SYNTHETIC BIOLOGY

Synthetic Biology, 2021, **6(1)**, 1–11
DOI: https://doi.org/10.1093/synbio/ysab030
Advance access publication date: 17 September 2021
Research Article

Standardization of inducer-activated broad host range expression modules: debugging and refactoring an alkane-responsive AlkS/P_{alkB} device

Alejandro Arce-Rodríguez[1,2], Ilaria Benedetti[1], José Manuel Borrero-de Acuña[2], Rafael Silva-Rocha[1,†], and Víctor de Lorenzo[1,*]

[1]Systems Biology Department, Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco, Madrid, Spain
[2]Institute of Microbiology, Technische Universität Braunschweig, Braunschweig, Germany
[†]Present address Ribeirão Preto Medical School (FMRP), University of São Paulo Ribeirão Preto 14049-900 Brazil.
*Corresponding author: E-mail: vdlorenzo@cnb.csic.es

Abstract

Although inducible heterologous expression systems have been available since the birth of recombinant DNA technology, the diversity of genetic devices and their coming together in the corresponding vectors often result in a lack of reproducibility and interoperability. In an effort to increase the predictability of expression of genes of interest in a variety of possible bacterial hosts, we propose a composition standard for debugging and reassembling all regulatory parts that participate in the performance of such devices. As a case study, we addressed the *n*-octane and dicyclopropyl ketone-inducible P_{alkB} promoter of the alkane biodegradation pOCT plasmid of *Pseudomonas putida*. The standardized expression module included an edited *alkS* transcription factor divergently expressed and separated from P_{alkB} by a synthetic buffer segment. The DNA sequence of the *alkS* gene was modified to alleviate the catabolite repression exerted by several carbon and nitrogen sources through the Crc/Hfq complex of some hosts. The P_{alkB} promoter and the *alkS* variants were then formatted as Standard European Vector Architecture cargoes, and their activity in *P. putida* was quantified with fluorescent and luminescent reporters. Despite considerable editing of the DNA sequences involved, the thereby refactored module basically kept the functioning parameters of the original configuration. The same qualities were inspected when the system was passed to *Escherichia coli* and *Pseudomonas aeruginosa*. We argue that the application of the compositional standard thereby implemented in the AlkS/P_{alkB} module to other promoter/regulator pairs will enable more complex genetic programming in non-model bacteria.

Key words: SEVA; AlkS; *Pseudomonas*; Crc; Hfq; cytometry; noise

1. Introduction

Expression of genes of interest in hosts (e.g. bacteria) different from their native origin and triggered by an external inducer is one of the basis of modern biotechnology (1). A large number of genetic devices to this end have been developed over the years following the pioneering use of the Isopropyl β-D-thiogalactoside (IPTG)-inducible *lac* promoter for expression in *Escherichia coli* (2). Typical modules (3) encompass one promoter that is activated or repressed by a cognate transcription factor (TF; either an activator or a repressor). The regulator then binds the target sequence or changes its activity in a fashion dependent on exposure of cells to a physical (e.g. heat and light) or chemical (e.g. inducer) signal. This basic scheme is the template for a large number of popular expression vectors based on a suite of regulator/promoter pairs (1, 4, 5). While they have been useful when the issue was to express one or few genes at a time in one host, the onset of synthetic biology in recent years has multiplied the need of multiple, regulatable promoters endowed with specific parameters and as independent as possible of the physiological state of the host (5). One step in that direction was the creation in 2013 of the so-called Standard European Vector Architecture (SEVA; 6), which comprises a large number of standardized antibiotic marker genes, broad host range origins or replication and functional cargoes, aimed at simplifying genetic programming of a wide variety of bacteria of industrial and environmental interest (7). One type of such cargoes includes expression modules. While the boundaries of the corresponding DNA segment within the plasmid vector or transposon vector frame are well defined in the SEVA format, the organization of the regulatory elements inside the cargo has not been standardized yet. In this work, we propose a specific arrangement for such inducible modules that attempts to preserve the inducibility of the TF/promoter pairs usable for heterologous expression while simplifying the native regulation of the cognate systems.

As a case study for such standardization effort, we have chosen the regulatory node that controls the expression of the *alk* genes for biodegradation of octane borne by the OCT plasmid of the soil bacterium *Pseudomonas putida* GPo1 (8). In its native arrangement,

Submitted: 26 December 2020; **Received (in revised form):** 8 September 2021; **Accepted:** 15 September 2021

two gene clusters are involved in the process. *alkST* encodes the transcriptional regulator of the pathway (AlkS, inducible by *n*-octane) and AlkT (a component of alkane hydroxylase). The second *alkBFGHJKL* cluster determines the rest of the activities, which are expressed from the upstream AlkS-dependent promoter P_{alkB} (9, 10). Once excised from its native context and assembled adjacent to each other in a single DNA segment, the *alkS*/P_{alkB} pair has been used to develop a number of biosensors for alkanes as well as heterologous expression vectors (11–15). The last is facilitated by the use of the gratuitous, soluble inducer dicyclopropyl ketone (DCPK). However, this simple rearrangement of functional segments with *alkS* and P_{alkB} does not eliminate the regulatory complexity embodied in them. *alkS* is transcribed through two promoters, P_{alkS1} and P_{alkS2}, which are negatively and positively regulated, respectively, by AlkS. In addition, translation of AlkS is subject to the post-transcriptional control of the Crc/Hfq complex, which introduces an additional regulatory layer (i.e. catabolite repression) in the system (16). Finally, the activity of AlkS seems to be influenced also by the cytochrome terminal oxidase Cyo (17). Such a regulatory density allows the extant system to compute many physiological signals other than the mere presence of pathway substrates. Yet, the same intricacy is also a nuisance for the predictability of the gene expression module. In the work presented below, we have constructed a refactored AlkS/P_{alkB} device in which any known regulatory control—other than induction by DCPK—has been eliminated and replaced by non-regulatory DNA sequences, following a defined composition standard. As shown below, the resulting inducible expression module keeps the functionality of the wild-type arrangement with very similar performance parameters. On this basis, we advocate the general application of the compositional standard used to assemble this device for increasing the reproducibility and interoperability of a large number of other devices made with regulatory parts mined from the genomes of environmental bacteria.

2. Materials and methods

2.1 Strains, plasmids and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 1. All *P. putida* specimens were derived from the reference isolate *P. putida* KT2440. *E. coli* strains DH5α and CC118 were used as hosts for maintenance of plasmids and used as recipients of the AlkS/P_{alkB} system as indicated in each case. Unless indicated otherwise, cells were grown at either 30°C (*P. putida*) or 37°C (*E. coli* and *P. aeruginosa*) in rich LB medium (18) amended, where necessary, with 100 μg/ml ampicillin (Ap), 50 μg/ml streptomycin (Sm) and 50 μg/ml chloramphenicol (Cm), to retain plasmids as necessary. In the case of *P. aeruginosa* PAO1 cultures, the concentration of Sm was increased to 500 μg/ml. For

Table 1. Strains and plasmids used in this work

Strain/plasmid	Description/relevant characteristics	Reference
E. coli strains		
CC118	F-, Δ(*ara-leu*)*7697*, *araD139*, Δ(*lac*)*X74*, *phoA*Δ*20*, *galE*, *galK*, *thi*, *rpsE*, *rpoB*, *argE*(*Am*), *recA1*	(49)
DH5α	F-, *supE44*, Δ*lacU169*, (*ϕ80 lacZDM15*), *hsdR17*, (*rk-mk+*), *recA1*, *endA1*, *thi1*, *gyrA*, *relA*	(50)
HB101	Sm^R, $hsdR^-$ M^+, *pro*, *leu*, *thi*, *recA*	(18)
P. putida strains		
KT2440	Prototrophic, wild-type strain derived from *P. putida* mt-2 without pWW0 plasmid	(51)
KT2440 *crc*::Gm	*P. putida* KT2440 with the chromosomal *crc* gene interrupted with a Gm^R cassette	(16)
P. aeruginosa strain		
PAO1	Prototrophic, wild-type reference strain	(52)
Plasmids		
pRK600	Cm^R; *oriV* ColE1, tra^+ mob^+ of RK2; helper plasmid for mobilization in tripartite conjugations	(53)
pMA	Cloning vector for synthetic DNA	GeneArt[a]
pAlkS3	Ap^R; pMA cloning vector bearing the optimized sequence of *alkS* gene	This work
pBAM1	Mini-Tn5 suicide delivery vector, source of P_{neo} promoter	(26)
pSEVA429 *crc*$^+$	Sm^R, oriRK2, *oriT*; pSEVA421-derivative carrying an $alkS^{ED}$/P_{alkB} expression system. The gene *alkS* is edited for SEVA-incompatible restriction sites, but encodes the wild-type primary amino acid sequence of the AlkS protein and keeps the Crc/Hfq-binding sequence in the cognate transcript	This work
pSEVA429[b]	Sm^R, oriRK2, oriT; pSEVA 421-derivative carrying the $alkS^{CR}$/P_{alkB} expression system. *alkS* sequence is same as $alkS^{ED}$ (Supplementary Figure S1) but 5′-end edited for removing the Crc/Hfq binding sequence of the corresponding mRNA	This work
pSEVA421	Sm^R, *oriV* RK2, *oriT*, standard MCS	(6)
pSEVA426	Sm^R, *oriV* RK2, oriT, *luxCDABE* reporter system	(6)
pSEVA429 → *luxCDABE*	Sm^R, oriRK2, oriT; pSEVA 429 cloned with the *luxCDABE* reporter system	This work
pSEVA427	Sm^R, *oriV* RK2, oriT, green fluorescent protein (GFP) reporter system	(6)
pSEVA429 *crc*$^+$ → GFP	pSEVA429 *crc*$^+$ with a promoterless *GFP* gene as a transcriptional reporter	This work
pSEVA429 *crc*$^+$ → *luxCDABE*	pSEVA429 *crc*$^+$ with a promoterless *luxCDABE* operon as a transcriptional reporter	This work
pSEVA429 → GFP	pSEVA429 with a promoterless *GFP* gene as a transcriptional reporter	This work
pJAMA30	Ap^R, *oriV* ColE1; native P_{alkST}-*alkST*/P_{alkB} expression system driving the transcription of *GFP tir*. This segment is flanked by NotI sites	(12)
pAR*alkS*	Sm^R, *oriV* RK2, *oriT*; pSEVA421 cloned with the NotI fragment from pJAMA30 carrying the native alkane/DCPK-responsive reporter system	This work

