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Transcription factor-based biosensors and inducible systems in non-model bacteria: current progress and future directions

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Genetic diversity within the geobiosphere encompasses enormous sensing capabilities and many non-model bacteria are of biotechnological interest. Biosensing, or more generally inducible, systems are a vital component of metabolic engineering, as they allow tight control of gene expression as well as the basis for high-throughput screens on non-growth-related phenotypes. While these inducible systems, primarily transcription factor/promoter pairs, have been utilized extensively in *Escherichia coli*, progress in other bacteria is limited because of differences in transcription machinery, physiological compatibility of parts and proteins, and other nuances. Here, we provide an overview of the available genetic biosensing elements in non-model organisms and state-of-the-art efforts to engineer them, and then discuss challenges preventing these methods from common use in non-model bacteria.

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Introduction

The geobiosphere's genetic diversity encompasses enormous catalytic and sensing capabilities. Despite this, testing and utilization of these abilities have been disproportionately focused on a choice group of model organisms, primarily *Escherichia coli*. For many industrial and other biotechnology applications, non-model bacteria have been historically used (e.g. acetone from *Clostridium acetobutylicum*, antibiotics from *Streptomyces*) and continue to have promising applications.

Biosensing, or more generally inducible systems, are vital in the context of genetic engineering. Inducible systems allow tight control of native and heterologous genes and biosynthetic pathways and enable the use of genetic engineering tools that require distinct on/off states. Biosensors are also used as the basis for performing high-throughput experiments on non-growth-related phenotypes by making the phenotype selectable or screenable with the aid of reporter genes.

While inducible systems, primarily transcription factor (TF)/promoter pairs, have been utilized extensively in *E. coli*, use in other bacteria is limited when appropriating existing biosensing systems because of differences in transcription machinery, physiological compatibility, and other nuances.

Here, we briefly provide an overview of the available genetic biosensing elements in non-model bacteria and current engineering efforts, and then discuss high-throughput and advanced methods to engineer biosensors with the main challenges and limitations that preventing these methods from common use in non-model bacteria. We call for the expansion of genetic parts, particularly biosensing elements, that operate across a spectrum of bacteria in order to improve the speed and ease of engineering less developed but situationally advantageous bacteria.

Current state of the art of inducible systems in non-model organisms

Non-model bacteria are an untapped reservoir of regulatory elements. Many manifest as allosteric TF/promoter pairs and have been repurposed as heterologous inducible systems and biosensors (listed in Table 1). Chemical inducers such as carbon sources, metabolites, and antibiotics, environmental signals such as light, pH, oxygen, or temperature, and autoregulatory quorum-sensing molecules can all be sensed by TFs (Figure 1).

Chemical induction is the most commonly used type of inducible system due to the natural abundance of small molecule-sensing TFs. Many bacterial strains rely on only one or a small handful of inducible systems due to the difficulty in expanding the availability of TF/promoter pairs [1,2]. Emerging industrial hosts like *Rhodococcus opacus* and *Halomonas* spp. have relied on one inducible promoter each, an antibiotic thiostrepton-inducible *TipA* promoter and an IPTG-inducible promoter (Ptrc), respectively [3•,4••].

