Standardization of regulatory nodes for engineering heterologous gene expression: a feasibility study

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Summary
The potential of LacI/Ptet, XylS/Pm, AlkS/Pakb, CprK/PDB3 and ChnR/PchnB regulatory nodes, recruited from both Gram-negative and Gram-positive bacteria, as the source of parts for formatting expression cargoes following the Standard European Vector Architecture (SEVA) has been examined. The cargoes following the Standard European Vector Architecture (SEVA) have been analysed in an Escherichia coli strain of reference within exactly the same plasmid backbone and bearing the different functional segments arrayed in an invariant DNA scaffold. Their performance was then analysed in an Escherichia coli strain of reference through the readout of a fluorescence reporter gene that contained strictly identical translation signal elements. This approach allowed us to describe and compare the cognate expression systems with quantitative detail. The constructs under scrutiny diverged considerably in their capacity, expression noise, inducibility and ON/OFF ratios. Inspection of such a variance exposed the different constraints that rule the optimal arrangement of functional DNA segments in each case. The data highlighted also the ease of standardizing inducer-responsive devices subject to transcriptional activation as compared to counterparts based on repressors. The study resulted in a defined collection of formatted expression cargoes lacking any cross talk while offering a panoply of choices to potential users and help interoperability of the specific constructs.

Introduction
Prokaryotic transcription factors (TFs) responsive to exogenous physicochemical signals and their target promoters have been an invaluable source of biological parts for designing heterologous gene expression (Baneyx, 1999; Terpe, 2006; Yin et al., 2007). Most heterologous gene expression designs rely upon placing the DNA of the TF and its promoter artificially close to the target sequence for transcription of the gene(s) of interest (Gol; Marschall, 2017). The literature contains a large number of variations on such a basic theme, most often in the form of dedicated plasmid vectors that may be also endowed with additional features to optimize expression of such GoI, i.e. by modifying copy number, toxicity, mRNA stability, translation efficiency and other features (Zerbs et al., 2009; Kaur et al., 2018; Kent and Dixon, 2020). The majority of the studies describing heterologous expression systems are based on Escherichia coli as the host and the TFs/promoters are often retrieved from regulatory networks of the same organism. Popular devices of this sort include many variants of Lac/P lac (Heyneker et al., 1976; Lanzer and Bujard, 1988; Glascock ad Weickert, 1998), TetR/P tet (Skerra, 1994; Korpela et al., 1998; Ramos et al., 2005), AraC/P araBAD (Guzman et al., 1995; Haldimann et al., 1998), Rha/P rhaBAD (Egan and Schleif, 1993; Haldimann et al., 1998; Kelly et al., 2016) and cI857/Pc (Elvin et al., 1990; Love et al., 1996), which can be combined with the use of the selective viral T7 RNA polymerase/P T7 transcription machinery (Studier and Moffatt, 1986; Sørensen and Mortensen, 2005). Signals for triggering expression with
these modules may vary from the natural inducers to synthetic structural alternatives as well as mere physical cues, e.g. temperature, cell density and medium composition (Andrews et al., 1996; Scholz et al., 2003; Henlifer et al., 2005; Tang et al., 2008; Choudhary and Schmidt-Dannert, 2010; Tang and Cirino, 2011; Kelly et al., 2016; Taylor et al., 2016; Ellefson et al., 2018; Meyer et al., 2019). In principle, these systems, along with specialized strains, have thus far covered most – if not all – of the necessities for engineering E. coli. However, while use of numerous different TF-based transcriptional control systems have enabled the engineered control of gene expression for many organisms, surprisingly – with few exceptions (Balzer et al., 2013) – little work has attempted to standardize the description of transcriptional devices or rigorously and quantitatively describe their function within disparate genetic contexts.

A growing number of expression devices have been inspected in recent years owing to the push for standardization brought about by synthetic biology (Canton et al., 2008; Beal et al., 2020). Nonetheless, the best studied modules aimed at engineering complex expression circuits are largely limited to E. coli. In contrast, many other useful expression systems – especially those intended for non-model Gram-negative microorganisms – are poorly characterized and subject to all types of capricious designs and vector architectures. As a consequence, despite the large number of potentially useful regulatory parts available for controlling heterologous gene expression (Calero et al., 2016), reliable metrics assessing the performance of these regulatory parts within different genetic contexts is lacking. Recent developments are improving this state of affairs. Since 2013, an expanding collection of genetic tools (including expression systems), following the assembly rules of the so-called Standard European Vector Architecture (SEVA; Silva-Rocha et al., 2013), enable reusability, portability and comparative assessment of promoter and genetic device performance in a variety of Gram-negative hosts (Calero et al., 2016; Tas et al., 2021). The SEVA standard has been further expanded (Arce-Rodríguez et al., 2021) to propose a specific architecture for heterologous expression modules consisting of a fixed DNA sequence that acts as the scaffold for incorporation of the TF gene and the target promoter. In this design, all elements are organized in an otherwise invariable array of segments within a SEVA vector frame. The value of this format for expression cargoes was shown in the design and performance of a module based on the AlkS/P* regulatory node. This device included parts from the OCT plasmid of Pseudomonas oleovorans (van Beilen et al., 2001; Makart et al., 2007; Rojo, 2009), and gene expression can be triggered by n-octane as well as by the gratuitous inducer dicyclopropyl ketone (DCKP).

In this work, we have extended the standardization of expression modules to three additional platforms based on regulatory nodes mined from Gram-negative bacteria, i.e. the E. coli, IPTG-inducible LacI/P* (Brosius et al., 1985), XylS/P* from Pseudomonas putida, which responds to m-toluol (3-methylbenzoate, 3-mBz; Ramos et al., 1987; Gawin et al., 2017), and ChnR/P* from Acinetobacter sp., a cyclohexanone-responsive system (Steigedal and Valla, 2008). One more expression module originated from the regulatory node CprK/P* of the Gram-positive organism Desulfotobacterium hafniense, which can be induced by chlorinated molecules, e.g. 3-chloro-4-hydroxyphenylacetic acid (CHPA; Kemp et al., 2013). In order to comply with the SEVA format, the biological parts employed in this study were edited to erase undesired restriction sites that could interfere with cloning and their DNA sequences debugged (Silva-Rocha et al., 2013; Arce-Rodríguez et al., 2021) in order to improve their performance without affecting their functionality. As explained below, the key parameters and compositional constraints that rule the performance of each system could then be rigorously determined and the data evaluated for fitting specific bioengineering goals. Since each of the pieces chosen for this study came from a different biological context and are controlled by diverse mechanisms, their formatting in a comparable genetic arrangement permitted a faithful appraisal of their expression output in a given host as well as the influence of the standardization. The work below showcases both the value and feasibility of standardizing five types of modules and paves the way to a much ampler repertoire of choices for designing regulatory networks with intended phenotypic outputs.

