A portable library of phosphate-depletion based synthetic promoters for customizable and automata control of gene expression in bacteria

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Summary

Industrial biotechnology gene expression systems relay on constitutive promoters compromising cellular growth from the start of the bioprocess, or on inducible devices, which require manual addition of cognate inducers. To overcome this shortcoming, we engineered an automata regulatory system based on cell-stress mechanisms. Specifically, we engineered a synthetic and highly portable phosphate-depletion library of promoters inspired by bacterial PHO starvation system (Pliar promoters). Furthermore, we fully characterized 10 synthetic promoters within the background of two well-known bacterial workhorses such as E. coli W and P. putida KT2440. The promoters displayed an interesting host-dependent performance and a wide strength spectrum ranging from 0.4- to 1.3-fold when compared to the wild-type phosphatase alkaline promoter (PphoA).

By comparing with available gene expression systems, we proved the suitability of this new library for the automata and effective decoupling of growth from production in P. putida. Growth phase-dependent expression of these promoters could therefore be activated by fine tuning the initial concentration of phosphate in the medium. Finally, the Pliar library was implemented in the SEVA platform in a ready-to-use mode allowing its broad use by the scientific community.

Introduction

Microbial growth generally hampers biotechnology objectives because it consumes resources that cease to be available for production processes. Decoupling microbial growth from production processes is seen by many as an optimal solution because it would enable temporary resource allocation, thus avoiding interferences between cell growth and production (Durante-Rodriguez et al., 2018; Stargardt et al., 2020). Fine control of biotechnological output(s) during the initial growth phase maximizes cell biomass by reducing metabolic burdens, while timely expression of biotechnological pathways in a subsequent phase will optimize production (Lo et al., 2016; Lemmerer et al., 2019). Growth-decoupling processes require highly regulated systems, often involving the addition of an inducer or repressor molecule to initiate the expression of the genes responsible for synthesizing the product of interest (Palomares et al., 2004). However, in many cases such inducers usually have a high cost and can become toxic, so post-treatment processes are required to remove them from both the final product and the waste (Menart et al., 2003; Nevoigt et al., 2007). The use of stress inducible promoters to control processes has emerged as an interesting solution to such problems (Hicks et al., 2020). The use of this type of promoter allows the engineering of transcription factor (TF)-based systems capable of detecting environmental changes that trigger gene expression once the cells have reached a desirable growth. Examples of stress-induced promoters include those controlled by temperature (Qing et al., 2004; Yin et al., 2007; Valdez-Cruz et al., 2010) changes in nutrient concentration (Sanders et al., 1998; von der Heyde et al., 2015) heavy metals (Blasi et al., 2012) and quorum sensing (Soma and Hanai, 2015). However, these systems are not free from important drawbacks. In the case of quorum sensing-based
systems, the difficulty of adjusting cell densities can reduce the system’s accuracy (Soma and Hanai, 2015), while systems based on carbon source starvation require adding extra glucose to complete the production process, albeit at reduced yields (Bothfeld et al., 2017). Heat-shock inducible promoters face challenging upscaling processes (Caspeta et al., 2009), whereas heavy metal-based inducible systems often trigger demanding metabolic responses impacting yield (Blasi et al., 2012; Bothfeld et al., 2017). However, stress inducible promoters allow less complex decoupling of growth from production cycles in fermentation processes (Lo et al., 2016). This advantage facilitates coordination when constrictive synthesis stages are required, delaying the appearance of toxic metabolites and reducing the occurrence of futile cycles that decrease productivity (Engstrom and Pfleger, 2017; Kent and Dixon, 2019). Therefore, the search and development of easily scalable and metabolically harmless portable promoters capable of fine tuning gene expression in a wide range of systems has become of great importance.

Inorganic phosphorus (Pi) is one of the most essential elements in living systems and a key player in their biochemical processes. Pi plays a role as a structural element in DNA, proteins, phospholipids, etc. and as an essential element in the energy metabolism (Santos-Beneit, 2015). Escherichia coli uses two different mechanisms to transport Pi into the cell depending on extracellular concentration: (i) a low-affinity Pi transport system (Pit) that operates when there are no extracellular Pi restrictions; and (ii) a specific Pi transport system (Pst). Pst is part of the PHO Pi-starvation system, a global regulatory two-component system involved in phosphate transport and assimilation that comprises over 30 genes in E. coli (Gao and Stock, 2013; Uluseker et al., 2019). The PHO Pi-starvation system is induced under extracellular Pi depletion and has been extensively studied in E. coli (Van Dien and Keasling, 1998). The PHO regulon is finely regulated by the PhoB/PhoR two-component system. The response regulator PhoB is phosphorylated by the histidine kinase PhoR under Pi depletion. Subsequently, the phosphorylated PhoB (PhoB-P) activates transcription of a large set of genes by binding to a conserved operator region, the PHT box, and recruiting the transcription of a large set of genes by binding to a consequently, the phosphorylated PhoB (PhoB-P) activates the histidine kinase PhoR under Pi depletion. Subsequently, we exploit the potential of synthetic promoters for decoupling growth and expression by developing a tunable and completely self-driven, two-stage bioprocess in P. putida. The new promoter library has been implemented in the standardized pSEVA platform for its use worldwide.