Table 1

Transcription factor-based inducible systems used in non-model bacteria

Genus	Inducer; TF; Source	Species	Operating range	Dynamic range	Reporter	Ref.
Bacillus	IPTG; LacI *E. coli*		10–1000 μM	11 000-fold	sfGFP	[21]
	Green Light (535 nm); CcaS/R *Synechocystis* PCC 6803	*B. subtilis*	0.1–10 μmol/m^2s^1 (526 nm)	70-fold	sfGFP	[16•]
	Xylose; XylR[a] Native	*B. megaterium*	0–0.4 mM		GFP	[38••]
Caldicellulosiruptor	Xylose; XylR Native	*C. bescii*	1–33.3 mM	32-fold	*ldh*	[19]
Clostridium	Anhydrotetracycline; TetR *E. coli*	*C. aceto-butylicum*	25–200 ng/mL	41-fold	*gusA*	[27]
	Arabinose; AraR *C. acetobutylicum*	*C. cellulolyticum*	0.001–about 1 g/L	800-fold	*gusA*	[9]
	Laminaribiose; GlyR3 Native	*C. thermocellum*	2–10 mM	40-fold	*spo0A*	[10]
	Tetracycline; TetR *E. coli*	*C. difficile*	20–500 ng/mL		*gusA*	[1;45]
	Xylose; XylR Native		0.03–3% (w/v)	100-fold	mCherry	[2]
Corynebacterium	*p*-Coumaric Acid; PadR *B. subtilis*	*C. glutamicum*	1–5 mM		YFP	[37]
	Shikimic acid; ShiR Native		about 1–500 mM		EGFP	[14]
Cupriavidus	3-hydroxypropionic acid; HpdR *Pseudomonas putida* KT2440		about 0.1–10 mM	517-fold	RFP	[6]
	L-arabinose AraC; *E. coli*	*C. necator* H16		1200-fold	RFP	[6]
	Itaconic acid; ItcR *Yersinia pseudotuberculosis*			105-fold	RFP	[30]
Cyanobacteria	Ammonium Sulfate; GlnR *Lactococcus lactis ATCC 19435*	*Synechocystis* spp. PCC 6803	0.3–3 mM	1.6-fold	eYFP	[13]
	IPTG; LacI *E. coli*	*Synechocystis* spp. PCC 7942	0.01–10 mM	24-fold	eYFP	[25]
	Oxygen; FNR *E. coli*			~3-fold	FbFP F37T	[18]
	Rhamnose; RhaS *E. coli*	*Synechocystis* spp. PCC 6803	0.25–1 mg/mL		YFP	[7]
Pseudomonas	3-hydroxypropionic acid; MmsR Native	*P. denitrificans* ATCC 13867	0.01–100 mM	100-fold	GFP RFP	[28]
	4-hydroxybenzoate; PobR-DM *Actinetobacter baylyi* ADP1	*P. putida* KT2440	about 30 μM–30 mM	12-fold	sfGFP fused with *ubiC* (*E. coli*)	[15]
	Ammonium Sulphate; GlnR *L. lactis ATCC 19435*	*P. putida* NRRL B14683	2 mM–about 10 mM	10-fold	RFP	[13]
	IPTG; pTrc *E. coli*	*P. putida* KT2440		80-fold		[8]
	Protocatechuate; PcaU *A. baylyi* ADP1	*P. putida* KT2440	About 3 μM–10 mM	12-fold	sfGFP	[29]
Rhodococcus	Acetamide; AceT *Mycobacterium smegmatis*	*R. opacus* PD630	0.01–10 nM	5-fold	GFP+	[3•]
	Anhydrotetracycline; TetR *E. coli*		0.05–100 ng/mL	67-fold	mCherry	[3•]
	Arabinose; AraC *E. coli*		0.1–100 mM	59-fold	EYFP	[3•]
	Tetramethylpyrazine; TpdR Native	*R. jostii* TMP1	1–5 mM		EGFP	[52]

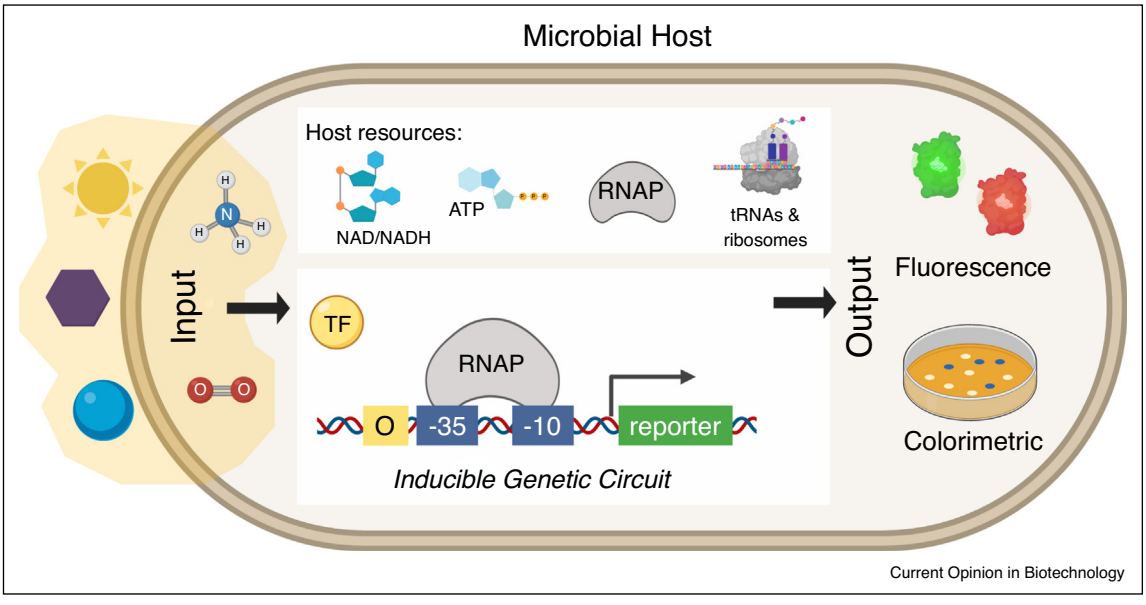
Table 1 (*Continued*)

