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Biosensing in Smart Engineered Probiotics

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Engineered microbes are exciting alternatives to current diagnostics and therapeutics. Researchers have developed a wide range of genetic tools and parts to engineer probiotic and commensal microbes. Among these tools and parts, biosensors allow the microbes to sense and record or to sense and respond to chemical and environmental signals in the body, enabling them to report on health conditions of the animal host and/or deliver therapeutics in a controlled manner. This review focuses on how biosensing is applied to engineer "smart" microbes for in vivo diagnostic, therapeutic, and biocontainment goals. Hurdles that need to be overcome when transitioning from high-throughput in vitro systems to low-throughput in vivo animal models, new technologies that can be implemented to alleviate this experimental gap, and areas where future advancements can be made to maximize the utility of biosensing for medical applications are also discussed. As technologies for engineering microbes continue to be developed, these engineered organisms will be used to address many medical challenges.

1. Introduction

Probiotic and commensal microbes are naturally valuable assets for the host. These microbes can prevent pathogen colonization, reduce the frequency and severity of various ailments, modulate the brain activity through the gut–brain axis, and selectively colonize tumor microenvironments.[1] For example, various strains of *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, and *Bacillus* inhibit the colonization of many pathogenic bacteria.[2–5] This inhibition occurs through a number of mechanisms, including reduction of the luminal pH, competition for nutritional resources, and excretion of bacteriocin.[6] Some microbes also exhibit tumor-specific colonization that can significantly inhibit the growth of the tumors. This property has been demonstrated and applied using several bacteria, including *Clostridium*,[7–9] *Bifidobacterium*,[10] *Escherichia coli*,[11] and an attenuated version of *Salmonella typhimurium* (aSt).[12,13]

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Many microbes also alleviate the symptoms or reduce the occurrence of various ailments, including diarrhea,[14] allergy,[15] and gut inflammation.[16] Often, the exact mechanisms of action for these microbes are not well understood. Many of these microbes improve health through interfacing with both host cells and other gut microbes. This communication largely occurs via the production and degradation of various proteins and metabolites that alter the composition of the microbiome, tune the pH of the gut, stimulate the function of the mucosal barrier, and modulate the activity of the immune system.[1]

Microbe-host interactions can also influence the activity of the brain through the gut–brain axis. Some probiotic and commensal microbes can synthesize and degrade brain-modulating neurotransmitters, including catecholamines and serotonin.[17–19] Microbes have also been shown to indirectly tune neurotransmitter levels by interacting with neurotransmitter-producing host epithelial and immune cells and by modulating the composition of other neurotransmitter-regulating microbes in the gut.[20–23] These connections make the gut–brain axis an avenue for microbes to interface with the nervous system to correct neurological malfunctions and help the host cope with stressors.[24]

The natural qualities of probiotic and commensal microbes provide an excellent starting point for engineering microbes with new capabilities. Through synthetic biology, a wide array of new genetic parts may be introduced into these organisms for various applications. A common approach to engineering microbes for health-related goals is to simply express therapeutic proteins from constitutive promoters. These promoters are always active, independent of external stimuli. This approach has been applied for engineering microbes to treat or prevent various diseases and disorders, including hyperammonemia,[25] phenylketonuria,[26] diabetes,[27] AIDS,[28,29] oral mucositis,[30] inflammatory diseases,[31,32] obesity,[33] cancers,[34] and bacterial infections.[28]

Although constitutively expressing proteins can be effective, a new class of engineered probiotics can be developed with biosensors. In contrast to constitutive protein expression, biosensor-regulated expression provides a means of intelligent control, where chemical cues (including those administered to the host and those naturally present in the body) and environmental cues (including oxygen level, pH, and temperature) determine when and where the probiotic produces the desired proteins (**Figure 1**A). There are several key advantages to controlling protein expression with biosensors. First, this expression

method maximizes the genetic stability of the engineered cell. Heterologous protein expression burdens the cell, increasing the probability of enriching for mutations (e.g., mutations in promoters leading to no expression) that render the cells therapeutically non-functional.[35] Second, limiting protein production to a specified location in the body can minimize potential off-target effects of the proteins, including toxicity and tolerance buildup.[36] Third, the use of biosensors enables microbes to be engineered for diagnostic applications (Figure 1B) as well as therapeutic applications (Figure 1C). Biosensors also allow for the implementation of biocontainment genetic circuits that let the user control the viability of the engineered microbe (Figure 1D).

The biosensors needed for engineered probiotics can be obtained through part mining of native systems or development of synthetic regulators. Microbes naturally respond to a wide variety of external stimuli, many of which are found in the gut, to control their RNA and protein levels.[37] These natural responses can be leveraged for biosensing in microbes. Alternatively, synthetic protein and RNA sensors can be created through a variety of techniques. For example, novel protein sensors have been developed by fusing the ligand-binding and DNA-binding domains of different proteins[38] and by evolving natural sensors for improved response or altered ligand specificity.[39,40] Similarly, chemical- or environmental condition-sensing RNA regulators have been created.[41,42] The topic of biosensor development has been broadly reviewed[43–45], and many approaches are being applied to the development of biosensors in various non-model gut microbes.[46,47]

The topic of engineered cells for medical applications has been reviewed in the past, with reviews broadly covering engineered microbes for medical applications,[48] focusing on developing engineered live therapeutics with increasing complexity,[49] discussing synthetic biology approaches to developing engineered bacterial and mammalian cells,[50] and focusing on applications of engineered microbes for combating pathogens,[51] treating cancers,[52] and developing biocontainment systems.[53] This review will focus on the application of "biosensing" for the development of smart designer probiotics engineered to sense the status and conditions of the host. Using this information, the probiotics can report on the health of the host (Section 2), respond by generating therapeutics (Section 3), or control their own viability for self-biocontainment (Section 4).