[a] https://www.thermofisher.com/content/dam/LifeTech/Documents/geneart/geneart-vector-map.pdf.
[b] Complete DNA sequence available in the SEVA database (http://seva-plasmids.com/).

solid media preparation, LB medium was supplemented with 1.5% (w/v) Bacto Agar (Pronadisa). Where indicated, the expression of P_{alkB} promoter was induced by the addition of (DCPK: Sigma, 95% purity) in solid and liquid media at the concentrations indicated.

2.2 Recombinant DNA techniques

General methods for DNA manipulation were performed with standard protocols described elsewhere (18). The amplification of DNA fragments by polymerase chain reaction (PCR) was implemented in 50-μl reactions containing ∼100 ng of genomic DNA or 10 ng of plasmid as template, 0.25 mM dNTPs, 25 pmol of each primer and 1 U of GoTaq DNA polymerase (Promega). Reactions were run by an initial denaturalization (5 min, 94°C) followed by 30 cycles of denaturalization (1 min, 94°C), annealing (1 min, 58°C–64°C), extension (1–3 min at 72°C) and final extension (10 min, 72°C). PCR products were purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and, when required, digested with restriction enzymes purchased from New England Biolabs. Plasmid DNA was isolated by means of the Wizard® Plus SV Minipreps DNA Purification system (Promega). *E. coli* cells were transformed with plasmids with the $CaCl_2$ method (18). In the case of *P. putida* and *P. aeruginosa*, plasmids were introduced by either conjugative triparental mating using the *E. coli* HB101 (pRK600) as helper strain (19) or electroporation of cells previously washed with 300 mM sucrose and concentrated in the same solution (20).

2.3 Construction of the P_{alkB}/AlkS expression modules compatible with the SEVA

In order to create an expression system that could fit the SEVA plasmid platform (6, 7), the gene *alkS* from *P. putida* GPo1 was edited to remove incompatible restriction sites (*alkS*^{ED}). The modified sequence (Supplementary Figure S1) was entirely synthesized by GeneArt/Thermo Fisher (Waltham, Massachusetts) and delivered as an insert in the pMA vector that was called pAlkS3. Both the AlkS regulatory protein and the P_{alkB} promoter were then arrayed with the other DNA segments indicated in Figure 1. The P_{alkB} promoter was PCR-amplified with primers aaPalkB1-F (5′**AGCGGATAACAATTTCACACAGGA**CGTGTTTTTCCAGCAGACGAC3′) and aaPalkB1-R (5′ATGACCTAGGCTCTCGACATCTTAAACCTGAGC3′), using as template genomic DNA from *P. putida* PBS4 (21). The P_{neo} promoter was also amplified by PCR with oligonucleotides aaPKm-NcoI1-F (5′TAGAACCATGGTTTTTCCTCCTTATAAAG3′; underlined sequence shows NcoI recognition site) and aaM13-R24-rev (5′ TCCTGTGTGAAATTGTTATCCGCT 3′) from pBAM1 (Table 1). The sequence of primer aaM13-R24-rev is complementary to the 5′ end of primer aaPalkB1-F (bold characters), allowing the assembly of P_{neo} with P_{alkB} by SOEing PCR (22). Insertion of the resulting product into the NcoI/AvrII restriction sites of a pSEVA frame gave rise to expression vector pSEVA429 *crc*$^+$ (Supplementary Figure S2).

2.4 Removal of the Crc-binding site in the *alkS* gene

The Crc-binding site in the 5′ end of the *alkS* gene in pSEVA429 *crc*$^+$ was modified with PCR-based site-directed mutagenesis. Briefly, the first 570 bp of *alkS* were amplified with the oligonucleotides 5-alkSmut-NcoI (5′GCGCCCATGGGCATGAA**G**AT**C**AA**G**AT**C**AT**C**AACAATGAT TTCCCGGTTGCCAAGATCG3′) and 3-alk Smut-XhoI (5′AGCGCCTGCAAGTTTAAGCC3′) using the pAlkS3 as template. The forward primer contains the recognition sequence of NcoI enzyme (underlined) and also six single-nucleotide mutations that eliminate the Crc site from *alkS* (in bold characters). The PCR fragment was then digested with NcoI and XhoI (the last within the *alkS* sequence), and the resulting 406 bp fragment was recloned into the same sites of pSEVA429 *crc*$^+$. In order to follow the SEVA nomenclature, this final expression vector was named pSEVA429 (i.e. Sm resistant, carrying a RK2 origin of replication and AlkS/P_{alkB} expression cargo; Supplementary Figure S2).

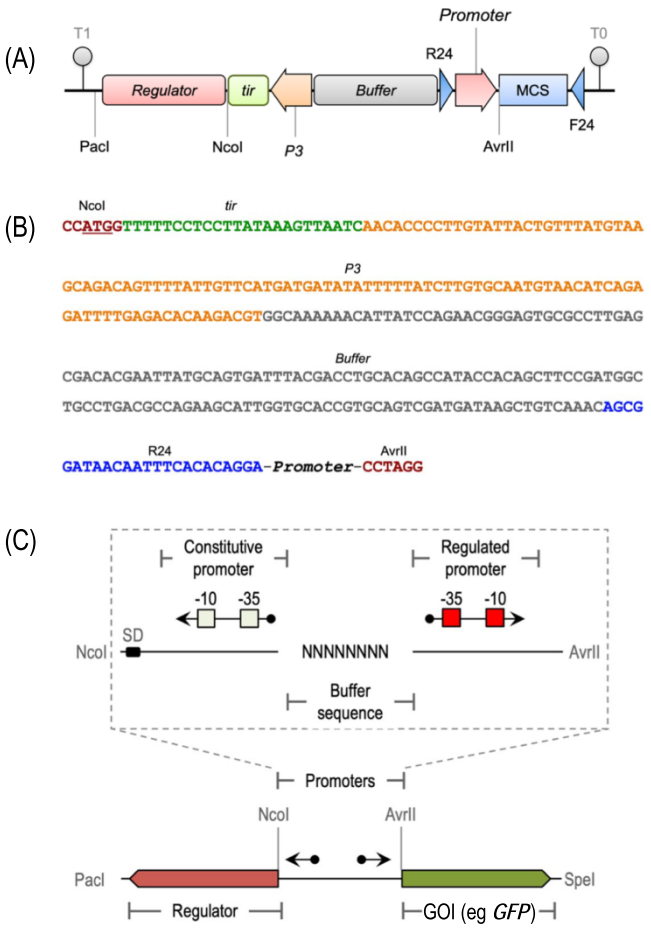

Figure 1. Organization of standardized inducible expression modules. (A) Arrangement of DNA portion, boundaries and their roles as the frame for inserting genes for inducer-responsive transcriptional regulators and cognate target promoters. (B) Blowup of the standardized DNA sequence that acts and the scaffold for the rest of the functional parts. (C) Configuration of reporter plasmids (with *GFP* or any other gene of interest, GOI) used in this work. Note constant and variable segments.

