

# Engineering Diagnostic and Therapeutic Gut Bacteria

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**ABSTRACT** Genetically engineered bacteria have the potential to diagnose and treat a wide range of diseases linked to the gastrointestinal tract, or gut. Such engineered microbes will be less expensive and invasive than current diagnostics and more effective and safe than current therapeutics. Recent advances in synthetic biology have dramatically improved the reliability with which bacteria can be engineered with the sensors, genetic circuits, and output (actuator) genes necessary for diagnostic and therapeutic functions. However, to deploy such bacteria *in vivo*, researchers must identify appropriate gut-adapted strains and consider performance metrics such as sensor detection thresholds, circuit computation speed, growth rate effects, and the evolutionary stability of engineered genetic systems. Other recent reviews have focused on engineering bacteria to target cancer or genetically modifying the endogenous gut microbiota *in situ*. Here, we develop a standard approach for engineering “smart probiotics,” which both diagnose and treat disease, as well as “diagnostic gut bacteria” and “drug factory probiotics,” which perform only the former and latter function, respectively. We focus on the use of cutting-edge synthetic biology tools, gut-specific design considerations, and current and future engineering challenges.

## INTRODUCTION AND BACKGROUND

Genetically engineered bacteria have the potential to diagnose and treat a wide range of diseases linked to the gastrointestinal tract, or gut. Such engineered microbes will be less expensive and invasive than current diagnostics and more effective and safe than current therapeutics. Recent advances in synthetic biology have dramatically improved the reliability with which bacteria can be engineered with the sensors, genetic circuits, and output (actuator) genes necessary for diagnostic and therapeutic functions. However, to deploy such bacteria *in vivo*, researchers must identify appropriate gut-adapted strains and consider performance metrics

such as sensor detection thresholds, circuit computation speed, growth rate effects, and the evolutionary stability of engineered genetic systems. Other recent reviews have focused on engineering bacteria to target cancer (1, 2) or genetically modifying the endogenous gut microbiota *in situ* (3, 4). Here, we develop a standard approach for engineering “smart probiotics,” which both diagnose and treat disease, as well as “diagnostic gut bacteria” and “drug factory probiotics,” which perform only the former and latter function, respectively. We focus on the use of cutting-edge synthetic biology tools, gut-specific design considerations, and current and future engineering challenges.

## Natural Probiotics

Natural probiotics are evolved organisms that are isolated, cultured, and consumed for a health benefit (5). Many bacterial strains have been developed as natural probiotics, most prominently those of the *Lactobacillus* and *Bifidobacterium* genera (6). These microbes have been used to treat *Clostridium* infection (7); inflammatory diseases such as obesity, diabetes, and inflammatory bowel disease (8); and neurological conditions such as anxiety, depression, and autism spectrum disorder (6), among other pathologies. Natural probiotics are known

**Received:** 27 June 2017, **Accepted:** 8 September 2017,

**Published:** 20 October 2017

**Editors:** Robert Allen Britton, Baylor College of Medicine, Houston, TX 77030, and Patrice D. Cani, Université catholique de Louvain, Louvain Drug Research Institute, Brussels 1200, Belgium

**Citation:** Landry BP, Tabor JJ. 2017. Engineering diagnostic and therapeutic gut bacteria. *Microbiol Spectrum* 5(5):BAD-0020-2017. doi:10.1128/microbiolspec.BAD-0020-2017.

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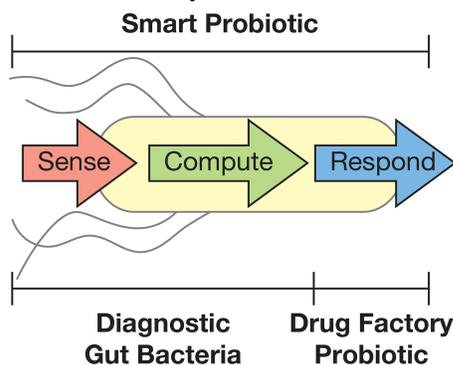
to exert beneficial effects by directly signaling with the human host through chemical or physical means or by altering the composition and metabolism of the gut microbiota (9). However, like all bacteria, natural probiotics have evolved complex gene regulatory networks that activate or silence therapeutic pathways in response to poorly understood intracellular and extracellular signals. Furthermore, the genetic and molecular mechanisms by which natural probiotics evolved to act are poorly understood (10). As a result, it has proven difficult to improve the efficacy of probiotic therapies, and natural probiotics have thus far yielded mixed success (11).

### Smart Probiotics

An exciting alternative is to genetically engineer “smart probiotics”: bacteria that sense the levels of one or more gut biomarkers, compute whether the profile of those biomarkers is diagnostic of disease, and respond by delivering a precise dose of one or more appropriate therapeutics (4, 12–14) (Fig. 1).

Smart probiotics offer benefits over traditional non-living therapeutics (i.e., chemical or biologically produced [biologic] drugs) and natural probiotics. First, both smart and natural probiotics can deliver drugs locally to diseased tissues, resulting in increased efficacy and decreased side effects relative to systemic administration of traditional therapeutics. Second, bacteria produce drugs on-site, eliminating the need for expensive purification steps involved in chemical drug

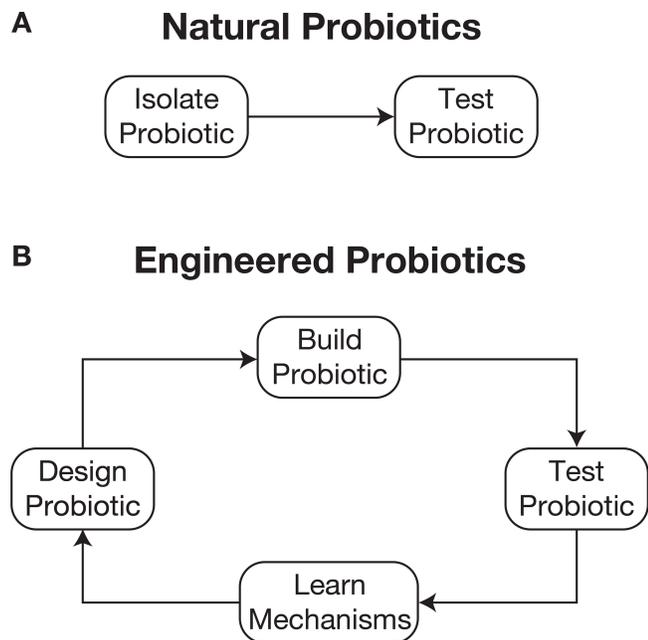
**FIGURE 1** The three classes of engineered gut bacteria. Smart probiotics sense one or more biomarkers, compute that those biomarkers are present in a combination indicative of disease, and respond by delivering a precise dose of one or more appropriate therapeutics at the diseased tissue. Diagnostic gut bacteria sense one or more biomarkers, compute that those biomarkers are present in a combination indicative of disease, and produce a reporter which can be externally measured by a clinician. Drug factory probiotics constitutively produce a therapeutic within the body.



synthesis or fermenter-based biologic production. Third, due to the use of well-characterized engineered gene-regulatory networks, rather than poorly characterized evolved versions, therapeutic pathways can be activated far more reliably *in vivo* with smart as opposed to natural probiotics. Fourth, the genetic pathways resulting in the production of the therapeutic compounds are well-defined in smart probiotics. This feature is unlike natural probiotics, which may produce undefined mixtures of bioactive compounds whose effects are difficult to disentangle. Fifth, in stark contrast to their natural counterparts which are simply taken “as-is” from nature, smart probiotics are developed using iterative cycles of design, construction, testing, and learning, which enables continual performance improvements (Fig. 2). Finally, future advances in our understanding of the biology of gut-linked diseases will result in continued improvements in smart probiotic designs and expand the number of diseases that can be treated.

The foundation for smart probiotics was laid at the turn of the current century when Remaut and colleagues genetically modified the natural probiotic *Lactococcus lactis* to constitutively secrete the human anti-inflammatory cytokine protein interleukin-10 (IL-10) (Fig. 3A). We classify this IL-10-secreting *L. lactis* strain, and any bacterium engineered to constitutively produce a therapeutic within the body, as a “drug factory probiotic.” Because drug factory probiotics perform only the “back end” delivery function, we consider them a simpler subclass of smart probiotics (Fig. 1). As the authors intended, oral administration of the IL-10-secreting *L. lactis* reduces colon inflammation (colitis) by 50% in mice (15) (Fig. 3B). Furthermore, 10,000-fold lower IL-10 concentrations are required to achieve a therapeutic effect when delivered in the gastrointestinal tract by *L. lactis* compared to intraperitoneal administration (15). This increase in efficacy decreases the potential for unwanted immunosuppressive side effects. These benefits are clear examples of several of the advantages of smart probiotics.