2. Biosensing for Reporting on the Health and Conditions of the Host

Many studies have utilized sensing modules in probiotics and commensals to generate living diagnostics (**Table 1**). One application of smart diagnostic microbes is the detection of gut inflammation. Riglar et al. modified the phage λ CI/Cro bistable switch developed by Kotula et al.[54] to detect tetrathionate, a compound associated with gut inflammation.[55] Specifically, they linked the expression of the cro memory element to the tetrathionate-sensitive, *S. typhimurium*-native two-component system (TCS) TtrSR and its cognate promoter P_{ttrBCA}. They engineered *E. coli* NGF-1, a strain capable of long-term colonization of the gut, to express a colorimetric enzymatic reporter from the memory switch. This system reliably reported the presence of tetrathionate in $IL10^{-/-}$ mice for up to six months after bacterial administration. Noting that the *S. typhimurium* P_{ttrBCA} promoter requires the oxygen-sensitive global regulator FNR for transcription, Daeffler et al. addressed this unwanted cross-regulation issue by adapting an alternative tetrathionate-responsive TCS (TtrSR-P_{ttrB}) from *Shewanella baltica* that is completely orthogonal to *E. coli* FNR.[56] They also derived a thiosulfate-sensitive TCS (ThsSR-P_{phsA}) from *Shewanella halifaxensis* as thiosulfate is another compound associated with gut inflammation. Both TCSs were linked to a fluorescent reporter in *E. coli* Nissle 1917 (EcN), and they found that only the thiosulfate sensor activated expression of the reporter when exposed to the inflamed mouse gut. Mimee et al. utilized the same thiosulfate sensor in a novel microelectronic-based luminescence detection system, but the thiosulfate sensor was only demonstrated in vitro.[57] As an in vivo demonstration of their technology, they integrated the heme-responsive transcriptional repressor HrtR into EcN to detect bleeding in porcine models. To facilitate heme diffusion into the cell, they also expressed the heme transporter ChuA. The heme-sensing bacteria were loaded into a reservoir in an ingestible capsule with microelectronics capable of detecting the signal from the microbes' luminescent reporter. This information was wirelessly transmitted outside the body, enabling a novel method of real-time diagnosis.

Another diagnostic target is host infection. Several efforts have utilized biosensors to detect pathogenic bacteria in vitro,[57,58] while others have advanced the systems to in vivo diagnostics. For example, Mao et al. engineered *Lactococcus lactis* to detect cholera infections in vivo.[4] They developed a library of chimeric TCSs with the cholera autoinducer 1 (CAI-1)-binding domain of *Vibrio cholerae* (Vc) CqsQ fused to the signal transduction domain of *L. lactis* NisK. The best-performing chimera successfully repressed the cognate promoter P_{NisR} in response to CAI-1. Next, they linked the promoter to a TetR-based inversion module to create an inducible CAI-1-sensing circuit. Using a colorimetric enzymatic reporter, the engineered strain could inform of Vc infection in mice after being isolated from fecal matter. Certain et al. studied the dynamics of microbial infection by employing an inducible CI/Cro memory switch.[54,59] They demonstrated that the inducer changed the memory state of the switch from OFF to

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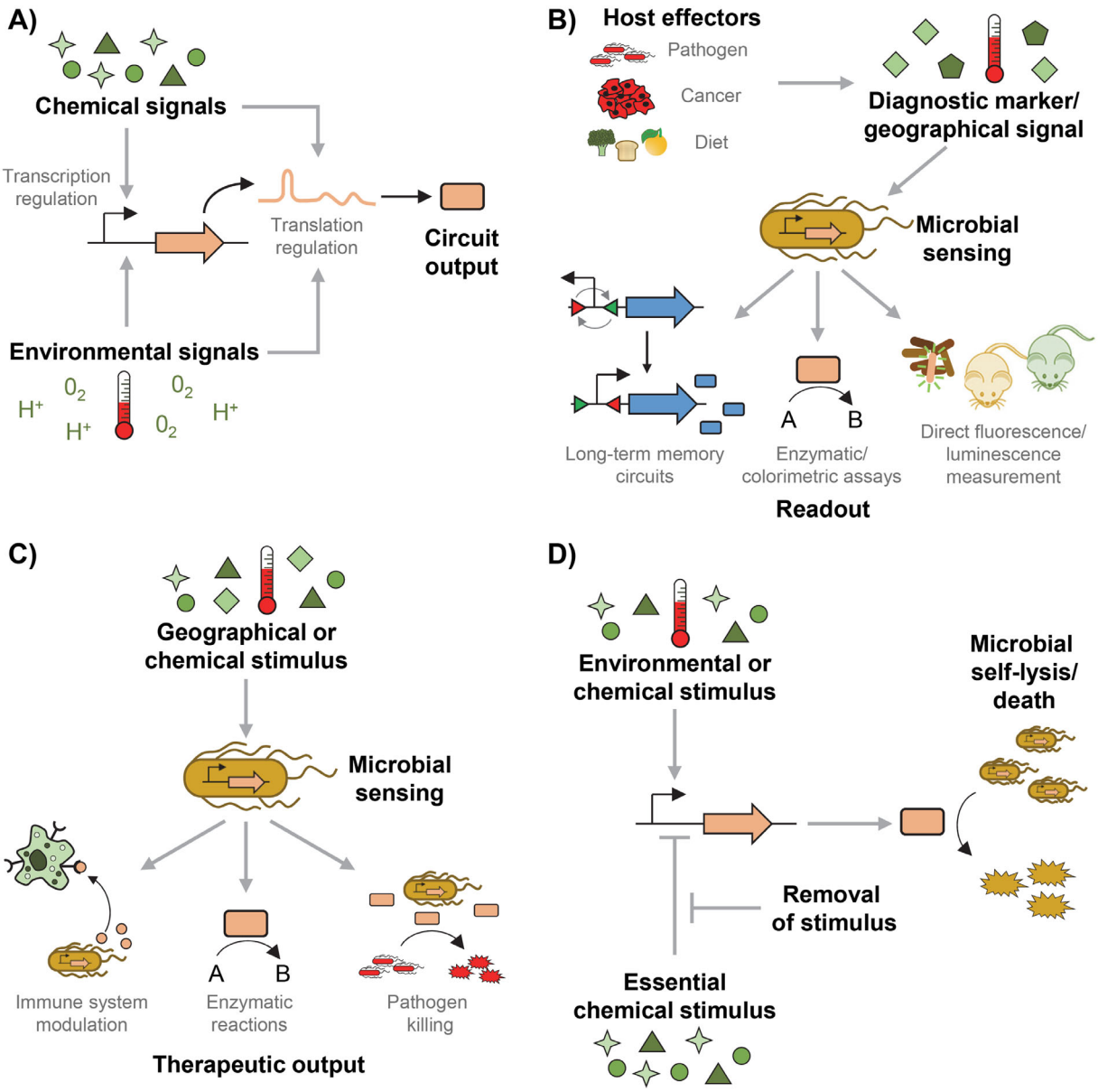