2.5 Parameterization of the AlkS/P_{alkB} module

For generating constructs that report transcriptional activity as a fluorescent emission, the *GFP tir* gene of pSEVA427 was cloned into plasmids pSEVA429 *crc*$^+$ and pSEVA429 as a HindIII/SpeI insert to generate plasmids pSEVA429 *crc*$^+$ → GFP and pSEVA429 → GFP, respectively. As a control, we excised the NotI fragment from pJAMA30 containing the original *alkST* genes of pOCT transcribed by their own P_{alkST} promoter and, in divergent orientation, the P_{alkB} driving the expression of the *GFP tir* gene (12) (Supplementary Figure S3). This ∼6.7-kb fragment was cloned into the NotI site of pSEVA421 to generate the control vector pAR*alkS*. The three *GFP*

tir reporter vectors described above, as well as the promoter-less pSEVA427, were transferred into wild-type *P. putida* KT2440 cells and into its isogenic *crc*::Gm derivative. Plasmid-bearing strains were then grown in LB medium at 30°C until mid-exponential phase, the cultures supplemented with 0.05% v/v DCPK and fluorescent emission for the next 6 h followed with a CyFlow® Cube 8 (Partec GmbH, Münster, Germany) flow cytometer. GFP was excited at 488 nm, and the fluorescence signal was recovered with a 536/40 BP filter. Note that monitoring performance of other expression devices in *E. coli* (23) and *P. putida* (24) has been done for much longer. Yet, we entertain that the most relevant characteristics of the promoter(s) become manifest when cells are still growing exponentially. This is because transcriptional activity relies only on the intrinsic characteristics of the engineered modules before entering stationary phase and become affected by physiological responses to nutrient starvation. The resulting data were processed using FlowJo v. 9.6.2 software (FlowJo LLC, Ashland, OR, USA) as follows. Forward and side scatter density plots were used to identify the bacterial cell population of interest and to exclude debris. Data were recorded for at least 10 000 bacteria, and the GFP specific output was calculated using population mean fluorescence intensities. For the parameterization of the edited AlkS/P_{alkB} module in other bacterial hosts, *E. coli* CC118 and *P. aeruginosa* PAO1 carrying pSEVA429 → GFP were grown in LB medium at 37°C until mid-exponential phase, induced with 0.05% v/v DCPK and analyzed by flow cytometry as described above. For monitoring activity of the AlkS/P_{alkB} module at a population level, plasmids pSEVA429 *crc*$^+$ and pSEVA429 were inserted with the promoterless luminescent reporter *luxCDABE* operon excised from pSEVA426 as a HindIII/SpeI fragment. This originated plasmids pSEVA429 *crc*$^+$ → *luxCDABE* and pSEVA429 → *luxCDABE*, respectively. As with the GFP counterparts before, these plasmids were passed to the wild-type *P. putida* host. For measuring light emission under various DCPK concentrations, cells grown overnight in LB were diluted in same medium, placed in a Microtest™ 96-well assay plate (BD Falcon), regrown to mid-exponential phase, added with the inducer and luminescence recorded after 4 h.

2.6 Promoter kinetics and statistical analyses

Promoter induction kinetics were calculated from fluorescence mean values at each timepoint using linear regression analysis. These analyses were used to obtain the slope values shown in Table 2. Promoter inducibility was calculated from the data collected 6 h postinduction, using the following equation: (signal ON population–signal promoterless pSEVA427)/(signal OFF population–signal promoterless pSEVA427). In the sporadic cases where the signal of cells carrying the promoterless pSEVA427 was slightly higher than the signal of OFF cells, this parameter was omitted from the equation. Statistical differences of the maximum inducibility values between the strains harboring the different AlkS/P_{alkB} modules were calculated by means of a two-tailed Student's *t*-test. All statistical analyses were performed with GraphPad Prism v.9.0.1 (GraphPad Software, San Diego, California USA).

Table 2. Promoter expression parameters of the different AlkS/P_{alkB} modules constructed in this study

Strain/plasmid	Fluorescence induction slope (m)[a]	Promoter inducibility (mean fluorescence fold change)[b]
P. putida KT2440		
pARAlkS (control)	247.6 ± 6.79	49.37 ± 4.59
pSEVA429 *crc*$^+$ → GFP	190 ± 4.87	80.53 ± 0.61***
pSEVA429 → GFP	187.90 ± 4.93	80.98 ± 14.70*
P. putida KT2440 *crc*::Gm		
pARAlkS (control)	105.3 ± 4.18	20.22 ± 10.01
pSEVA429 *crc*$^+$ → GFP	135.2 ± 4.58	59.01 ± 2.86**
pSEVA429 → GFP	139.3 ± 9.738	81.36 ± 6.08***
E. coli CC118		
pSEVA429 → GFP	11.72 ± 0.82	7.07 ± 1.74
P. aeruginosa PAO1		
pSEVA429 → GFP	69.98 ± 3.75	27.24 ± 8.15

[a]The slope value was calculated using the linear regression of the induction kinetics as explained in Section 2.6.
[b]Promoter inducibility was calculated 6 h after the first timepoint, as explained in Section 2.6. The statistical differences between strains harboring the pARAlkS control plasmid versus the *crc*$^+$/*crc*$^-$ standardized versions were calculated by the Student's *t*-test.
*P-values: <0.05; **<0.01; ***<0.005.

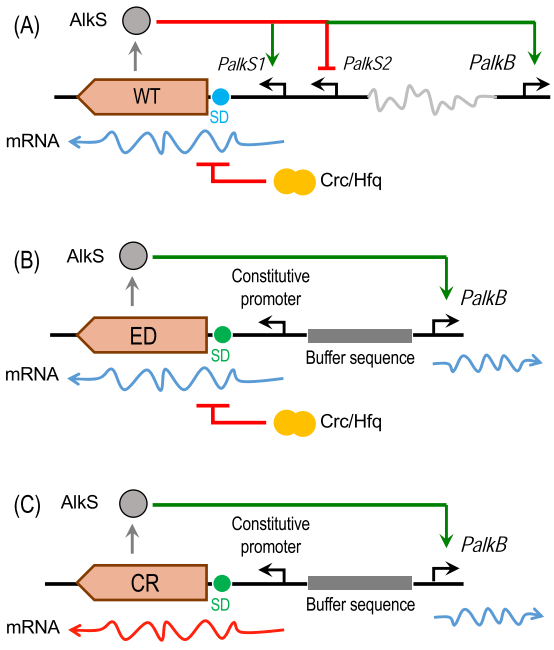

Figure 2. Functional segments of the naturally occurring and standardized AlkS/P_{alkB} device. (A) Native organization of regulatory parts in the original context of pOCT plasmid. Note complex arrangement of transcriptional and post-transcriptional signals (e.g. inhibition of *alkS* mRNA translation by the Crc/Hfq complex) and dual effect of inducer-activated AlkS on self-promoters P_{alkS1} and P_{alkS2}. (B) Constitution of the standardized AlkS P_{alkB} expression module. The wild-type DNA sequence of the regulator has been edited to remove restriction sites incompatible with the SEVA standard but keeping the same primary amino acid sequence (AlkS^{ED}). *alkS* expression is now under the control of a heterologous Shine-Dalgarno (SD) sequence and a constitutive promoter (P_{neo}, see text) and closer to target, divergent promoter P_{alkB}—albeit separated by the buffer sequence indicated in Figure 1B. (C) AlkS/P_{alkB} device bearing a regulator variant devoid of its Crc-binding site. As before, this change in the DNA of *alkS* keeps the primary amino acid sequence of the protein identical to the wild-type regulator.

