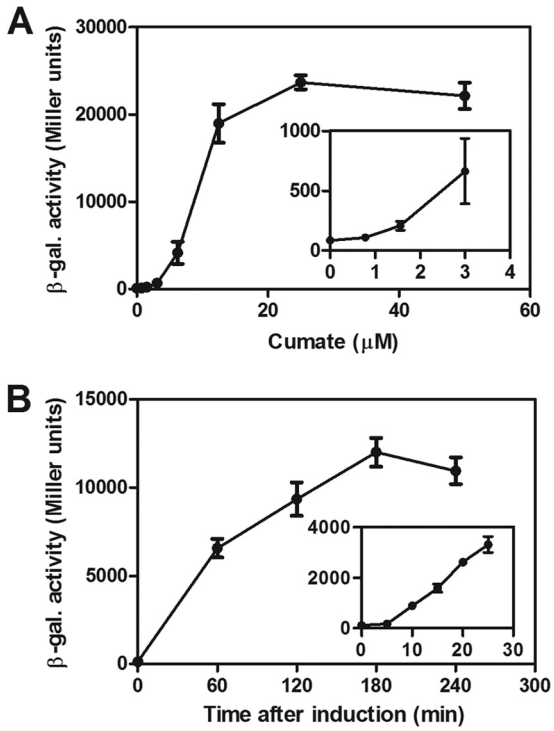

FIG 3 (A) Dose-response curve of P_{Q5} assayed as β-galactosidase (β-gal.) activity of the P_{Q5}-*lacZ*$^+$ transcriptional fusion in the *Sphingomonas* Fr1 wild type (strain JVZ857/pQF-lacZ) grown with different cumate concentrations (no cumate and 2-fold increments from 0.78 to 50 μM cumate). The inset is a closeup of the same dose-response curve illustrating responsiveness to low cumate concentrations. (B) Induction kinetics of P_{Q5} was assayed by adding 50 μM cumate to a noninduced culture of strain JVZ857/pQF-lacZ in the mid-exponential phase and following β-galactosidase activity of P_{Q5}-*lacZ*$^+$ over time. The inset represents a set of independent experiments focusing on the first 25 min after inducer addition. Data represent the means ± standard deviations of the results of three independent biological replicate experiments, each performed with two technical replicates.

TABLE 2 P_{Q5} activity in different *Alphaproteobacteria*

Strain	β-Galactosidase activity (Miller units)[a,c]		Ratio[b,c]
	Without cumate	With cumate	
Sphingomonas sp. strain Fr1 (JVZ857)	87 ± 6.9	24,000 ± 800	270
Sphingomonas aerolata DSM 14751	61 ± 7.0	16,000 ± 1,300	260
Sphingomonas melonis DSM 14444	75 ± 3.6	6,800 ± 1,400	90
Sphingobium indicum DSM 16412	1.2 ± 2.1	1,700 ± 36	1,400
Novosphingobium rosa DSM 7285	28 ± 24	13,000 ± 3,000	480
Caulobacter crescentus CB15	10 ± 3.1	11,000 ± 1,200	1,000
Paracoccus denitrificans JVZ2585	1.8 ± 0.6	8,700 ± 1,300	4,700

[a] Data represent means ± standard deviations of the results of three independent biological replicates.
[b] Data represent ratios of unrounded mean values (induced results/uninduced results).
[c] Values are given with two significant digits.

deleted unless *phyR* is also deleted or replaced by the *phyR*^{D194A} allele encoding a nonphosphorylatable version of PhyR, suggesting that phosphorylation of PhyR is the cause of lethality. Accordingly, it has been proposed that *phyP* is essential because its deletion would lead to overactivation of the GSR (18).