The Remaut group later iterated on the IL-10-secreting *L. lactis* strain several times to improve its performance. One concern is uncontrolled growth of engineered bacteria within the gut. This may be detrimental to the health of the host or result in unintended escape into the environment and corresponding spread of synthetic DNA into natural populations. To overcome this problem, the authors introduced a genomic mutation resulting in thymidine auxotrophy, making their drug factory probiotic dependent on thymidine added to laboratory growth media for survival. Indeed, this biocontainment strategy greatly decreases survival in the porcine gut



**FIGURE 2** A comparison of the development process for natural and engineered probiotics. **(A)** Natural probiotics are isolated and then tested for efficacy without an ability to methodically improve their capabilities. **(B)** In contrast, engineered probiotics undergo a design-build-test-learn cycle which allows for continual probiotic improvement and knowledge gain with each iteration.

(16). Additionally, a phase I clinical trial for treatment of Crohn's disease revealed that this second-generation IL-10-secreting *L. lactis* does not generate systemic or long-term side effects and that the biocontainment strategy functions as intended in humans (17). However, a subsequent phase IIA trial failed to demonstrate statistically significant beneficial effects in humans. Unfortunately, the reasons for this failure are unknown (18). Nonetheless, to improve efficacy, the strain has since been re-engineered to secrete single-chain antibodies that bind tumor necrosis factor alpha (TNF $\alpha$ ) (i.e., anti-TNF $\alpha$  nanobodies), preventing its ability to stimulate inflammation (19). Clinical trials using the anti-TNF $\alpha$  nanobody-secreting *L. lactis* to treat inflammatory bowel disease are now being pursued (20; <https://www.dna.com/Technologies/ActoBiotics>).

Undoubtedly inspired by the *L. lactis* work, other groups have since engineered drug factory probiotics to treat colitis (17, 19, 21–28), diabetes (29–32), obesity (33), and numerous pathogen infections (34–38). These efforts are all based on traditional genetic engineering approaches wherein a heterologous genetic pathway is simply introduced into a gut-relevant bacterial host. On the other hand, exciting advances are being made by

incorporating modern synthetic biology technologies into smart probiotic designs.

### Synthetic Biology

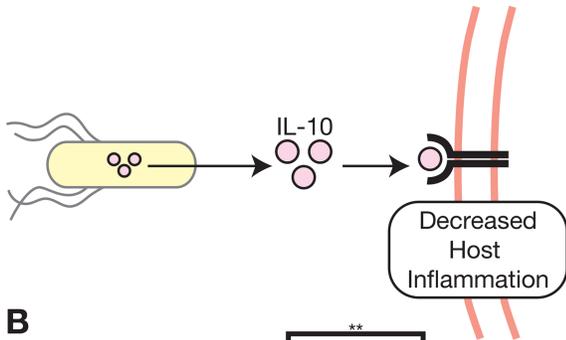
Synthetic biology is a new engineering discipline wherein living organisms are genetically programmed to carry out unnatural behaviors, with applications in medicine, agriculture, energy, manufacturing, and fundamental biology research, among other enterprises (14, 39–41). In contrast to traditional genetic engineering approaches, synthetic biologists program cellular behaviors using the “sense-compute-respond” paradigm adapted from electrical engineering (42) (Fig. 4). Here, organisms are engineered to express genetically encoded sensors, such as signal transduction pathways, that detect specific chemical or physical inputs inside or outside the cell. Sensors convert chemical or physical inputs into biological outputs such as gene transcription. These biological outputs, in turn, serve as inputs into engineered genetic circuits, to which the sensors are linked (43, 44). Genetic circuits are networks of interacting regulatory molecules, such as transcription factors (TFs) and their target promoters, that perform computations such as multi-input logic or memory. Finally, the circuits control the activity of actuator genes, such as metabolic pathways or secreted proteins, which program the cell to change its own state or the state of its environment. Importantly, sensors, circuits, and actuators (i.e., devices) are designed to be modular, such that they function in the same manner in different genetic, organismal, or environmental contexts. This modularity enables a large number of new designs to be rapidly imagined, constructed, and tested, while increasing predictability and improving performance.

### Genetically Encoded Sensors

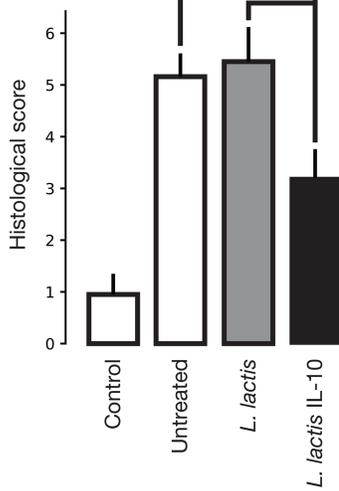
Bacteria have evolved thousands of genetically encoded sensors, many of which can be repurposed to diagnose disease. These fall into two major categories: one-component systems (OCSs) and two-component systems (TCSs).

OCSs, cytoplasmic TFs that directly interact with and are allosterically modulated by chemical and physical inputs, are the largest class of bacterial sensors (45) and have been used for gut-relevant sensing applications. In *in vitro* work, synthetic biologists have utilized OCSs that respond to acyl-homoserine lactone quorum-sensing autoinducers to program *Escherichia coli* to monitor communication between pathogenic *Pseudomonas aeruginosa* on solid agar (46) and in biofilms (47). In an *ex vivo* study, NorR, an OCS activated by

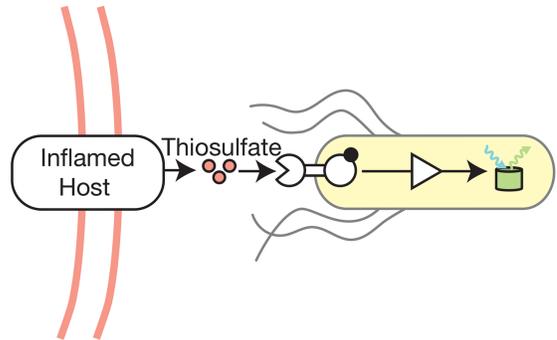
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IL-10 Treatment of Inflammation**



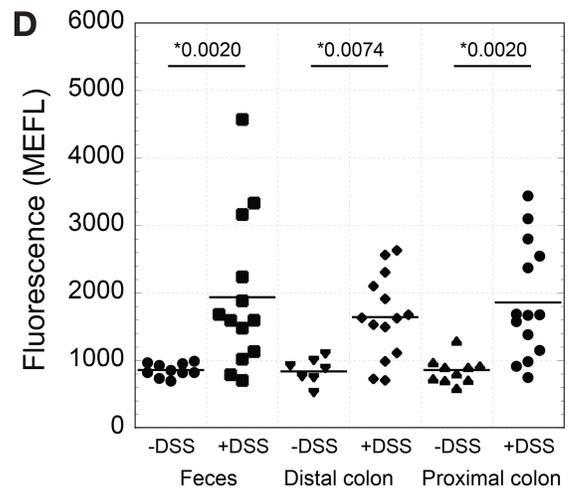
**B**



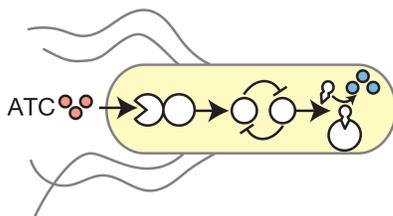
**C Diagnostic Gut Bacteria:  
Host Inflammation**



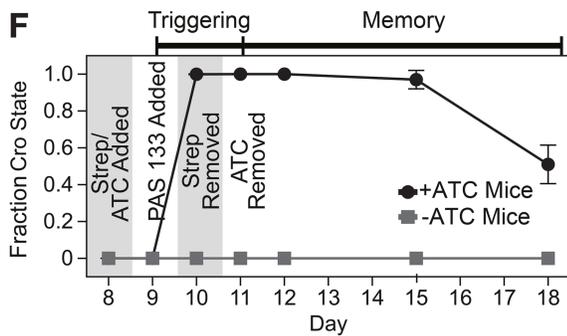
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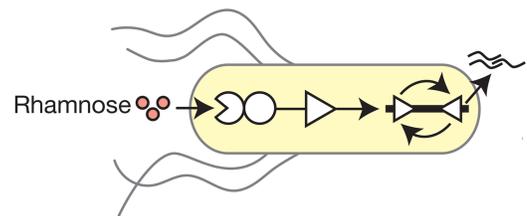
**E Diagnostic Gut Bacteria:  
Diet Administered Antibiotic**



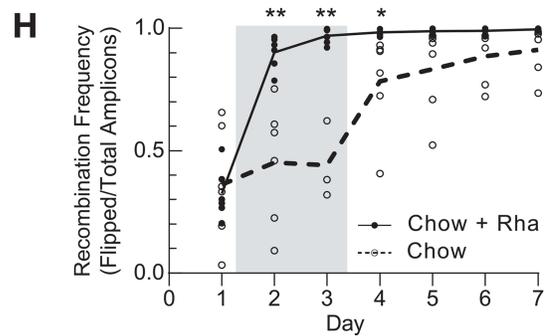
**F**



**G Diagnostic Gut Bacteria:  
Diet Administered Sugar**



**H**



nitric oxide, was used to detect the presence of inflammatory signaling in an epithelial cell coculture of intestinal explants (48). Finally, various orally administered gut-adapted bacteria have been used to sense dietary lactose (49), xylan (50), and rhamnose (51), as well as fucose produced by the gut microbiota (52) in the mouse gastrointestinal tract. Though they will surely play a major role in future diagnostic efforts, OCSs are limited by the fact that they cannot sense disease biomarkers that are not transported across the membrane or do not induce secondary chemical or physical changes inside the cell.