3. Results and discussion

3.1 A compositional standard for engineering inducer-dependent heterologous gene expression

Figure 1A sketches the organization of the inducible expression module proposed in this work for SEVA cargoes (6) aimed at heterologous expression of genes of interest in a variety of bacterial hosts. First, the standard asks for constitutive expression of the gene(s) encoding the effector-responsive regulator. In their natural context, TFs are often subject to a degree of self-regulation, either positive or negative (25). This introduces an excessive complexity that the arrangement shown in Figure 1A mitigates if not entirely eliminates. The standardized sequence that holds the various functional parts of the device (Figure 1B) is assembled as follows. The source of transcription of the signal-responsive TF gene is the 106 bp minimal promoter P3/P_{neo} that drives expression of the kanamycin resistance gene of pBAM1 (26). Following this promoter, default translation efficiency is also fixed by means of a 24-bp translation initiation region (TIR) retrieved from the *GFP* variant borne by pGreenTIR plasmid (27). This is an unusual ribosome-binding sequence known to act as a translational enhancer that is expected to curb the expression noise that could originate from a poor translation. The segment for constitutive expression/translation of the TF is followed upstream by a 150-bp segment of DNA with no known function or activity retrieved from the $lacI^q$–P_{trc} expression system of plasmid pTrcA (28). This sequence has no known regulatory elements and it functions as a buffer region to ease mutually negative supercoiling that could stem from transcription of divergent promoters (29). As shown in Figure 1A, the adjacent piece of DNA is the one that bears the promoter targeted by the inducible regulator and orientated opposite in respect to the sequences for expression of the TF gene. The specific DNA sequence of this promoter changes from case to case, but it should by default be accommodated within a segment of not more than 100 bp. The 3′ of this promoter sequence is bound by an AvrII site, which links this segment to the start of the SEVA polylinker (6). Note that two sites at the boundary buffer sequence/promoter and at the end of the MCS have target sequences for oligonucleotides R24 and F24 (6). These are used for amplification and analyses of possible inserts. Once the gene encoding the inducer-responsive TF is placed in this arrangement as a NcoI (overlapping the leading ATG)–PacI DNA fragment, the whole expression module becomes inserted in the SEVA frame as a

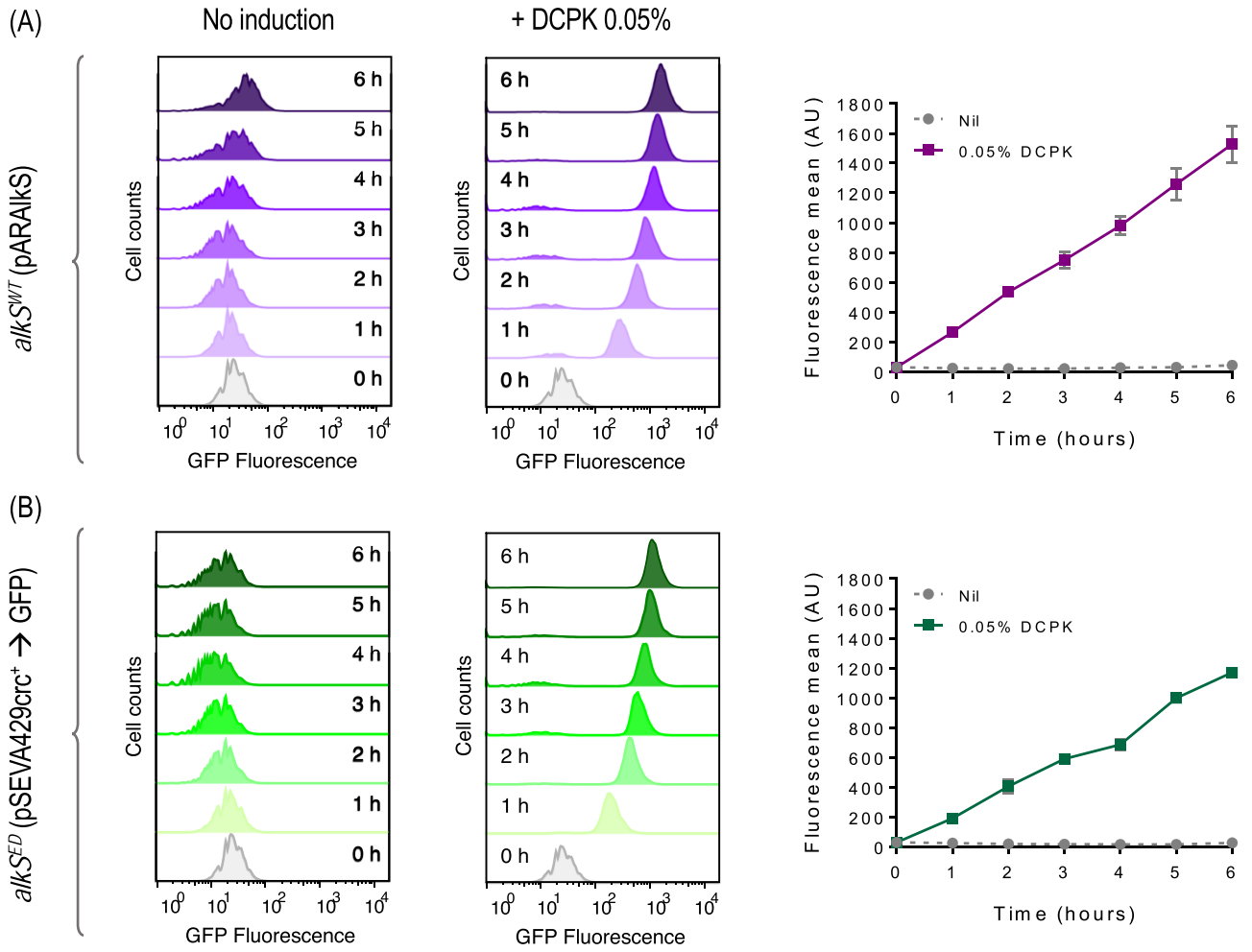

Figure 3. Transcriptional activity of AlkS/P_{alkB} node before and after standardization as a SEVA cargo. *P. putida* transformants with either (A) pARalkS (wild-type *alk*S) or (B) pSEVA429 crc^+ → GFP (edited *alk*S variant ED) were grown in LB until mid-exponential phase and treated or not with 0.05% AlkS effector DCPK. Fluorescent emission was then measured in a cytometer for the next 6 h as explained in Section 2. The right panels show the mean GFP fluorescence signals with their respective standard deviation calculated from the populations showed in (A) and (B). The experiments were carried out in biological triplicates.

PacI–AvrII addition (Figure 1C), shielded both upstream and downstream by transcriptional terminators contributed by the vector structure and ready to be inserted with any gene of interest cloned in the corresponding polylinker.

3.2 Reshaping the *alkS* and P_{alkB} pair as an inducible expression device

As a case study of formatting a naturally occurring inducible promoter into a standardized expression cargo, we picked the regulatory elements that control transcription of the *alk* genes of the OCT plasmid of *P. putida* GPo1 (8, 9). The choice was motivated by the exemplary regulatory density of the native system that includes transcriptional and post-transcriptional control layers checking expression of *alkS* (16, 30; Figure 2A). This provided an archetypal case to inspect the impact of the simplified formatting explained above on the behavior of the resulting expression module. To this end, we first edited the wild-type DNA sequence of the regulator to eliminate restriction sites incompatible with the SEVA rules while preserving the amino acid sequence. The resulting DNA segment was then produced as a 5′ → 3′ 2668-bp NcoI–PacI DNA fragment. The complete list of nucleotide changes entered in the sequence of this *alkS* variant (that we term $alkS^{ED}$ for edited) is compiled in Supplementary Figure S1. The $alkS^{ED}$ variant was then coupled to the spacer shown in Figure 1B, which was added with the wild-type 90-bp sequence of the target P_{alkB} promoter (see Section 2). The resulting cargo was subsequently placed in plasmid pSEVA421 (7) as a PacI–AvrII insert, resulting in expression vector pSEVA429 *crc*+ (Table 1; Supplementary Figure S2). For parameterization of the activity of the thereby refactored expression device, the construct was added with the promoterless *GFP tir* gene of pSEVA427 (7) downstream of P_{alkB} (Section 2) resulting in reporter plasmid pSEVA429 *crc*+ → GFP. To have a reference of the wild-type expression device borne by plasmid pOCT with native regulatory parts, we excised the ∼6.7-kb NotI fragment of plasmid pJAMA30 (12) containing *alkST* and a divergent P_{alkB} promoter upstream of a *GFP* reporter (Supplementary Figure S3). This DNA was then inserted into the same plasmid frame of pSEVA421 used for the edited devices, thereby originating control plasmid pAR*alkS* (Table 1; note that the *alkS* variant in this case is the original wild-type $alkS^{WT}$ as shown in Supplementary Figure S1). Once equivalent constructs with $alkS^{WT}$ (pAR*alkS*) and $alkS^{ED}$ (pSEVA429 *crc*$^+$ → GFP) were constructed, we were able to evaluate the effect of the standardization of the architecture of the regulatory node on transcriptional performance.