Results and discussion

Construction and analysis of a PphoA expression system and its validation in E. coli W and P. putida KT2440

In E. coli the basic structure of PphoA includes a PHO box located around the −35 box (5’-CTGTCA-TAAAGTTGTCACTAC-3’). By taking advantage of its well-known promoter structure, we constructed a PphaA-based Pi depletion expression system. The device comprised the PphoA promoter and the msf (GFP) translated by a strong bicistronic RBS (BCD2) as the reporter module (Mutalik et al., 2013) (Fig. 2A). As might be expected when using the E. coli DH10B strain as a host, PphoA showed maximum activity at limited phosphate concentration (< 50 μM), while no activity was registered at higher levels of Pi (≥ 1 mM) (Fig. 2A).

Most traditional studies involving design and characterization of synthetic promoters use laboratory-derived strains, which accumulate growth and functionality-limiting mutations under real-life biotechnological conditions. In order to assess our device with relevant bacterial workhorses, we decided to monitor the performance of PphoA in E. coli W and P. putida KT2440. E. coli W (ATCC 9637) has properties that make it a highly

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preferred strain for industrial applications: (i) it produces low amounts of acetate, (ii) it can be grown to high-cell density during fed-batch culture, (iii) it exhibits good tolerance to environmental stresses, and (iv) it is a very fast growing strain with superior growth rate compared to classical K-12-derived strains (Archer et al., 2011; Park et al., 2011; Felpeto-Santero et al., 2015). On the other hand, *P. putida* is a soil bacterium whose value as a workhorse for industrial applications has recently drawn the attention of many authors, mostly due to its high robustness against environmental stress and its capacity to degrade a large array of natural and xenobiotic chemicals and pollutants (Nikel and Lorenzo, 2018; Poblete-Castro et al., 2012). As expected, *PphoA* was also active in *E. coli* W and *P. putida* KT2440 at limited Pi conditions. However, slight host-dependent differences were found: *PphoA* activation required less incubation time in *E. coli* (0.5 vs. 1.5 h), while reduced basal activity (promoter leaking) and higher absolute activity (up to 1.5 times higher) were recorded for *P. putida* (Fig. 2B, Fig. S1A, B). As previously reported for other PHO-related promoters (Gao and Stock, 2018) the activity of *PphoA* as a function of external Pi followed an inhibitory Hill’s function (Eq. 1, Fig. 2C). Using this function, we further estimated several functional parameters of *PphoA*, including basal and maximum activities, the operational (OR) and dynamic (DR) ranges, IC50 (Pi concentration supporting 50% of maximum activity), and its Hill’s constant (K), which is dependent on the binding cooperativity between TF PhoB-P and the PHO box (Fig. 2D) (Ang et al., 2013). *PphoA* activity in *E. coli* W showed higher activity at Pi concentrations below 0.1 mM, peaking at concentrations ranging from 0 to 50 µM. This is in good agreement with previous reports (Lübbe *et al.*, 1995). *PphoA* activity significantly decreased at higher concentrations and was completely repressed at concentrations above 0.2 mM (Fig. 2C). The estimated OR value was 0.2 mM for IC50 0.12 mM, while DR showed an 11-fold activity increase for the promoter. The value of Hill’s constant was 2, which is within the range of K values calculated elsewhere for the *phoB* (1.9) (Ritzefeld *et al.*, 2013) and *phoA* (1.6) (Gao and Stock, 2015) promoters. This seems to be related to a TF PhoB-P dimer binding to the promoters’ single PHO box (Bianco *et al.*, 2012; Gao and Stock, 2018).
The behaviour of P\textit{phoA} in \textit{P. putida} KT2440 showed interesting differences when compared with \textit{E. coli} W (Fig. 2C). The promoter retained peak activity at phosphate concentrations that doubled those for \textit{E. coli} (0.1 mM). In addition, P\textit{phoA} remained active at Pi concentrations as high as 0.6 mM, which resulted in a
threefold increase in OR and a higher IC$_{50}$ value (0.43 vs. 0.12 mM). PphoA also displayed higher DR and K values in *P. putida* (10 and 2 times higher, respectively). The calculated K value was also over twice as high in *P. putida* than in *E. coli* W. This steeper sensitivity could be due to an increase in cooperative binding of TF PhoB-P to the PHO box or to the existence of a cellular process that increases sensitivity (Ang et al., 2013).