To test this hypothesis, we used a strain deleted for chromosomal *phyP* and *phyR* (strain JVZ1357) (18) complemented in *trans* with a functional PhyP-3×FLAG fusion expressed under the control of P_{Q5} (plasmid pAK200-QPhyP) and *phyR* under the control of its own promoter (plasmid pCM62-PhyR). As a readout for activation of the GSR, this strain also harbored a plasmid carrying the *nepR*p-*lacZ*$^+$ transcriptional fusion (plasmid pAK501-nepRp) in which *lacZ* expression is driven by the previously mapped, PhyR-dependent *nepR* promoter (18). Growth of this strain on LB agar or in liquid LB required the addition of cumate (data not shown), suggesting that in the absence of cumate, PhyP-3×FLAG was depleted below the level required for viability. When grown in liquid culture containing cumate, removal of the inducer by extensive washing and dilution into fresh medium lacking cumate (OD_{600} of 0.025) resulted in growth arrest in the exponential phase (OD_{600} of ca. 2) after 6 to 7 generations; in contrast, when the same culture was diluted into fresh medium containing cumate, growth was normal and continued until it reached the stationary phase (OD_{600} of >10). Western blots confirmed that PhyP-3×FLAG levels were dramatically decreased in the depleted strain compared to the nondepleted strain (Fig. 4A). Decreased levels of PhyP-3×FLAG correlated with increased *nepR*p-*lacZ*$^+$ activity as measured with β-galactosidase assays (Fig. 4B), demonstrating that, indeed, loss of PhyP leads to GSR overactivation. As expected, a control strain expressing nonphosphorylatable PhyR^{D194A} [plasmid pCM62-PhyR(D194A)] instead of wild-type PhyR displayed reduced *nepR*p-*lacZ*$^+$ activity and showed no dependence on cumate with respect to growth (data not shown) and GSR activation (Fig. 4). Similar results were obtained for otherwise isogenic strains lacking pAK501-nepRp (data

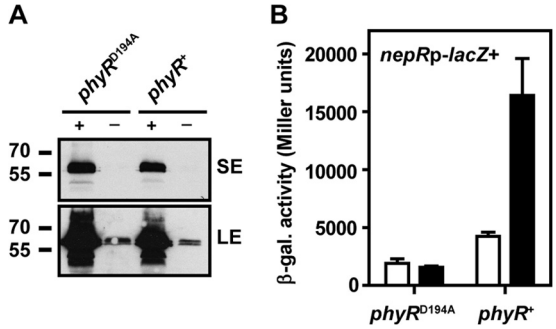

FIG 4 (A) Western blot of the PhyP-3×FLAG-depletion strain harboring the *phyR* wild-type (*phyR*$^+$) or D194A mutant (*phyR*^{D194A}) allele grown with (+) or without (−) cumate (see the main text for details). PhyP-3×FLAG was detected using primary mouse monoclonal anti-FLAG M2 antibodies and secondary goat anti-mouse horseradish peroxidase-coupled IgG antibodies and ECL Western blotting detection reagent. Membranes were exposed to X-ray films for a short exposure of 20 s (SE) as well as a long exposure of 15 min (LE) to visualize residual PhyP-3×FLAG in the depleted strains. Numbers on the left indicate molecular mass markers (in kDa). (B) *nepR*p-*lacZ*$^+$ β-galactosidase activity assayed in the same strains as represented in panel A grown with cumate (white bars) or without cumate (black bars). Three independent biological replicate experiments were performed, and data are displayed as means ± standard deviations. Results in panel A are representative of the results of three replicate experiments.

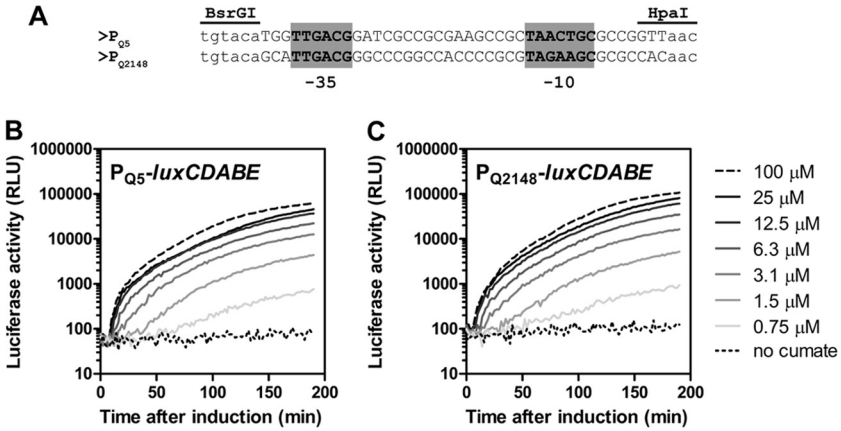

FIG 5 (A) Alignment of core promoter regions in P_{Q5} and P_{Q2148}. The sequence between BsrGI and HpaI is the only difference between the two reporter plasmids pQ5-lux and pQ2148-lux. (B and C) Time courses of cumate-dependent induction of P_{Q5}-*luxCDABE* and P_{Q2148}-*luxCDABE* reporter constructs. Cumate concentrations used are indicated on the right. Luciferase activity is given in arbitrary units (RLU [relative luminescence units]). Data are from a single experiment that is representative of three independent biological replicates.

not shown), indicating that the observed phenotypes were not an artifact of *lacZ* expression. Altogether, these results verify that *phyR* is epistatic to *phyP* (18) and support the idea that *phyP* is essential because it prevents detrimental overactivation of the general stress response through prevention of constitutive PhyR phosphorylation. More generally, they demonstrate the utility of P_{Q5} for studying essential gene function.