Results and discussion

Rationale for formatting heterologous gene expression devices

The five expression systems under examination have each a different type of mechanism for controlling transcription of their target promoters. LacI/P* is a representative of the plethora of devices based on the LacI repressor, the DNA binding ability of which to its cognate operator depends not only on inducer addition (e.g. IPTG) but also on the intracellular levels of the regulator itself (Calos, 1978). A somewhat equivalent situation but with an activator is embodied in XylS/P*, the functioning of which depends as much on the inducer 3-mBz as on the levels of the transcriptional factor: overproduction of XylS activates P* without any effector (Inouye et al., 1987; Mermod et al., 1987; Ramos et al., 1987; Spooner et al., 1987). In contrast, ChnR/P* (Steigedal and Valla, 2008; Benedetti et al., 2016a,b), in which the TF belongs to the same family of regulatory proteins as
XylS (Iwaki et al., 1999; Tobes and Ramos, 2002), is transcriptionally silent unless cells face the cognate inducer (cyclohexanone). The transcriptional factor of the CprK/P<sub>DCPK</sub> node belongs to the CRP/FNR group of regulators and, following binding to the CHPA effector, binds a target sequence (termed dehalobox) within the sequence of the promoter region (Kemp et al., 2013; Benedetti et al., 2016a,b). Finally, the regulator r-octane/dicyclopropylketone (DCPK)-responsive AlkS/P<sub>alkB</sub> (Canosa et al., 2000; van Beilen et al., 2001; Rojo, 2009) is a member of the unusual MalT family of regulators, which require ATP binding for activity. In addition, alkS expression is subject in *Pseudomonas* to catabolite repression mediated by Crc, but the variant used in this work has been erased of that feature (Arce-Rodríguez et al., 2021). Although the five regulatory nodes under study do not exhaustively cover all possible architectures for inducible transcription, they represent many of those which have been used to this end in various expression vectors available thus far (Brautaset et al., 2009; Balzer et al., 2013; Marschall, 2017).

The first stage of this study involved the editing of individual components of each of the regulatory nodes and their assembly to meet SEVA standards (Silva-Rocha et al., 2013; Arce-Rodríguez et al., 2021; Fig. 1A), so that all share the following common features, namely, (i) a constitutive promoter driving the expression of (ii) the gene encoding the regulatory protein that is transcribed divergently with respect to its target promoter, (iii) a spacer sequence (buffer) that insulates expression of the relevant elements from adjacent DNA features and (iv) the cognate promoter that is activated upon addition of the inducer molecule. All expression systems were flanked by SEVA-compatible Pac-I-AvrII sites, allowing for easy swapping between SEVA vector backbones. Once inserted into these restriction sites, a downstream multiple cloning site (MCS) facilitates the addition of any gene(s) of interest and it is followed by a universal M13 (F24) priming site added to the backbone for sequencing/checking purposes. The functional cargo ends up with a unique SpeI site such that, upon insertion into any SEVA vector as a PacI-SpeI DNA segment, the expression cargoes described above are bracketed by the strong terminators T0 and T1. This structure ensures transcriptional insulation of the DNA segments encoding the TF and its cognate promoter (Fig. 1A). The basic architecture described above facilitates modular DNA assembly and eliminates potential effects on expression

![Fig. 1. Main features of the standardized expression systems.](image)

A. Schematic representation of the main features of a standardized pSEVA-compatible expression cargo, indicating the relevant elements involved in the regulatory device, i.e. transcriptional regulator, cognate promoter and a buffer sequence that separates both features. A handy MCS for cloning procedures is located downstream of the regulatory device. In addition, there are R24 and F24 oligonucleotide priming sites that can be used for checking/sequencing purposes. The unique restriction sites PacI, AvrII and SpeI allow for module exchange within SEVA platform backbones, in such a way that the whole module is flanked by appropriate transcriptional terminators T0 and T1. Note that the standard does not include an RBS for expression of the GoI inserted in the MCS.

B. Implementation of the standard in modules AlkS/P<sub>alkB</sub>, ChnR/P<sub>chnB</sub> and CprK/P<sub>DCPK</sub> and incorporation in vector pSEVA231 originated plasmids pSEVA239, pSEVA2311 and pSEVA2312, respectively. As indicated in the text, the standard could be applied partially to the XylS/P<sub>xylS</sub> expression cargo, resulting in plasmid pSEVA238. This construct bears a spacer that is longer and different of the others and has the native P<sub>alc</sub> promoter driving expression of the xylS.

C. Composition of LacI/P<sub>trc</sub> module variants. The IPTG-dependent expression module [LacI/P<sub>trc</sub>] borne by pSEVA234C is the only one that follows all the terms of the standard. In the partially standardized device pSEVA234, lacI is expressed from P<sub>alc</sub> and is transcribed in the same orientation as the target promoter P<sub>alc</sub>. Two additional expression devices were constructed based on this non-standardized expression cargo by flipping the lacI segment, including the promoter P<sub>alc</sub> (pS634r) or substituting it by the P<sub>neo</sub> one (pS634rk).

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unrelated to the functional elements of the regulator/promoter systems themselves – provided that they are inserted at the same point of a given replicon and thus share an identical backbone.