Figure 1. Chemical and environmental sensors allow microbes to be engineered for diagnostic, therapeutic, and biocontainment applications. A) Chemical signals (including various sugars, host-produced metabolites, and synthetic compounds) and environmental signals (including oxygen level, pH, and temperature) can regulate the rate of protein production at the transcriptional and translational levels. B) Many host effectors, including pathogenic bacteria, cancers, and diets, affect the levels of various chemical and environmental signals in the host's gut. Probiotic and commensal microbes can be engineered with sensors that measure and report on the levels of these stimuli. Example reporting methods include uses of memory circuits that maintain their state long-term for measurement outside the body, enzymatic or colorimetric assays that are correlated to the sensed levels, and direct in vivo (e.g., by imaging or electronic sensors) or ex vivo (e.g., using fecal samples) measurements of fluorescence or luminescence. C) Microbes can also be engineered to produce therapeutic outputs only when the microbes sense disease-relevant stimuli. The signals can include those naturally present in the target location, or ones externally administered to the host. Under the induced conditions, the microbe can produce therapeutic proteins for the treatment of diseases and infections. D) Chemical and environmental sensors can also be applied to microbial biocontainment. Some circuits control cell viability by inducing cell death with the addition of a chemical or in response to environmental stimulus. With these circuits, the cell grows until the stimulus is applied. Other circuits instead initiate cell death when a chemical is removed. This growth-supporting chemical is added to the cultures when the cell is grown in vitro.

ON only in actively dividing cells. Using this circuit, they sought to interrogate the replication state of memory switch-containing *E. coli* that had infected mice. Specifically, they exposed the bacterial cells to the inducer and to levofloxacin, an antibiotic that preferentially kills dividing bacteria. They discovered that while levofloxacin treatment reduced bacterial burden at the infection site, the proportion of actively dividing bacteria increased, contrary to the result from in vitro levofloxacin treatment.

Smart diagnostics have also been used to report on other physiological conditions and diseases. Takahashi et al.

Table 1. Bacteria engineered as diagnostics.

Diagnostic application	Detected compound or condition	Sensor type	Sensor genetic part	Circuit	Reporter	Strain	Ref.
Gut inflammation	Tetrathionate	TCS	*Salmonella typhimurium* TtrSR-P_{ttrBCA}	CI/Cro memory switch	β-galactosidase	*Escherichia coli* NGF-1	[55]
Gut inflammation	Tetrathionate	TCS	*Shewanella baltica* TtrSR-P_{ttrB}	NA	GFP	EcN	[56]
Gut inflammation	Thiosulfate	TCS	*Shewanella halifaxensis* ThsSR-P_{phsA}	NA	GFP	EcN	[56]
Gut bleeding	Heme	T-TF	*E. coli* O157:H7 ChuA-*Lactococcus lactis* HrtR-$P_{L(HrtO)}$	NA	luxCDABE combined with microelectronics	EcN	[57]
Cholera	Cholera autoinducer 1 (CAI-1)	TCS	Engineered chimera[a)]	TetR (inverter)	β-lactamase	*L. lactis*	[4]
Cell division	aTc	TF	TetR-P_{tet}	CI/Cro memory switch[b)]	β-galactosidase	*E. coli* NGF-1	[59]
Cancer	Tumors colonized by engineered EcN	NA	NA	NA	β-galactosidase converting LuGal into luciferin	EcN	[65]
Inflammation	Nitrogen oxides	TF	*E. coli* NsrR-P_{YeaR}	BxbI or TP901-9 recombinases	GFP	*E. coli* DH5αZ1	[64]
Diabetes	Glucose	TCS	*E. coli* CpxAR-P_{CpxP}	BxbI or TP901-9 recombinases	GFP	*E. coli* DH5αZ1	[64]

AHL, *N*-acyl homoserine lactone; aTc, anhydrotetracycline; EcN, *E. coli* Nissle 1917; GFP, green fluorescent protein; TCS, two-component system, consisting of a histidine kinase and a response regulator; TF, transcription factor; T-TF, transporter-transcription factor; LuGal, a soluble conjugate of luciferin and galactose; NA, not applicable. If NA is present in the "Circuit" column, the sensor directly regulates expression of the reporter; otherwise, there is an additional circuit layer. [a)]The TCS is an engineered chimera where the CAI-1-binding domain of *Vibrio cholerae* CqsQ is fused to the signal transduction domain of *L. lactis* NisK. The chimeric histidine kinase interacts with the response regulator NisR; [b)] Switching occurs only in actively dividing engineered *E. coli*.