Despite observed host-dependent differences in behaviour, we proved the potential of PphoA (and likely other PphoA-based promoters) as suitable gene expression systems for different biotechnological bacterial workhorses. These results and the inferred broad universality of the PHO two-component system (Fig. 1) pave the way for the construction of highly portable gene expression systems in bacteria. In order to generate tools to support optimization and tight regulation of biotechnological processes based on the PHO Pi-starvation system, we generated a set of new-to-the-nature synthetic promoters to expand the spectrum of promoter activity strengths.

**Combinatorial construction and screening of a library of synthetic Pi-dependent promoters (Pliar)**

Common strategies used to engineer synthetic promoters entail: (i) random and site directed mutagenesis of natural promoters by using error-prone DNA polymerases and/or degenerated (randomized) oligonucleotides, and (ii) the construction of hybrid promoters combining structural parts of different promoters to obtain the desired functionality (Johnson et al., 2018; Yang et al., 2018). To engineer synthetic Pi-starvation promoters we used a combined strategy, where sequence-randomized PHO boxes were introduced in the −35 box of a strong constitutive promoter (BG42) (Zobel et al., 2015). The overall workflow is shown in Fig. 3. In order to design a fully portable synthetic PHO Box, we analysed known PHO boxes from several Gram positive and negative bacteria, thus generating a consensus sequence which included multiple variable positions (Fig. 3A, Fig. S2). The consensus sequence was further used as a template to design a set of degenerate primers, including thousands of alternative PHO Boxes. Using these primers, we amplified the constitutive promoter BG42 to deliver a large library of putative synthetic promoters that included alternative PHO Boxes (Pliar promoters). These synthetic promoters included the −10 region from BG42 and a variable PHO box located around the −35 region (Fig. 3B). To facilitate library screening and the characterization of the new synthetic Pliar promoters, and following the procedure for PphoA, they were fused to BCD2 and the reporter gene msfgfp, thus generating the pSEVA23_Pliar vectors, Fig. 3C.

The initial screening step was performed directly using the cloning strain *E. coli* DH10B. After transformation, colonies showing GFP fluorescence only under Pi limitation but not under Pi excess were selected for further analysis. In a second screening step, the GFP fluorescence of positive colonies was quantitatively evaluated in MOPS minimal medium at plenty and limited Pi concentrations. Remarkably, the single inclusion of a −35 box by PHO boxes in the −35 position was enough to become the constitutive BG42 in a Pi-depleting regulated promoter. The inclusion of alternative PHO boxes guided a large distribution of promoter strengths (Fig. 3D), ranging from promoters exhibiting similar activity to that found in PphoA at limited Pi concentration, for example Pliar 52 and 53, to promoters displaying weak promoter activities (Pliar 15, 3, 56) and even stronger activity (Pliar 51).

With plenty Pi, similar to PphoA, most of the Pliar promoters showed reduced activity. However, several promoters retained elevated basal activity, for example Pliar 51 and 68. From this first set of analysed promoters, we selected ten covering the full spectrum of activity strengths for further studies and characterization with relevant industrial strains such as *E. coli* W and *P. putida* KT2440. The selected synthetic Pliar promoters were: 1, 10, 15, 17, 51, 52, 53, 59, 68 and 70 (Fig. 3D).