Use of P_{Q5} in other *Alphaproteobacteria*. To broaden the potential use of our system, we first tested it in a number of other sphingomonads using plasmid pQF-lacZ. As seen from Table 1, in all cases high induction ratios of approximately 100- to 1,000-fold between noninduced and cumate-induced cultures were obtained as measured by β-galactosidase assays. In the cases where the induction ratio was even higher than in *Sphingomonas* Fr1, this stemmed from a tighter repression rather than from higher promoter activity. Indeed, *Sphingomonas* Fr1 displayed the highest absolute β-galactosidase activity of all strains tested. In general, we speculate that strain-specific differences in induction ratios and absolute β-galactosidase activity can be attributed to different strengths of P_{Q5} and/or the promoter driving expression of CymR, $P_{bla\text{-mut1T}}$, although we cannot exclude the possibility that there are other factors involved.

We next tested P_{Q5} in other alphaproteobacterial species representing the orders *Caulobacterales* (*C. crescentus*), *Rhodobacterales* (*P. denitrificans*), and *Rhizobiales* (*M. extorquens* and *A. tumefaciens*). For all species except *M. extorquens*, P_{Q5} activity was monitored using plasmid pQF-lacZ; for *M. extorquens*, a fragment containing P_{Q5} and *cymR** was subcloned in a previously described bacterial luciferase (*luxCDABE*) transcriptional reporter plasmid (27). Whereas P_{Q5} was essentially inactive in *A. tumefaciens* (<2 Miller units with or without cumate; data not shown), the promoter was inducible in the other species tested with induction ratios of approximately 1,000- to 5,000-fold (Table 2 and Fig. 5B). As discussed above for sphingomonads, these high induction ratios are due to tighter repression rather than to higher activity of P_{Q5}. Similar to results obtained from *Sphingomonas* Fr1, in *M. extorquens* the response to inducer addition was rapid (apparent after approximately 10 min) and expression was tunable over a wide range of inducer concentrations. We also tested a derivative of P_{Q5}, P_{Q2148}, in which the P_{syn2} core promoter had been replaced by a minimal promoter from *M. extorquens* AM1 (Fig. 5A) naturally driving expression of *rpsL* (META1_2148). This promoter was chosen because it shows a clear single transcriptional start site and is active in both methanol- and succinate-grown bacteria according to transcriptome sequencing (RNA-Seq) data (A. Francez-Charlot and J. A. Vorholt, unpublished data). Similar to P_{Q5}, P_{Q2148} was rapidly and highly inducible and showed approximately 2-fold-higher activity than P_{Q5} at the highest cumate concentration tested (Fig. 5C).

Altogether, these results indicate that P_{Q5} can be used as a versatile inducible expression system in *Sphingomonadales* and also in members of other orders of the *Alphaproteobacteria*. In addition, results with P_{Q2148} suggest that P_{syn2} may be easily exchanged for other species-specific promoters to achieve, for example, tighter repression or higher expression levels in cases where P_{syn2} does not provide the desired characteristics.

A comprehensive set of cumate-inducible plasmids. In addition to pQF, a number of P_{Q5}-based plasmids have been constructed (see Table 1) that carry, in addition to the carboxy-terminal 3×FLAG tag, different amino-terminal epitope tags (HA, *myc*, fluorescent proteins) commonly used for immunodetection or protein localization studies. In all constructs, the original reading frame displayed in Fig. 2B is maintained. Besides conventional cloning vectors, for most plasmids derivatives compatible with the Gateway cloning system (Invitrogen) have also been constructed. Last, we have constructed a P_{Q5}-based plasmid carrying the broad-host-range pBBR origins of replication and transfer that may be used in bacteria for which pCM62-based plasmids are not suitable, such as *Bradyrhizobium japonicum* (H.-M. Fischer, personal communication). Plasmids are available through the Addgene plasmid repository (www.addgene.org).

Conclusions. We developed a tight and highly inducible gene expression system for sphingomonads, for which this is the first such system described, and demonstrated its successful application for depletion analysis. This system adds a valuable part to the genetic toolbox of sphingomonads, and we anticipate that it will be of broad use for both basic and applied research. In addition, the system was demonstrated to work in a number of other *Alp-*

haproteobacteria in which it may be used to complement already existing inducible gene expression systems (3, 6, 40–42).

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