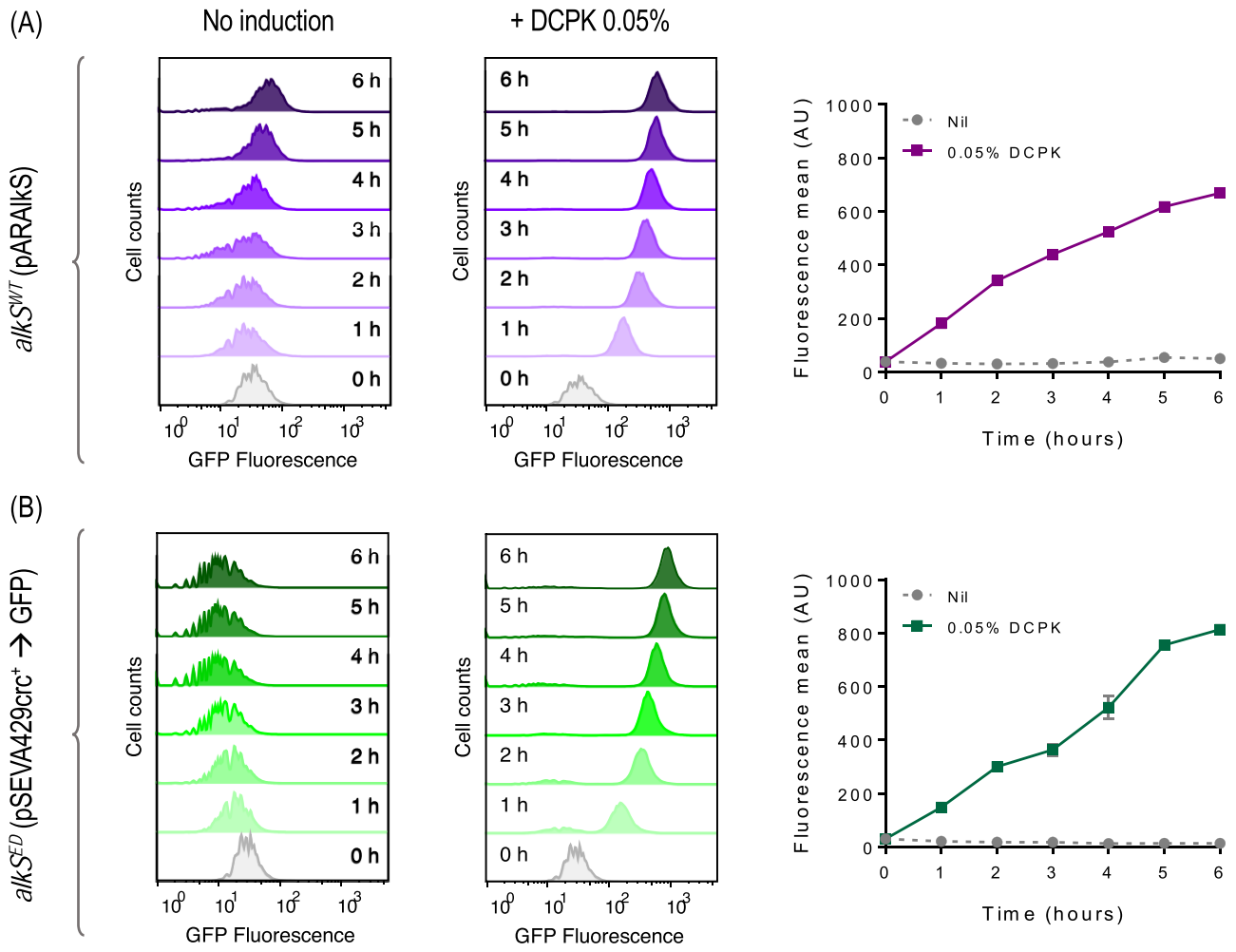

Figure 4. Effect of Crc on performance of the standardized AlkS/P_{alkB} module. Plasmids (A) pARalkS ($alkS^{WT}$) and (B) pSEVA429*crc*$^+$ → GFP ($alkS^{ED}$) were placed in a *crc*::Gm mutant of *P. putida* KT2440, the transformants grown in LB and treated as indicated in the legend of Figure 3. The right panels show the mean GFP fluorescence signals with their respective standard deviation calculated from the populations shown in (A) and (B). The experiments were carried out in biological triplicates.

3.3 The effect of formatting AlkS/P_{alkB} on the performance of the expression system

In the first series of experiments, we compared the behavior of the AlkS/P_{alkB} pair assembled with all the native regulatory systems borne by the pOCT plasmid (Figure 2A) and recreated in plasmid pAR*alk*S versus that of the same regulatory system arranged with the composition standard of Figure 1 as implemented in pSEVA429 *crc*$^{+}$ → GFP. Differences included a synthetic expression segment for transcription and translation of the regulator and an upstream buffer DNA sequence that was followed by the divergent AlkS target promoter P_{alkB} as sketched in Figure 2B. *P. putida* KT2440 was transformed with each of these two plasmids, and transformants were grown in LB medium with Sm and fluorescent readout followed as explained in Section 2. The cytometry results of these experiments are shown in Figure 3. Inspection of the resulting graphs revealed some features of both the native and the formatted system that are worth considering for handling the expression device. First, whether formatted or not, both P_{alkB} systems had a comparable dynamic range (i.e. absolute promoter output; 31) and a similar quasi-linear induction kinetics (differences were ∼20%). Second, both devices displayed an increasingly sharp unimodal expression pattern (32) along the induction period with low noise and limited cell-to-cell variation at the time of full induction. Finally, a closer inspection of cells not added with DCPK revealed a degree of non-induced basal transcription (in particular in the wild-type, non-edited configuration; Supplementary Figure S4) that spontaneously increased with growth (Figure 3A). Although such a basal level is not high (it remains within the same order of magnitude than the baseline expression), it has to be taken into account when expression of toxic proteins or coupling with other devices is pursued (33). These results confirmed the correct functioning of the expression device following the reassembly of its DNA parts. Yet, we wondered about the less predictable effects of other physiological inputs that operate on the AlkS/P_{alkB} in its native context.

3.4 Effect of Crc on the formatted and not-formatted AlkS/P_{alkB} device

Ideally, for engineering-reliable genetic devices, regulatory parts should deliver their function in a context-independent manner (34). Such a context includes not only genomic locations (35) and availability of resources (36) but also physiological signals (37, 38) that orchestrate the induction hierarchy. One of these is catabolite repression (30), which in the case of *P. putida* operates through a complex interplay between the proteins Crc and Hfq with small RNAs to inhibit translation of messenger RNAs (mRNAs) of target