**Activity and performance of Pliar synthetic promoters in E. coli W**

The selected Pliar promoters were transferred to *E. coli* W and their behaviour monitored as a function of the Pi concentration (Fig. 4A). All of them showed peak activity in the absence of extracellular phosphate, (Fig. 4A, Fig. S3). Similar to PphoA, at extracellular Pi concentrations higher than 0.2 mM all of them were repressed, showing only basal activity. The broad variability of basal activities displayed by the different Pliar promoters is quite remarkable. The strongest (Pliar 51, 59 and 68) were also those displaying the highest basal activities (up to 5 times higher than in PphoA). On the contrary, the weakest promoters (Pliar 1, 15 and 10) had lower basal activity at high-Pi concentrations (over 5 times lower than PphoA). The data from the dose–response curve was subsequently adjusted to Hill’s function (Fig. 4 B, Fig. S4). Despite the wide range of activity strengths displayed by the Pliar promoters, it should be noted that all of them had OR (~ 0.2 mM) IC$_{50}$ (~ 0.12 mM) and K (~ 2) values similar to those estimated for the wild-type PphoA. In fact, most of the variability observed was due to differences in their basal and peak activities, which promoted a broad spectrum of DRs. It would thus be reasonable to think that the nucleotide sequence of the synthetic promoters determines their strength. In fact, we noted that the weakest promoters were those with the
highest percentage of GC in the PHO box (Fig. 4B). This effect has been previously described for variants of the Plac promoter in *E. coli*. Authors analysed different spacer sequences between the −35 and −10 regions and it seemed that higher GC percentages increased the resistance to separate DNA strands (Liu *et al.*, 2004). However, some exceptions were found, for example Pliar 70, which has a low-GC content (17%) and still exhibits medium-strength activity similar to that of *P*phoA.

Overall, based on the estimated functional parameters, the synthetic Pliar promoters could be clustered in four groups using clustergram analysis (Eisen *et al.*, 1998) (Fig. 4B):

i. *PhoA*-like Promoters: Promoters that showed regulatory activity akin to P*phoA* (Pliar 59, Pliar 53 and Pliar 70).

ii. High-strength Promoters: Promoters with high-activity irrespective of Pi concentrations and showing lower dynamic range (Pliar 51, and 68).

iii. Medium-strength Promoters: Promoters exhibiting medium strength at low Pi and not completely repressed at Pi concentration above 200 µM (Pliar 17 and 52).

iv. Low-strength Promoters: They are the weaker promoters, with peak activity more than two times lower than *PhoA*-like promoters. Interestingly, these were the promoters with higher DR (Pliar 1, 10 and 15).

**Activity and performance of Pliar promoters in *P. putida* KT2440**

The Pliar library was further analysed in *P. putida* KT2440 and, similar to our findings with *E. coli* W, the promoters displayed a Pi-dependent repression and an extended spectrum of activity (Fig. 5A, Fig. S5). However, notable differences were found. For instance, Pliar promoters had null leaking activity at high-Pi concentrations, which was in contrast to the behaviour exhibited in *E. coli*. Remarkably, Pliar 51, 59 and 68’s basal activity was over 10-times lower in *P. putida* and they triggered higher activity than in *E. coli* W (Figs S4 and S6). On the whole, these results argue in favour of a major control of Pliar promoters’ gene expression in *P. putida*.

Overall, the synthetic promoters showed significantly larger DR when compared with *E. coli*. Greater OR, around 0.6 mM, were also estimated and they consistently showed higher IC₅₀ values (up to 4 times higher for most analysed promoters) (Fig. 5B, Fig. S6). Estimated K values showed wide variability, ranging from 1.5 in Pliar 15 to the steeper slope of 8.1 observed in Pliar 52. These values confirmed the higher sensitivity of
Pliar promoters in *P. putida*. Therefore, since Pi is necessary for many cellular processes, the extended OR and higher sensitivity observed in *P. putida* opens the possibility of regulating biotechnological processes at a wider range of Pi concentrations than in *E. coli*, thus avoiding extreme conditions of phosphorus stress (Givskov et al., 1994). No direct relationship could be established between the nucleotide sequences of PHO boxes and promoters’ behaviour. As was done for *E. coli*, they were clustered into four groups according to their performance (Fig. 5B).

i. **PhoA-Like Promoters**: Promoters that showed regulatory activity similar to PhoA (Pliar53 and Pliar52).

ii. **High-strength Promoter**: A Promoter with high activity at low-Pi concentrations, showing the higher dynamic range (Pliar 1).

iii. **Medium-strength Promoters**: Promoters displaying medium strength at low Pi (Pliar17, 51 and 68).

iv. **Low-strength Promoters**: Weaker promoters with peak activity more than 2 times lower than PhoA-like promoters (Pliar10, 15, 70 and 59).

Interestingly, some promoters displayed a very different behaviour depending on the host: Pliar 1, which was a weak promoter in *E. coli*, becomes the strongest in *P. putida*. On the contrary, while Pliar 70 and 59 showed a PhoA-like behaviour in *E. coli*, they displayed low activity in *P. putida*. Overall, these data stress the need for a broad library of promoters in order to cover optimal gene expression requirements for different bacterial hosts.