**Implementing the SEVA standard into expression cargoes**

The general formatting of regulated transcription devices according to SEVA rules explained above had to be adapted case by case to the specific features of each expression module. As mentioned above, four out of five of the expression systems tested in this work are positively regulated. Among these, three (AlkS, ChnR and CprK) were exclusively dependent on inducer addition. This greatly facilitated streamlining their components, following a standard layout that encompasses (i) a P3/Pneo promoter and a translation initiation region (TIR) to drive the constitutive production of the regulatory proteins AlkS, ChnR and CprK and (ii) a neutral, 150-bp long DNA segment that separates the TF gene from its target promoter, identical in length and sequence in all three cases (Fig. 1B). This DNA spacer contains no known regulatory elements and serves to alleviate the negative supercoiling from the transcription of the flanking promoters (Wu et al., 1988). As indicated above, the alks variant used in this study had its Crc recognition removed to mitigate catabolite repression in Pseudomonas hosts (Hernández-Arranz et al., 2016; Arce-Rodríguez et al., 2021). The organization of the constructs resulting from implementing these criteria to Alks/Psi, CprK/PDB3 and ChnR/PchnB is shown in Fig. 1B. The other positively regulated device (XylS/Pm) required a singular handling as its activity relies both on inducer and intrinsic levels of the TF (Inouye et al., 1987; Mermod et al., 1987; Spooner et al., 1987; Ramos et al., 1990; Michan et al., 1992; Kessler et al., 1994; Zwick et al., 2013). In order to leave the system dependent on inducer-only we kept the relatively weak native Psa2 promoter driving the expression of xylS (Gallegos et al., 1996; González-Pérez et al., 2004) instead of replacing it by the stronger P3/Pneo as in the other cases. Furthermore, instead of the standard spacer used for formatting Alks/Psi, CprK/PDB3 and ChnR/PchnB we entered a 0.8-kb synthetic DNA segment derived from vector pJ653 (Blatny et al., 1997) that was modified to eliminate restriction sites conflicting with the SEVA standard. Since this buffer sequence was merely meant to insulate the TF/promoter pair, we anticipated little or no influence of its length or sequence on the functionality of the device (Fig. 1B). Finally, the LacI/Ptrc expression cargo was the only one examined based on a repression mechanism. Although a positive control may in principle deliver a tighter transcriptional regulation (Yin et al., 2007; Engstrom and Pfleger, 2017), IPTG-inducible devices remain to this day the most widely used small-scale expression systems. In the best-case scenario, when the operator in the cognate promoter is occupied by the repressor, transcription cannot proceed. The main issue is thus reducing basal expression without affecting inducibility of the system. A large number of lac-derived expression systems are based on the lacI allele, a promoter-up mutation of lacI that increases production of the repressor by 10-fold (Calos, 1978). Having these considerations in mind, we engineered two versions of this expression system. First, a fully SEVA-standardized LacI/P_trc expression cassette (named hereafter [LacI/P_trc]) that fulfilled all the specifications described for ChnR/P_chnB, Alks/Psi and CprK/PDB3 (Fig. 1C). And second, a LacI*-based variant derived from the widely used plasmid pTrc-99A (Amann et al., 1988), which was edited to meet SEVA compositional rules while maintaining the features of the original construct. These involved the lacI* gene oriented in the same direction as its target P_trc promoter, but separated by a neutral 231-bp DNA fragment (Fig. 1C).

In sum, the [LacI/P_trc], Alks/Psi, ChnR/PchnB and CprK/PDB3 modules were considerably reshaped in respect to their naturally occurring configuration, while (owing to the reasons explained above) XylS/Pm pair was only partially reformed. In the four fully standardized expression devices, the gene encoding the TF is preceded by a TIR, and the whole is transcribed from the P3/Pneo promoter. Also, the TF gene is divergently located and isolated from its target promoter by means of a common DNA insulator sequence. In addition, the partially modified but still SEVA-compatible LacI*/P_trc variant with co-directional TF gene and target promoter was also included as indicated above. With this panel of constructs at hand, we set out to investigate the functioning parameters delivered by each system as explained next.

**Analysis of expression dynamics by flow cytometry**

To prevent the occurrence of effects unrelated to the features of the regulation/promoter systems themselves, all replicons used in this study shared an identical backbone, i.e. plasmid pSEVA231. This vector harbours the following functional modules: a medium copy-number pBBR1 origin of vegetative replication, a kanamycin resistance gene and a multiple cloning site (MCS) default cargo (Silva-Rocha et al., 2013). Following SEVA composition rules, the expression cargos were inserted in exactly the same location, i.e. between the Pad-AvII sites, so that they were placed right upstream the MCS. This originated plasmids pSEVA234 (containing LacI*/P_trc), pSEVA234C (encompassing the fully standardized
[LacI/P\textsubscript{trc}\textsuperscript{C} derivative], pSEVA238 (harbouring the XylS/P\textsubscript{m} cassette), pSEVA239 (carrying the regulator/promoter regions of AlkS/P\textsubscript{alkB}), pSEVA2311 (bearing the ChnR/P\textsubscript{chnB} regulatory pair) and pSEVA2312 (accommodating the CprK/P\textsubscript{DB3} expression system). In order to monitor, quantify and compare their activity, a reporter gene encoding the monomeric and superfolder GFP (msfGFP), bearing a TIR motif (Miller and Lindow, 1997), was cloned downstream of each of the regulator/promoter systems under study at the same position of the MCS (see Experimental procedures). The resulting constructs (termed pS234\textsubscript{M}, pS234C\textsubscript{M}, pS238\textsubscript{M}, pS239\textsubscript{M}, pS2311\textsubscript{M} and pS2312\textsubscript{M}) carried the LacI\textsuperscript{q}/P\textsubscript{trc}, [LacI/P\textsubscript{trc}\textsuperscript{C}], XylS/P\textsubscript{m}, AlkS/P\textsubscript{alkB}, ChnR/P\textsubscript{chnB} and CprK/P\textsubscript{DB3} expression systems, respectively. In all instances, the strain of reference \textit{E. coli} CC118 (Manoil and Beckwith, 1985) was adopted as the host and the resulting transformants were grown under identical conditions. Also, all experiments were run in LB medium in shaken-flask cultivations as a standard culture condition widely used in common laboratory practices.