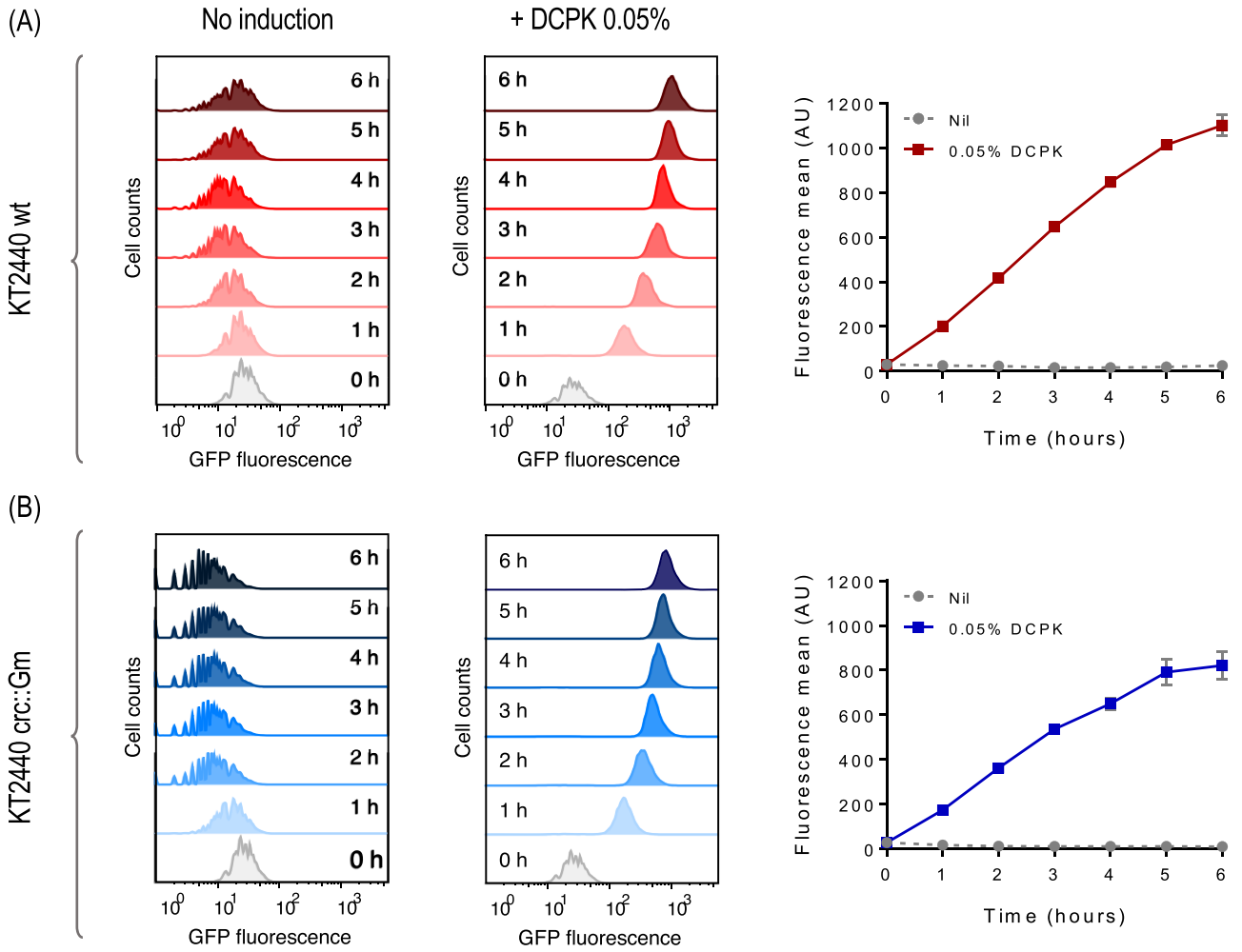

Figure 5. Behavior of an *alkS* gene variant devoid of the Crc/Hfq-binding site to its mRNA. Plasmid pSEVA429 → GFP (with the AlkS/P_{alkB} device bearing the regulator gene devoid of the Crc-binding site in its mRNA) was transformed into *crc*$^{+}$ (A) and crc^{-} (B) strains of *P. putida*. Transformants were grown in LB and treated as indicated in the legend of Figure 3. The right panels of the figure show the mean GFP fluorescence signals with their respective standard deviation that were calculated from the populations shown in (A) and (B). The experiments were carried out in biological triplicates.

genes (16). AlkS is subject to such post-transcriptional regulation when placed in *P. putida* but not in *E. coli* (39). In order to calibrate the effect of such a control layer and whether it was kept or not in the standardized construct, we run the experiments shown in Figure 4. To this end, pAR*alkS* and pSEVA429 *crc*$^+$ → GFP were placed in an isogenic *crc*::Gm mutant of *P. putida* (Table 1) known to be blind to catabolite repression caused by many of the components of LB medium (40, 41). As shown in Figure 4, the overall behavior of the standardized and non-standardized expression devices remained very similar in dynamic range and inducibility, although their expression kinetics was slower as compared to the data shown in Figure 3.

The data above were welcome results, as refactoring of an existing regulatory node with a different architecture often results in devices that perform significantly worse than the naturally occurring setup (42, 43). But the question still remained of whether we could erase altogether the effect of *crc* on the performance of the AlkS/P_{alkB} module, not by moving to a different genetic background but by rewriting the DNA sequence of the regulator. To address this, an additional *alkS* derivative was synthesized in which the Hfq/Crc-binding site of the corresponding mRNA was edited as described in Section 2 and Supplementary Figure S1. These changes replace the original codons by synonym triplets in a way that keeps the amino acid sequence of the AlkS protein but breaks the target site for Hfq/Crc within the cognate mRNA. As before, the resulting DNA (that bears the variant hereafter called *alkS*^{CR} for Crc-free) was formatted as a NcoI–PacI fragment and coupled to the spacer shown in Figure 1B and the P_{alkB} promoter. The resulting cargo was subsequently placed in the frame of plasmid pSEVA421 as PacI–AvrII insert, resulting in expression vector pSEVA429 (Supplementary Figure S2). For the sake of comparing its performance, a promoterless *GFP* gene identical to that of pAR*alkS* and pSEVA429 *crc*$^+$ → GFP was added to pSEVA429, thereby generating pSEVA429 → GFP. This *alkS*^{CR}-containing plasmid was then placed in isogenic *crc*$^+$ and *crc*$^-$ strains of *P. putida*, and the readout of the fluorescent reporters followed in LB medium with or without DCPK induction as before. As shown in Figure 5, removal of the *crc* site from the *alkS* sequence had only a moderate effect on the performance of the expression device, as the induction patterns were quite similar when pSEVA429 → GFP was placed in isogenic *P. putida* strains with or without the factor.

To gain some insight into this apparently minor influence of removing the Crc site in *alkS*, we constructed additional derivatives of pSEVA429 *crc*$^+$ and pSEVA429 with a promoterless *luxCDABE* operon. This luminescent reporter is considerably more sensitive than GFP (44) and therefore a better proxy of transcriptional output from P_{alkB} at a population level. The new construct was placed in *P. putida* KT2440 and the cognate transformants grown in LB with different inducer concentrations. In this case (Figure 6), the overall effect of removing the Crc site from the *alkS* mRNA became more evident. As shown in Figure 6, the construct with *alkS*^{CR} produced luminescence levels approximately twice as high those as the same with *alkS*^{ED}. This accredited the role of Crc on *alkS* expression and its improvement with the edited gene, although we cannot rule out that factors other than the transcriptional regulator can also influence physiological control.

3.5 Interoperability of the *alkS*^{CR}/*PalkB* device in other bacteria

Once the expression module engineered in pSEVA429 was characterized in *P. putida*, we next examined its performance in *E. coli* and *P. aeruginosa* (Table 1). Note that the first host lacks altogether

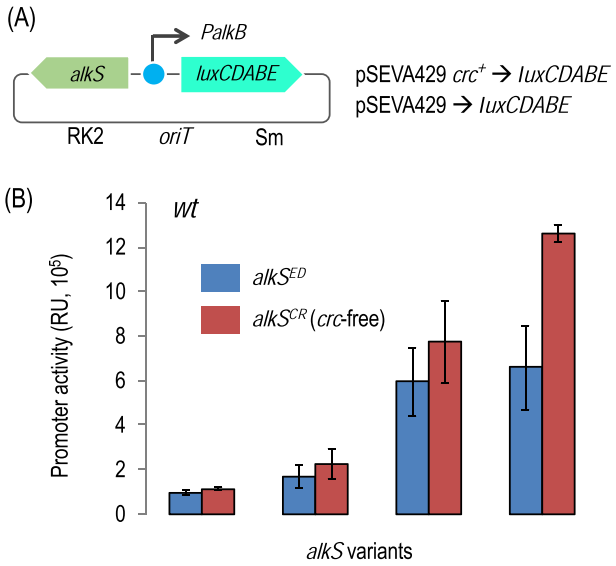

Figure 6. Influence of different inducer concentrations in *P. putida* populations bearing the AlkS/P_{alkB} module with the regulator gene with or without the Crc-binding site. Plasmids pSEVA429 *crc*$^+$ → *luxCDABE* (*alkS*^{ED}, with Crc-binding site) and pSEVA429 → *luxCDABE* (*alkS*^{CR}, without Crc site) were transformed into *P. putida* KT2440, grown in LB until mid-exponential phase and added with the DCPK concentrations indicated in each case. Luminescent emission was then recorded after 4 h as a proxy of transcriptional activity as explained in Section 2. (A) Sketch, not to scale, of functional segments in reporter plasmids. (B) Readout of reporter constructs borne by *P. putida*. Data shown for three technical replicates.

the Crc/Hfq-based system of catabolic repression that is typical of the Pseudomonads (34), while the second is a close—but by no means identical—relative of *P. putida*. For the experiments, *E. coli* CC118 and *P. aeruginosa* PAO1 were transformed with pSEVA429 → GFP bearing the edited the *alkS*^{CR}/*PalkB* device described above, grown and induced with DCPK as before. The results are shown in Figure 7. While the gross inducibility pattern was kept in either case, the parameters changed significantly, especially when *E. coli* was used as the host of the construct (Figure 7A). In this case, the inducibility and the dynamic range of the promoter were worse than *P. putida* with the same plasmid (Figure 5A), and the expression pattern was noisy—as compared to the basically monomodal display of the same device in the original host. In contrast, when the host of pSEVA429 GFP was *P. aeruginosa* (Figure 7B) and despite a conspicuous increase of phenotypic diversity, the expression module behaved within the same parameter set than that observed using *P. putida* as the host (Figure 5A). This was not a surprise given the physiological and regulatory similarity between the two *Pseudomonas* strains used.