Poly phosphate accumulation drives the different behaviour of Pliar promoters in *P. putida*. Due to the essential role that Pi plays in growth, most microorganisms accumulate it in the form of...
polyphosphate (poly-Pi). However, this poly-Pi storage ability is largely species-dependent (Kulakovskaya, 2015). For instance, it is well-known that *E. coli* accumulates poly-Pi to a very low extent (Nesmeyanova, 2000), while *P. putida* has a very active poly-Pi accumulation system that drives Pi accumulation in large quantities throughout their whole life cycle (Nikel et al., 2013). Therefore, differential phosphate accumulation abilities of these two strains could likely be responsible for differences in behaviour exhibited by the PHO-based promoters. In the case of *E. coli*, the concentration of extracellular Pi should remain more stable overtime, thus resulting in repressed promoters at concentrations greater than 0.2 mM. On the contrary, *P. putida*’s higher capacity to accumulate Pi could result in a significant reduction of extracellular Pi, thus leading to promoter activation, even at high initial phosphate concentrations.

To test this hypothesis, we grew both strains in the presence of 2.5 mM phosphate and monitored the amount of Pi in the supernatant over time. In both cultures, we observed a growth-dependent decrease in the levels of Pi. However, while in the case of *E. coli* this was observed only in the exponential phase, in *P. putida* the reduction of Pi extends throughout the complete growth curve (Fig. 4A and B). *E. coli* was only able to reduce the initial Pi concentration by 27%, which is in good agreement with previous reports indicating that *E. coli* only removed extracellular Pi during the exponential growth phase (Lübke et al., 1995). On the other hand, *P. putida* KT2440 was not only able to eliminate almost 50% of the initial phosphate during the exponential growth phase, but it continued to remove Pi in the stationary phase and reduced the concentration by up to 80% after 24 h (Fig. 4B). Therefore, active Pi removal could explain why Pliar promoters displayed activity in *P. putida* even at high-phosphate concentrations. In fact, it is reasonable to think that *P. putida* efficiently reduces the concentration of extracellular Pi to a critical level, thus triggering promoter expression irrespective of the initial Pi concentration in the culture medium.

Fig. 5. A. Comparison of the Pliar promoters’ activity in *P. putida* KT2440 at increasing Pi concentration. Pliar activity in terms of GFP fluorescence was measured in resting cells and normalized considering PphoA’s activity as the reference (value of 1). Readings were taken after 3.5 h’ incubation at 30°C in MOPS minimal medium, 0.2% glucose supplemented with phosphate.

B. Activity parameters or the synthetic Pliar promoters in *E. coli* W were calculated using Hill’s function, where *IC* is half maximal inhibitory concentration, *K* is Hill’s constant, *normalized basal activity*, *normalized maximum activity*, *dynamic range*, *operational range*, *guanine and cytosine percentage*. Promoter clusters were ordered according to their parameters using clustergram analysis.
A Pliar-based gene expression system for decoupling growth and production in *P. putida* KT2440

The ability of *P. putida* KT2440 to linearly reduce the external Pi concentration along its whole life cycle paved the way for designing a novel growth-decoupled gene expression system based on PHO-related promoters. Furthermore, Plier promoters could be used to engineer a pre-programmed, two-phase, growth-decoupled biotechnological process. In order to address this challenge, we monitored both GFP-based fluorescence and OD600 over time in a culture of *P. putida* harbouring the *msfgfp* gene under the control of Plier 53, a promoter that showed strong activity and suitable control in this strain (Fig. 5). When the cells grew in the presence of 1 mM of Pi, the poly-Pi accumulation abilities of *P. putida* promoted the appearance of two well-defined phases: i) a growth phase in which *P. putida* grew by consuming the available Pi, and ii) an expression phase following Pi depletion (Fig. 6C). During the growth phase, Pi in sufficient amounts promoted the full repression of promoter Plier 53, thus avoiding undesirable metabolic loads and facilitating optimal resource allocation for robust growth. Following Pi depletion, *P. putida* not only suddenly stopped growing, but the activation of Plier 53 drove an intense GFP-derived fluorescence that triggered the expression phase.

