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Current Opinion in Microbiology

Design of synthetic bacterial biosensors

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Novel whole-cell bacterial biosensor designs require an emphasis on moving toward field deployment. Many current sensors are characterized under specified laboratory conditions, which frequently do not represent actual deployment conditions. To this end, recent developments such as toolkits for probing new host chassis that are more robust to environments of interest, have paved the way for improved designs. Strategies for rational tuning of genetic components or tools such as genetic amplifiers or designs that allow *post hoc* tuning are essential in optimizing existing biosensors for practical application. Furthermore, recent work has seen a rise in directed evolution techniques, which can be immensely valuable in both tuning existing sensors and developing sensors for new analytes that lack characterized sensors. Combined with advancements in bioinformatics and capabilities in rewiring two-component systems, many new sensors can be established, broadening biosensor use cases. Last, recent work in CRISPR-based dynamic regulation and memory mechanisms, as well as kill-switches for biosafety and innovative output integration concepts, represents promising steps toward designing bacterial biosensors for deployment in dynamic and heterogeneous conditions.

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Current Opinion in Microbiology 2023, **76**:102380

This review comes from a themed issue on **Microbial Systems and Synthetic Biology**

Edited by **Victor Sourjik** and **Kiran Patel**

For complete overview of the section, please refer to the article collection, "Microbial Systems and Synthetic Biology 2023"

Available online 11 September 2023

https://doi.org/10.1016/j.mib.2023.102380

1369–5274/Published by Elsevier Ltd.

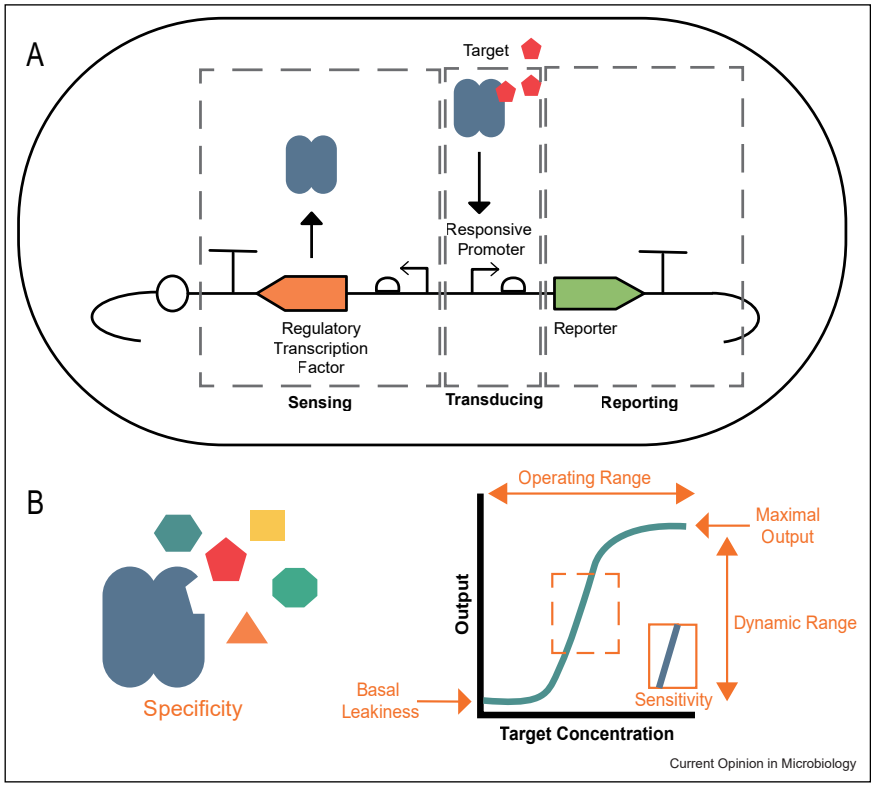
Introduction

Bacterial biosensors can provide real-time information in dynamic environments [1–5]. Bacteria's natural ability to withstand and adjust to frequently stressful conditions makes them strong candidates for applications such as human health diagnostics or pollution detection in terrestrial and marine environments. Furthermore, bacterial biosensors offer a low-cost, compact, environmentally sustainable, and robust option for continuous detection of analytes [6]. Despite progress in creating precise and sensitive biosensors, there are still challenges to overcome before they can be widely deployed in medical or environmental settings.