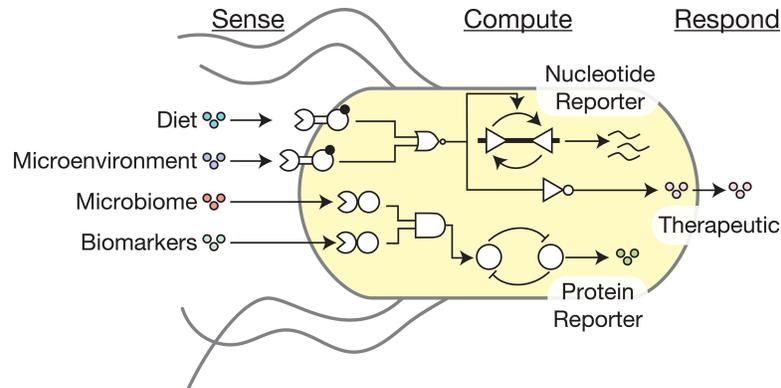
TCSs are the largest family of signal transduction pathways in biology (53, 54) and the primary means by which bacteria sense extracellular stimuli. TCSs are composed of a membrane-bound or cytoplasmic sensor histidine kinase (SK) and a cytoplasmic response regulator protein. In the presence of a specific stimulus, the SK phosphorylates the response regulator, which then binds DNA and activates or represses transcription from one or more output promoters.

In recent work, we hypothesized that thiosulfate ( $S_2O_3^{2-}$ ) could serve as a novel biomarker of colitis and set out to engineer a gut-adapted bacterium to sense it in mouse models. However, no genetically encoded thiosulfate sensor had previously been reported. Therefore,

we developed a combined computational and experimental approach to discover the first known biological thiosulfate sensor, a TCS from the marine bacterium *Shewanella halifaxensis* (55). We expressed this TCS, which we named ThsSR, in the natural probiotic *E. coli* Nissle 1917 (also known as Nissle), and encoded the reporter gene superfolder green fluorescent protein (*sfGFP*) as the output (Fig. 3C). We then orally administered the thiosulfate-sensing Nissle strain to control mice and mice administered the colitis-inducing compound dextran sodium sulfate (DSS) and performed flow cytometry on bacteria sampled from the colon and feces 6 hours later. As hypothesized, we observed that ThsSR is activated in mice with, but not without, colitis (Fig. 3D). Furthermore, the greater the extent of inflammation, the greater the ThsSR activity, suggesting that thiosulfate may be a novel colitis biomarker (55).

Importantly, our flow cytometry method enables identification and analysis of sensor strains without eliminating the native gut bacteria via broad-spectrum antibiotic treatment or germ-free methods. This feature enables minimally invasive measurements of biomarkers in native gut pathways and could generate more physiologically relevant results that are more likely to translate to clinical conditions. We are now investigating whether thiosulfate is a colitis biomarker in humans and

**FIGURE 3** Examples of current engineered gut bacteria. **(A)** A drug factory probiotic was made by engineering *L. lactis* to constitutively produce IL-10. IL-10 is secreted by the bacteria and then bound by the IL-10 receptor in the gut, resulting in downregulation of host inflammation. **(B)** DSS was used to induce inflammation in mice, and disease pathology was measured with histological scores. Treatment with the IL-10 drug factory probiotic was found to decrease symptoms by 50% compared to untreated mice or mice administered the natural probiotic *L. lactis*. **(C)** A diagnostic gut bacterium was created by engineering *E. coli* Nissle to sense thiosulfate, which is produced in the gut during inflammation. The thiosulfate is sensed by the ttrSR TCS, which activates expression of the fluorescent protein sfGFP. **(D)** sfGFP fluorescence of bacteria isolated from the feces and distal and proximal colon was measured and found to be significantly increased in mice experiencing inflammation in each location. Panel adapted from *Mol Syst Biol* (55) with permission of the publisher. **(E)** *E. coli* NGF-1 was modified to express the ATC sensor TetR, which controlled expression of the *cro* TF. The *cro* TF is one component of the lambda phage *cro/cI* toggle switch. ATC altered the start of the switch to become *cro*-dominant, and therefore produce LacZ protein, which produces a blue pigment. **(F)** The ATC-sensing diagnostic gut bacteria were administered to mice which were administered ATC via drinking water. Temporary administration of ATC was found to activate LacZ production, and the memory was retained for up to 1 week. Panel adapted from *Proceedings of the National Academy of Sciences* (56) with permission of the publisher. **(G)** The native RhaR rhamnose sensor in *B. thetaiotaomicron* was used to control expression of the *Int12* recombinase, which permanently inverts a barcode segment of DNA in the genome. **(H)** The rhamnose diagnostic gut bacteria switched the state of the barcode as detected via qPCR when administered rhamnose in drinking water. However, even without administration of rhamnose, the sensor toggled states, due to either leaky recombination or residual rhamnose in the plant-based chow. Panel reprinted from *Cell Systems* (51) with permission of the publisher.



**FIGURE 4** An outline of the types of sense, compute, and respond behavior an engineered gut bacterium can exhibit. Chemicals from a variety of sources within the gut may be of interest to a smart bacterium, including the host diet, compounds produced locally by the host in the bacterium's microenvironment, signals from other components of the microbiome, and general biomarkers of host disease. Computation is performed with a variety of logic gates and memory elements. The bacterium actuates a response with a therapeutic molecule in the case of a smart probiotic, or by producing a nucleotide or protein-based reporter for a diagnostic gut bacterium.

whether our engineered bacterium could be advanced as a clinical diagnostic.

Our thiosulfate-sensing Nissle strain is an early example of a “diagnostic gut bacterium.” This class of engineered gut bacteria utilize sensors, or sensors linked to genetic circuits (discussed below), to diagnose a gut-linked disease via expression of a reporter gene (Fig. 2).

### Genetic Circuits

Genetic circuits can be inserted between sensors and reporter genes to increase the quality, accuracy, or duration of diagnostic gut bacterial readouts. In a pioneering proof-of-principle study, Silver and coworkers used the *E. coli* tetracycline repressor (TetR) to express the phage  $\lambda$  transcriptional repressor *cro*, which in turn flipped the *cI/cro* transcriptional toggle switch from a *cI*- to a *cro*-dominant state in the presence of the tetracycline derivative anhydrotetracycline (aTc) (Fig. 3E). In this design, the *cI*-repressible promoter  $P_r$  is used to drive expression of the reporter gene *lacZ*, which is detected via production of a blue pigment indicator in X-gal agar plate growth media. First, the group transformed the mouse gut-derived *E. coli* strain NGF-1 with this sensor-circuit device. The device was integrated into the genome of NGF-1 to enable long-term evolutionary stability. Then they pretreated mice with the antibiotic streptomycin to clear the native gut flora, and orally administered the aTc-sensing NGF-1 strain to the mice, enabling long-term colonization (i.e., persistent growth in the gut without repeated oral administration). Once colonized, the engineered NGF-1 strain reports

exposure to aTc supplied in drinking water by flipping to the *cro*-dominant state, which is detected by plating the fecal matter on X-gal-containing media (56). Impressively, the aTc sensor device remains activated for over 1 week after exposure to aTc due to the stability of the  $\lambda$  genetic switch (Fig. 3F).

Tetrathionate ( $S_4O_6^{2-}$ ), the oxidative dimer of thiosulfate, has also been implicated as a colitis biomarker (57, 58). Recently, the Silver group modified their aTc-sensing gut bacterium to function as a tetrathionate sensor by replacing *tetR* with the tetrathionate-sensing *Salmonella enterica* serovar Typhimurium TCS *trrSR* (59, 60). The tetrathionate-sensing NGF-1 was orally administered to mice pretreated with streptomycin, resulting in stable levels of engineered bacteria in the feces after 6 months. Impressively, DNA sequencing and functional screening of fecal isolates showed that the tetrathionate sensor device had not lost the ability to respond to tetrathionate. For diagnosis, the strain was then administered to control mice and the *Salmonella* infection and IL-10 mouse models of colitis. A significant portion of the sensor bacteria switched to the activated *cro*-dominant state in the colitis compared to control mice. However, activation only occurred in an average of 1% and 5% of bacteria for the *Salmonella* and IL-10 models, respectively, compared to 100% activation of the sensor *in vitro*, with some diseased mice not giving rise to any activated colonies. This limitation demonstrates the need for further design-build-test-learn cycles to improve the performance of these diagnostic gut bacteria.