Since Pi depletion associated to cellular metabolism drives the activation of the PHO promoters, it is reasonable to think that the activation of Plier promoters could be finely controlled by tuning the level of initial Pi in the culture medium. In other words, using Plier promoters it would be possible to select the desirable cell biomass responsible for a given biotechnological output in a two-phase, growth-decoupled bioprocess just by adjusting the initial concentration of Pi. This controlled expression could be pre-programmed and would avoid the need for costly downstream processes to remove inducers while supporting self-induction bioprocesses. To test the feasibility of this interesting system, the fluorescence of *P. putida* cells harbouring the *msfgfp* gene under the control of several Plier promoters was monitored over time in MOPS minimal medium using 0.05, 0.5, 1, 1.5 and 2.5 mM phosphate concentrations (Fig. 6D). In good...
agreement with our reasoning, the higher the initial Pi concentration in the medium, the higher bacterial growth was required to activate the synthetic promoters. In fact, at extracellular Pi concentrations of up to 1.5 mM, we observed a linear correlation between initial Pi concentration and cellular biomass (measured as OD_{600}) and the Pliar promoters became active regardless of the promoter analysed (Fig. 6D). At Pi concentrations between 1.5 and 2.5 mM, the Pliar promoters became active once P. putida reached its peak growth (OD_{600} > 3). Using these linear correlations, the Pliar library became a very effective tool not only for decoupling growth from production but also to control, ad hoc, the biomass required to trigger production according to the needs of the process. For instance, in the case of the production of toxic metabolites, the decoupled bioprocesses could be initiated using high-phosphate concentrations, for example 1.5 mM. In this scenario, the production stage could only start once enough biomass has accumulated (OD ≥ 3). On the contrary, when just a limited amount of cellular biomass is required, the use of lower initial Pi concentrations might facilitate swift induction of Pliar promoters, thus delivering greater levels of production from the start of the process. In addition, the large range of activities and functional parameters contained in the Pliar library supports a wide choice of optimal promoters based on the requirements of biotechnological processes. In addition, the ability to select suitable promoters from the Pliar library will further expand gene expression-based control of target bioprocesses.

Finally, we contextualized the Pliar library within gene expression systems widely used in P. putida by comparing the performance of Pliar 53 promoter with XylS/P_{m}, RhaRS/P_{rhab} and lacIq/P_{irc} (de Lorenzo et al., 1993; Gawin et al., 2017; Jeske and Atenbuchner, 2010). For this, we constructed a series of plasmids expressing the fluorescent reporter RFP under the control of Pliar 53, P_{m}, P_{rhab} and P_{irc} and monitored the RFP-based fluorescence in P. putida cells under growing conditions (Fig. 7, Fig. S7). Noteworthy, we found interesting advantages when using the Pliar 53 system including: i) a lower basal activity and higher dynamic range than in the other systems, despite similar maximum activity was monitored and, ii) the decoupling of growth from production using the Pliar 53 had a negligible effect on growth rate (≈ 10% reduction), while higher reduction was found using RhaRS/P_{rhab} (20%) and XylS/P_{m} (40%). In the case of the lacIq/P_{irc} the elevated basal expression displayed affected the bacterial growth, even in absence of inductor (Fig. 7). In addition, it’s remarkable the fact that since Pliar system does require adding exogenous inductor, the fermentation cost of a putative bioprocess based on this system would be significantly cheaper than using either RhaRS/P_{rhab}, the XylS/P_{m} or lacIq/P_{irc} system.

Fig. 7. Comparison of Pliar 53, RhaRS/P_{rhab}, XylS/P_{m}, and LacIq/P_{irc} gene expression systems in P. putida KT2440. The activity, in terms of RFP fluorescence was measured in 96-well plates using growing cells at 30°C. The cultures were initiated at 0.2 OD_{600}^-1 Pi, rhamnose, m-toluic acid and IPTG Concentrations in mM. ^0 Growth rate in h^-1. ^1 Dynamic range, measured as the activity increase between the induced and repressed state.

Ready-to-use Pliar promoters synbio tool

Taken together, Pliar promoters have proved to be a very useful synbio tool to support automatic control of gene expression in biotechnological endeavours. In order to facilitate its portability and use by the community, we implemented several Pliar promoters in the context of the Standard European Vector Architecture, SEVA, platform (Martinez-Garcia et al., 2019). SEVA is a synthetic biology platform aimed at assisting the choice of optimal genetic tools for de-constructing and re-constructing complex prokaryotic phenotypes. Since its launching, SEVA has become a widely used platform and its standards are well-known and largely accepted by the synbio community. Therefore, standardizing Pliar promoters within SEVA makes them available to a wide community of potential end users in a wide range of applications. Promoters displaying a high range of activity in E. coli and P. putida were selected to construct the ready-to-use tools. They included Pliar 53 (PphoA-like activity in both microorganisms); Pliar 15 (weak activity in both); Pliar 17 (medium strength in both); Pliar 51 (high strength in E. coli and medium strength in P. putida); Pliar 1 (low strength in E. coli and high strength in P. putida); and Pliar 70 (medium strength in E. coli and low strength in P. putida). Pliar promoters were
inserted between restriction sites PacI and AvrII on the cargo site of a pSEVA231 vector, kanamycin resistant and with a broad range pBRR1 replication origin (Fig. S8). The final plasmids were named according to SEVA nomenclature: pSEVA2317 (Pliar 53); pSEVA2317A (Pliar 15); pSEVA2317B (Pliar 17); pSEVA2317C (Pliar 51); pSEVA2317D (Pliar 70); pSEVA2317E (Pliar 1). pSEVA2317 plasmids are available on the SEVA repository (http://seva-plasmids.com).