A whole-cell bacterial biosensor is typically composed of a sensing module — frequently a transcription factor or sensor kinase — that regulates a transducing module, such as a promoter, that drives a reporting module, but can also be nucleic acid-based (i.e. riboswitches, aptamers) (Figure 1a) [6,7]. Many canonical biosensors have been based on transcriptional repressor topologies [8]. Characteristics that contribute to a bacterial biosensor's efficacy are its specificity, sensitivity, and dynamic and operational ranges, which are often fitted to a Hill Curve (Figure 1b) [9]. A biosensor must be able to sense a desired target analyte in a dose-dependent manner with minimal, if any, cross-talk or antagonistic interaction with other structurally similar analytes or analytes that share transcriptional regulators or promoter constructs. Optimal dynamic and operational ranges speak to a biosensor's ability to detect biologically relevant concentrations of the target analyte and to exhibit a distinguishable response gradient. Furthermore, the absence of target analyte should yield minimal 'leaky' expression.

Designing whole-cell bacterial biosensors requires consideration of the context of their application, as well as identification of the sensing components for the target analyte(s). While rational forward engineering approaches have dominated strategies for optimizing bacterial biosensor genetic elements, numerous recent studies have turned toward directed evolutionary tactics [10]. Recent work has focused on the development of biosensors that can withstand and sense under contextually relevant conditions, uncovering uncharacterized biosensing modules for new targets, optimization of existing biosensors, adjusting for spatiotemporally heterogeneous environments, and innovative integration of output. This review focuses mainly on design of transcription factor-based whole-cell bacterial biosensors for small-molecule ligands, but it must be noted that RNA-based biosensors have also achieved significant recent improvement [7,9,11].

Figure 1



Transcription factor-based biosensor. **(a)** General structure of a transcription factor-based biosensor, with the transcription factor sensing element, its corresponding promoter(s) as the transducing element, and an output-reporting element. **(b)** Qualities and metrics in characterizations of biosensors, including specificity and dynamic and operating ranges.

Exploring novel host chassis

For deployment of whole-cell bacterial biosensors into their intended contexts, the environment of application must be considered. Different host chassis offer expanded functionalities and robustness in challenging environments that standard laboratory strains may not. More importantly, biosensor constructs characterized and tuned in model organisms may not be easily portable or exhibit the same behaviors in new organisms or environments [12].

Important considerations for host chassis outside well-characterized model organisms, such as *Escherichia coli*, include biosafety, robustness in spatiotemporally heterogeneous biosensing environments, and genetic tractability [13]. In a recent biosensing system, Cooper et al. harnessed horizontal gene transfer capabilities of *Acinetobacter baylyi* to detect tumor DNA, serving as encouraging incentive for mining potential utilities offered by new host chassis for biosensing applications [14]. To facilitate exploration of new host chassis, recent work has included new toolkits and approaches for different host chassis [15]. Broad-host range and portable expression tools, such as the Universal Bacterial Expression Resource, which decouples biosensing machinery from host expression by producing T7 polymerase for expression of the genetic components on the plasmid, have also been developed [16,17].

Standardization of genetic components and new toolboxes for new chassis has given rise to computational-automated rational design of complex synthetic circuits [18]. *Cello* was designed for researchers to specify logic gate circuit modules using Verilog with a user constraint file that includes user-defined host organism, strain, and context [6,19]. Recently, Taketani et al. demonstrated the capacity of *Cello* for designing multi-input and -output biosensors by integrating bile acid and aTc sensing promoters in *Bacteroides thetaiotaomicron* to identify three distinct states (either aTc or bile acid, or neither) with a luminescence reporter [5].