In a first series of experiments, we inspected the gross performance of these expression systems in terms of bacterial growth, inducibility and reporter output by means of flow cytometry as a way to expose expression noise and cell-to-cell variability. Fig. 2 shows the fluorescence patterns of cells harbouring the expression system under study along time after the addition of saturating amounts (1.0 mM) of the corresponding inducer, as indicated (increasing inducer > 1.0 mM does not have an effect on the expression level, data not shown). \textit{E. coli} CC118 carrying vector pSEVA23M with a promotor-less msfGFP gene inserted in the same backbone as the constructs under analysis was used as negative control.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Analysis of fluorescence distribution with flow cytometry. Cultures of \textit{E. coli} CC118 harbouring the corresponding expression plasmids, as indicated in each plot, were grown under the conditions explained in Experimental Procedures up to the mid-exponential phase (OD\textsubscript{600} \approx 0.4). At this point, gene expression was induced by addition of the corresponding effectors (t = 0) as indicated in each case. \textit{E. coli} CC118 carrying the promoter-less pSEVA23M plasmid (C-, grey plots) was used as negative fluorescence control. Samples were collected every hour after induction (t = 1, 2, 3 and 4 h), cells were fixed and the msfGFP signal was analyzed with a flow cytometer. Plots show the results of representative samples.}
\end{figure}
expression control. Perusal of the frequency distribution histograms for green fluorescent cells revealed significant differences among the regulator/promoter systems examined. In the absence of inducer, cells remained mostly non-fluorescent in the strains with plasmids bearing the XylS/P<sub>m</sub>, CprK/P<sub>DB3</sub> and ChnR/P<sub>chnB</sub> modules. In contrast, a detectable fraction of bacteria with the AlkS/P<sub>alkB</sub> device produced fluorescence under non-induced conditions. This was exacerbated in the case of both IPTG-inducible expression systems, in which a quite significant part of cells showed a detectable GFP signal which was indicative of a significant basal level. As expected, Fig. 2 shows that all expression systems responded to the addition of the corresponding inducer, as reflected by the movement of fluorescence signals towards higher intensity values over time. In all cases, fluorescence values fell in a single peak – suggesting a mono-modal expression pattern. But the results also showed non-identical shapes (indicating varying degrees of expression heterogeneity) and the fluorescence accumulated with different kinetics depending on time course and the system under analysis. In all instances, 4 h of induction appeared to bring about saturation of GFP output of cells with any of the expression systems.

**ON/OFF expression levels of regulator/promoter nodes**

Further inspection of the results obtained by means of the flow cytometry experiments described in the previous section allowed us to draw additional features of the expression systems under consideration, both in the OFF and ON states. First, in order to assess the tightness of regulation, we compared basal, uninduced levels of fluorescent emissions in each case. This is an important quality of expression systems, as even low basal activity can be detrimental for the host’s physiology if the product of the GoI is toxic and/or causes metabolic or regulatory burden. Ideally, inducible heterologous expression devices should have the lowest possible background. Fig. 3 shows a detailed analysis of the fluorescence values measured during mid-exponential growth without any inducer addition (t = 0). The data exposed significant differences among the x-mean values of fluorescence basal levels. The LacI-dependent expression systems were by far the leakiest device. As displayed in Fig. 3, the LacI<sup>P<sub>trc</sub></sup> device delivered a basal expression approximately 70-fold higher than ChnR/P<sub>chnB</sub> which behaved as the tightest device under non-inducing conditions (fluorescence x-mean of 1.39 versus 0.02 arbitrary units, respectively). Full standardization of the IPTG-inducible device imposed lacI to be expressed through a heterologous promoter and being divergently transcribed with respect to the Gol. Both transcripational units were also separated by a standardized DNA buffer sequence. This arrangement reduced the background fluorescence of this regulator/promoter pair to one-third (x-mean fluorescence decreased to 0.41 arbitrary units in the [LacI/P<sub>trc</sub><sup>C</sup>] derivative). Yet it still roughly doubled that of the AlkS/P<sub>alkB</sub> expression system, which still showed a considerable basal activity of around 0.24 arbitrary units. In contrast, XylS/P<sub>m</sub>, CprK/P<sub>DB3</sub> and ChnR/P<sub>chnB</sub> proved to be the most stringently regulated expression systems under study, with basal activities of 0.075, 0.041 and 0.02 arbitrary units, respectively (Fig. 3).

Other important parameters included induction kinetics and net promoter strength. Fluorescence accumulated steadily along time in cells bearing the regulatory/promoter devices upon addition of the corresponding inducer. But significant differences were observed among them with respect to their transcriptional capacity (i.e. maximal GFP output) in the activated ON state (Fig. 4A). The XylS/P<sub>m</sub> cassette produced the highest GFP signal, followed by the AlkS/P<sub>alkB</sub> system. In contrast, the lowest reporter levels were obtained with CprK/P<sub>DB3</sub> and either of the IPTG-inducible expression devices. Taking the XylS/P<sub>m</sub> expression system as reference, both CprK/P<sub>DB3</sub> and LacI<sup>P<sub>trc</sub></sup> produced fourfold less fluorescence. This was followed by [LacI/P<sub>trc</sub><sup>C</sup>], which reached only one-seventh of the maximum XylS/P<sub>m</sub> range. ChnR/P<sub>chnB</sub> occupied an intermediate position, i.e. about half of the capacity exhibited by the best performers XylS/P<sub>m</sub> and AlkS/P<sub>alkB</sub>. In terms of absolute reporter output, the ranking of fully induced transcriptional strength under the specific

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Fig. 4. Dynamic range and inducibility of expression cargoes.

A. The means of the geometric mean (x-mean) of msfGFP fluorescence ± standard deviation of the activated expression of cultures of E. coli CC118 cells carrying the corresponding expression plasmids (as indicated), obtained from three independent cytometry experiments, were plotted for comparison of their expression levels along time (t = 0, before induction; t = 1, 2, 3 and 4 h after adding the corresponding inducer, respectively).