implemented paper-based platforms using *E. coli* lysates and toehold switches[60–62] to identify specific species of microbes in the gut microbiome.[63] Fluorescent reporters *cis*-repressed by toehold hairpin formation were *trans*-activated by species-specific RNAs from ten different microbes found in human microbiomes. By incorporating the toehold switch into a cell-free paper system, the authors demonstrated a low-cost method of analyzing and quantifying microbiome composition. Courbet et al. utilized whole-cell biosensors to detect clinically relevant biomarkers in urine and plasma samples, including nitrogen oxide for inflammation and glucose for diabetes.[64] Danino et al. relied on EcN's proclivity to preferentially colonize cancerous masses to detect liver cancer from urine samples.[65] They engineered tumor-colonizing EcN to stably express β-galactosidase (LacZ), and upon tumor colonization, the engineered strain cleaved a luciferin-galactose conjugate using LacZ, releasing luciferin. A luminescence-based assay was then used to detect luciferin in urine.

3. Biosensing for Smart Expression of Therapeutics

3.1. Cancer Treatments

One promising application of microbial therapeutics is the treatment of cancer. Engineered cells specifically targeted to tumors can avoid the systemic toxicity of chemotherapeutic agents and enable repeated dosing of a therapeutic at the cancerous site. Many efforts to develop cancer therapies have involved engineering microbes that preferentially colonize hypoxic tumors to deliver constitutively expressed therapeutic proteins.[8,66–69] Other efforts have focused on engineering the cell to recognize and target the acidic tumor microenvironment[70] or to preferentially bind to cancer cell surfaces.[71] However, these microbes relied on constitutive expression of the therapeutic. Here, we discuss smart therapies that utilize an additional layer of control over the expression of the therapeutic (**Table 2**A).

The most common strategy to control cancer therapeutic expression is to express the proteins only when the cell is in the hypoxic tumor microenvironment. For example, He et al. controlled expression of an anti-angiogenesis tumstatin gene in EcN using the oxygen-dependent *E. coli* global regulator FNR and the *Vitreoscilla* P_{vhb} promoter.[72] He et al. later improved the therapeutic by fusing a p53 cell cycle checkpointing agent to tumstatin.[73] Ryan et al. placed the cytotoxin HlyE under the control of the *S. typhimurium* (St) oxygen-sensitive promoter P_{fnrS} in aSt.[74] The cytotoxin was only expressed following colonization of the tumor microenvironment by aSt. Zheng et al. also took advantage of aSt's ability to selectively colonize the hypoxic regions of tumors by expressing the *Vibrio vulnificus* FlaB flagellin gene under the control of an arabinose-inducible promoter.[75]. Upon colonization of the hypoxic tumor and supply of exogenous arabinose, the engineered strain significantly increased immune cell recruitment to the tumor site as compared

Table 2. Biosensors coupled to therapeutic production.

Detected compound or condition	Sensor type	Sensor genetic part	Additional circuit component or mechanism of action	Therapeutic	Therapeutic mechanism of action	Strain	Ref.
A) Cancer:							
Low oxygen	TF	*Escherichia* coli FNR-*Vitreoscilla* P_{vhb}	NA	Tumstatin (Tum 5)	Anti-angiogenesis	EcN	[72]
Low oxygen	TF	*E.* coli FNR-*Vitreoscilla* P_{vhb}	NA	Tumstatin-p53 fusion	Anti-angiogenesis and cell cycle checkpointing	EcN	[73]
Low oxygen	TF	Engineered St FNR-P_{fnr}	NA	HlyE	Cell lysis	St	[74]
Arabinose[a)]	TF	AraC-P_{BAD}	NA	*Vibrio vulnifcus* FlaB	Immune cell recruitment	St	[75]
Cell density (AHL)	TF	LuxR-P_{luxR}	P_{luxR}-*luxI*, P_{luxR}-φX174E[b)]	HlyE, CCL21, and Bit1-iRGD chimera	Cell lysis and immune cell recruitment	St	[76]
B) Metabolic disorder:							
Low oxygen	T-TF	PheP-FNR-P_{fnrS}	FNR-P_{fnrS}-*pheP*	Phenylalanine ammonia-lyase	Phenylalanine removal	EcN	[77]
Low oxygen	TF	FNR-P_{fnrS}	NA	Feedback resistant-ArgA	Ammonia removal	EcN	[78]
C) Inflammatory bowel disorder/colitis:							
Non-permissive condition	NA	NA	Self-lysis[c)]	IL-1Ra	IL1-receptor antagonism	*Bacillus subtilis*	[79]
Xylan[a)]	TF	Putative *Bacteroides ovatus* xylanase-inducible promoter	*Bacteroides fragilis*-derived peptide sequence-mediated secretion	TGF-β_1	Immune suppression	*B. ovatus*	[80]
D) Infection:							
AHL (from PA)	TF	PA LasR-P_{las}	P_{las}-Lysin E7[d)]	Pyocin S5	Cell lysis	*E. coli* TOP10	[81]
AHL (from PA)	TF	PA LasR-P_{las}	P_{las}-Lysin E7[d)]	Pyocin S5 and Dispersin B	Cell lysis and anti-biofilm hydrolase	EcN	[82]
AHL (from PA)	TF	PA LasR-P_{las}	FlgM-mediated secretion	CoPy (Colicin E3-Pyocin S3 chimera)	RNase and cell lysis	*E. coli* MG1655	[83]
AHL (from PA)	TF	PA LasR-P_{las}	P_{las}-*cheZ*-mediated chemotaxis and YebF-mediated secretion	Microcin S and DNaseI	Biofilm prevention and biofilm destruction	*E. coli* RP437 ΔcheZ	[84]
Cholera autoinducer 1 (CAI-1)	TCS	*Vibrio cholerae* CqsS-LuxU-LuxO-P_{tpQrr4}	P_{tpQrr4}-gRNA, P_{con}-*dCas9*, AraC-P_{BAD}, and YebF-mediated secretion[e)]	Artilysin	Cell lysis	*E. coli* MG1655	[85]
Pathogenicity and antibiotic resistance	TCS	*V. cholerae* ToxRS-P_{ompU}	*V. cholerae* SetR-P_L-*ccdA*[f)]	CcdB	DNA gyrase inhibition	*E. coli* β3194	[86]
cCF10	TF	*Enterococcus faecalis* PgrX-P_{pgrQ}	NA	Enterocin A, Hiracin JM79, and Enterocin P	Cell lysis	*Lactococcus lactis*	[87]
Tetrathionate	TCS	*Salmonella typhimurium* TtRS-P_{ttrBCA}	NA	Microcin H47	ATP synthase inhibition[g)]	EcN	[88]
HPA (from *Candida albicans*)	T-TF	*E. coli* HpaX-HpaA-P_{BC}	NA	*Burkholderia cenocepacia* RpfF-synthesized cis-2-dodecenoic acid	Hypha formation inhibition	*E. coli* NGF-1	[89]