Genus	Inducer; TF; Source	Species	Operating range	Dynamic range	Reporter	Ref.
Shewanella	3-oxo-C12-HSL; LasR *P. aeruginosa*	*S. oneidensis* MR1	1 nM–1 μM		Gas ratio of methyl bromide/ ethylene	[53]
Streptomyces	Cumate; CymR *P. putida* F1	*S. albus* J1074	about 2.5–5 nM	66-fold	*indC*	[12]
	Oxytetracycline; OtrR *Streptomyces rimosus*	*S. venezuelae*	0.01–4 μM		GFP	[20]
	Paramycins; PamR *Streptomyces alboniger*	*S. alboniger* DSM-40043	0.1–5 mg/L		*gusA*	[31]
	Anhydrotetracycline; TetR[a] *E. coli*	*S. venezuelae*	1–about 10 μM		sfGFP	[40]

ldh, lactose dehydrogenase; *gusA*, β-glucuronidase; FbFP, Flavin mononucleotide-binding fluorescent proteins; *ubiC*, chorismate pyruvate-lyase; *Bxb1*, serine-integrase; *indC*, indigoidine synthetase.
[a] Cell-free system.

To overcome the limited number of inducible systems available within non-model strains, a common strategy is to appropriate a handful of well-characterized *E. coli*-derived inducible systems (e.g. pBAD, pTET, pLac) that can function in non-model bacteria [5–7]. This strategy, most often simply adjusting expression levels of the TF to improve fold induction in non-model organisms, has varying degrees of success. Reducing TetR expression by mutating the −10 box of the promoter led to an increase in dynamic range by 67-fold upon induction of anyhydrotetracycline-inducible system (pTET) in *R. opacus* PD630 [3•]. Likewise, controlling LacI repressor concentration with a weak promoter increased the overall change in expression by 80-fold of IPTG-inducible *E. coli* (pTrc) promoter in *Pseudomonas putida* [8]. While useful in the lab, these systems are often limited industrially due to the expense, varying half-lives of inducers or complications from serving a dual role as inducer and carbon source. Sugar-based inducers are simultaneously utilized as carbon sources in hosts, and thus depleted over time and are complicated by carbon catabolite repression [9,10]. While IPTG is often used as a substitute for lactose, certain transcription factors like BgaR in *C. acetobutylicum*, only respond to lactose [11].

Figure 1



Context dependence of genetic circuits.
A wide range of chemical, physical, and environmental inducible systems have been engineered and tested in non-model organisms for biotechnological applications. However, engineered inducible systems often lead to unpredicted behavior when transferred from one host to another, due to various cellular resources and machinery (e.g. RNAP, ribosomes), thus requiring further engineering and optimization in the new host chassis.