Additionally, though thiosulfate and tetrathionate are both anaerobic electron acceptors for bacterial respiration, tetrathionate is preferred and may be rapidly consumed by the gut microbiota. This fact may have contributed to the low tetrathionate signal observed by Silver and coworkers. Indeed, we did not observe colitis-dependent activation of a different tetrathionate-sensing TCS in DSS-treated mice with a native microbiota (55). Taken together, these results suggest that tetrathionate may not be an ideal colitis biomarker.

Recombinases that invert promoter-containing DNA segments (61) or engineered barcode sequences (62) are an alternative circuit design for long-term memory in diagnostic gut bacteria. In a recent study by Lu, Voigt, and coworkers, a recombinase was used to invert a genomic DNA barcode in the human commensal *Bacteroides thetaiotaomicron* to remember exposure to diet-derived rhamnose in the mouse gut (51) (Fig. 3D). However, appreciable background recombination was observed in this study, highlighting the need for future engineering cycles to improve performance. Ultimately, though, all of these early studies clearly demonstrate the exciting potential of diagnostic gut bacteria, particularly as more biomarkers are identified and bacterial sensors developed.

### Genetically Encoded Therapeutic Actuators

Drug factory probiotic design efforts have generated over 25 genetically encoded therapeutic actuators targeting diverse diseases (Table 1). A major class is bacterially secreted human proteins that augment human signaling networks. These include IL-10 (15) and anti-TNF $\alpha$  nanobodies (19), which treat colitis, and glycolipoprotein-1 (63), which treats diabetes. A second important class is bacterial proteins that augment human signaling networks. Examples of this class include the Toll-like receptor 2 stimulator LcrV protein from *Yersinia pseudotuberculosis* (25) and the superoxide dismutase SodA protein from *L. lactis* (22), both of which decrease symptoms of colitis. A final class is genetic pathways that produce therapeutic metabolites. For example, expression of At1g78690, an *N*-acyltransferase, led to production of *N*-acylphosphatidylethanolamines (NAPEs), which reduced food intake and obesity in mice (30). In another example, expression of the *Propionibacterium acnes* linoleic acid isomerase coPAI led to production of conjugated linoleic acid, which altered fatty acid composition and decreased body fat (33). The synthetic biology approach described above will enable integration of these and other therapeutic actuators into smart probiotics to increase their therapeutic efficacy and decrease associated side effects.

## ENGINEERING SMART PROBIOTICS

When setting out to construct a smart probiotic, one must make a series of basic design decisions. These include selecting a chassis organism and gut disease biomarkers, identifying the computations that need to be performed, and determining the appropriate therapeutic response. A toolkit of chassis organisms and genetic parts has been developed to accomplish these goals. Furthermore, synthetic biology techniques are available for developing new chassis and parts when current options are insufficient. Finally, deploying smart probiotics within the complex and dynamic gut environment presents additional challenges that require further design considerations.

### Chassis Selection

The criteria for selecting smart probiotic chassis partially diverge from those used when selecting natural probiotics. Similar to natural probiotics, one must consider the ability of the chassis to survive transit through the gastrointestinal tract, colonize particular macroscopic or microscopic geographical locations within the gut, and reach desired densities therein. On the other hand, the amenability of the chassis to DNA transformation, the availability of tools to modify its genome, and the repertoire of sensors, genetic circuits, and actuators compatible with it are also essential to consider.

Previous studies have established a number of “off-the-shelf” chassis with different properties. Multiple strains of the Gram-negative facultative anaerobe *E. coli*, the traditional workhorse of synthetic biology, have been deployed in mice for different purposes. These include laboratory strains (35, 37, 52, 64), Nissle (34, 38, 55), and NGF-1 (56, 60, 65). The Gram-negative anaerobe *B. thetaiotaomicron* has recently been developed as a new smart probiotic chassis (51). This bacterium belongs to the phylum *Bacteroidetes*, which is one of the two dominant phyla in human stool (66). Gram-positive facultative anaerobe alternatives include *Lactobacillus casei* (23, 26–28, 34), *Lactobacillus gasseri* (24, 32), *Lactobacillus paracasei* (33, 36), *Lactobacillus plantarum* (22), and *L. lactis* (15, 17, 19, 22–28, 31, 32, 34, 67–69). Many of these lactic acid bacteria, along with *Bacteroides xylanisolvens*, a relative of *B. thetaiotaomicron*, have Generally Recognized As Safe recognition by the U.S. Food and Drug Administration (70), facilitating their use in humans.

### Colonization

The ability of an organism to colonize a specific biogeographical gut location may be necessary for effective

**TABLE 1** Examples of engineered gut bacteria<sup>a</sup>

Type	Disease targeted	Compound sensed	Therapeutic produced	Chassis	In vivo	Reference(s)
Drug factory probiotic	Autoimmune diseases		Ovalbumin	<i>L. lactis</i>	X	<a href="#">67</a>
	Cholera		Chimeric lipopolysaccharide	<i>E. coli</i> E56b	X	<a href="#">37</a>
	Cholera		CAI-1	<i>E. coli</i> Nissle 1917	X	<a href="#">38</a>
	Celiac		DQ8 gliadin epitope	<i>L. lactis</i>	X	<a href="#">68</a>
	Colitis		IL-10	<i>L. lactis</i>	X	<a href="#">15, 17</a>
	Colitis		Trefoil factors	<i>L. lactis</i>	X	<a href="#">21</a>
	Colitis		DNA for TGF- $\beta$ 1	<i>E. coli</i> BM2710	X	<a href="#">64</a>
	Colitis		SOD	<i>L. plantarum</i>	X	<a href="#">22</a>
	Colitis		SOD	<i>L. lactis</i>	X	<a href="#">22</a>
	Colitis		Manganese-dependent catalase	<i>L. casei</i>	X	<a href="#">23</a>
	Colitis		SOD	<i>L. gasseri</i>	X	<a href="#">24</a>
	Colitis		LcrV	<i>L. lactis</i>	X	<a href="#">25</a>
	Colitis		Anti-TNF $\alpha$ nanobodies	<i>L. lactis</i>	X	<a href="#">19</a>
	Colitis		SOD	<i>L. casei</i>	X	<a href="#">26</a>
	Colitis		SOD	<i>L. casei</i>	X	<a href="#">27</a>
	Colitis		CAT	<i>L. casei</i>	X	<a href="#">27</a>
	Colitis		Elafin	<i>L. casei</i>	X	<a href="#">28</a>
	Colitis		Elafin	<i>L. lactis</i>	X	<a href="#">28</a>
	Diabetes		IL-10	<i>L. lactis</i>	X	<a href="#">29</a>
	Diabetes		NAPEs	<i>E. coli</i> Nissle 1917	X	<a href="#">30</a>
	Diabetes		Glutamic acid decarboxylase	<i>L. lactis</i>	X	<a href="#">31</a>
	Diabetes		IL-10	<i>L. lactis</i>	X	<a href="#">31</a>
	Diabetes		GLP-1	<i>L. gasseri</i>	X	<a href="#">32</a>
	<i>E. coli</i>		Lactoferrin	<i>L. casei</i>	X	<a href="#">34</a>
	<i>E. coli</i>		Chimeric lipopolysaccharide	<i>E. coli</i> E56b	X	<a href="#">35</a>
	<i>Listeria monocytogenes</i>		Listeria adhesion protein	<i>L. paracasei</i>	X	<a href="#">36</a>
	Obesity		Conjugated linoleic acid	<i>L. paracasei</i>	X	<a href="#">33</a>
Diagnostic gut bacteria	<i>Citrobacter rodentium</i>	Fucose		<i>E. coli</i> BW25113	X	<a href="#">52</a>
	Colitis	NO		<i>E. coli</i>		<a href="#">48</a>
	Colitis	Tetrathionate		<i>E. coli</i> NGF-1	X	<a href="#">60</a>
	Colitis	Tetrathionate		<i>E. coli</i> Nissle 1917	X	<a href="#">55</a>
	Colitis	Thiosulfate		<i>E. coli</i> Nissle 1917	X	<a href="#">55</a>
	Diet sensor	Lactose		<i>Streptococcus thermophilus</i>	X	<a href="#">49</a>
	Diet sensor	ATC		<i>E. coli</i> NGF-1	X	<a href="#">56</a>
	Diet sensor	Arabinogalactan		<i>B. thetaiotaomicron</i>	X	<a href="#">51</a>
	Diet sensor	IPTG		<i>B. thetaiotaomicron</i>	X	<a href="#">51</a>
	Diet sensor	Rhamnose		<i>B. thetaiotaomicron</i>	X	<a href="#">51</a>
Smart probiotic	Colitis	Xylan	IL-2	<i>B. ovatus</i>		<a href="#">156</a>
	Colitis	Xylan	Keratinocyte growth factor-2	<i>B. ovatus</i>	X	<a href="#">50</a>
	Colitis	Xylan	TGF- $\beta$ 1	<i>B. ovatus</i>	X	<a href="#">157</a>
	Diabetes	Nisin	Insulin	<i>L. lactis</i>		<a href="#">158</a>
	Diabetes	Glucose	GLP-1	<i>E. coli</i> Nissle 1917		<a href="#">63</a>
	Diabetes	Glucose	PDX-1	<i>E. coli</i> Nissle 1917		<a href="#">63</a>
	<i>Pseudomonas aeruginosa</i>	3OC12HSL	E7 lysis	<i>E. coli</i>		<a href="#">47</a>
	<i>P. aeruginosa</i>	3OC12HSL	Bacteriocin	<i>E. coli</i> MG1655		<a href="#">46</a>