B. Fold induction of the different expression systems calculated from results of the cytometry experiments. Mean ± SD data were ordered from the highest to the lowest dynamic range. Results were statistically tested by means of a one-way variance analysis (ANOVA) for multiple comparisons. Significant differences between the expression systems are represented in the graph with * (P-value ≤ 0.05), ** (P-value ≤ 0.01), *** (P-value ≤ 0.001) or **** P-value ≤ 0.0001). The rest of comparisons were not statistically significant.

conditions tested was XylS/P$_m$ > AlkS/P$_{alkB}$ > ChnR/ P$_{chnB}$ > CprK/P$_{D83}$ = LacI$^9$/P$_{trc}$ > [LacI$^9$/P$_{trc}$]$^C$. The ratio between the fully induced output and the basal level enabled calculation of inducibility, i.e. the dynamic range of the system. As shown in Fig. 4B, the ChnR-regulated device displayed the highest ratio with 464-fold increase of GFP signal following full induction (Fig. 4B, green bar). High figures were also observed in XylS/P$_m$ (Fig. 4B, orange bar) with a 291-fold increase. This was followed by CprK-dependent and AlkS-regulated expression systems (134-fold and 64-fold changes, respectively). Once more, the LacI-regulated expression systems were by far the least performing of the devices upon induction. In this case, GFP fluorescence barely increased by sevenfold with the [LacI$^9$/P$_{trc}$]$^C$ system and as little as fourfold in LacI$^9$/P$_{trc}$. Under the conditions of the assays and taking into account the dynamic range as a descriptor of the expression capabilities, the regulatory devices analysed from best to poorest would be ChnR/P$_{chnB}$ > XylS/P$_m$ > CprK/P$_{D83}$ > AlkS/P$_{alkB}$ > [LacI$^9$/P$_{trc}$]$^C$ > LacI$^9$/P$_{trc}$

**Issues with standardization of the LacI/P$_{trc}$ system**

The clearly non-ideal behaviour of the [LacI/P$_{trc}$]$^C$ and LacI$^9$/P$_{trc}$ in the tests above prompted us to examine in more detail additional circumstances that could influence their activity and therefore make their standardization more problematic. In both cases, P$_{trc}$ is a chimaera of the E. coli trp operon and lacUV5 promoters, including a single proximal lacO1 operator from the lac operon and the optimally spaced E. coli consensus –35 and –10 motifs. However, in the case of pSEVA234C (Table S1, GenBank accession number OM855579) production of the LacI repressor is driven from a synthetic P$_{neo}$ promoter. This one is stronger than the counterpart used in plasmid pSEVA234 and thus predictably affecting the repressor-operator ratio in P$_{trc}$ in a way that decreases the basal level (Figs 3 and 4; Fig. S1A) as seen also in other instances (Oehler et al., 1990, 1994; Bertram and Hillen, 2008), but also hindering full transcription of the promoter (Schuller et al., 2020). Yet, another notable difference between the [LacI/P$_{trc}$]$^C$ and LacI$^9$/P$_{trc}$ is the orientation of the lacI gene in respect to the promoter (Fig. 1C). This arrangement can produce readthrough transcription of the regulator in one case and divergent, mutually inhibitory activity in the other (Masulis et al., 2015). In order to rigorously dissect these variables, two more constructs based on the LacI/P$_{trc}$ layout (originated from plasmid pTrc99a) were built. In both cases, the lacI gene was placed in the reverse orientation with respect to its target P$_{trc}$ promoter. Expression of the LacI repressor was kept under the control of its own P$_{lac}$ promoter in vector pS634r, while the native promoter was substituted by the stronger P$_{neo}$ promoter in pS634rk (Table S1, Fig. S1 and Experimental procedures). Insertion of the msfGFP gene in each vector led to plasmids pS634r-M and pS634rk-M respectively, which were transformed in E. coli CC118 and their fluorescence compared to those of the parental plasmid pS634-M.
The results of the corresponding induction experiments are shown in Fig. S1. Flipping lacIq gene orientation in respect to \( P_{trc} \) led to a remarkable increase of msfGFP expression from p634r-M, both in the basal and in the induced state, i.e. repression was less efficient in this case. This could be due to a lower production of the LacI regulator and/or to a poorer binding to its target promoter. The reasons for this unexpected result are not clear, but led us to discard the hypothesis of a readthrough scenario when lacI and its target promoter \( P_{trc} \) are colocalized and placed in the same orientation. Repression was, however, recovered upon substitution of the \( P_{lacI} \) promoter by the stronger promoter \( P_{neo} \) in vector pS634r. Differences between these constructs expose still more ingredients in the functioning of expression devices subject to negative regulation; not only innate promoter strength and stoichiometry of the repressor/operator interplay, but also the spatial arrangement of the functional segments in the construct. These surely cause proximal and distant effects which are difficult to predict (Kolesov et al., 2007) and therefore hardly amenable to standardization.

**Gene expression noise: phenotypic diversification**

Flow cytometry profiles embody valuable information on cell-to-cell homogeneity: A sharp peak indicates low dispersion and high uniformity, while a wider peak indicates variation of expression levels among individual cells. A robust indicator of the level of heterogeneity is the so-called coefficient of variation (CV), a parameter that relates the standard deviation with the mean value which is usually expressed as a percentage. In practical terms, the lower the CV, the less noise there is. As CV is unitless and dimensionless, this proxy can be used to compare experimental results over time as well as those obtained in different platforms.

Fig. 5 shows the CVs (expressed as percentage) of *E. coli* CC118 harbouring the corresponding expression systems along time, expressed as the mean of the percentage of the coefficient of variation (CV’100) ± standard deviation at each time point of the flow cytometry experiments (t = 0 before induction, t = 1, 2, 3 and 4 h after addition of the corresponding inducers). The noisiest systems were the standardized version of the [LacI/P\(_{neo}\)]\(_{C}\) and the XylS/P\(_{m}\) regulatory devices, while the ones with the lowest cell-to-cell heterogeneity were CprK/P\(_{DB3}\) and AlkS/P\(_{alkB}\).