Beyond exogenously introduced chemicals, dynamic gene regulation can also occur in response to the host's metabolic state, potentially alleviating toxic product accumulation and optimizing production pathways. Dynamic sensor-regulator systems enable dynamic control of metabolic imbalances that often occur in engineering cellular factories. Modulation of actinorhodin metabolite concentrations from becoming toxic in *Streptomyces* by cumate-inducible regulatory system improved its production by 60% [12]. Biosensors independent of the host's native nitrogen regulatory system can be used to enhance production of metabolites produced under low or high nitrogen conditions [5]. An ammonium biosensor was developed to enhance biosynthesis of a nitrogen-rich polymer in *E. coli*, *P. putida*, and *Synechocystis* spp. [13]. Nitrogen-responsive biosensors, identified through part mining, have enabled dynamic regulation of heterologous genes in lipid biosynthesis to improve biodiesel precursor production in *R. opacus* [3•].

Biosensing intracellular concentrations of metabolites can provide an efficient high-throughput screen of hyperproducing strains. A biosensor monitoring intracellular shikimate (central to phenolic amino acid biosynthesis) in *Corynebacterium glutamicum* allowed for RBS library screenings via fluorescence-activating cell sorting (FACS) [14]. To alleviate product inhibition of the pathway, sensor-enabled positive feedback-control mediated by the product can be used to increase production. The 4-hydroxybenzoate-responsive biosensor in *P. putida* enabled an enzyme library screening and selecting for enzyme *ubiC* from *E. coli* that converts chorismite to 4-hydroxybenzoate with 60% higher catalytic efficiency compared to the wild-type [15].

The ability to control gene expression with physical or environmental cues (e.g. light, pH), is attractive for many engineering applications. Green light-activated CcaS/red light-deactivated CcaR TF pair (CcaSR) derived from *Cyanobacteria* is the most commonly used light-inducible system. Green light phosphorylates a chromophore group covalently bound to CcaS, activating CcaR to initiate transcription. While light-based sensors naturally have a limited detection range, a 70-fold activation and rapid dynamic response were achieved via promoter and protein engineering in *Bacillus subtilis* [16•].

A particular problem with many cyanobacterial promoters is their relation to daily light-dark cycles; they are commonly more active when exposed to light, thus making their functionality context-dependent. Further complicating matters, otherwise ideal inducible promoters, like the anhydrotetracycline-responsive promoter yielding a 230-fold induction in *Cyanobacteria*, are limited by the inducer's light sensitivity [17]. To expand the *Cyanobacteria* toolkit, low oxygen-responsive FNR (fumarate and nitrate reduction) TF from *E. coli* that was engineered for use in *Synechocystis* spp. PCC 6803 has been applied for expressing oxygen-sensitive enzymes or at low oxygen conditions [18].

Optimizations of inducible systems are often species-specific due to the knowledge gap concerning the dependences of genetic circuits on the host organism. Thermophiles pose special challenges in constructing inducible systems, as common mesophilic-derived systems are not physiologically compatible, requiring identification of host-specific inducible elements (e.g. part mining of xylose-inducible promoter in thermophile *Caldicellulosiruptor bescii* [19]) to obtain function at high temperatures. Antibiotic-inducible promoters lack potential orthogonality due to their limitation to hosts with native antibiotic resistance (commonly *Streptomyces*) [20]. A bacitracin-inducible promoter has been applied in a few bacitracin-resistant *B. subtilis* strains, but even then expression decreases after two hours likely due to a bacitracin stress response [21].

Current methods to engineer inducible systems in non-model organisms

Metabolic engineering strategies to improve titer of target products in specific non-model organisms have often relied on trial-and-error optimization based on the host's machinery to control gene expression leading to laborious and organism-specific design-build-test cycles, mirroring previous methods demonstrated in *E. coli*. As seen above, the most common method involves engineering the parts in *E. coli* and then transferring them into the strain of interest.

