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

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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_{alk} 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_{alk} 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_{alk} 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_{alk} 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,
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 alkBFGHJL cluster determines the rest of the activities, which are expressed from the upstream AlkS-dependent promoter P_{alk} (9, 10). Once excised from its native context and assembled adjacent to each other in a single DNA segment, the alkS/P_{alk}, 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 dicyclopentyl ketone (DCPK). However, this simple rearrangement of functional segments with alkS and P_{alk} does not eliminate the regulatory complexity embodied in them. alkS is transcribed through two promoters, P_{alk1} and P_{alk2}, which are negatively and positively regulated, respectively, by AlkS. In addition, translation of the alkS promoter (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_{alk} 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_{alk} 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, phoAΔ20, galE, galK, thi, rpsE, rpoB, argE(Am), recA1 | (49) |
| DH5α | F-, supE44, ΔlacU169, (pho80 lacZDM15), hisdR17, (rK-MK+), recA1, endA1, thi1, gyrA, relA | (50) |
| HB101 | SmR, KanR M*, pro, leu, thi, recA | (18) |
| **P. putida strains** | | |
| KT2440 | Prototrophic, wild-type strain derived from P. putida mt-2 without pWW0 plasmid | (16) |
| KT2440 crc::Sm | P. putida KT2440 with the chromosomal crc gene interrupted with a SmR cassette | (51) |
| **P. aeruginosa strain** | | |
| PAO1 | Prototrophic, wild-type reference strain | (52) |
| **Plasmids** | | |
| pRK600 | CmR, oriV ColE1, truB mobC of RK2; helper plasmid for mobilization in tripartite conjugations | (53) |
| pMA | Cloning vector for synthetic DNA | GeneArt<sup>a</sup> |
| pAlkS3 | Ap<sup>R</sup>; PMA cloning vector bearing the optimized sequence of alkS gene | This work |
| pBAM1 | Mini-Tn5 suicide delivery vector, source of P_{alk} promoter | (26) |
| pSEVA429 crc<sup>+</sup> | SmR, oriRK2, oriT; pSEVA421-derivative carrying an alkS<sub>D</sub>/P_{alk} 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<sup>b</sup> | SmR, oriRK2, oriT; pSEVA421-derivative carrying the alkS<sub>C</sub>/P_{alk} expression system. alkS sequence is same as alkS<sub>D</sub> (Supplementary Figure S1) but S<sub>2</sub>-end edited for removing the Crc/Hfq binding sequence of the corresponding mRNA | This work |
| pSEVA421 | Sm<sup>R</sup>, oriV RK2, oriT, standard MCS | (6) |
| pSEVA426 | Sm<sup>R</sup>, oriV RK2, oriT, luxCDABE reporter system | (6) |
| pSEVA429 → luxCDABE | Sm<sup>R</sup>, oriRK2, oriT; pSEVA429 cloned with the luxCDABE reporter system | This work |
| pSEVA427 | Sm<sup>R</sup>, oriRK2, oriT, green fluorescent protein (GFP) reporter system | (6) |
| pSEVA429 crc<sup>+</sup> → GFP | pSEVA429 crc<sup>+</sup> with a promoterless GFP gene as a transcriptional reporter | This work |
| pSEVA429 crc<sup>+</sup> → luxCDABE | pSEVA429 crc<sup>+</sup> 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<sup>R</sup>, oriV ColE1, native P_{alk}−alkST/P_{alk} expression system driving the transcription of GFP | (12) |
| pAlkRK5 | Sm<sup>R</sup>, oriV RK2, oriT; pSEVA421 cloned with the NotI fragment from pJAMA30 carrying the native alkane/DCPK-responsive reporter system | This work |

<sup>a</sup>https://www.thermofisher.com/content/dam/LifeTech/Documents/geneart/geneart-vector-map.pdf.

<sup>b</sup>Complete DNA sequence available in the SEVA database (http://seva-plasmids.com/).
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 denaturation (5 min, 94°C) followed by 30 cycles of denaturation (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 CaCl2 method (15). In the case of P. putida and P. aeruginosa, plasmids were introduced by either conjugative triparental mating using the E. coli 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 PalkB/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 GP01 was edited to remove incompatible restriction sites (alkS	extsuperscript{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 PalkB promoter were then arrayed with the other DNA segments indicated in Figure 1. The PalkB promoter was PCR-amplified with primers aaPalkB1-F (5′-AAGGATTAACATTTACGACGACGAAGTTTTCCTACGAGCGGAGGAGC3′) and aaPalkB1-R (5′-TGACTACTGTGCTTGAACTCTTAATACGAACACTTGAACCTGAC3′), using as template genomic DNA from P. putida PBS4 (21). The PalkB promoter was also amplified with PCR by oligonucleotides aaPKm-NcolI-1-F (5′-TAGAACCATCGGTCTTTTCCTCCTCTGTAATAAG3′; underlined sequence shows Ncol recognition site) and aaPalkB1-R (5′-TCCTGTGTGAAATTGTTATCCGCT3′) 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 PalkB with PalkB by SOEing PCR (22). Insertion of the resulting product into the Ncol/AvrII restriction sites of a pSEVA frame gave rise to expression vector pSEVA429 crc	extsuperscript{+} (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	extsuperscript{+} was modified with PCR-based site-directed mutagenesis. Briefly, the first 570 bp of alkS were amplified with the oligonucleotides 5′-alkSmut-NcolI (5′-GGCCCTCTGCGATGAAATGACTAA GATCATGAACATGATTTCCCGTTGCGCAAGATCG3′) and 3′-alkSmut-XholI (5′-AGCCGCTGCAAGTATTGAGGCC3′) using the pAlkS3 as template. The forward primer contains the recognition sequence of Ncol 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 Ncol and Xhol (the last within the alkS sequence), and the resulting 406 bp fragment was recloned into the same sites of pSEVA429 crc	extsuperscript{−}. 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/PalkB expression cargo, Supplementary Figure S2).