Considering dynamic and transient contexts in characterization and optimization of biosensors is crucial, as many biosensor phenotypes are not conserved across diverse environments or hosts. Even altered growth rates can influence the dynamic range of well-characterized biosensors [20]. Simulating the environment of intended biosensor deployment, such as tuning oxygen availability or using gut models, can be informative [5,21,22].

Depending on the assay, modifying samples themselves for detection of small molecules can also aid application-centered biosensor design [23].

Sensing new analytes

One of the largest concerns impeding the route to practical deployable biosensors is the lag of natural characterized and specific biological sensing elements behind target analytes of interest. To this end, high-throughput identification of potential transcriptional regulators and structure-guided mutagenesis have been immensely illuminating [24].

Multiple methods have been explored to hack sequence-based knowledge of transcriptional regulator topologies to find sensors for molecules with no characterized transcriptional regulators. Hanko et al. screened GenBank annotations for the common conserved LysR-type transcriptional regulator divergent pattern and cross-referenced. The Comprehensive Enzyme Information System to systematically uncover 16 previously uncharacterized inducible sensors [24]. Screening gene clusters upregulated in response to progesterone using RNA-seq, followed by validation with *in vitro* ChIP-seq and biolayer interferometry with predicted binding sequences, also resulted in the first documented and characterized progesterone transcriptional bacterial biosensor [25].

Other methods include tapping into existing characterized allosteric transcription factors to develop sensors for adjacent molecules. Some researchers have combined structure-guided mutagenesis and directed evolution approaches with fluorescence-activated cell sorting (FACS) to select for the desired biosensor phenotypes [4,26]. Taylor et al. used LacI as a scaffold to assemble mutational libraries with a combination of Rosetta-aided protein variant designs, amino acid saturation mutagenesis, and error-prone Polymerase chain reaction (PCR) (Figure 2a and b). Assembling these variants to drive Green fluorescent protein output upon activation, variants specifically responsive to fucose, lactitol, sucralose, and gentabiose and less promiscuous to the original ligand, Isopropyl ß-D-1-thiogalactopyranoside, were selected for using FACS [26]. Similar studies with transcriptional regulator scaffolds have yielded a caffeine-inducible system using error-prone PCR with FACS, and a selective sensor for aromatics ethylbenzene and m-xylene using structure-guided mutagenesis [4,27]. Not only have these mutational library studies yielded specific biosensors for new target analytes, but analysis of the mutations common across variants exhibiting desired behavior also elucidated previously uncharacterized structural functions.

To facilitate building new sensing modules from existing sensing pathways, Chang et al. developed a modular receptor scaffold called Engineered Modularized Receptors Activated via Ligand-induced Dimerization to repurpose the sensing component of a two-component sensing system from *Vibrio* species to a standard DNA-binding domain in *E. coli* that drives expression of a fluorescent reporter gene to build a sensor for bile salts [28].

RNA-based biosensors, such as riboswitches and toehold switches, particularly in the identification of aptamers for new target analytes without known biosensors, have also seen significant recent developments [7,29,30]. High-throughput pipelines for RNA biosensor libraries have been developed and continuously improved, such as the *de novo* rapid *in vitro* evolution of RNA biosensors, which uses a self-cleaving ribozyme system to distinguish aptamers with ligand-binding affinity [29,31].