T7 bacteriophage expression systems, as well as homologues of TetR and LacI, are the most commonly used tool to build host-independent orthogonal systems in both Gram-negative and Gram-positive bacteria [21–25]. However, high T7 RNAP levels can quickly exhaust cellular resources [22,26]. Some have used protein degradation tags or engineered negative feedback-controlled genetic circuits mediated by TetR to control T7 RNAP levels in *Synechocystis* spp. PCC 6803 [22], and also in *E. coli*, *P. putida*, and *B. subtilis* [26]. Interestingly, failure due to T7 RNAP mRNA degradation was observed in halophile *Halomonas* spp. TD01 [4••]. Instead, an orthogonal, broad-host inducible system was engineered using part-mined T7-like RNAPs in industrial strains *E. coli* S17-1, *Halomonas* spp. TD01, and *Pseudomonas entomophila* LAC31. This T7-like/lacO system was used to control cell morphology and polyhydroxybutrate production pathway in *Halomonas* spp. TD01, resulting in a 100-fold increase in cell length and 36% increase in polyhydroxybutrate production [4••].

Engineering inducible systems based on viewing promoter elements (i.e. −10 and −35 boxes, TF binding site) as modular is a common strategy for non-model organisms [12]. Proper placement of the TF binding site on a strong native constitutive promoter or phage

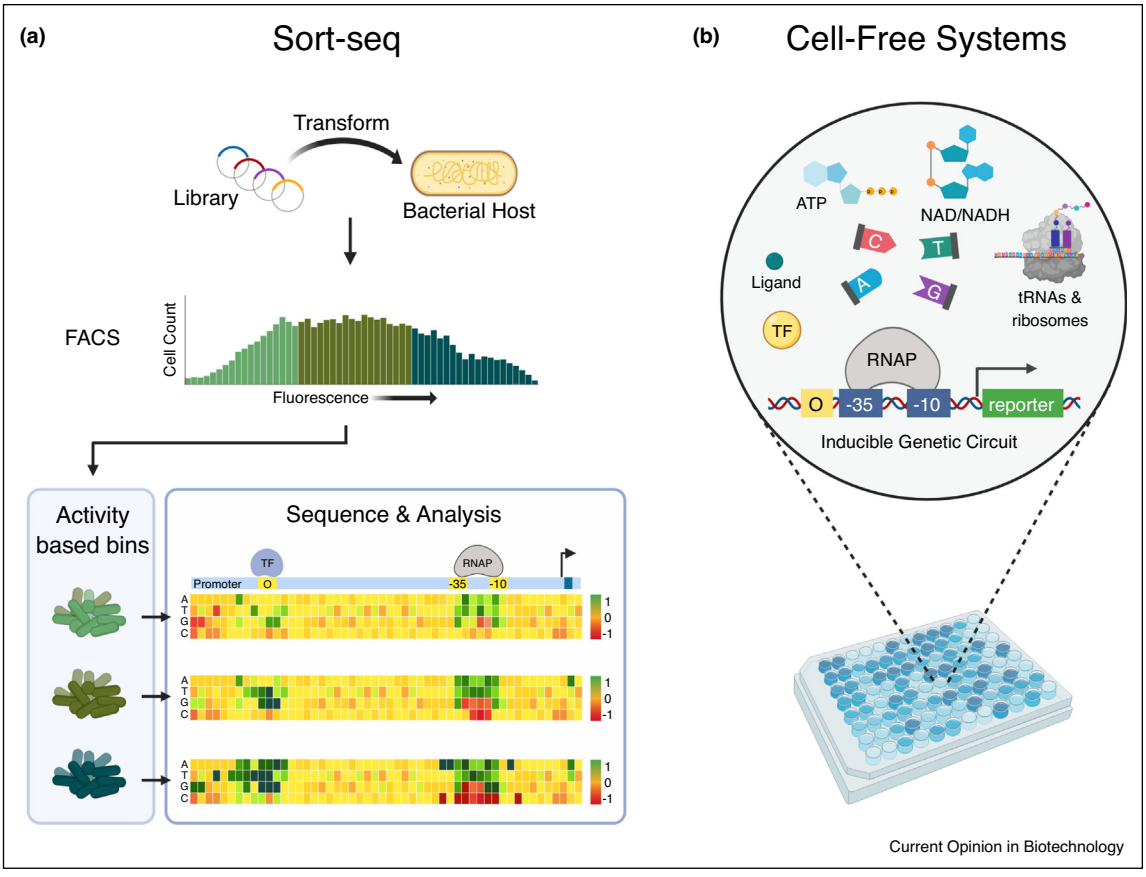
promoter blocks RNAP from binding to the promoter [4••,23]. Optimization to achieve a 'true off' state can be tuned by the number, location, and sequence of TF binding sites. The insertion of two TetR binding sites, for example, surrounding or within the −35 and −10 boxes of native constitutive *C. acetobutylicum* thiolase promoter resulted in threefold improved repression than with one operator [27]. Similar architectural strategies are also applicable for activators. Addition of two TF binding sites in pBAD promoter lowered basal expression and increased induction levels by twofold compared to one binding site in *Synechocystis* [5]. Alternatively, multi-input hybrid promoters built using a repressor and an inducible system provide a high signal-to-noise ratio by lowering basal activity while retaining expression strength in the presence of both inducers [28]. Altering DNA sequence of promoter elements, as well as the TF itself, can further fine-tune the biosensor performance to respond with the appropriate sensitivity and signal output according to the growth conditions to increase metabolite productivity [29–31].

