Synthetic vanillate-regulated promoter for graded gene expression in Sphingomonas

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Regulated promoters are an important basic genetic tool allowing, for example, gene-dosage and gene depletion studies. We have previously described a cumate-inducible promoter (PQ5) that is functional in diverse Alphaproteobacteria. This promoter has been engineered by combining a synthetic minimal promoter, Psyn2, and operator sites and the repressor of the Pseudomonas putida F1 cym/cmt system. In the present study, we engineered a vanillate-regulated promoter using Psyn2 and the regulatory elements of the Caulobacter crescentus vanR-vanAB system. We show that the resulting promoter, which we called PV10, responds rapidly to the inducer vanillate with an induction ratio of about two orders of magnitude in Sphingomonas melonis Fr1. In contrast to the switch-like behavior of PQ5, PV10 shows a linear dose-response curve at intermediate vanillate concentrations, allowing graded gene expression. PV10 is functionally compatible with and independent of PQ5 and cumate, and vice versa, suggesting that both systems can be used simultaneously.

Regulated promoters are an essential genetic tool for studying bacterial physiology as well as for synthetic biology and industrial applications. For example, they provide means to study essential gene function by depletion analysis and to conditionally express toxic genes. Although multiple of such systems are usually available for a particular model organism, they are often underdeveloped for many non-model organisms, and even constitutive (minimal) promoters are not always available. We have previously described a synthetic approach that allowed us to develop a cumate-inducible expression system that is functional in diverse Alphaproteobacteria, including several sphingomonads, Methylobacterium extorquens and Caulobacter crescentus. In this approach, we first identified a minimal promoter consensus based on alignment of several Sphingomonas melonis Fr1 housekeeping gene promoters, then screened for mutations in non-conserved positions in the −10 element of this minimal promoter for increased expression, and finally combined this mutant promoter (termed Psyn2) with operator sequences and the repressor of the heterologous cym/cmt system, which naturally controls cumate and cymene catabolism in Pseudomonas putida F1. This engineered promoter (called PQ5) was cumate-regulated and resulted in induction ratios of two- to three orders of magnitude in the different organisms tested. For sphingomonads, a group of bacteria with great potential in bioremediation, industrial biotechnology and plant protection, this was the first dedicated inducible gene expression system described to date.

Because certain applications call for more than one inducible promoter, we wondered whether it would be possible to combine Psyn2 with yet other heterologous operator sequences and repressors so it would be regulated by a stimulus other than cumate. Here we describe such a promoter, termed PV10, that combines Psyn2 with the vanO operator sequences of the vanAB operon and the vanillate-responsive repressor VanR naturally involved in vanillate degradation in the freshwater bacterium Caulobacter crescentus. Our experiments demonstrate that PV10 is vanillate-inducible and shows a high dynamic range of gene expression in S. melonis Fr1, and that PV10 and PQ5 are orthogonal, with each promoter only responding to its designated stimulus, vanillate and cumate, respectively.

Results

Design of PV10. A scheme of the organization of vanR and PV10 is shown in Fig. 1a. The design rationale is described in the following. In Caulobacter crescentus, vanAB is divergently transcribed from vanR, encoding the GntR-type transcriptional repressor of the vanAB operon. The vanAB promoter (vanABp) has been mapped and shows −35 (TTGACG) and −10 (AAGATT) boxes indicative of a housekeeping, σ70-dependent promoter.
vanR1 was placed under control of the constitutive promoter \( P_{\text{syn2}} \) (see below), we reasoned that, at the same time, this configuration would allow tight repression. In fact, \( P_{\text{syn2}} \) is very strong and such strong expression is probably not needed in most cases where expression levels in the physiological range are desired. vanR was placed under control of the constitutive promoter \( P_{\text{syn2}} \), which we have used before to drive expression of the \( P_{Q5} \) repressor CynrR\(^+\). The basic plasmid for vanillate-regulated gene expression is \( pVH \), a derivative of the broad-host-range plasmid \( pCM6211 \), in which downstream of \( PV10 \) a multiple cloning site (MCS) on plasmid \( pVH \).

Characterization of \( PV10 \). In order to characterize \( PV10 \)-dependent gene expression in \( S. melonis \) Fr1, \( PV10 \) was transcriptionally fused to \( E. coli \) lacZ (plasmid \( pVH\)-lacZ), and \( PV10\text{-lac}^+ \) activity was followed in strain JVZ857/\( pVH\)-lacZ grown with different vanillate concentrations using \( \beta \)-galactosidase assays. As shown in Fig. 2a, \( PV10\text{-lac}^+ \) activity was dependent on the inducer concentration, showing a low basal activity (48 +/- 7 Miller units) without vanillate and high activity (3600 +/- 280 Miller units) at the highest vanillate concentration tested (250 \( \mu \)M). This represents a maximal induction ratio of 74-fold. In the range of 6.5 to 74 \( \mu \)M vanillate, the dose-response curve was essentially linear (\( R^2 = 0.994 \)), indicating that \( PV10 \) allows graded gene expression, rather than showing switch-like behavior. To follow induction dynamics, JVZ857/\( pVH\)-lacZ was grown to mid-exponential phase, \( PV10 \) was induced by addition of 250 \( \mu \)M vanillate and \( PV10\text{-lac}^+ \) activity was repeatedly measured over 4.5 h. As seen from Fig. 2b, the response is rapid and sustained. To see whether \( PV10 \) could be used simultaneously with the previously characterized cume-inducible promoter \( P_{O5} \), we tested both promoters for their response to vanillate and/or cumate. Like for \( PV10 \), \( P_{O5} \) activity was followed using strain JVZ857 harboring a plasmid-borne \( P_{O5}\text{-lac}^+ \) transcriptional fusion described previously. As shown in Fig. 2c, \( PV10 \) did not respond to cumate, and cumate had no effect on the induction by vanillate. Similarly, \( P_{O5} \) showed no response to vanillate, and vanillate did not affect the capacity of \( P_{O5} \) to respond to cumate.