**Cross-regulation among expression systems**

Since genetic constructs often require altogether separate transfer functions of the various regulatory devices, time, recovering in most cases the initial values after 4 h of effector addition. Yet, XylS/P\(_{m}\) and [LacI/P\(_{neo}\)]\(_{C}\) showed a conspicuously uneven signal distribution in the ON state throughout the whole period, especially at early times after induction. In particular, their CV value reached ~ 100% dispersion, i.e. the standard deviation was similar to the mean value after the addition of the inducer. Although the spreading of GFP signals decreased over time in both XylS/P\(_{m}\) and [LacI/P\(_{neo}\)]\(_{C}\) devices, it remained higher than any of the other expression systems staying at a good CV of 80% at late induction times. The general behaviour of the expression systems in terms of cell-to-cell heterogeneity was coherent with earlier observations with equivalent transcriptional devices (Balzer et al., 2013). Since noise stems in some cases from low intracellular levels of the TF involved (Silva-Rocha and de Lorenzo, 2010), the unexpected variability of the XylS/P\(_{m}\) regulatory system could be explained, at least in part, by the low XylS protein abundance when expressed from its natural, low-activity promoter \( P_{as} \) (Marquès and Ramos, 1993), which results in strong stochastic effects (Goni-Moreno et al., 2017). Repressor levels and geometrical/topological effects (Kolesov et al., 2007; Chong et al., 2014) can also account for the considerable increase in noisy GFP levels of cells bearing the [LacI/P\(_{neo}\)]\(_{C}\) expression system (Fig. 5).
we next analysed potential crosstalk among effectors of the regulatory devices. To this end, cultures of *E. coli* CC118 harbouring each of the expression cargoes, were independently supplemented with the corresponding inducers and the fluorescent output after 3 h was inspected as before with flow cytometry (Fig. 6). A control strain carrying the promoterless msfGFP plasmid pSEVA237M (Table S1), was also included in the assay. As expected, each expression system responded to its *bona fide* effector. In contrast, none of the inducers affected either the control or strains carrying plasmids with non-associated transcriptional devices, which showed fluorescence patterns indistinguishable from untreated samples. The growth curves were independent of whether the cultures were supplemented with the cognate inducer or a different one: none of the inducers at the concentrations employed in this work (1.0 mM) inhibited growth of *E. coli* or changed the overall growth profiles – neither specific growth rates nor final optical densities (not shown). These experiments accredited that expression systems under study are not influenced by inducers other than their cognate effectors and therefore they are mutually orthogonal.

**Inducer-mediated release of reactive oxygen species**

To further characterize the expression systems under analysis, we investigated if any of the effectors of the regulatory proteins caused physiological stress in the host. As different types of insults ultimately converge in generation of reactive oxygen species (ROS; Cabicos et al., 2000; Zhao and Drlica, 2014) we inspected their formation in cells following exposure to the corresponding inducers. This scenario is not devoid of precedents, as toxicity of some typical inducers (e.g. IPTG), has been documented (Dvorak et al., 2015). This holds true also for aromatic molecules and other organic compounds, which often cause redox stress (Nikel et al., 2016). On this basis, potential toxic effects and intracellular accumulation of ROS upon addition of the inducers to cells with each of the expression systems was assessed by analysing growth profiles and by direct quantification of ROS by flow cytometry (Fig. 7). As shown in Fig. 7A, the addition of inducers at the concentration employed in this work (1.0 mM) did not inhibit or changed the overall growth profiles of *E. coli*. In particular, neither the specific growth rate nor the final optical density was affected by the addition of any inducer, compared to the growth
pattern of a non-treated control culture. In contrast, diamide (a drainer of NADPH; Kosower and Kosower, 1995) and hydrogen peroxide (H₂O₂) acted as bacteriostatic agents in cell exposed to either chemicals, leading to growth arrest by approximately one hour after addition. Redox stress monitoring was then set up using 5-(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) as the ROS-sensitive probe. The results shown in Fig. 7B exposed an expected ROS or H₂O₂, but no significant oxidative stress in the others.

In sum, the results above indicated that the inducers of the expression modules had neither ROS effects nor an impact in the growth of the bacterial host. Therefore, they can be considered as safe agents in this context, at least at the concentrations used in the experiments.

Conclusion

Inducible expression of heterologous genes is not only at the basis of recombinant DNA-based Biotechnology but also an essential component of the genetic circuits which are at the core of contemporary Synthetic Biology (Engstrom and Pfleger, 2017). The literature contains dozens of regulatory nodes of existing bacterial gene networks which have been refactored in the shape of a conditional expression device. As is the case with other DNA segments encoding properties of interest, standardization of such constructs may bring less flexibility but can also add a considerable value in terms of portability and reusability (Canton et al., 2008; Rakke et al., 2014; Beal et al., 2020). In this work we have chosen five types of inducible expression modules intended to be used as transcriptional SEVA cargoes and we have examined the feasibility of applying a rigorous composition standard to them. The results clearly indicate that not every TF/promoter pair can be reformatted to this end – or not so easily. Because of being dependent on a minimal number of factors, those most amenable to standardization are those (i) with promoters which have little or no activity under uninduced conditions, (ii) are subject to positive control, (iii) are regulated by TFs independent of intracellular TF abundance. The only two cases in our study that duly meet all these criteria are the ChnR/ChnB and CprK/CprD modules, and therefore they were the ones where a rigorous format could be applied. To a lesser extent, but still amenable to standardization is the AlkS/Palkg module, the flaw in this case being its significant basal level in the absence of induction. The issue can be traced to Palkg, not the regulator, and therefore could be plausibly fixed with other promoter variants. The XylS/Pm device is a popular expression system in Gram-negative bacteria which can be found in the literature in diverse configurations. Complete standardization is however complicated by the ability of XylS to activate Pm in the absence of inducers if over-produced (Ramos et al., 1987). The partial formatting embodied in pSEVA238 is nevertheless enough for an excellent performance as an expression tool. Finally, the data above with LacI and Pm showcases the difficulty of standardizing expression modules based on effector-
responsive repressors. The results shown in Fig. 2 and Fig. S1 expose a large number of variables at issue that influence operation of such systems and thus make them less amenable to a rigorous configuration paired with others thereof. In sum, we argue that the outcome of the experiments above on the one hand consolidate some expression devices as full-fledged SEVA cargoes and in the other they can guide future attempts of standardizing transcriptional regulation tools for Synthetic Biology and Biotechnology in general.