4. Conclusions

In this work, we have used the inducer-dependent and AlkS-mediated activation of the P_{alkB} promoter of the pOCT plasmid as an example of the roadmap that could be followed for reshaping a naturally occurring regulatory node into a standardized device for engineering heterologous expression (45). As with any standard, there is an arbitrary but still reasonable and scientifically justifiable choice of a given composition rule (46). The one we propose in this work is summarized in Figure 1 and explained in detail above. As is also the case of other standards, this particular choice will certainly limit flexibility but will foster interoperability,

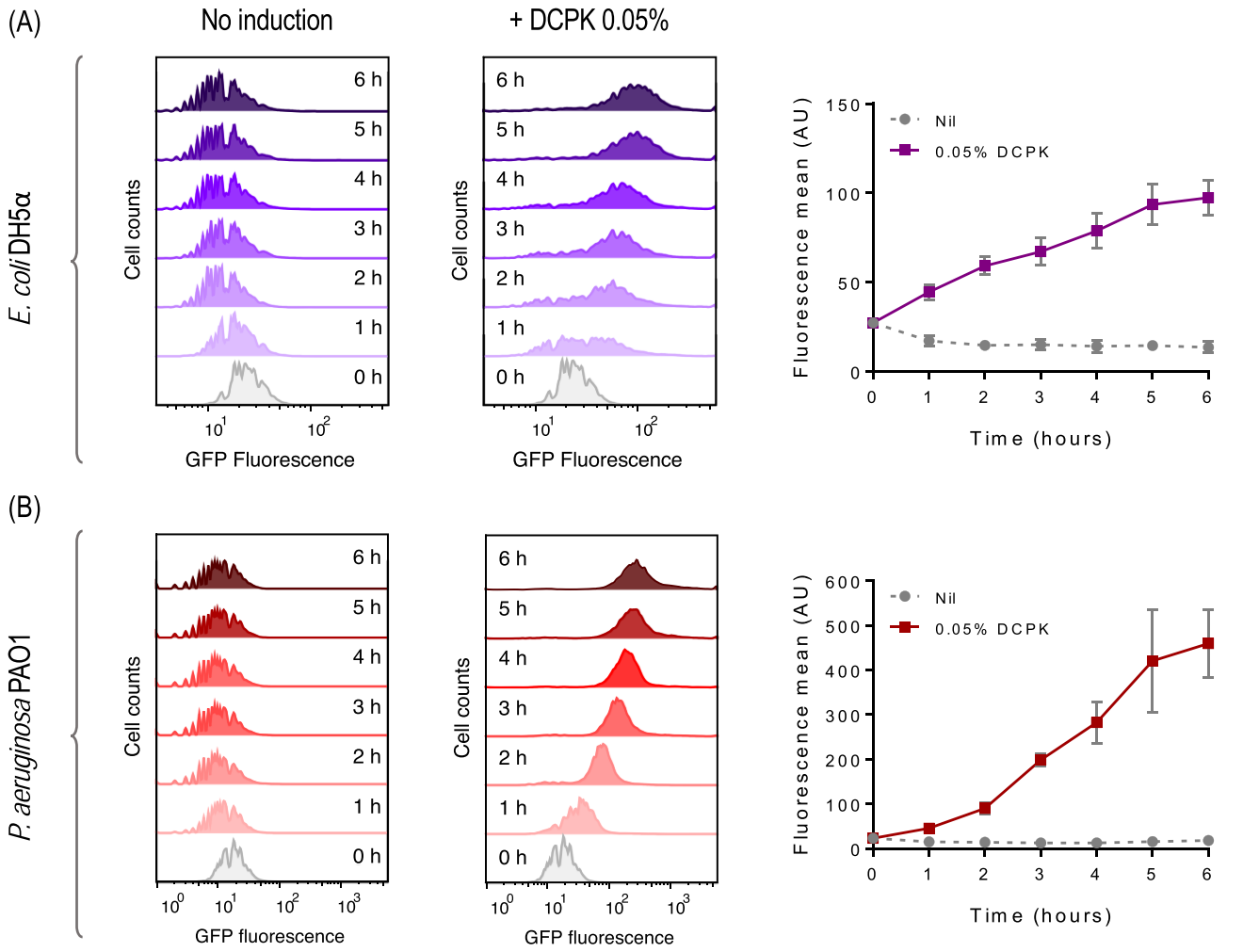

Figure 7. Behavior of the *alkS*^{CR} gene variant devoid of the Crc/Hfq-binding site in other bacterial species. Plasmid pSEVA429 → GFP was transformed into (A) *E. coli* DH5α or (B) *P. aeruginosa* PAO1. Transformants were grown in LB and treated as indicated in the legend of Figure 3. The right panels of the figure show the mean GFP fluorescence signals with their respective standard deviation that were calculated from the populations shown in (A) and (B). Each experiment was carried out in biological triplicates.

parameterization and comparative metrology (47, 48). The work above exemplifies how the same device, still after formatting, may go through successive, improved versions of the same functional DNA segments even if a prefixed arrangement is kept constant. In the cases examined above, we can consider plasmids pAR*alkS* and pSEVA429 *crc*$^{+}$ as beta versions of what we propose to be an standardized AlkS/P_{alkB} expression device apt for inclusion as a cargo in the SEVA collection: pSEVA429 (Supplementary Figure S2). A comparative summary of the more relevant properties of the key constructs with the *alkS*^{CR}/*PalkB* module used in this work in the hosts of interest is shown in Table 2. Despite the considerable edits of the regulatory parts involved and their relative position within the same DNA segment, it is noteworthy that the basic properties were kept in the standardized version. Note however that—as shown above—there is still room for improvement, and it is likely that other versions will follow, an issue that is contemplated in the updated nomenclature of the SEVA collection (7). For instance, the system could be refactored to make it more digital (e.g. lowering its basal expression (33)), making it more independent of physiological control. But, in the meantime, pSEVA429 is an altogether standardized off-the-shelf expression vector with a large number of benefits, including the possibility of comparing faithfully its performance with other expression modules that follow the same arrangement. We ultimately expect such standardization to ease the engineering of complex systems and encourage other genetic tool developers to follow suit.

Supplementary data

Supplementary data are available at SYNBIO Online.

Data availability

All materials described in this article are available upon reasonable request.

Funding

SETH (RTI2018-095584-B-C42) (MINECO/FEDER); SyCoLiM (ERA-COBIOTECH 2018-PCI2019-111859-2) Projects of the Spanish Ministry of Science and Innovation; MADONNA (H2020-FET-OPEN-RIA-2017-1-766975); BioRoboost (H2020-NMBP-BIO-CSA-2018-820699); SynBio4Flav (H2020-NMBP-TR-IND/H2020-NMBP-BIO-2018-814650); MIX-UP (MIX-UP H2020-BIO-CN-2019-870294)

Contracts of the European Union; InGEMICS-CM (S2017/BMD-3691) Project of the Comunidad de Madrid-European Structural and Investment Funds (FSE, FECER).

Acknowledgments

Authors are indebted to Fernando Rojo and Renata Moreno for strains and valuable materials.

Conflict of interest statement. None declared.