AHL, *N*-acyl-homoserine lactone; EcN, *E. coli* Nissle 1917; HPA, hydroxyphenylacetic acid; PA, *Pseudomonas aeruginosa*; St, *S. typhimurium*; TF, transcription factor; T-TF, transporter-transcription factor; NA, not applicable. [a)] Exogenous inducer not directly related to disease state; [b)] P_{luxR}-*luxI* forms a positive feedback circuit where LuxI synthesizes AHL, promoting a buildup of the therapeutic protein. P_{luxR}-φX174E forms a negative feedback loop, inducing self-lysis of the cell once the population reaches a certain threshold. The lysis enables release of the therapeutic protein. The combined positive-negative feedback results in a bacterial population that completes oscillatory cycles of therapeutic synthesis and lytic release; [c)] Upon reaching the gut, *Bacillus subtilis* natively senses unfavorable growth conditions and lyses, releasing the therapeutic protein of interest; [d)] Lysin E7 expression induces cellular lysis to more effectively deliver the therapeutic protein to the pathogen; [e)] The CRISPR interference circuit (P_{tpQrr4}-gRNA and P_{con}-*dCas9*) represses the arabinose-inducible promoter that control the expression of artilysin. Under high CAI-1, no gRNA is transcribed from P_{tpQrr4}-gRNA, allowing for arabinose-inducible artilysin production. Localization to the periplasm by YebF causes artilysin to lyse the host cell (*E. coli*), enabling efficient delivery of artilysin to the pathogen; [f)] In a pathogenic and antibiotic-resistant *V. cholerae* cell, SetR inhibits the expression of the CcdA antitoxin (by binding to the P_L promoter) which prevents CcdB toxin-mediated killing. This therapy is dependent on whole-plasmid conjugation from an *E. coli* carrier strain to *V. cholerae*. After conjugation, CcdB toxin-mediated killing occurs only in pathogenic and antibiotic-resistant bacteria that harbor SetR (antibiotic resistance indicator) and ToxR (pathogenicity indicator); [g)] Hypothesized mechanism of action.

to a non-engineered aSt control. Din et al. also engineered a quorum-sensing circuit in aSt to accumulate and release HlyE in the tumor.[76] Therapeutic protein expression was controlled by the *N*-acyl homoserine lactone (AHL)-sensitive transcription factor LuxR and its cognate promoter P_{lux}. LuxR-P_{lux} also controlled the expression of the AHL-synthesis protein LuxI in a positive feedback loop and cell lysis protein φX174E in a negative feedback loop. As the aSt cell density in the tumor increased, the cells synthesized increasing amounts of AHL, HlyE, and φX174E. Upon reaching a critical threshold, most cells lysed and released their therapeutic payload at the tumor site, while a few surviving cells began the cycle again. They also created engineered strains that replaced HlyE with a cytokine or apoptotic peptide and determined that a mixture of all three strains was most effective at preventing tumor growth. Finally, they demonstrated that the combination of both chemotherapy and a mixture of all three engineered strains significantly increased the survival of tumor-bearing mice relative to either therapy alone.

3.2. Metabolic and Inflammatory Disorder Treatments

Efforts to treat metabolic disorders with biosensor-augmented engineered probiotics have relied on the detection of the anaerobic gut environment (Table 2B). For example, Isabella et al. constructed a strain of EcN to overexpress the phenylalanine transporter PheP and phenylalanine ammonia lyase (PAL) and tested its ability to reduce phenylalanine levels in an animal model for phenylketonuria.[77] In order to ensure therapeutic expression in the gut, both PheP and PAL were expressed using the *E. coli* oxygen sensitive promoter P_{fnrS}. Kurtz et al. implemented the same strategy to treat hyperammonemia using EcN.[78] In this therapeutic, P_{fnrS} controlled the expression of the *E. coli* enzyme *N*-acetylglutamate synthase, leading to improved consumption of free ammonia.

Gut inflammation is another promising target for smart engineered microbes (Table 2C). Porzio et al. relied on the inherent ability of *Bacillus subtilis* to lyse in the gut upon sensing the unfavorable growth conditions.[79] They engineered their strain to produce IL-1 receptor antagonist (IL-1Ra), and upon lysis in the gut, free IL-1Ra reduced symptoms of inflammation. Hamady et al. engineered human commensal *Bacteroides ovatus* to express human transforming growth factor beta 1 (TGF-β1) to treat a murine model of colitis.[80] They utilized the putative *B. ovatus* xylanase promoter to express TGF-β1 in the gut only in the presence of xylan, a dietary fiber, and incorporated an *N*-terminal *Bacteroides fragilis*-derived peptide secretion tag onto TGF-β1 to induce therapeutic release.