Experimental procedures

Strains, plasmids, culture media and growth conditions

The relevant properties of the strains and plasmids used in this work are listed in Table S1. E. coli DH10B, DH5α and CC118 strains were used for cloning procedures. E. coli CC118, harbouring the corresponding plasmid(s) under analysis, was used for assessing msfGFP activity driven by the different expression systems. Bacteria were grown routinely at 37°C in LB medium (10 g l⁻¹ of tryptone, 5 g l⁻¹ of yeast extract and 5 g l⁻¹ of NaCl). When required, Km (75 μg ml⁻¹) was added to the media. The necessary inducers, isopropyl-β-D-1-thiogalactopyranoside (IPTG), 3-methylbenzoate (3-mBz), cyclohexanone (CHx), di-cyclopropyl ketone (DCPK) or 3-chloro-4-hydroxyphenylacetic acid (CHPA), were added where indicated to a final concentration of 1.0 mM. For growth curves, E. coli CC118 cells transformed with the plasmids indicated in each case were grown overnight in LB medium at 37°C under standard conditions as described above and then diluted 1:20 in multiwell microtiter plates containing 190 μl of LB medium supplemented with kanamycin. Cells were then grown with rotatory shaking in a Victor2 plate reader (PerkinElmer, Shelton, CT, USA) at 30°C to avoid excessive condensation on the plate lids during 2.5 h. After reaching mid-exponential growth (OD600 ~ 0.4), cells were treated with the effector of choice or the oxidative stress-inducing agent at the indicated concentrations (see below) and the incubation at 30°C resumed for 24 h. Bacterial growth was recorded as the OD600 measured every 15 min. Data shown corresponds to two independent experiments with eight technical replicates each. Unless otherwise indicated, DNA synthesis was performed by GeneArt (Thermo Fisher Scientific; Wallingham, MA, USA). Primers used in this work were provided by Sigma-Aldrich (St. Louis, MO, USA). Restriction enzymes and Phusion DNA polymerase, used for PCR amplifications, were purchased from New England Biolabs (Beverly, MA, USA). DNA AmpliTools Green Master Mix (Biotools B&M Labs SA, Madrid, Spain) was routinely used for colony PCR verifications. The fidelity of DNA fragments was later confirmed by restriction mapping and DNA sequencing of relevant features (Secugen Sequencing and Molecular Diagnostics, Madrid, Spain).

DNA constructs

General methods for DNA manipulation were performed as described elsewhere (Sambrook et al., 1989). Oligonucleotides used in PCRs are listed in Table S2. The ITPG-inducible expression system was obtained from the plasmid pTrc99a. The expression cassette containing the lacI² gene and the Pną promoter was amplified by PCR using primers TRC-F and TRC-R. These primers introduced PacI and AvrI restriction sequences in the flaking region of the fragment. A 1.4-kb PCR fragment was gel-purified and cloned in vector pSEVA221 previously digested with PacI/AvrI, leading to plasmid pSEVA224. The 3-mBz-inducible XylS/Pm expression system was constructed based on a cassette present in vector pJB653 and derivatives thereof (Blatny et al., 1997). In that system, the gene encoding the XylS activator is placed in a divergent orientation relative to its target Pm promoter. As the XylS/Pm DNA fragment presented many restriction sites used in SEVA multi-cloning site, the cassette sequence was edited in silico and the resulting variant was synthesized de novo by GeneArt. The 2.0-kb DNA fragment with the edited XylS/Pm sequence was cloned as a PacI/AvrI fragment into the pSEVA226 reporter vector, generating plasmid pSEVA226-XylS-Pm, which was then used to produce plasmid pSEVA238 (Calles et al., 2019). The AlkS/Pαβ expression cassette based on the cognate system of P.putida GP01 was fully synthesized by GeneArt in order to fulfill SEVA standards and was cloned into vector pSEVA421 to produce pSEVA429 (Arce-Rodríguez et al., 2021). Then, a PacI-AvrI fragment containing the complete regulatory device was re-cloned in vector pSEVA231 digested with the same enzymes, leading to plasmid pSEVA239. The design and construction of a standardized vector carrying the ChnR/PchnB expression system is described elsewhere (Benedetti et al., 2016b). Finally, the CprK/PDB3 expression system was assembled (Benedetti, 2014) by amplifying cprK1 from plasmid pMLK1 (Kemp et al., 2013) by PCR with primers 5’-CPRK1 and 3’-CPRK1, harbouring AvrI and PacI sites, respectively. The resulting DNA fragment was then digested with both enzymes and cloned into vector pSEVA231 digested with the same endonucleases, producing the intermediate plasmid pSEVA231-CPRK1. A separate DNA fragment contained the cognate promoter PDB3, which was followed by a buffer sequence that separated the promoter from the divergent promoter P3’_neo, which was introduced along with the TIR sequence 5’-GAT TAA CTT TAT AAG GAG GAA AAA-3’ (Miller
and Lindow, 1997), to drive the constitutive expression of cprK. This fragment, containing the CHPA-inducible P_DB3 promoter plus the constitutive P3/P_neo promoter, was cloned into the Nhel/AvrII sites of plasmid pSEVA231-CPRK1, producing plasmid pSEVA2312 that harbours the complete CprK/P_DB3 expression cassette. The corresponding reporter plasmid pS2312-M was obtained as indicated below. The promoterless msfGFP reporter was obtained from plasmid pGA-LacI, containing an edited PstI-less version of msfGFP, in a PCR reaction using oligonucleotides msfGFP-HindIII-F, which contained a TIR motif and an HindIII site, and msfGFP-Spel, bearing a SpeI restriction site. The amplified fragment was then digested with HindIII and SpeI, gel purified and ligated into the corresponding sites of pSEVA231, producing plasmid pSEVA237M (Benedetti et al., 2016b; Calles et al., 2019). All plasmids used to monitor expression activity arising from the different systems, i.e. pS234-M, pS234C-M, pS238-M, pS239-M, pS2311-M and pS2312-M, were constructed by restriction of pSEVA237M with HindIII-Spel and ligation into the corresponding expression system previously cleaved within the same endonucleases.