2.5 Parameterization of the AlkS/PalkB module

For generating constructs that report transcriptional activity as a fluorescent emission, the GFP tir gene of pSEVA427 was cloned into plasmids pSEVA429 crc	extsuperscript{+} and pSEVA429 as a HindIII/SpeI insert to generate plasmids pSEVA429 crc	extsuperscript{+} ↔ GFP and pSEVA429 → GFP, respectively. As a control, we excised the NotI fragment from pAMA30 containing the original alkST genes of pocT transcribed by their own PalkB promoter and, in divergent orientation, the PalkB 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 pARKlS. 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 DCCK 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<sub>alk</sub> 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 DCCK and analyzed by flow cytometry as described above. For monitoring activity of the AlkS/P<sub>alk</sub> module at a population level, plasmids pSEVA429 crc<sup>+</sup> and pSEVA429 were inserted with the promoterless luminescent reporter luxCDABE operon excised from pSEVA426 as a HindIII/SpeI fragment. This originated plasmids pSEVA429 crc<sup>-</sup> → 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<sub>alk</sub> 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<sub>alk</sub> modules constructed in this study

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

The slope value was calculated using the linear regression of the induction kinetics as explained in Section 2.6. 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<sup>+</sup>/crc<sup>-</sup> 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<sub>alk</sub> 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<sub>alkS1</sub> and P<sub>alkS2</sub>. (B) Constitution of the standardized AlkS P<sub>alk</sub> 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<sup>STD</sup>). alkS expression is now under the control of a heterologous Shine-Dalgarno (SD) sequence and a constitutive promoter (P<sub>lacI</sub>, see text) and closer to target, divergent promoter P<sub>alk</sub>—albeit separated by the buffer sequence indicated in Figure 18. (C) AlkS/P<sub>alk</sub> 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/Pneo 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–Ptet 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](image-url)
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_{alkS} 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_{alkS} (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 NsiI 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](image-url)

**Figure 4.** Effect of Crc on performance of the standardized AlkS/P_{alkB} module. Plasmids (A) pAR_{alkS} (alkS^{WT}) and (B) pSEVA429crc^+ → 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<sub>alkB</sub> on the performance of the expression system

In the first series of experiments, we compared the behavior of the AlkS/P<sub>alkB</sub> pair assembled with all the native regulatory systems borne by the pOCT plasmid (Figure 2A) and recreated in plasmid pAR<sub>alkS</sub> versus that of the same regulatory system arranged with the composition standard of Figure 1 as implemented in pSEVA429 <sup>crc+</sup> → 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<sub>alkB</sub> as sketched in Figure 2B. <i>P. putida</i> 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<sub>alkB</sub> 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<sub>alkB</sub> in its native context.

3.4 Effect of Crc on the formatted and not-formatted AlkS/P<sub>alkB</sub> 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 <i>P. putida</i> operates through a complex interplay between the proteins Crc and Hfq with small RNAs to inhibit translation of messenger RNAs (mRNAs) of target

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**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<sub>alkB</sub> device bearing the regulator gene devoid of the Crc-binding site in its mRNA) was transformed into crc<sup>+</sup> (A) and crc<sup>−</sup> (B) strains of <i>P. putida</i>. 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, pARalkS and pSEVA429 \( crc^{+} \rightarrow \) 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\( \rightarrow \)lux 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 \( P_{\text{alk}} \) promotor. 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 pARalkS and pSEVA429 \( crc^{+} \rightarrow \) GFP was added to pSEVA429, thereby generating pSEVA429 \( \rightarrow \) 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 \( \rightarrow \) 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 \( P_{\text{alk}} \) 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 as those with the same with alkS\( ^{ED} \). This accented 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 the Crc/Hfq-based system of catabolite 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 \( P. aeruginosa \) PA01 were transformed with pSEVA429 \( \rightarrow \) 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 \( P_{\text{alk}} \) 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<sup>CR</sup> 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 PA01. 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 pARalkS and pSEVA429<sup>cr</sup>c+ as beta versions of what we propose to be an standardized AlkS/P<sub>alkB</sub> 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<sup>CR</sup>/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.

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