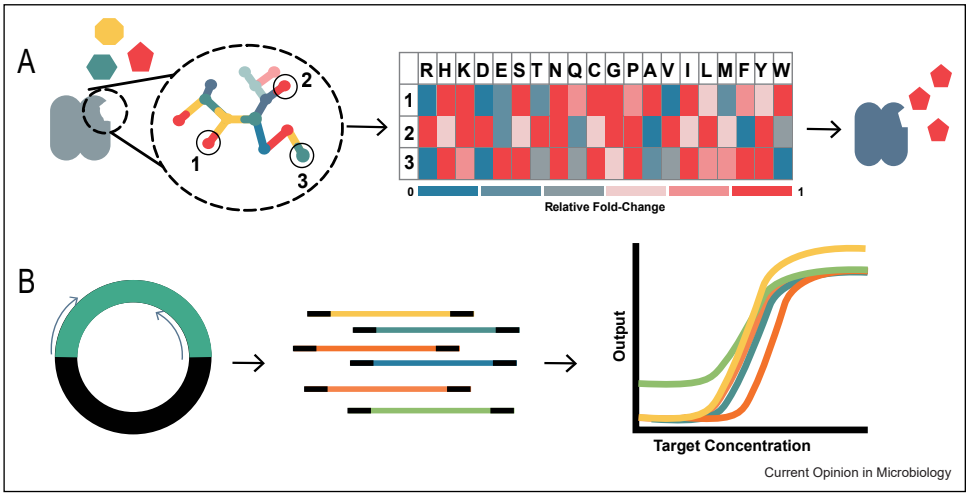
Characterizing and optimizing biosensors

Optimization of bacterial biosensor parameters, including specificity, sensitivity, dynamic range, and basal expression, has led to rational, semirational, and evolutionary strategies [9]. Multiple tuning forks for biosensors exist, including the transcriptional regulator that binds the target, and the genetic elements responsible for its expression (i.e. promoter, ribosomal-binding site (RBS), etc.) or those in the transducing element regulated by the transcription activator/repressor.

Commonly, assembling a mutational library can help fine-tune a biosensor with desired characteristics for a particular application, similarly to finding new transcriptional biosensors in 'Sensing New Analytes'. Tuning sensitivity or narrowing specificity of a promiscuous transcription factor can be achieved by identifying key residues of a transcriptional sensor to create a structure-guided variant mutational library for directed evolution (Figure 2a) [2,22,28]. Snoek et al. recently generated a variant library with error-prone PCR on the effector-binding domain of BenM, a transcriptional activator that binds *cis–cis* muconic acid, to obtain biosensors exhibiting greater specificity, sensitivity, and inversion of function in high-throughput (Figure 2b). Variants with desired characteristics, including one with more than 1000× increase in maximum fold-change induction, were selected using FACS [32]. Instead of FACS, Rottinghaus et al. used antibiotic resistance and sucrose counterselection as positive and negative reporters, respectively, to screen responses of a variant library of promiscuous transcription factors TyrR and FeaR to phenylalanine, tyrosine, phenylethylamine, and tyramine. These selective markers allowed selection of variants that both respond to the desired amine and not to others [4]. Microfluidic technology can be useful with high-throughput screens of biosensors' spatiotemporal dynamic characteristics [33].

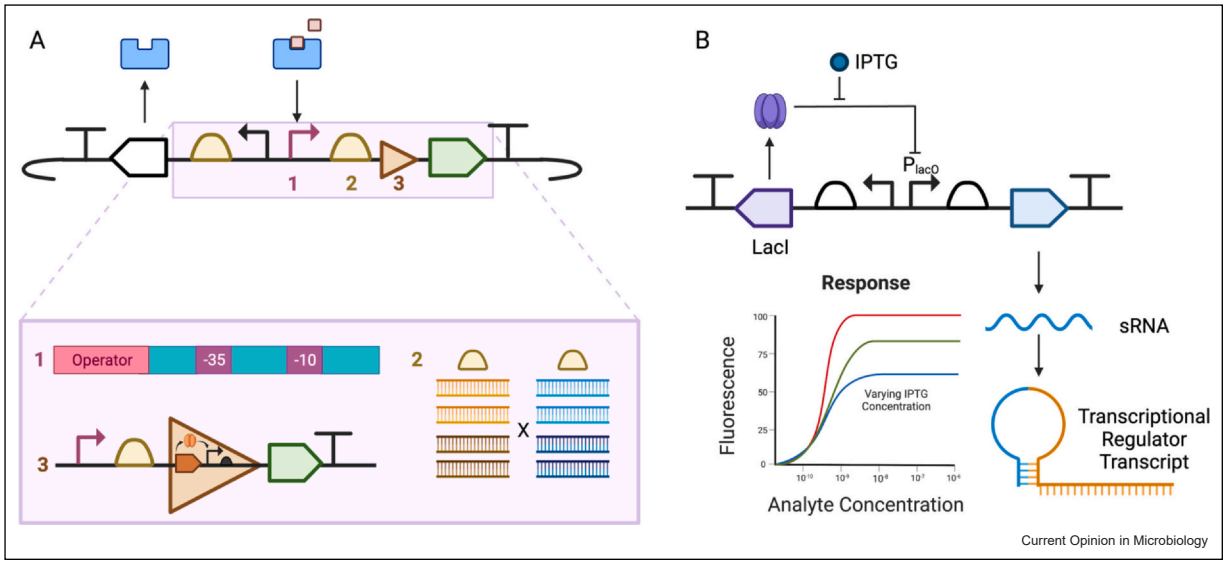
The origin of replication, promoter, RBS, and protein degradation tags are frequently genetic parts effective in initial tuning of a biosensor's sensitivity and dynamic range (Figure 3a) [21,34–36]. Hybrid promoters can be engineered