Abbreviations: CAT, catalase; GLP-1, glucagon-like peptide 1; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; NAPE, N-acylphosphatidylethanolamine; PDX-1, pancreatic and duodenal homeobox gene 1; SOD, superoxide dismutase; TGF, transforming growth factor

diagnosis or treatment of disease. For example, when targeting a patient with ulcerative colitis, which impacts the large intestine, it may be beneficial for the organism to colonize the large intestine (71). On the other hand, if treating the related Crohn's disease, wherein pathology extends from the mouth to anus (71), colonization of the small intestine may also improve the therapeutic effects. Members of the *Bacteroidaceae* family, which are found in higher numbers in the colon, may be better suited for the former disease, whereas members of the *Lactobacillaceae* family, which are found in both the small intestine and colon, may be better suited for the latter (72). Furthermore, specific genetic determinants within a species can result in different colonization capabilities (72), which opens the door for engineering chassis bacteria with specific colonization patterns.

Alternatively, when engineering diagnostic gut bacteria, it may be beneficial to have bacteria that do not colonize the gut. For example, our thiosulfate-sensing Nissle strain (55) survives passage through the gut but does not appear to colonize or reach high numbers when the native gut microbiota is intact (73). These features result in minimal perturbations to the native gut microbiota relative to other strains which may outcompete native flora for specific niches and thereby alter gut microbiota or host physiology. For example, alterations to gut microbiota can lead to the highly undesirable side effects of inflammation (74) or opportunistic pathogen infection (75).

### Genetic Tractability

The capacity to perform genetic modifications that yield desired outcomes is fundamental for smart probiotic design but varies widely between gut bacterial chassis. Genetic tractability requires tools for reliable DNA transformation, control of gene expression, and post-translational control of protein processes such as secretion. A wide array of sensors, circuit components, and actuators are also needed and will be covered in depth in later sections.

*E. coli* has an unrivaled genetic toolbox, which we will use to exemplify the parts required to engineer smart probiotics. DNA transformation can be accomplished via chemical or electrical disruption of the membrane, phage-mediated transduction, or conjugation from another bacterium. To persist, the transferred DNA requires self-replicating elements (76) or genomic integration (77). Selectable markers such as antibiotic resistance genes are important for obtaining and maintaining transformants in the laboratory but do not appear to be required for retention of plasmid DNAs in

short-term *in vivo* experiments (55, 73). The transcription rate of sensor, circuit, or actuator mRNAs can be precisely controlled using well-characterized constitutive promoter libraries (78, 79) or chemically inducible promoters (80, 81). Termination of transcription is important to prevent unwanted cross-talk between different transcribed elements on a contiguous piece of engineered DNA. Previous work has generated large libraries of well-characterized transcription terminators, many of them very efficient (82, 83). The mRNA translation rate is commonly used to control the protein expression level. The translation rate of a target gene can easily be tuned in *E. coli* by computational design of custom ribosome binding site (RBS) sequences via the RBS calculator (84–86) or through the use of the more modular “bicistronic design” RBSs developed by Endy, Arkin, and coworkers (79). Translated *E. coli* proteins can be functionalized with tags that facilitate their secretion from the cell (87) or rapid degradation (88). These *E. coli* parts have primarily been characterized in laboratory strains. However, recent work demonstrated that a complex circuit could be transferred from a laboratory strain to a native gut isolate while maintaining complete functionality (56) despite possible differences in individual part performance between strains.

When using a chassis other than *E. coli*, many of the aforementioned tools will be unavailable or poorly developed. Thus, techniques to rapidly generate reliable new genetic tools in non-*E. coli* chassis are needed. This goal was recently demonstrated by Lu, Voigt, and colleagues in *B. thetaiotaomicron* (51). To transform this less tractable organism, a conjugative plasmid containing the genetic construct was assembled in *E. coli* and subsequently transferred to *B. thetaiotaomicron* via conjugation. Once transformed, the plasmid expressed an integrase (89) that catalyzed its integration into a fixed site in the genome. The authors then designed a library of synthetic promoters resulting in a 20-fold range of reporter gene expression levels by inserting a 26-bp DNA sequence in different regions of the native housekeeping sigma factor's promoter, P<sub>BT1311</sub>. They also developed three carbohydrate-inducible promoters by taking advantage of natively expressed proteins and a synthetic IPTG-inducible promoter from *E. coli*. To vary translation strength, they modified the native RBS of the 50S ribosomal protein gene *rplL*\* by targeting conserved positions, resulting in a 1,000-fold range of gene expression levels.

Several parts have been developed for *Lactobacilli* as well (90). These include nisin- (91), peptide pheromone SppIP- (92), IPTG-, and xylose- (93) inducible

promoters and constitutive promoter libraries (94). A range of replicative plasmids (90) also exist, as do single-stranded DNA recombining techniques to generate point mutations in chromosomal DNA (95, 96). However, the current lack of a consensus organism in this genus will require many of these parts to be recharacterized in the specific species selected as a chassis.

### Sensor Design

The simplest way to engineer a smart probiotic sensor entails expressing nonnative output genes (e.g., reporters, genetic circuit components) from endogenous chemical or physical input-responsive promoters with no additional engineering of the chassis (49, 51, 52). Though convenient, this approach can produce false negatives or positives due to the poorly understood and complex gene regulatory networks that control the activity of endogenous promoters. This method can be improved upon by knocking out the sensor genes that control the promoter of interest and expressing codon-altered versions of their open reading frames under nonnative promoters, RBSs, and so on. This process, termed genetic refactoring (97–100), can break the key connections in the native control networks, making sensor performance more reliable. However, sensor proteins may still be subject to post-translational regulation, which can interfere with their function. An even more reliable approach is to port sensors from unrelated organisms into the chassis of interest (55, 101, 102) and optimize their genetic encoding and expression levels for optimal sensor performance (103).

### Sensor portability

In many instances, sensors of targeted disease biomarkers have not evolved in the chassis of interest. Thus, sensors often need to be ported between organisms. The successful transfer of LacI (51, 93) and TetR homologs (104, 105) between evolutionarily distant organisms demonstrates the portability of OCSs. OCS portability is improved by the fact that a single protein performs both the sensing and transcriptional control functions.

This feature stands in contrast to TCSs, which have different sensing and effecting proteins, resulting in the need to perform a two-dimensional gene expression optimization (55, 102, 103). SKs are often membrane bound, which could compromise TCS functionality when transported between organisms with drastically different membrane structures. Additionally, SKs often require poorly characterized accessory sensor proteins that allosterically modulate the SK in the presence of the input. If known, such accessory proteins must also be expressed and optimized in the new chassis. If unknown,

the TCS will fail to function if the chassis lacks them. Thus, despite their benefits in sensing diverse extracellular signals, TCSs are sometimes more difficult to engineer than OCSs.

### Sensor performance characteristics

Most synthetic biological sensors have been characterized in bench experiments where the growth media, growth phase, density, and other factors that influence the function of synthetic genetic systems are controlled. However, chemical, microbial, and host biological aspects of the gut are poorly understood, spatially heterogeneous, and vary over time in a single individual or between individuals. Gut environment variations impact the growth and physiology of engineered gut bacteria and may therefore introduce variability in sensor performance.