References

1. Terpe,K. (2006) Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotech.*, **72**, 211–222.
2. Browning,D.F., Godfrey,R.E., Richards,K.L., Robinson,C. and Busby,S.J.W. (2019) Exploitation of the *Escherichia coli lac* operon promoter for controlled recombinant protein production. *Biochem. Soc. Trans.*, **47**, 755–763.
3. Fernandez-López,R., Ruiz,R., de la Cruz,F. and Moncalián,G. (2015) Transcription factor-based biosensors enlightened by the analyte. *Front Microbiol.*, **6**, 648.
4. Marschall,L., Sagmeister,P. and Herwig,C. (2017) Tunable recombinant protein expression in *E. coli:* promoter systems and genetic constraints. *Appl. Microbiol. Biotech.*, **101**, 501–512.
5. Martínez-García,E. and de Lorenzo,V. (2017) Molecular tools and emerging strategies for deep genetic/genomic refactoring of *Pseudomonas*. *Curr. Opin. Biotechnol.*, **47**, 120–132.
6. Silva-Rocha,R., Martinez-Garcia,E., Calles,B., Chavarria,M., Arce-Rodriguez,A., de las Heras,A., Paez-Espino,A.D., Durante-Rodriguez,G., Kim,J., Nikel,P.I. *et al.* (2013) The Standard European Vector Architecture (SEVA): a coherent platform for the analysis and deployment of complex prokaryotic phenotypes. *Nucleic Acids Res.*, **41**, D666–D675.
7. Martínez-García,E., Goñi-Moreno,A., Bartley,B., McLaughlin,J., Sánchez-Sampedro,L., Del Pozo,H.P., Hernández,C.P., Marletta,A.S., De Lucrezia,D., Sánchez-Fernández,G. *et al.* (2020) SEVA 3.0: an update of the Standard European Vector Architecture for enabling portability of genetic constructs among diverse bacterial hosts. *Nucleic Acids Res.*, **48**, 3395.
8. Rojo,F. (2009) Degradation of alkanes by bacteria. *Environ. Microbiol.*, **11**, 2477–2490.
9. van Beilen,J.B., Panke,S., Lucchini,S., Franchini,A.G., Röthlisberger,M. and Witholt,B. (2001) Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. *Microbiology*, **147**, 1621–1630.
10. Canosa,I., Sánchez-Romero,J.M., Yuste,L. and Rojo,F. (2000) A positive feedback mechanism controls expression of AlkS, the transcriptional regulator of the *Pseudomonas oleovorans* alkane degradation pathway. *Mol. Microbiol.*, **35**, 791–799.
11. Sticher,P., Jaspers,M.C., Stemmler,K., Harms,H., Zehnder,A.J. and van der Meer,J.R. (1997) Development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples. *Appl. Environ. Microbiol.*, **63**, 4053.
12. Jaspers,M.C., Meier,C., Zehnder,A.J., Harms,H. and van der Meer,J.R. (2001) Measuring mass transfer processes of octane with the help of an *alkS alkB:: gfp*-tagged *Escherichia coli*. *Env. Microbiol.*, **3**, 512–524.
13. Makart,S., Heinemann,M. and Panke,S. (2007) Characterization of the AlkS/*PalkB*-expression system as an efficient tool for the production of recombinant proteins in *Escherichia coli* fed-batch fermentations. *Biotechnol. Bioeng.*, **96**, 326–336.
14. Reed,B., Blazeck,J. and Alper,H. (2012) Evolution of an alkane-inducible biosensor for increased responsiveness to short-chain alkanes. *J. Bacteriol.*, **158**, 75–79.
15. Bahls,M.O., Kardashliev,T. and Panke,S. (2017) Novel sensors for engineering microbiology. In: Lee SY (ed). *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals*. Springer, New York, pp. 331–357.
16. Hernández-Arranz,S., Sánchez-Hevia,D., Rojo,F. and Moreno,R. (2016) Effect of Crc and Hfq proteins on the transcription, processing, and stability of the *Pseudomonas putida* CrcZ sRNA. *RNA*, **22**, 1902–1917.
17. Dinamarca,M.A., Ruiz-Manzano,A. and Rojo,F. (2002) Inactivation of cytochrome o ubiquinol oxidase relieves catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. *J. Bacteriol.*, **184**, 3785–3793.
18. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. de Lorenzo,V. and Timmis,K.N. (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.*, **235**, 386–405.
20. Choi,K.H., Kumar,A. and Schweizer,H.P. (2006) A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J. Microbiol. Methods*, **64**, 391–397.
21. Yuste,L., Canosa,I. and Rojo,F. (1998) Carbon-source-dependent expression of the *PalkB* promoter from the *Pseudomonas oleovorans* alkane degradation pathway. *J. Bacteriol.*, **180**, 5218–5226.
22. Horton,R.M., Hunt,H.D., Ho,S.N., Pullen,J.K. and Pease,L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*, **77**, 61–68.
23. Nielsen,A.A.K., Der,B.S., Shin,J., Vaidyanathan,P., Paralanov,V., Strychalski,E.A., Ross,D., Densmore,D. and Voigt,C.A. (2016) Genetic circuit design automation. *Science*, **352**, aac7341.
24. Thompson,M.G., Pearson,A.N., Barajas,J.F., Cruz-Morales,P., Sedaghatian,N., Costello,Z., Garber,M.E., Incha,M.R., Valencia,L.E., Baidoo,E.E.K. *et al.* (2020) Identification, characterization, and application of a highly sensitive lactam biosensor from *Pseudomonas putida*. *ACS Synth. Biol.*, **9**, 53–62.
25. Alon,U. (2019) *An Introduction to Systems Biology: Design Principles of Biological Circuits*. CRC Press, Boca Raton, FL.
26. Martínez-García,E., Calles,B., Arévalo-Rodríguez,M. and de Lorenzo,V. (2011) pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. *BMC Microbiol.*, **11**, 38.
27. Miller,W.G. and Lindow,S.E. (1997) An improved GFP cloning cassette designed for prokaryotic transcriptional fusions. *Gene*, **191**, 149–153.
28. Amann,E., Ochs,B. and Abel,K.-J. (1988) Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene*, **69**, 301–315.
29. Wu,H.-Y., Shyy,S., Wang,J.C. and Liu,L.F. (1988) Transcription generates positively and negatively supercoiled domains in the template. *Cell*, **53**, 433–440.
30. Rojo,F. (2010) Carbon catabolite repression in *Pseudomonas:* optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol. Revs.*, **34**, 658–684.

31. Snoek,T., Chaberski,E.K., Ambri,F., Kol,S., Bjørn,S.P., Pang,B., Barajas,J.F., Welner,D.H., Jensen,M.K. and Keasling,J.D. (2020) Evolution-guided engineering of small-molecule biosensors. *Nucleic Acids Res.*, **48**, e3.
32. Kærn,M., Elston,T.C., Blake,W.J. and Collins,J.J. (2005) Stochasticity in gene expression: from theories to phenotypes. *Nat. Revs. Genet.*, **6**, 451–464.
33. Calles,B., Goñi-Moreno,Á. and de Lorenzo,V. (2019) Digitalizing heterologous gene expression in Gram-negative bacteria with a portable ON/OFF module. *Mol. Syst. Biol.*, **15**, e8777.
34. Costello,A. and Badran,A.H. (2021) Synthetic biological circuits within an orthogonal central dogma. *Trends Biotechnol.*, **39**, 59–71.
35. Bryant,J.A., Sellars,L.E., Busby,S.J.W. and Lee,D.J. (2014) Chromosome position effects on gene expression in *Escherichia coli* K-12. *Nucleic Acids Res.*, **42**, 11383–11392.
36. Borkowski,O., Ceroni,F., Stan,G.-B. and Ellis,T. (2016) Overloaded and stressed: whole-cell considerations for bacterial synthetic biology. *Curr. Op. Microbiol.*, **33**, 123–130.
37. Cases,I. and de Lorenzo,V. (2005) Promoters in the environment: transcriptional regulation in its natural context. *Nat. Revs. Microbiol.*, **3**, 105–118.
38. Shingler,V. (2011) Signal sensory systems that impact σ54-dependent transcription. *FEMS Microbiol. Revs.*, **35**, 425–440.
39. Yuste,L. and Rojo,F. (2001) Role of the crc gene in catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. *J. Bacteriol.*, **183**, 6197–6206.
40. Molina,L., La Rosa,R., Nogales,J. and Rojo,F. (2019) Influence of the Crc global regulator on substrate uptake rates and the distribution of metabolic fluxes in *Pseudomonas putida* KT2440 growing in a complete medium. *Environ. Microbiol.*, **21**, 4446–4459.
41. Molina,L., Rosa,R.L., Nogales,J. and Rojo,F. (2019) *Pseudomonas putida* KT2440 metabolism undergoes sequential modifications during exponential growth in a complete medium as compounds are gradually consumed. *Environ. Microbiol.*, **21**, 2375–2390.
42. Chan,L.Y., Kosuri,S. and Endy,D. (2005) Refactoring bacteriophage T7. *Mol. Sys. Biol.*, **1**, 2005.0018.
43. Temme,K., Zhao,D. and Voigt,C.A. (2012) Refactoring the nitrogen fixation gene cluster from Klebsiella oxytoca. *Proc. Natl. Acad. Sci. USA*, **109**, 7085–7090.
44. Sagi,E., Hever,N., Rosen,R., Bartolome,A., Premkumar,J., Ulber,R., Lev,O., Scheper,T. and Belkin,S. (2003) Fluorescence and bioluminescence reporter functions in genetically modified bacterial sensor strains. *Sens. Actuators*, **90**, 2–8.
45. Martínez-García,E., Benedetti,I., Hueso,A. and de Lorenzo,V. (2015) Mining environmental plasmids for synthetic biology parts and devices. *Microbiol. Spectr.*, **3**, Plas-0033-2014.
46. Porcar,M., Danchin,A. and de Lorenzo,V. (2015) Confidence, tolerance, and allowance in biological engineering: the nuts and bolts of living things. *Bioessays*, **37**, 95–102.
47. de Lorenzo,V. and Schmidt,M. (2018) Biological standards for the Knowledge-Based BioEconomy: what is at stake. *New Biotechnol.*, **40**, 170–180.
48. Beal,J., Goñi-Moreno,A., Myers,C., Hecht,A., de Vicente,M.D.C., Parco,M., Schmidt,M., Timmis,K., Baldwin,G., Friedrichs,S. *et al.* (2020) The long journey towards standards for engineering biosystems: are the molecular biology and the biotech communities ready to standardise? *EMBO Rep.*, **21**, e50521.
49. Manoil,C. and Beckwith,J. (1985) TnphoA: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA*, **82**, 8129–8133.
50. Hanahan,D. and Meselson,M. (1983) Plasmid screening at high colony density. *Methods Enzymol.*, **100**, 333–342.
51. Nelson,K.E., Weinel,C., Paulsen,I.T., Dodson,R.J., Hilbert,H., Martins dos Santos,V.A., Fouts,D.E., Gill,S.R., Pop,M., Holmes,M. *et al.* (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Env. Microbiol.*, **4**, 799–808.
52. Holloway,B.W. (1955) Genetic recombination in *Pseudomonas aeruginosa*. *J. Gen. Microbiol.*, **13**, 572–581.
53. Kessler,B., de Lorenzo,V. and Timmis,K.N. (1992) A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol. Gen. Genet.*, **233**, 293–301.