Figure 2



Directed evolution strategies for improvement of biosensors. **(a)** Structure-guided amino acid saturation mutagenesis entails identifying key residues of a transcriptional sensor either computationally with predictive software or X-ray crystallography. Key residues are swapped with all other possible amino acids to select variants with desired responses (i.e. greatest dynamic range). **(b)** Error-prone PCR harnesses error-prone polymerases to generate a mutant library of a specific sequence of interest (i.e. operator sites, RBS, etc.), so variants with desired responses can be selected in high-throughput.

Figure 3



Rational strategies for optimizing biosensor output. **(a)** Colored box highlights genetic parts frequently tuned in tuning biosensor output. (1) Denotes the responsive promoter, which includes transcriptional activator-binding site (operator), the –35 and –10 RNAP-binding sites, and the spacers in-between these parts. Repositioning operator and RNAP-binding site positions and varying their strengths by altering nucleotide sequences themselves can influence biosensor output. In (2), varying combinations of the RBSs of the transcriptional regulator and the output can produce varied dynamic range. (3) Conceptually shows the mechanism of amplifier elements upstream of the reporting element to improve output amplitude and detection sensitivity. **(b)** Tuner mechanism for a biosensor that uses a toehold switch regulated by an external inducer, so a single strand of RNA is produced that binds to the biosensor transcriptional regulator transcript to modulate its translation rate. This allows for post-construction modulation of biosensor dynamic range. (Created with BioRender.com).

from integrating operator sequences to a known promoter sequence to tune a promoter responsive to a desired regulator, as Zhang et al. did with *hrtO*, a promoter regulated by the heme-responsive transcription factor HrtR [2]. Repositioning and altering promoter components on the sensing construct can change biosensor behaviors, as shown with the arsenic-responsive p_{ars} promoter, among others (Figure 3a) [1,2,37].

New methods and information for predictive tuning have recently emerged. The effects of operator site spacing, combinations of operator sequences and RNA polymerase (RNAP)-binding sequences, and alternative promoter architectures on fold-change response and leakiness were recently evaluated [38]. RNAP-binding site offers another tuning fork for predictively building hybrid multi-input promoters [39]. Another study used a design-of-experiments statistical method to analyze reporter expression as a function of expression strength of the transcription factor and the regulated promoter and RBS, resulting in 'rules' for transcription factor-based biosensor design [40]. For example, a weaker promoter driving the transcriptional regulator generally yields a greater dynamic range, likely due to steric interference [22,40,41]. A library of cross-RBSs ($RBS_{regulator}$ × RBS_{output}) was also recently evaluated for glucarate transcription factor-based biosensor cdaR and sfGFP to increase dynamic range and develop a deep learning algorithm for predictively tuning regulator and output translation (Figure 3a) [42]. Taken together, these studies set the stage for rational engineering approaches to optimization.