In summary, our result demonstrate that \( PV10 \) is a rapidly responding, vanillate-inducible promoter in \( S. melonis \) Fr1. Furthermore, they suggest that \( PV10 \) is orthogonal to the previously described cume-inducible promoter \( P_{O5} \), and both promoters can be used simultaneously without interference.

Other vanillate-regulated expression plasmids. In addition to the basic vanillate-inducible expression plasmid \( pVH \), we have also constructed two destination plasmids for Gateway cloning, \( pVHD \) and \( pVYD \), that allow C-terminal fusions to the HA tag and SYFP2, respectively, and three plasmids for N-terminal fusions to mCherry, SYFP2, and mTq2 (\( pVCY \), \( pVCTq \), and \( pVCTq \), respectively). Plasmids will be made available from Addgene (www.addgene.org).
ducibly and carefully regulated. In contrast, compared to PV10, PQ5 shows both higher absolute expression and relative induction upon inducer addition\(^1\), making PQ5 more suitable when very high gene expression levels are desired, e.g. for overexpression studies. Thus, the two promoters are complementary and one or the other might be better suited depending on the biological question. Importantly, because there is no crosstalk between the CymR*/PV10 and VanR/PV10 systems, i.e. they are orthogonal, both can be used simultaneously, allowing more sophisticated genetic studies of bacterial physiology.

**Methods**

**Strains and growth conditions.** *Escherichia coli* TOP10 (Invitrogen) or “cold survival” (Invitrogen) were used for cloning and routinely grown in LB-Lennox at 37°C. *S. melonis* Fr1 wild-type strain JVZ875\(^2\) was grown in LB-Lennox at 28°C. Plasmids were transformed in *S. melonis* by electroporation as previously described\(^11\). When appropriate, antibiotics were added at the following concentrations: tetracycline (10 μg/ml) and chloramphenicol (34 μg/ml). Vanillate (4-hydroxy-3-methoxybenzoic acid) was purchased from Sigma-Aldrich (Cat. No. W398802-25G) and dissolved in ethanol to give 1000× stock solutions for final concentrations indicated in the figure legends. Cmumate stocks were prepared as described previously\(^1\). For “no vanillate” and “no cumamate” controls, cultures were mock treated with 0.1% (vol/vol) ethanol.

**Plasmid construction.** Standard molecular biology protocols were followed\(^4\). Plasmid DNA polymerase for PCR and restriction enzymes were from Thermo Scientific, and T4 DNA ligase was from New England Biolabs. pVH was constructed using Phusion DNA polymerase for PCR and restriction enzymes were from Thermo Scientific, and T4 DNA ligase was from New England Biolabs. pVH-lacZ was constructed from pAK127lacZ(MCS)\(^1\) using XbaI/Acc65I. pVYD was obtained by subcloning a PsiI/SpeI fragment of pVH containing cymR\(^*\) and cloned in pQH1 via PciI/NheI, replacing cymR\(^*\). Then, a synthetic fragment (Eurofins, MWG Operon, Germany) containing P\(_{bla-moot}\)\(^2\) for vanH expression and P\(_{cum}\) for vanillate-regulated expression was amplified using primers PV10_F (5'-ATT TTC TAG ATG TTT TCA GTC GGC GCG AAT GC-3') and PV10_R (5'-ATT TTC TAG CAT CAG GGT TAT TG-3') and cloned between NheI/HindIII. For dose-response curves and cross-induction experiments, Reporter assays. Promoter activities were measured essentially as described previously\(^1\). For dose-response curves and cross-induction experiments, *S. melonis* Fr1 carrying pHV-lacZ or pQ\(_{pQ5}\)-lacZ were grown in LB-Lennox containing different concentrations of vanillate and/or cumamate overnight to mid-exponential phase and β-galactosidase activity was measured according to Miller\(^11\). To follow induction kinetics, *S. melonis* carrying pHV-lacZ was grown to mid-exponential phase and induced by the addition of 250 μM vanillate, and β-galactosidase was measured at different time points. All results are presented as mean ± SD of three biological replicates. Linear regression analysis to evaluate linearity of the dose-response curve was performed in GraphPad Prism 5 (version 5.04, Graphpad Software Inc., USA).

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Acknowledgments
We thank Martin Thanbichler, Dominic Esposito, Joachim Goedhart and Theodorus W. J. Gadella for plasmids. This work was supported by Swiss National Science Foundation (SNF) grant 31003B-152835.

Author contributions
A.K. designed and performed experiments. A.K., J.A.V. and A.F.-C. conceived the project and wrote the manuscript.

Additional information
Competing financial interests: The authors declare no competing financial interests.
How to cite this article: Kaczmarczyk, A., Vorholt, J.A. & Francez-Charlot, A. Synthetic vanillate-regulated promoter for graded gene expression in Sphingomonas. Sci. Rep. 4, 6453; DOI:10.1038/srep06453 (2014).