Emerging high-throughput discovery and engineering methods for inducible systems

Predictable behavior of a biosensor in a different cellular context is crucial, but current engineering methods that rapidly engineer inducible systems in *E. coli* often have unpredictable outputs when transferring the system into another species. The dependence on host resources, machinery, and environmental factors (e.g. temperature, pH) greatly affects the output. Therefore, conducting the development and engineering within the target host can improve predictability in designing inducible systems.

Massively Parallel Reporter Assays (MPRAs) provide a high-throughput interrogation of genotype-to-phenotype relationships through sorting and deep DNA sequencing (Figure 2a). In the sort-seq method, mutagenized promoter or 5'-UTR libraries, including TF binding sites, are coupled to a fluorescent reporter and sorted into activity-based bins with subsequent sequencing analysis revealing important features not obvious *a priori*. Minor changes in the TF binding site, promoter, or 5'-UTR (including secondary

Figure 2



Emerging high-throughput engineering methods in non-model microorganisms.
(a) Sort-seq experiments enable high-throughput screening and assembly of genetic parts in non-model organisms with improved predictability in a context-dependent manner. **(b)** Cell-free transcription/translation (TXTL) systems shows promise in rapid development and characterization of inducible systems in an *in vitro* environment.

structures) can have drastic effects on translation efficiency [32]. Screening a >10 000 member library comprising *E. coli* and *Vibrio cholerae* sigma54-like promoters showed that short, CT-rich sequence motifs complementing the AG-rich Shine-Dalgarno sequence causes an inhibitory translation mechanism across sigma54 promoters [33•], highlighting how systematic surveying of the sequence space can reveal poorly understood mechanisms.

Sort-seq experiments can also enable high-throughput screening and assembly of genetic parts in non-model organisms when genome annotation is lacking [34]. FACS-seq and metagenomic approaches have been used to identify and characterize regulatory sequences from prokaryotic genomes both at transcriptional and translational levels to create universal and orthogonal host-specific genetic circuits [35••,36]. Analysis of ~30 000 putative regulatory sequences from 184 prokaryotic genomes, via combinations of RNA-seq, DNA-seq, and FACS-seq, found a positive correlation between transcriptional activity and sigma70 binding affinity, while translation rates varied across *Pseudomonas aeruginosa*, *E. coli*, and *B. subtilis* [35••]. Alternatively, high-throughput screening using droplet microfluidics-based cell sorting techniques where the production strain and an *E. coli* biosensing strain are co-encapsulated avoids challenges associated with engineering cross-species TF-based biosensors [37].

Cell-free transcription/translation (TXTL) systems applied as an *in vitro* tool show promise in the rapid development and characterization of regulatory sequences and proteins in non-model organisms (Figure 2b). To overcome constraints working within the organism, cell-free extracts of the giant Gram-positive *Bacillus megaterium* were used to characterize a native xylose-inducible promoter both at the transcription and translation level [38••]. While primarily used with model bacteria, the successes of optimizing protein yields and expression in cell-free TXTL platforms indicate the potential this method has for emerging non-model systems [39,40].