Efforts toward standardization and modularization have expedited biosensor tuning [18]. For example, characterized promoters can be easily swapped to improve fold-change response and limit of detection [22]. Notably, Meyer et al. developed a set of optimized 'Marionette' strains using an elegant dual-selection directed evolution scheme to achieve biosensor transcription factors and their corresponding responsive promoters that exhibit low basal leakiness, improved sensitivity and dynamic range, and high specificity [43].

Amplification elements have been explored to boost biosensor output. Wan et al. added amplifier elements to the previously characterized arsR-p_{ars} arsenic biosensor, drastically improving output and sensitivity (Figure 3a) [41]. Sensor-driven expression of T7 RNAP, with the T7 promoter driving fluorescence output, opens another promising avenue for biosensor amplification, as demonstrated with previously characterized biosensors for copper and glucose [44]. More recently, RepL was integrated into biosensor response to increase copy number to amplify biosensor output [45]. In some cases, the reporting segment can even be manipulated to reduce basal leakage and improve dynamic range. For instance, splitting the large and transcriptionally inefficient *lux* luminescence cassette so that luxABDE was integrated into the *E. coli* Nissle genome and luxC was expressed on the plasmid in response to heme resulted in improved limit of detection and dynamic range [46,47].

Though most efforts at optimizing biosensors have focused on the biosensor itself or its regulatory genetic components, modulating the intracellular concentration of the target ligand also can serve as a tuning fork by regulating its diffusion out of or uptake into the cell [36,40]. Miller et al. improved a sensor for erythromycin A (EryA) by expressing a macrolide resistance protein to phosphorylate EryA and keep it from diffusing out of the cell, improving the sensor's sensitivity [36].

Finally, Bartoli et al. proposed a method for tuning a biosensor post construction with a toehold switch regulated by an external inducer to modulate the translation rate of the transcriptional biosensor. Although the expanded range of detection limits came at the expense of fold change in the output driven by the regulated promoter, this poses a promising approach for building biosensors that are adaptive to various sensing environments or host chassis (Figure 3b) [34].

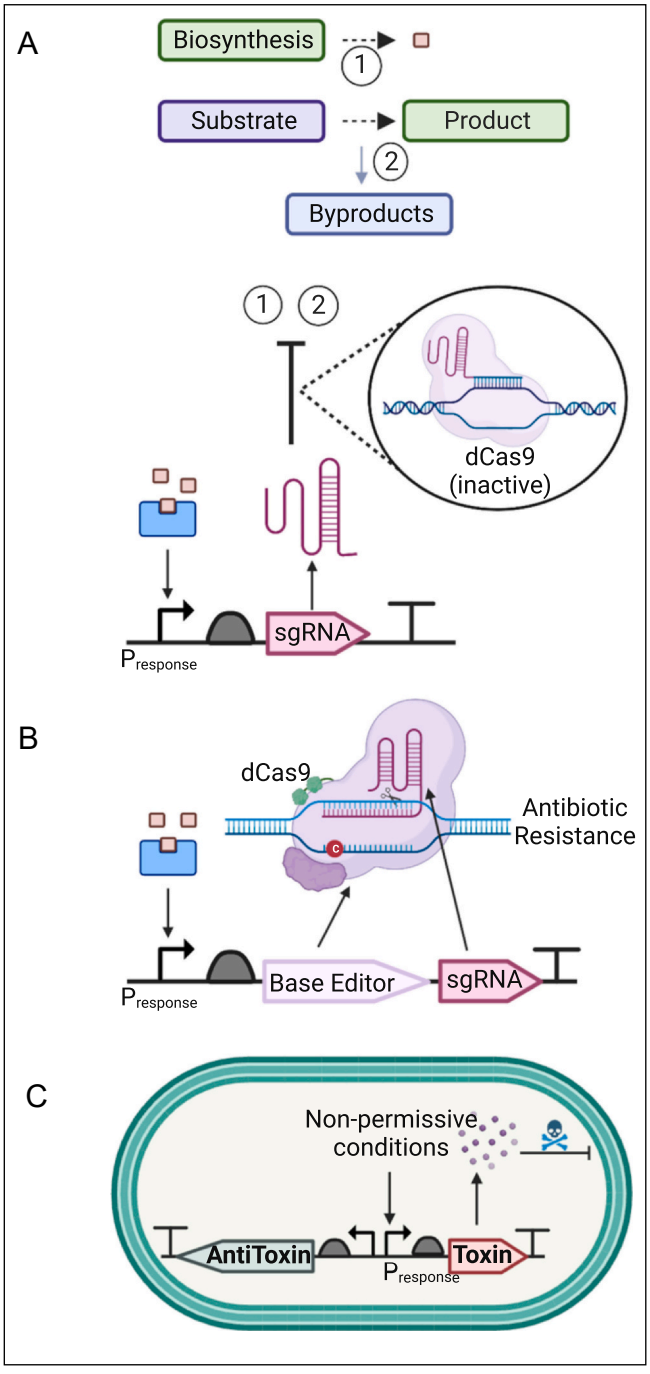
Incorporating features for spatiotemporally dynamic environments