3.3. Infection Treatments

Microbes can be engineered to sense and kill pathogens by exploiting genetic parts from the pathogen of interest (Table 2D). A commonly used sensor is the *Pseudomonas aeruginosa* (PA) transcription factor LasR that binds to the PA-specific AHL and induces expression from its cognate promoter, P_{las}. Saeidi et al. demonstrated that *E. coli* TOP10 engineered to express the bacteriocin Pyocin S5 from LasR-P_{las} could selectively kill PA in vitro.[81] They also placed the E7 lysis protein under control of P_{las} so that the engineered *E. coli* would lyse and more efficiently deliver its therapeutic payload in response to PA. In a follow-up work, Hwang et al. implemented the same circuit in EcN with the addition of the anti-biofilm enzyme Dispersin B also controlled by P_{las}.[82] They demonstrated that this engineered strain could work as a prophylactic and therapeutic in *C. elegans* and mouse infection models. Gupta et al. also targeted PA in vitro using its quorum sensing system to express the chimeric bacteriocin CoPy (Colicin E3-Pyocin S3) in *E. coli* MG1655.[83] To increase therapeutic efficiency, CoPy was secreted via FlgM. In a separate work, Hwang et al. engineered *E. coli* RP437 ΔcheZ to move toward and kill PA in vitro.[84] The expression of the chemotaxis protein CheZ, the bacteriocin Microcin S, and the anti-biofilm enzyme DNaseI were all controlled by LasR-P_{las} to enable movement toward and killing of PA. Microcin S and DNaseI were engineered to be secreted by YebF for extracellular delivery of the pathogen-killing agents.

Vc is another common target for pathogen-killing smart microbes. Jayaraman et al. built a CAI-1-responsive circuit in *E. coli* to kill Vc.[85] Because CAI-1 binding to CqsS represses the promoter P_{tpQrr4}, a CRISPR interference circuit was used to instead induce the expression of the therapeutic lysis protein artilysin in response to CAI-1. Artilysin was also secreted via YebF to enable efficient delivery, inhibiting the growth of Vc in vitro. Mazel et al. designed a system to specifically kill only a pathogenic and antibiotic-resistant Vc cell using plasmid conjugation from *E. coli* β3194.[86] Upon plasmid conjugation, the TCS ToxRS (associated with pathogenicity) activates transcription of the intein-split toxin CcdB from the cognate promoter P_{ompU}. In an antibiotic resistant strain, the resistance-associated transcription factor SetR represses transcription of the CcdA antitoxin from the P_L promoter. This application of Boolean AND logic to specifically kill only a pathogenic and antibiotic-resistant Vc cell was demonstrated in a zebrafish model.

Other pathogens have been targeted as well. Borrero et al. engineered *L. lactis* to detect the *Enterococcus faecalis* sex pheromone cCF10.[87] They adapted the cCF10-sensitive transcription factor PgrX to drive the expression of three bacteriocins from the promoter P_{pgrQ}, thus killing multi-drug resistant *E. faecalis* in vitro. Palmer et al. targeted St with a strain of EcN designed to detect tetrathionate, which is associated with St infections in the gut.[88] They used the tetrathionate-responsive St TCS to control the expression of the bacteriocin Microcin H47, which inhibited St growth in vitro. Tscherner et al. utilized the hydroxyphenylacetic acid (HPA) transporter (HpaX), transcription factor (HpaA), and cognate promoter (P_{BC}) to detect HPA, which is produced by the fungus *Candida albicans*.[89] Upon HPA detection, an enzyme was expressed to synthesize *cis*-2-dodecenoic acid which inhibits the formation of *C. albicans* hypha. They demonstrated hypha formation inhibition ex vivo, resulting in reduced filamentation, virulence factor expression, and epithelial damage.

4. Biosensing for Biocontainment

While in the host or after released into the environment, both engineered and wild type microbes can evolve and exchange genes with other organisms.[90–93] This adaptation can lead to

Table 3. Genetic circuits applied to the biocontainment of microbes.

Strain	Controlling inducer	Killed by addition/removal or killing condition	Log10 escape frequency	Tested in vivo?	Generations of stability	Ref.
A) Chemical sensing:						
Saccharomyces cerevisiae	Galactose	Removal	−7.7	No	ND	[100]
S. cerevisiae	Estradiol and galactose	Removal of galactose or addition of estradiol	−10	No	ND	[101]
Escherichia coli MC1000	IPTG	Addition	−4.3	No	ND	[102]
E. coli MG1655	aTc and IPTG	Removal of aTc or addition of IPTG	−7	No	≈14	[103]
E. coli MG1655	Cellobiose, galactose, and IPTG	Survival in only one condition out of the 8 (2^3) possible inducer combinations[a)]	−8	No	≈57	[103]
E. coli MG1655	Arabinose, aTc, and IPTG	Addition	−3	No	ND	[104]
E. coli MG1655	aTc, IPTG, and biotin (auxotroph)	Removal	−11.9[b)]	No	110	[95]
E. coli MG1655	Arabinose	Addition	−7.7	No	1700[c)]	[105]
B) Quorum and environmental sensing:						
E. coli TOP10 and MG1655	AHL	Cell density decrease	NA	No	ND	[106]
E. coli NEB10*β*	Temperature	Temperature decrease	−4 to −3	Yes	ND	[108]
E. coli MG1655	Temperature	Temperature decrease	−5	Yes	140[d)]	[109]