Conclusions

Synthetic biology largely aims to develop tools to speed up optimization of industrial processes. In this context, gene promoters play a major role because they are responsible for controlling gene transcription, thus providing the adequate protein load for target processes. In this work, we present a new set of nutrient-stress inducible promoters, the Pliar library. These new synthetic promoters are inspired by the PHO Pi-starvation system and were engineered by turning a strong constitutive promoter into a set of inducible promoters through the addition of PHO boxes at position −35. Pliar promoters increase the spectrum of activity strength from 0.4-fold to 1.3-fold compared to the wild-type PphoA and support fine control of biotechnological processes through selection of promoters that are best suited to the desired outputs.

Portability is one key aspect to consider when seeking and engineering new expression systems. Promoter portability supports the development of functional synbio tools in a large range of microorganisms. Extra regulatory elements are often required in the final constructs when developing these portable expression systems, which can entail subsequent increases in metabolic burdens in the host microorganism (Kushwaha and Salis, 2015). Due to the wide distribution of the PHO Pi-starvation system among bacteria (Fig. 1), synthetic Pliar promoters potentially represent a suitable tool to control gene expression in a large number of microorganisms without the need to introduce extra regulatory mechanisms. In this regard, the validation of the Pliar library in two biotechnological workhorses’ microbial systems, E. coli W and P. putida KT2440, underpin potential portability of this system. In addition, SEVA-standardization of our library will facilitate future use of Pliar promoters in almost any bacterial strain with a functional PHO Pi-starvation system.

Widely used expression systems such as Xyls/Pm, RhaRS/Pnmab and LacIq/Ptrc require expensive and/or toxic chemical inducers that limit their use in biotechnological applications. Furthermore, unlike Pliar promoters, they do not support the design of self-inducible biotechnological processes. Using P. putida KT2440 as a model, we proved here that Pliar promoters can be used to automatically induce any biotechnological process of interest. When using these promoters the reduction of extracellular Pi associated to bacterial growth and polyPi accumulation triggered a pre-programed Pi-starvation scenario, thus inducing the activation of the Pliar promoters without extra addition of chemical inducers. In addition, Pliar promoters are an interesting synbio tool for decoupling microbial growth from production, therefore avoiding the appearance of futile cycles and reducing metabolic burdens by simply adjusting phosphate concentrations in the fermentation medium.

Experimental procedures

The bacterial strains, plasmids and oligonucleotides used in this work are listed in Table 1.

For cloning, propagation and phosphate biosensor activity assays, E. coli, and P. putida strains were grown in LB medium or MOPS minimal medium (Neidhardt et al., 1974) with 0.2% glucose as sole carbon source, with or without KH₂PO₄. Kanamycin (100 µg ml⁻¹) was added when necessary. E. coli were grown at 37°C and P. putida KT2440 at 30°C. Phosphate concentrations were calculated using Merck’s phosphate assay kit and following the manufacturer’s instructions.

DNA work

E. coli DH10B was used for cloning. DNA polymerases and other DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA) and oligonucleotides were synthesized by Sigma-Aldrich (St. Louis, MO, USA). DNA purification kits were purchased from Nyztech (Lisboa, Portugal).

Construction of the PhoA-based phosphate biosensor

The phoA promoter from E. coli MG1655 was PCR-amplified and transcriptionally fused to the bicistronic translational coupler BCD2 (Mutalik et al., 2013). The fluorescent msfgfp gene (GFP) from plasmid pBG42 (Zobei et al., 2015) was used as reporter module. Subsequently, the PphoA::BCD2::msfgfp fragment was cloned using the PacI/SpeI restriction sites in the broad host range pSEVA231 vector (Antoine and Locht, 1992; Martinez-Garcia et al., 2019) and generating the pSEVA23_phoA vector Fig. 2A.