Other concerns of existing biosensor constructs that preclude deployment include dynamic regulation and recording sensing events in a transient environment, long-term stability, and biocontainment [48].

Dynamic regulation is essential for the constantly fluxing conditions many applications of biosensing call for. In many recent studies, researchers have added CRISPRi to optimize a pathway (Figure 4a) [1,2,49]. For instance, one study coupled a transcription factor-based biosensor for GlcN6P to expression of single-guide RNA (sgRNAs) that blocked transcription of proteins integral to pathways competitive to GlcNAc, to optimize GlcNAc synthesis [1]. The capacity for low-burden, orthogonal, and predictable regulation offered by CRISPRi has been demonstrated as Santos-Moreno, J et al. built some of the hallmark circuit designs of synthetic biology using CRISPRi: the bistable toggle switch and the repressilator [49–51].

Owing to the transient nature of many biosensing contexts, genetic recording and memory have become an important area of research. Previously, recombinase-based memory circuits were commonly used as a memory feature, but recently, Zou et al. integrated a base editor enzyme along with sfGFP into the output of the arsenic biosensor construct arsR-p_{ars}, with an interference sgRNA that edited the

Figure 4



Recent developments in preparing bacterial biosensors for spatiotemporally dynamic environments. **(a)** CRISPRi-mediated dynamic regulation. dCas9 protein is inactivated, so the sgRNA can be used to interfere with transcription of enzymes along biosynthetic (1) or metabolic (2) pathways. In the former, CRISPRi can be integrated into a biosensor to regulate production of the target ligand and maintain it at a threshold level. In the latter, it can be used to optimize production of an end product by inhibiting pathways for by-products. **(b)** CRISPR-based memory mechanism via base editing on the genome allows for recording of sensing events. This is erasable when coupled with a selective marker, such as kanamycin resistance, so edited cells lose resistance and can be 'reset' by applying selective pressure. **(c)** Toxin–antitoxin switch for biocontainment, which terminates the bacterial sensor under specific cues. The antitoxin is expressed constitutively at low levels to counter basal expression of the toxin under permissive conditions, but nonpermissive conditions increase expression of the toxin, leading to cell death. (Created with BioRender.com).

sfGFP. Not only was this a heritable genomic memory mechanism, but it also was erasable when coupled with a selective marker, so that recording cells can be 'reset' (Figure 4b) [52,53]. This was employed in a two-component biosensor to detect thiosulfate, along with a colorimetric reporter and regulated delivery of immunomodulator Av-Cystatin [35]. In another memory circuit, Riglar D et al. coupled a Cro/CI-based transcriptional switch memory system to a TtrR/TtrS two-component system to record tetrathionate sensing events, and evaluated the construct's long-term stability *in vivo*, establishing its potential as a living diagnostic [54,55]. Stirling et al. applied the same switch to implement a pulse counter [56].