One way to overcome sensor variability is to increase the dynamic range (i.e., the ratio of sensor output in the maximally versus minimally active states) (106). A larger dynamic range allows for more distinguishable bits of information to pass through a sensor, resulting in more accurate measurements of the input. Sensor dynamic range can be increased by optimizing the expression level of sensor proteins or the sequence of sensor output promoters to eliminate cryptic constitutive transcriptional start sites that increase leakiness (103). Additionally, TCSs utilize phosphorylation/dephosphorylation cycles that make them more robust than OCSs to fluctuations in the expression levels of sensor proteins (107). This benefit may make TCSs more reliable sensors than OCSs in the gut.

The threshold sensitivity of a sensor to its input is an important and often overlooked factor that is fundamental to gut performance. Clinically relevant biomarker levels are likely to fall within specific ranges, and sensors must match these ranges to successfully diagnose a patient. Few studies have examined general methods of tuning the threshold sensitivities of sensors (108), and much work remains to address this problem. Additionally, many biological sensors are sensitive to only a small range of input concentrations and do not respond outside of that range, which makes them ill suited for quantifying a wide range of biomarker levels. Recent techniques enable the expansion of the range over which a sensor is sensitive, making it capable of measuring a broad range of input concentrations (109).

### Sensor outlook

Currently, only a tiny fraction of evolved bacterial sensors have been characterized. Furthermore, it is likely

that off-the-shelf sensors will not be available for new disease biomarkers identified in forthcoming gut metabolomic or similar studies. To develop new sensors, new OCS and TCS sensor genes can be easily identified via bioinformatic analyses (45, 54). However, their inputs can seldom be predicted, and there are few efficient methods for discovering them experimentally. In some cases, sensor domain homology can be utilized to predict the class of inputs (110, 111), or the local genomic context can be analyzed to identify nearby metabolic clusters which may be linked to the sense compound (55). Alternatively, the transcriptome response of a natural gut microbiome can be probed for sensors that are upregulated in a diseased state, which often indicates activation of the sensor (112). This may allow identification of sensors for a particular disease without prior knowledge of the compound that is being detected. These sensors can then be refactored and characterized in a defined environment to determine the specific disease biomarker sensed. Future advances in synthetic biology methods which allow many OCSs and TCSs to be screened for responses to inputs in high throughput should greatly increase the number of available sensors (Fig. 5A).

### Genetic Logic Circuits

Genetic logic circuits can be used to increase the accuracy of smart probiotic diagnoses. For example, dietary nitrate is quickly consumed by the microbiota in the proximal gut (113), whereas nitrate produced due to inflammatory diseases can be found within the colon (114). Therefore, if nitrate was detected at the same time as a large intestine biomarker, then colitis would be likely, and it may be appropriate to secrete an anti-inflammatory compound. However, if nitrate was sensed alongside a small intestine biomarker, it would likely be of dietary origin rather than due to inflammation and should therefore be ignored. To execute such higher-order sensing, smart probiotics require multiple sensors and genetic logic circuits capable of integrating the biological inputs from those sensors simultaneously.

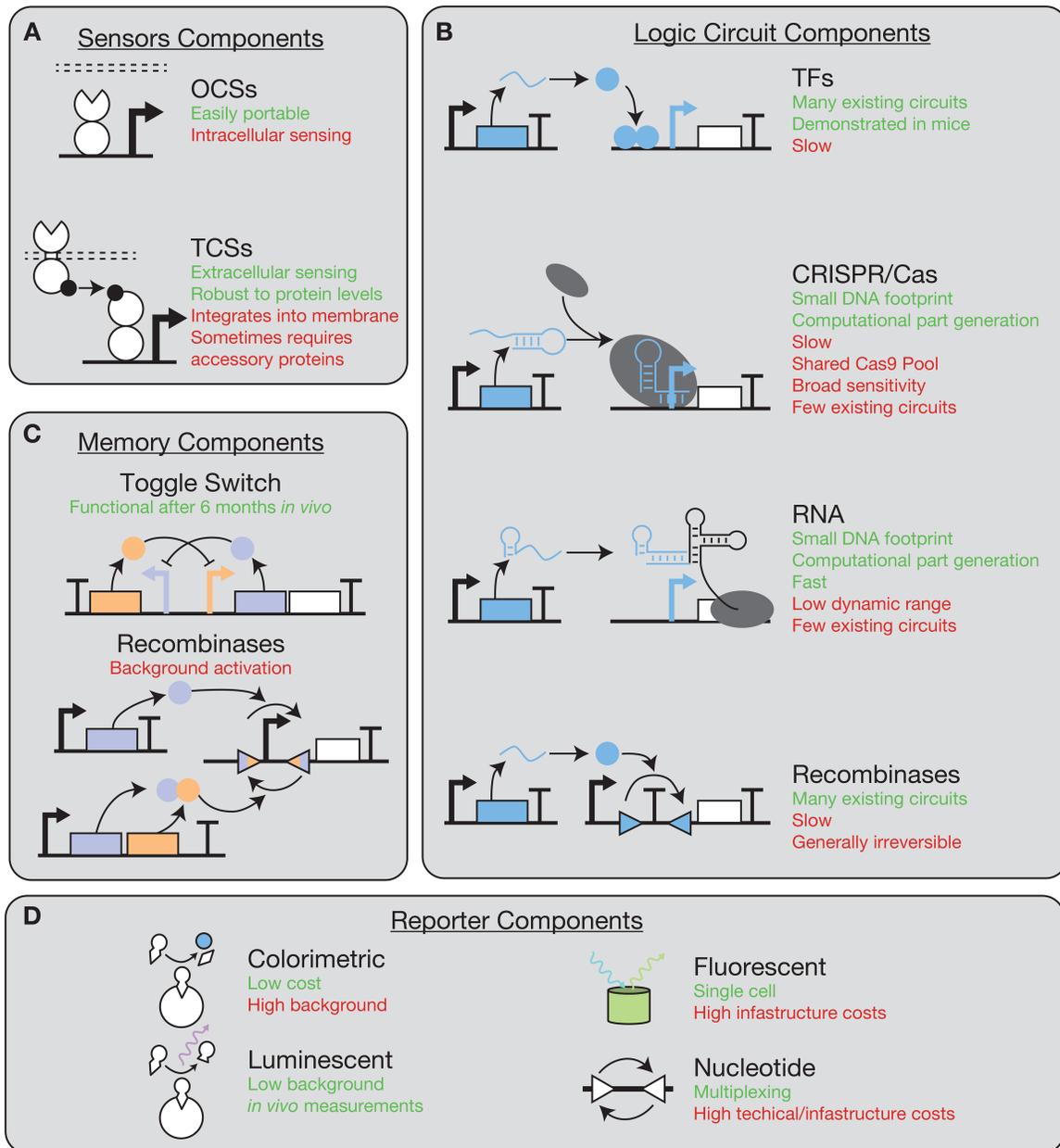
### Parts to implement logic

TFs are proteins that bind DNA operator sites to activate or repress transcription from promoters. TFs are the primary means by which synthetic biologists engineer logic. Several TF-based part families have been engineered and used to construct a remarkable range of genetic logic circuits.

Recently, Voigt and colleagues engineered a library of over 10 orthogonal homologs of the TetR transcrip-

tional repressor (104). Here, orthogonality refers to the fact that each TetR homolog represses only its target promoter, and not the promoter of any other TetR homolog in the library. Each orthogonal TetR/promoter pair comprises a simple transcriptional “inverter” circuit wherein high expression of a TetR homolog from an upstream (input) promoter results in low transcription from the corresponding repressible (output) promoter (104). In follow-up work, the group used the library to construct a series of NOR logic circuits, which are active only in the absence of two different TetR homologs. The group then wrote a new *in silico* program for automated genetic logic circuit design (named Cello, for cellular logic). Cello was used to computationally design the DNA sequences encoding 52 different genetic logic circuits, many that perform complicated operations typically associated with electronic systems. Initially, 94% of their genetic circuits failed due to undefined interactions between neighboring promoters and the genes they transcribed (115). To overcome this challenge, the group standardized the 15 bp upstream of the promoter with a random DNA sequence to remove variable effects of the upstream sequence. They also placed a ribozyme between the promoter and RBS which cleaves off any mRNA nucleotides encoded by the promoter to prevent the promoter from influencing translation initiation from the RBS (116). When these genetic parts were added alongside restrictions on the physical ordering of genetic parts along a DNA sequence to further reduce context-dependent performance changes, the success rate increased to a remarkable 71% (115). The vast majority of logical operations that one may want to engineer into an *E. coli* smart probiotic could now be implemented using this approach.