Construction of the synthetic phosphate promoters (Pliar) library

The PHO box logo sequence (Fig. 3A) was used as a template to design a set of degenerated primers that...
include randomized PHO Boxes (Table 1). These primers were used to construct the combinatorial library of synthetic Prilari promoters by PCR amplification. The constitutive promoter BG42 (5'-GCCCATTTGA-CAAGGCCTGCGCGCCAGGTATAATTGGCA-3') was used as a template. The structure of the synthetic promoters included promoter BG42's −10 region (5'-TATAAT-3') and a variable PHO box (5'-NTKTMY-HW-WHYNKT-MAY-3') located around the −35 region (Fig. 3B). Figure 3C shows the strategy implemented to construct Prilari promoters.

Construction of the synthetic Prilari 53_rfp, RhaRS/PrhaB_rfp, XylS/Pm_rfp and LacIq/Ptrc_rfp expression systems

The Codifying sequence for red fluorescence protein, RFP (E1010m (RFP)_CD) from the CIDAR Modular Cloning (MoClo) kit (Iverson et al., 2016) was cloned using the SMOOTH MoClo developed in our lab (paper in preparation) under the control of the promoters Prilari 53, RhaRS/PrhaB, XylS/Pm and LacIq/Ptrc, generating the plasmids pCMClv2_90, pCMClv2_91, pCMClv2_92 and pCMClv2_95, respectively. The synthetic constructs were then introduced in P. putida KT2440 by electroporation.

Fluorescence-based promoter activity assay

The Pi depletion promoter activity assays were carried out as in Uluseker et al. (2019). Briefly, the E. coli and P. putida strains carrying the Prilari or PrphoA plasmids were precultured overnight in MOPS minimal medium containing 5 mM KH2PO4 at 37°C and 30°C, respectively. Bacterial pre-cultures were used to inoculate the same fresh medium at 0.1 OD600 and were grown until mid-late exponential phase (0.6–0.9 OD600). Bacterial cells were then

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Conflict of interest

The authors declare no conflict of interests.

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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.** Sequence alignment of PHO boxes from diverse sources and PHO box consensus sequence used for the design of degenerated primers.

**Fig. S2.** Time course of PphoA activity at limited (50 μM) and plenty (1 mM) phosphate concentration in *E. coli* W (A) and *P. putida* KT2440 (B). PphoA activity was measured as GFP expression in MOPS minimal medium with 0.2% glucose supplemented with phosphate at 37°C for *E. coli* and 30°C for *P. putida*.

**Fig. S3.** Time course of PphoA and Pliar promoters’ activity over the time in *E. coli* W resting cells at increasing phosphate concentrations. Promoters’ activity was measured as GFP expression in MOPS minimal medium with 0.2% glucose at 37°C.

**Fig. S4.** Dose–response curves (Hill’s function) of synthetic Pliar promoters in *E. coli* W resting cells after 3.5 h incubation. Activity was measured as GFP expression and normalized considering PphoA’s peak activity as the reference (value of 1) in MOPS minimal medium with 0.2% glucose at 37°C.

**Fig. S5.** Time course of PphoA and Pliar promoters’ activity in *P. putida* KT2440 resting cells at increasing phosphate concentrations. Promoters’ activity was measured as GFP expression in MOPS minimal medium with 0.2% glucose at 30°C.

**Fig. S6.** Dose–response curves (Hill’s function) of synthetic Pliar promoters in *P. putida* KT2440 resting cells after 3.5 h incubation. Activity was measured as GFP expression and normalized considering PphoA’s peak activity as the reference (value of 1) in MOPS minimal medium with 0.2% glucose at 30°C.

**Fig. S7.** Time course of growth and activity of the Pliar 53, RhaRS/Pmab, XylS/Pm, and LacI/Prec promoter systems in *P. putida* KT2440. The activity in terms of RFP fluorescence was measured in 96-well plates using growing cells at 30°C in MOPS minimal medium 0.4% glucose, supplemented with 5 mM Pi. The cultures were initiated at 0.2 OD600. For induction, the cells carrying the Pliar 53 promoter were grown at 0.5 mM Pi; cells carrying RhaRS/P_mab were grown at 1 mM rhamnose; cells carrying XylS/Pm were grown at 1 mM m-toluic acid and cells carrying the LacI/Prec promoter system were grown at 1 mM IPTG.

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**Fig. S8.** Up, Schematic structure map of pSEVA1317 vectors. The position of the Pliar promoter is highlighted in dark green. KmR (kanamycin resistant). oriT is highlighted in purple and the origin of replication pBBR1 in blue. Down, nucleotide sequence of the Pliar promoters selected: pSEVA2317 (Pliar 53); pSEVA2317A (Pliar 15); pSEVA2317B (Pliar 17); pSEVA2317C (Pliar 51); pSEVA2317D (Pliar 70); pSEVA2317E (Pliar 1). Underline the sequence of the restriction enzymes PacI and AvrII.