Including built-in biosafety features is important in ensuring that biosensors can be safely deployed. This has been addressed in recent efforts with coupling the biosensor to essential metabolic functions, as Chien et al. did to promote localization of bacterial biosensors in tumor microenvironments, which exhibit a variety of physicochemical gradients, including pH, oxygen, and lactate [21]. Another strategy for biocontainment is kill-switches, which terminate the bacterial sensor under specifically engineered cues, such as certain molecules or physicochemical properties. The challenge for this is genetic stability due to the strong selective pressure of kill-switches. Stirling et al. employed a pH-sensitive switch using a toxin–antitoxin system, in which the toxin is produced under acid-shock conditions, thus permitting the strain to exist only under neutral pH (Figure 4c)

[56]. More recently, a CRISPR-based switch was developed, which causes double-stranded genomic cleavage in response to specified stimuli. Genomic redundancy of the kill-switch cassette improved efficiency, and coupling the plasmid to an essential gene enhanced its stability [57]. Acoustic and mechanical stimuli, such as magnetic waves, also pave the way for innovative methods for localization [58].

Integrating output

For translation into practical applications, recent work has been done in exploring unique output integration that demonstrates their practical use. For example, Din et al. paired an arsenic biosensor with the seminal synchronized lysis circuit (SLC) to couple electric signal admittance with the release of charged ions from sensor strain population oscillations [59,60]. Single-photon-avalanche photodiode technology has also been coupled to biosensors for a low-cost, portable sensing platform [46]. Some studies have used colorimetric outputs as diagnostic devices and microfluidic platforms for fluorescent readouts [37,41]. Me et al. recently built an ingestible capsule for wireless biosensing, demonstrating its efficacy in a murine and porcine gut [61].

Conclusion

Numerous advancements have been made in designing whole-cell transcription-based bacterial biosensors. Bacterial biosensors offer an affordable, dynamic, and environmentally friendly solution for detection, optimization of biosynthesis, and regulation of metabolic pathways. However, safe and effective deployment still calls for many developments. Most bacterial biosensors have been engineered and characterized within laboratory conditions, making them unsuitable for the frequently stressful heterogeneous conditions of applications and subject to off-target sensing. New host chassis can serve as more robust sensors; notable recent developments have included toolkits and portable methods for exploring less-characterized bacterial species. Furthermore, many analytes of interest do not yet have characterized sensors. To this end, researchers have developed techniques for mining genomic sequences to uncover new sensors, evolving existing biosensors for new target ligands, and rewiring known two-component systems for molecules that cannot diffuse into the cell. Many existing sensors must also be optimized, especially when considering deployment environments, which can result in a noisy signal. Their dynamic range, limit of detection, and specificity need to be tuned to biologically relevant levels. Standardization of genetic parts continues to be essential in initial rational tuning. Directed evolution approaches have also risen in importance for optimizing sensors. Some researchers have added additional genetic components for signal amplification or post-construction tuning with external inducers. Other future prospects include dynamic regulation, heritable recording mechanisms, and fail-safe approaches to biocontainment. CRISPRi can be employed in dynamic regulation to minimize byproducts of biosynthetic pathways or to regulate the production of target ligand by using an inactivated dCas9 protein with a sgRNA to interfere with the transcription of enzymes producing undesired byproducts or the target ligand to limit its production, respectively. Clustered Regularly Interspaced Short Palindromic Repeats technology can also be harnessed for its base editing capabilities in recording sensing events. For biocontainment, kill-switches can be developed so that bacteria will only exist under permissive conditions, but will self-destruct under nonpermissive ones, promoting their localization. Finally, creative methods for output integration into the final intended contexts, such as ingestible capsules, will be crucial in ensuring safe deployment of bacterial biosensors.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests: J.H. is a cofounder of GenCirq Inc, which focuses on cancer therapeutics. He is on the Board of Directors and has equity in GenCirq.

Acknowledgements

This work was supported by the National Institute of General Medical Sciences of the National Institutes of Health (Grant no. R01GM069811). A.C. was supported by the National Science Foundation Graduate Research Fellowship Program under Grant no. DGE-2038238. Any opinions, findings, and conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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