The CRISPR (clustered regularly interspaced short palindromic repeats) interference (CRISPRi) TF-design technology offers several benefits for genetic logic circuit design. In CRISPRi, a nuclease-deficient *Streptococcus pyogenes* Cas9 protein (dCas9) binds to an engineered guide RNA, which drives it to bind and repress virtually any promoter in a sequence-specific manner (117). Voigt and coworkers also engineered an orthogonal library of transcriptional inverters using CRISPRi. In particular, they designed a series of synthetic promoters with divergent spacer regions between the -35 and -10 elements, and cognate small guide RNAs (sgRNAs) that target each promoter sequence specifically. These inverters were then combined to create NOR (output is on only when no inputs are present), OR (output is on when any of multiple inputs are present), and AND (output is on only when all inputs are present) logic using one, two,



**FIGURE 5** The different components used to construct an engineered gut bacterium. The pros and cons of each component are listed in green and red text, respectively. **(A)** Sensors can be selected from OCSs or TCSs. **(B)** Logic circuits can be constructed using TFs, CRISPR/Cas repressors, RNA-based sRNA transcriptional activators, or serine recombinases. **(C)** Genetic memory can take the form of a toggle switch or recombined DNA. **(D)** The state of a circuit can be assayed using colorimetric, luminescent, fluorescent, or nucleotide reporters.

and four interacting gRNAs, respectively (118). Unlike the TetR work, where the operator sites of most repressor homologs had to be discovered via arduous high-throughput protein/DNA binding array methods, these CRISPRi logic gates could be designed largely using Watson-Crick base-pairing rules. Indeed, dozens

or hundreds of CRISPRi circuits can be easily engineered due to this feature (119). CRISPRi circuits are also promising because each sgRNA is on the order of 60 bp, resulting in a small and therefore inexpensive, simple to assemble, and possibly more evolutionarily stable DNA footprint. However, each sgRNA competes for a single

pool of Cas9, creating a point of possible retroactivity and thus circuit failure (14). Retroactivity occurs when the activity of downstream components of a circuit alters the performance of upstream components in an unexpected fashion (120). Lastly, sgRNA-regulated promoters, when compared to traditional TF-regulated promoters, are sensitive over a broader range of regulator concentrations, resulting in a less digital-like response. This broad sensitivity can result in propagation of variability or stochastic noise through the genetic circuit, which can also cause circuits to fail (121).

RNA molecules can also be used to engineer transcriptional logic circuits. This class of designs was originally demonstrated by Arkin and coworkers, who utilized the copy number control mechanism of the pT181 plasmid to engineer an antisense RNA that promotes the formation of a transcriptional terminator on a target RNA, preventing downstream transcription. Orthogonal regulatory RNAs were created and used to construct a NOR gate and a three-step cascade (122). Lucks and coworkers built upon this work by inverting the effect of the antisense RNA through the addition of an anti-anti-terminator RNA sequence and creating the first sRNA transcriptional activators (123), which have been employed to create a two-input AND gate (123) and a three-step cascade (124). The ability to computationally generate novel regulatory RNAs (124), combined with their small size, facilitates the creation of circuits containing a large number of nodes. However, this has yet to be demonstrated and is possibly hampered by the low dynamic range frequently observed with RNA-based regulation.

Finally, serine recombinases, proteins that bind DNA at specific sequences and invert or excise intervening DNA (125), have been used to orient transcriptional control elements on DNA to control the flow of RNA polymerase. These have enabled circuits that can count to 3 (126), form a large number of two-input Boolean logic gates (61, 127), and create state machines that remember both the presence and order of input signals (128). However, it can be difficult to balance the expression levels of all circuit components, particularly when reversible serine recombinases are used (129).

### Computation speed

The gut environment changes rapidly in space and time, and noncolonizing bacteria pass through the mouse gastrointestinal tract in several hours (55, 65, 73, 130). Moreover, colonizing bacteria face temporal changes in response to diet, exercise, and biogeographic position. Current transcriptional circuits exhibit dynamics that

are likely to be too slow for many diagnostic and therapeutic applications. In particular, changes in stable protein concentrations are limited by the rate of dilution due to cell growth, and it may take many doublings to dilute or produce enough protein to traverse the sensitive region of a downstream node in a logic circuit (131). Circuits containing four (115) and nine TFs (132) have both been shown to take 5 hours to compute a final state. Recombinase circuits are even slower; a single inversion step has been measured to take 2 (62), 3 (127), and 6 hours (132), whereas two-part gates were found to take 8 (62) and 9 hours (127) to fully respond. A single CRISPRi inverter has been shown to have a 10- to 90-minute delay and take 4 to 5 hours to switch between on and off states when controlling a stable protein (117, 118). Furthermore, genetic circuits transiently produce incorrect outputs (115), which could lead to improper diagnoses and therapeutic responses by smart probiotics.

The response times of transcriptional logic circuits can be accelerated by adding degradation tags to TF components (133). However, this approach can generate a new problem where TF components compete for a finite supply of degradation machinery (134) and therefore create a potential point of retroactivity. This problem could be overcome by engineered orthogonal degradation systems (135), though this work is still at early stages. Unlike proteins, RNA is naturally labile, and RNA-based circuits should therefore compute solutions more quickly. *In vivo* rates of RNA circuits have not been measured, but *in vitro* work suggests they could be as low as 5 minutes for each computational step (136).

### Robustness to environmental variability

A smart probiotic will experience a highly variable environment as it traverses the host from the mouth through the gut, but its circuits must function consistently to diagnose and treat disease reliably (137, 138). Growth rate is likely to change depending on factors such as gut location and variations in diet. These growth rate changes can dramatically alter circuit performance, particularly with stable proteins whose steady-state levels are linked to protein dilution. This challenge can be addressed by integrating feedback mechanisms such as negative autoregulation into computational nodes to compensate for growth effects (139, 140). Fluctuations in circuit performance appear particularly problematic for recombinase-based circuits because most implement irreversible computations. Temporary environments during administration or transit through the gut could prematurely result in permanent computation, giving

false results. This may be addressable with reversible recombination (129), but this technique has not been explored in detail.

### Long-term functionality

In some cases, it may be beneficial for smart probiotic circuits to function for long periods of time in the gut. To accomplish this goal, colonizing smart probiotics need to compete with the native microbiota while maintaining the ability to compute new treatments in response to alterations in disease state. If an engineered genetic circuit decreases the growth rate of the smart probiotic chassis, it could cripple the ability of the engineered organism to compete with the native gut microbiota. Such toxicity can occur with virtually all engineered genetic systems and has been reported in CRISPRi (118), TetR homolog (104, 115), and recombinase (62) circuits. Additionally, irreversible recombinase computation is particularly ill suited for long-term computation since it can only compute a response once and cannot respond to a changing environment. The only long-term study of computational circuit persistence showed that the genomic  $\lambda$ -derived toggle switch constructed using protein-based TFs (Fig. 3E) is capable of surviving for 6 months in an antibiotic pretreated mouse gut without losing functionality, highlighting the stability of this design strategy (60).

### Logic circuit outlook

Given current technologies, we believe that the most promising method for engineering genetic logic into smart probiotics is to use TetR-based TFs (Fig. 5B). Benefits include the availability of a large library of parts and the ability to design complex circuits in an automated fashion. Additionally, there are no significant drawbacks such as the toxicity associated with CRISPRi circuits since nontoxic TetR homologs have been identified. Furthermore, TetR-based circuits are reversible (albeit slow), unlike most recombinase-based systems.

An interesting unexplored alternative is to replicate the computation observed in posttranslational networks such as phosphorelays (141). Bacteria have been shown to contain multistep histidine kinase phosphorelays which can integrate signals from different sensors with AND logic (142). The protein components of these phosphorelays have evolved in a highly modular nature (53), suggesting that they may be generally engineerable (143, 144). Such a network would provide fast post-translational computation that may also be robust to changes in protein levels resulting from the complex gut environment (107).

## Memory Circuits

For diagnostic gut bacteria to report the state of diseases within the gut after passage in feces, they require the ability to remember previous exposure to disease biomarkers. The two current approaches used to form memory in the gut are TF-based toggle switches (56, 60) and recombinases (51) (Fig. 5C).

### Toggle switches

In a foundational paper in the field of synthetic biology, Collins and colleagues engineered a genetic toggle switch composed of two mutually repressing TFs, each of which prevented the other from being transcribed. They demonstrated that this circuit can remember exposure to different environmental signals even after the signals are removed (145, 146). This work was inspired by the  $\lambda$  cI/cro toggle switch that controls the bacteriophage's decision to stay in a lysogenic state or to switch to a lytic state after transduction of its host (147). The previously discussed aTc-sensing (Fig. 3E) (56) and tetrathionate-sensing (60) diagnostic gut bacteria utilized the  $\lambda$  toggle switch "as-is" to implement memory within the gut.

### Recombinases

Recent work has focused on the use of recombinases as a form of memory (51, 62, 129), in addition to their capability as logic circuit components. Recombinases have been used to record the presence of lactose (148) and rhamnose (51) within the gut. However, in both cases large amounts of background activation of the system were observed, limiting the time window in which there were identifiable differences between treatment and control mice (Fig. 3H) (51, 148). This may result from the fact that recording of the memory is irreversible, causing low levels of noisy recombination that accumulate false-positive outcomes over time. To address the irreversibility of recombination, Endy and colleagues expressed an excisionase, which reverses the recombination event, restoring DNA to its original sequence. After exhaustive tuning of expression levels, they created a strain which was able to toggle its memory between exposure to two different inducers for over seven cycles of induction (129).