Escape frequency is the ratio of colony forming units obtained for the strain when grown in the killing condition to the non-killing condition; generations of stability is the maximum number of cell generations before observing a significant increase in escape frequency. AHL, *N*-acyl homoserine lactone; aTc, anhydrotetracycline; IPTG, isopropyl-*β*-D-1-thiogalactoside; NA, value is not applicable to the respective genetic circuit; ND, values were not determined or could not be approximated from the methods of the cited paper. [a)] The authors developed three genetic circuits where the cells survive only in the presence of inducers A and B but not C. Each of the three circuits had a different inducer, IPTG, galactose, or cellobiose, as inducer C; [b)] Killing ratio value was obtained when the genetic circuit was paired with auxotrophy; [c)] Long-term stability was determined by applying the CRISPR-based circuit to targeted plasmid degradation rather than cell death caused by targeting the genome; [d)] Long-term stability was tested in vitro.

organisms acquiring competitive growth advantages that disrupt the ecosystem, acquiring metabolic traits that disturb the health of the gut, or developing pathogenic characteristics.[90,94] These potential adaptations necessitate stringent controls for the biocontainment of engineered organisms used in medical applications. One common indicator for the efficiency of biocontainment methods is the escape frequency, or the ratio of cell counts in the killing condition to the non-killing condition. The NIH guidelines for the escape frequency of engineered organisms is a ratio of less than 10^{-8}.[95] One common approach to achieve this goal with engineered microbes is auxotrophy, the removal of an essential metabolite-producing gene from the genome of the organism.[77,78,96] The deficient metabolite can be exogenously provided to maintain growth of the organism. A similar approach is to engineer the cell to be reliant on a nonstandard amino acid.[97,98] Although these methods can be effective, the engineered strains require additional care or supplementation to grow in vitro and can escape through cross-feeding. A complementary approach includes the use of biosensors, where the organism controls its own viability in response to chemical or environmental signals.

4.1. Chemical Sensing to Control Microbial Cell Viability

Numerous circuits for directly controlling cell viability with chemical biosensors have been developed for diverse organisms (**Table 3**A). One common method of controlling cell viability with chemical sensing is to link expression of an essential gene to a chemical-inducible promoter. Kong et al. expressed the essential genes *asdA* and *murA* in aSt using an arabinose-inducible promoter, providing a potential *Salmonella* vaccine strain with biocontainment circuits.[99] Similar circuits have also been developed for *Saccharomyces cerevisiae*.[100,101] Agmon et al. used a galactose sensor to control the expression of many essential genes individually and demonstrated an escape frequency of less than 10^{-7} upon removal of galactose.[100] When Cai et al. added a second layer of control, which involved excising the expression cassette for an essential gene in response to estradiol, the escape frequency was below 10^{-10}.[101]

Many chemical sensing circuits have also been developed for the biocontainment of various *E. coli* strains. One early biocontainment method for *E. coli* involved expressing the RelF toxin from an Isopropyl-*β*-D-1-thiogalactoside (IPTG)-inducible promoter.[102] To protect the system from inactivation by random mutations, the authors used two parallel RelF expression cassettes and demonstrated an escape frequency of 5×10^{-5}. Chan et al. developed a genetic circuit, termed "Deadman" using an IPTG-inducible biosensor that paired toxin expression with inducible degradation of an essential protein.[103] In the presence of IPTG, both the EcoRI endonuclease and the *mf*-Lon protease were expressed. The protease then quickly degraded the tagged essential protein MurC as well as the LacI repressor to further induce the system. Cells with the circuit displayed an escape frequency of less than 10^{-7}, but with poor long-term stability. They also developed an additional set of three-input circuits, termed

"Passcode" where biosensors for IPTG, galactose, and cellobiose controlled the same killing mechanisms. Cells with the "Passcode" circuits could survive under only one of the eight possible input conditions. When the authors paired this circuit with an *E. coli* strain deficient in recombinogenic and mobile elements, they achieved an escape frequency of less than 10^{-8} with an improved long-term stability.

Riboregulator-based biocontainment has also been developed in *E. coli*. The first use of riboregulators for biocontainment was a proof-of-concept demonstration of cell lysis, with the expression of two *cis*-repressed phage genes being regulated by anhydrotetracycline (aTc).[104] The expression of the cognate *trans*-activating RNAs was regulated by arabinose and IPTG. Gallagher et al. used riboregulators to control the expression of two essential genes in parallel, making cell survival dependent on the presence of the IPTG and aTc inducers.[95] Additionally, they combined the riboregulator system with biotin auxotrophy and EcoRI expression. To control the activity of EcoRI, they expressed the EcoRI-inhibiting EcoRI methylase with an aTc sensor. Removal of aTc and IPTG from the culture repressed the expression of the essential genes and EcoRI methylase, allowing EcoRI to cleave the genome. This system achieved a field-best escape frequency of lower than 1.3×10^{-12}.

Caliando and Voigt developed another biocontainment method using CRISPR-based genomic DNA degradation.[105] This system utilized CRISPR-Cas3 and CasABCDE to control the viability of *E. coli*. They integrated three arabinose-controlled Cas expression cassettes into the genome and constitutively expressed the cognate RNA on a plasmid. Using this system, the authors achieved an escape frequency of 1.9×10^{-8}, nearly meeting the NIH criteria. While they did not demonstrate long-term stability of the genome-targeting system, they showed stable maintenance of a plasmid-cleaving system for 1700 generations.

4.2. Quorum and Environmental Sensing to Control Microbial Cell Viability

Several recent works have implemented biosensing to engineer microbes that control their own viability to prevent survival when released into the environment (Table 3B). To accomplish this goal, Huang et al. utilized the LuxR/LuxI quorum sensing system.[106] When the engineered *E. coli* cells sense high concentrations of AHL, signifying a high cell density, the cells produce the antibiotic resistance gene, allowing them to survive in the presence of the respective antibiotic. However, when the cells leave the general population, the reduced concentration of AHL turns off expression of the antibiotic resistance gene, causing antibiotic-mediated cell death. Although this version of the system cannot be applied in vivo because of the antibiotic-based killing mechanism, it can be modified by applying quorum sensing to an alternative method of viability control, including cell lysis as demonstrated in vivo by Chowdhury et al.[107] Using a quorum-lysis system, Chowdhury et al. also demonstrated localized release of immunotherapeutics and an abscopal effect in a mouse tumor model.