Plasmid pSEVA634 was built by moving the cargo module from pSEVA234 onto pSEVA631 via restriction digestion with Pael and AvrII and ligation. Plasmids pS634r and pS634rX were constructed with the uracil-excision (USER) cloning method. To this end, a 1,227-bp fragment containing P_lacI and lacI* was PCR-amplified from vector pSEVA234 with primers lacIq-revert_F/lacIq-revert_R and incorporated into the linearized, 3,249-bp pSEVA634 backbone (separately amplified with primers pSEVA634_F/pSEVA634_R) to yield plasmid pS634r. In the case of plasmid pS634rX, a 304-bp DNA fragment containing the standard SEVA translation initiation sequence (TIR), P_neo, and a buffer sequence was commercially synthesized (Integrated DNA Technologies, Leuven, Belgium). This fragment was amplified with primers std_reg_F/std_reg_R and fused to the linearized 4,211-bp pS634r backbone. The fluorescence-reporter plasmid variants pS634-M, pS634r-M, and pS634rX-M were created by amplifying the msfGFP gene from pSEVA237M with primers msfGFP_F/msfGFP_R, followed by restriction-digestion of the PCR amplicon as well as pSEVA634, pS634r, or pS634rX with EcoRI and HindIII and ligation.

Fluorescent flow cytometry
Quantification of msfGFP expression was made with fluorescence flow cytometry on strains containing the relevant constructs. In each case, bacteria were inoculated into filtered LB medium and pre-grown to stationary phase. Overnight cultures were then diluted to an OD_600 of 0.05 in fresh filtered LB medium and incubated at the appropriate temperatures to an OD_600 ~ 0.4, when the corresponding inducer was added. This time point was considered as t = 0. Following exposure to the inducer, 1 ml samples were harvested at various time points, as indicated in the text, and spun down in a tabletop centrifuge at 13,000 g during 1 min; then, the cells were washed with 500 μl of filtered phosphate buffered saline, centrifuged again as indicated above and resuspended in 300 μl of 0.4% (w/v) paraformaldehyde and finally incubated at room temperature for 10 min. After cell fixation, cells were washed twice in 500 μl of filtered phosphate buffered saline, resuspended in 600 μl of the same buffer and stored on ice until analysis. The final OD_600 was adjusted to a value < 0.4 in all samples before flow cytometry analysis. Single-cell fluorescence of the samples was then analysed by flow cytometry using a GALLIOS cytometer (Perkin Elmer; Shelton, CT, USA) or with a MACSQuant™ VYB cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). GFP was excited at 488 nm, and the fluorescence signal was recovered with a 525/40 nm band pass filter. At least 25,000 events were counted in each aliquot. The GFP signal was quantified under the experimental conditions tested by firstly gating the cells in a side scatter against forward scatter plot, and then the GFP-associated fluorescence was recorded in the FL1 channel (515-545 nm). Data processing was performed using the FlowJo™ software (www.flowjo.com), which was used to calculate the geometric mean of fluorescence per bacterial cell (x-mean, n√x1·x2·...·xN) and the coefficient of variation expressed as percentage (CV = σ/μ,100, where σ is the population standard deviation and μ de population mean). All experiments were carried out at least three times with two technical replicates.

Monitoring of msfGFP expression at the population level
Quantification of green (msfGFP) fluorescence in cultures of E. coli CC118 harbouring either plasmid pSEVA634M, pS634r-M, or pS634rX-M was performed in 96-well plates in a Synergy Max plate reader (BioTek Instruments; Winooski, VT, USA). Overnight precultures in LB medium were diluted 1:100. The production of msfGFP was induced via the addition of IPTG at 1 mM after 2.5 h of cultivation (corresponding to the early exponential phase in microtiter plate cultures). Fluorescence was measured at an excitation wavelength of 483/9 nm and an emission wavelength of 514/9 nm, with the gain set at 80. The expression performance of the IPTG-inducible standardized device was likewise analysed in cultures of E. coli CC118 carrying plasmid pS234-M or pS234C-M, treated under different conditions as described. In this case, cells were grown in 96-well microtiter plates at
30°C in a Victor-2 multireader spectrophotometer (Perkin Elmer, Shelton, CT, USA). At time intervals of 15 min, the green fluorescence and the OD$_{600}$ of each culture were simultaneously recorded. Non-inoculated LB and cells harbouring the pSEVA234 empty plasmid were used as blank to adjust the baseline for measurements. The expression system activity was calculated by normalizing the green fluorescence to the OD.

**Flow cytometry detection of ROS (peroxides and hydroperoxides) by dichlorofluorescein derivatives**

To detect ROS formation by means of flow cytometry analysis, cultures of *E. coli* CC118 transformed with vector pSEVA237M were incubated overnight as indicated. The following day, samples were diluted in 25 ml of filtered LB medium to an initial OD$_{600}$ $\approx 0.4$. The mid-exponential phase of growth (OD$_{600}$ $\approx 0.4$), the culture was divided into 7 ml aliquots that were treated either with the stressors H$_2$O$_2$ or diamide (as positive controls of oxidative stress) or with 1 mM of each of the inducers under inspection. One more aliquot was kept untreated as negative control. After an additional 2 h incubation, 1 ml of the cultures was spun down at 13 000 $g$ for 2 min at room temperature, washed once with 1 ml of filtered phosphate buffered saline by centrifugation and resuspension and diluted in filtered phosphate buffered saline such that the OD$_{600}$ was under 0.4 in a final volume of 1 ml. Then, a freshly prepared solution of 5-(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA; Sigma-Aldrich, St. Louis, MO, USA) in DMSO was added to the samples at 20 $\mu$M and the green fluorescence to the OD.

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**Conflict of interest**

Authors declare no conflict of interest.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Expression level of populations harbouring diverse IPTG-inducible devices.

**Table S1.** Strains and plasmids used in this study.

**Table S2.** Primers used in the PCR reactions.