Genetic reporters for varying cellular contexts

Biosensors require an observable proxy for the inducing chemical or condition. While the preferred genetic reporters for high-throughput applications, *gfp* and related fluorescent proteins, have been used successfully in a range of aerotolerant bacteria (Table 1) [3•,4••,15], many genera cannot use such proteins due to the oxygen requirement of common fluorescent proteins or thermostability. Therefore, many alternative reporter systems ranging from colorimetric (e.g. *lacZ*, *gusA*) to anaerobic fluorescent proteins (e.g. iLOV, Y-FAST) have been developed.

The wide variety of fluorescent proteins available have enabled high-throughput analysis via flow cytometry of specific aerotolerant bacteria. Split-GFP reporter system enables high-throughput analysis of libraries to study secretion mechanism of *B. subtilis* [41], while a pH-sensitive fluorescent protein, *pHluorin2*, offers a non-invasive method to assess cytoplasmic pH in *Pseudomonas* spp. [42]. Red fluorescent protein (RFP) and its variants have been shown to outperform GFP in acidic conditions in *Pseudomonas denitrificans* [28]. Furthermore, RFP has a longer excitation and stronger emission wavelength over GFP that led to higher signal-to-noise ratio in *Cupriavidus necator* [43].

Obligate anaerobes and metabolic pathways under low oxygen requirements (e.g. in *Cyanobacteria*) pose a challenge due to the requirement for molecular oxygen for the proteins' fluorophore to mature. Flavin-based fluorescent proteins (FbFPs) overcome the oxygen requirement and instead the maturation requires flavin co-factors such as flavin mononucleotide or flavin adenine dinucleotide [44]. Light, Oxygen, Voltage (LOV)-based FbFPs such as *phi*LOV2.1 and iLOV are small anaerobic fluorescent proteins (~14-kDa) with fast folding kinetics that have successfully been used in *Clostridium* spp. for confocal microscopy [45]. Compared to GFP, however, the fluorescence of FbFPs is often masked by cellular autofluorescence (due to NADPH, flavins, siderophores), interfering with the signal-to-noise ratio measured by flow cytometry, pushing researchers toward low-throughput enzymatic colorimetric reporters (e.g. *gusA*) [46]. Alternatively, fluorescence-activating and absorption-shifting tag (FAST) strongly fluoresce upon binding to commercially available fluorogenic ligands in oxygen-limited environments. Function of FAST and fluorogenic ligands under both aerobic and anaerobic conditions enable analysis via flow cytometry and confocal microscopy [47••], and may enable high-throughput methods with FACS or plate-based assays for *Clostridium*.

Fluorescent proteins from mesophiles are not functional in thermophiles, requiring thermostable reporters (commonly the enzymatic *pheB* and *BgaB* isolated from *Geobacillus stearothermophilus* [48]). While mCherry fluoresces up to ~50°C, increased thermostability of superfolder GFP (sfGFP) and species-specific codon-optimization have been demonstrated in aerobic extreme thermophiles [49,50]. Thermostable FbFPs have been developed for aerobic and anaerobic thermophiles; however, FbFP variants such as codon-optimized *hot*LOV from *Thermosynechococcus elongatus* and *Ytv*ALOV from *B. subtilis* were non-functional in *Geobacter* spp. [49]. More thermostable LOV-based reporters, including MrFbFP and YN3FbFP Y116F from *Thermus thermophilus* isolated from Yellowstone National Park hot springs [44] and CagFbFP from thermophilic anaerobic bacterium *Chloroflexus aggregans* [51] are becoming available through genome mining approaches.

Conclusion

Research and engineering of non-model microorganisms have expanded greatly in recent years due to the expanding capabilities and methods of genetic editing. However, progress is still hampered by the lack of genetic parts,

specifically biosensors and inducible promoters. While numerous inducible promoters have been published recently (Table 1), many bacteria still rely on only one or a few each. Current methods of engineering such systems happens primarily in *E. coli*, requiring another cycle of engineering to adapt the genetic parts to comply with the host expression machinery. We propose that high-throughput screening methods, now enabled through novel genetic reporters functional in a broad range of bacteria, can and should be used in future engineering of genetic parts for non-model organisms. These methods will enable rapid development of advanced genome engineering methods and open the emerging synthetic biology field to new frontiers.

Conflict of interest statement

Nothing declared.

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