Temperature sensing was also used to control bacterial survival in the environment after excretion from the body.[108,109] When in the gut, microbes experience a relatively stable temperature near 37 °C, but after excretion from the body, the cells usually experience a reduced temperature. Piraner et al. engineered *E. coli* to sense this temperature downshift using a mutant version of the St-native TlpA temperature sensor.[108] Native TlpA showed half-maximal expression from the P_{tlpA} promoter at ≈43.5 °C, well above physiologically relevant temperatures, while the optimized mutant demonstrated half-maximal expression at ≈36 °C. To control cell viability in a temperature-dependent manner, they used the engineered temperature sensor to express the antitoxin CcdA of the CcdB-CcdA toxin-antitoxin system. At high temperatures (>36 °C), CcdA is expressed, preventing cell death. At low temperatures, CcdA is no longer expressed, allowing constitutively expressed CcdB to kill the cell. They demonstrated an escape frequency of 10^{-4}–10^{-3} using this system in mice. Alternatively, Stirling et al. used the *E. coli*-native P_{cspA} temperature-sensing promoter, which is activated below ≈30 °C, to differentiate between conditions inside and outside of the gut.[109] In this system, the antitoxin CcdA was constitutively expressed, and the toxin CcdB was expressed by P_{cspA}. They demonstrated that the system can maintain its efficiency for 140 generations in vitro. They also tested the system in vivo, achieving an escape frequency of less than 10^{-5}. These reports have displayed valuable proof-of-concept circuits for environmental biocontainment, with improvements still needed to achieve the NIH recommended escape frequency.

5. Conclusions

Incorporating biosensing into engineered probiotics has provided significant advances in live diagnostics and therapeutics. However, there is still vast potential for improvements and new directions. The synthetic biology technologies to mine and screen for native sensing capabilities of microbes and to design and build novel synthetic sensors will provide a boundless collection of biosensors. Significant advances continue to be made for in vitro biosensing, and many sensors for application-relevant environmental conditions and chemicals have yet to be demonstrated in vivo.[42,110–114] Importantly, the in vitro development of a sensor does not guarantee successful implementation in the complex gut environment. To be useful in the gut, sensors need to be tuned to respond to physiologically relevant chemical concentrations and conditions and to provide an effective therapeutic level upon activation without imposing a high metabolic burden on the cell.

The throughput of in vitro sensor development is orders of magnitude higher than the throughput of in vivo functionality testing, limiting the rate of applying microbial sensors to health-related applications. To improve the process of selecting strains for in vivo testing, new methods will need to be used that mimic the gut environment. For example, 3-D intestinal scaffolds have been developed that mimic the crypt-villus axis of the small intestine.[115] As a proof-of-principle, this technology was applied to study both the adhesion of common pathogens to the intestine and the efficiency at which different probiotic strains displace said pathogens. In addition, co-culture techniques with intestine-mimicking media can be used to simulate the complex microbial communities of the gut and to improve the relevance of the microbes' metabolic state.[116] Microfluidic "intestine-on-a-chip"

devices have also been developed that foster microenvironments with gut-relevant oxygen gradients, chemical diffusion rates, differential pH regions, microbial communities, and living human intestinal epithelial cells.[117,118] These technologies can be applied to improve the development of many engineered probiotics.

As the screening rate for engineered microbes improves with in vivo-simulating devices, microbes can be engineered with increasing complexity, stability, and safety. Currently, microbes are being engineered either as diagnostics or as therapeutics (but not as both), often for the same issues such as cancer[65,74] and inflammation.[55,56,79,80] However, the tools exist to engineer a single microbe with genetic circuits that perform both diagnostic and therapeutic functions simultaneously, to sense the ailment, report on the issue, and solve the problem. Similarly, microbes can be engineered to sense multiple gut-relevant conditions and/or to perform multiple therapeutic functions in parallel, as multiple ailments often accompany each other (e.g., pathogenic infection and gut inflammation).[119] To increase specificity, however, new sensors need to be developed and integrated into multi-input circuits (e.g., AND gates). For example, while oxygen-dependent sensors have been shown to be useful for targeted delivery of therapeutics to both hypoxic tumor cells (Section 3.1) and the anaerobic gut (Section 3.2), better gene circuits would respond to cancer-specific molecules and disease-specific chemicals, respectively, in addition to low oxygen levels. Importantly for each case, biocontainment circuits and technologies should be incorporated into the probiotics to prevent release into the environment.[120] However, increasing the complexity of genetic circuits also increases the burden on the cell. The generation of loss-of-function mutations can enrich for non-functional cells that out-compete the functional microbes. Methods need to be developed and implemented that reduce this burden and increase the genetic stability.[121,122]

Engineered probiotics have the potential to drastically improve the care of patients with difficult-to-diagnose and -treat disorders and conditions. However, engineering living organisms involves unique potentials and risks as discussed, and many factors must be carefully considered and studied, including 1) in vivo sensor sensitivity, selectivity, and robustness under fluctuating environmental conditions, 2) the effect of many variables such as diet, microbiome composition, and animal host cells on the performance of engineered microbes, 3) mutational stability of engineered circuits in vivo, and 4) biosafety measures. The recent advancements of engineered microbes in clinical trials is providing a valuable precedent for applying synthetic biology to solving health problems.[123] As this progress continues to be made, clinical data can guide the construction of future engineered microbial diagnostics and therapeutics. New problems will continue to be solved with engineered probiotics, and current issues will be tackled with improved efficiency and efficacy.

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Conflict of Interest

The authors declare no conflict of interest.

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