### Memory circuits outlook

The toggle switch has demonstrated clear superiority in its ability to accurately record a memory for a long period of time in the gut. However, if future diagnostic gut bacteria require memory of many different signals, the large number of available recombinase modules (62) may make them an attractive alternative. Additionally,

future diagnosis may seek to report many different levels of exposure compared to the binary nature of the previously discussed memory elements. An interesting alternative is the use of retrons, which perform 6-bp modifications of DNA sequences at a much lower rate than serine recombinases. This has enabled continuous integration of both signal amplitude and duration over many days at a population level (149) and may be useful for diagnosis of diseases with highly dynamic biomarkers.

### Genetic Actuators: Reporters

A range of reporter genes allow for measurement of sensor or genetic circuit outputs (Fig. 5D).

#### Colorimetric reporters

Colorimetric reporters have a long history of use in biotechnology, most prominently through the enzymatic activity of the *lacZ* gene on X-gal, producing a blue pigment (150), which has been employed in diagnostic gut bacteria (Fig. 3E) (56). Colorimetric assays are an attractive reporter system since they are low cost and do not require special instrumentation. When applied in a clinical setting, fecal coloration could enable simple in-home readouts of the state of engineered bacteria exiting the gut, but this approach may be limited by high background fecal pigmentation.

#### Luminescent reporters

Luminescent reporter genes, such as bacterial luciferase, typically yield increased sensitivity over colorimetric reporters due to very low background luminescence in fecal samples (151). Luminescence requires the addition of a luciferin substrate or its metabolic production by the bacteria themselves. Numerous luciferases are available, and each has different brightness, luciferin requirements, and output wavelengths. All luciferases require a luminometer instrument for measurement, making them less convenient than colorimetric reporters. However, high signal-to-background makes luminescent reporters desirable for clinical diagnostic gut bacteria.

#### Fluorescent reporters

Fluorescent measurement of bacteria from the gut allows single cell data to be collected from bacterial populations via flow cytometry (55, 65) or microscopy (52). Such analyses can be valuable in the research setting to detect more complex population-level changes such as bimodality, which are likely to occur in the complex gut environment. Single cell measurements can also be critical for troubleshooting engineered circuit perfor-

mance (115). However, fluorescent reporter genes are less suited to clinical applications due to large expensive instruments and processing requirements, including a postgut aerobic incubation to allow for the oxygen-dependent maturation of the fluorophores (55).

#### Nucleic acid reporters

Direct sequencing of nucleic acid changes resulting from engineered bacterial recombination (51) may be the most promising diagnostic modality. A unique challenge of characterization of bacteria in the gut is the extreme cost of a single replicate compared to test tube experiments. Sequencing of barcoded DNA allows for measurement of entire libraries of bacteria in a single heterogeneous culture (152). This approach could be applied to single mice to test thousands of candidate synthetic probiotic sensors in parallel. Similarly, it would allow for the clinical use of many different probiotic diagnostics, enabling multiplexing of diagnostic gut bacteria. This approach has higher technical and infrastructure costs than other reporter methods, but these are likely to decrease in the future, making this a very promising avenue of investigation.

## EXPERIMENTAL VALIDATION

Testing engineered gut bacteria in animal models or human trials is slow and expensive. Thus, it is prudent to validate and debug smart probiotic prototypes in a series of increasingly complex *in vitro* environments before final *in vivo* environments.

#### Defined Media

Defined media are useful for testing well-defined hypotheses on the performance of synthetic sensing, computing, or responding components. Questions about specific components may include what compounds a sensor binds or what levels of actuator can be produced. Broader system-wide questions include the following: Does expression of the synthetic system affect growth rates of the chassis (i.e., toxicity)? How do the expression levels of system proteins affect probiotic performance? and How does the growth rate of the chassis affect circuit performance? The high throughput and low cost of defined media allow for in-depth interrogation of these parameters. In addition, their relevance to more complex models may be improved by the development of standard defined media which replicate the gut environment through the inclusion of more complex carbohydrate and protein sources, as well as the use of anaerobic chambers.

## Bioreactors

The effect of the microbiome can be incorporated by using *in vitro* bioreactors which utilize defined media seeded with murine or human microbiome samples (137, 153). The presence of a microbiome allows for testing of interactions between the synthetic probiotic constituents or by-products of the microbiota. This involves sensing by-products of the microbiome or affecting the constituents of the microbiome. Additionally, it provides a competitive environment where basic stability questions of the synthetic constructs can be preliminarily investigated. There exists a range of microbiome bioreactors which may be suited for different chassis organisms. It will be important to standardize the use of particular models and verify that they reflect *in vivo* performance for specific chassis organisms by comparing parameters such as growth rate and protein production levels.

## Mouse Models

As with other therapeutics, mouse models are the most effective method for testing the performance of synthetic probiotics at diagnosing and treating disease. Probiotic bacteria can be integrated into existing murine disease models to demonstrate either sensing of relevant compounds (55, 60) or alleviation of disease markers (15). In addition to testing direct interaction of the synthetic system with the host, this can demonstrate the effect of the complex gut environment on the synthetic system (51, 55, 56, 60). Additionally, it can allow for investigation of differential effects due to different environments spatially throughout the gut.

A range of microbiome models are used in mouse studies, including native, antibiotic-treated, gnotobiotic, and exogenous microbiomes. Many strains of bacteria, including most *E. coli* used in engineered gut bacteria, have difficulty competing against the native microbiota, so antibiotic treatment is used to facilitate colonization with antibiotic-resistant engineered gut bacteria. Likewise, gnotobiotic mice, which are raised in germfree conditions and do not contain a gut microbiome, are easily colonized. However, large-scale perturbations of the gut environment may confound the study of gut-related diseases. For example, the commonly used colitis model, DSS treatment of mice, has been observed to be ineffective when conducted in the absence of a microbiome (154). Gnotobiotic mice can also be colonized with an exogenous microbiome either in the form of a defined consortium (155) or transplanted from human microbiome samples. The benefits and drawbacks of each of these models in testing engineered gut bacteria are largely unknown, making the best course of action

replicating the methods traditionally used in the disease model which is being studied to allow comparison of the treatment effectiveness to more traditional therapeutic methods. Finally, pigs, whose gastrointestinal tract closely resembles that of humans, have been used to test the performance and biosafety of engineered probiotics prior to proceeding to human trials (16).

## OUTLOOK

The application of tools developed by synthetic biologists to engineered gut bacteria will result in a step change in their efficacy and complexity. The *ad hoc* genetic implementations of drug factory probiotics over the past 2 decades will be improved through the use of well-characterized, high-performance expression systems in a wide variety of chassis organisms. This will enable higher expression of therapeutic compounds in more gut-relevant probiotic organisms, increasing their therapeutic efficacy.

Initial diagnostic gut bacteria (55, 60) will be improved upon by discovering more disease-relevant sensors and by studying their localized activation *in vivo*. New sensors can be generated using bioinformatic mining of microbiome data sets coupled with high-throughput library screening in mouse models using nucleotide-based reporters. Study of the biogeographic activation of disease-relevant sensors will determine the specifics of disease activity. In addition to their clinical applications, diagnostic gut bacteria will serve as scientific tools to locally measure specific metabolites within gut models.

These advances in engineering gut bacteria will enable the creation of the first smart probiotics capable of sensing and treating human disease within the gut. The engineering design-build-test cycle will allow for continual integration of knowledge gained from the study of host-microbiome interactions, natural probiotics, and synthetic biology to iteratively build upon existing smart probiotics. This will result in the bacteria being capable of sensing multiple disease states and localized environmental signals, computing a desired course of treatment, and administering this treatment from within the human gut. Localized diagnosis and treatment will make smart bacteria more effective and safer therapeutics.

## DEFINITIONS

**Actuator:** A molecule which changes the bacterial cell state or the state of its environment, such as metabolic enzymes or secreted signaling proteins.

**Biological output:** A bacterially produced molecule such as an RNA, transcription factor, or therapeutic molecule.

**Cellular memory:** The ability of a cell to maintain a response to a sensed input after removal of that input.

**Chassis:** A bacterial species used to host a synthetic DNA to create an engineered gut bacterium.

**Chemical or physical inputs:** The signals that an engineered bacterium detects to compute an appropriate response. Examples include traditional inducers, disease biomarkers, and quorum-signaling molecules.

**Colonization:** The ability of bacterial species to survive in an environment for an extended period of time without repeated oral administration.

**Device:** A biological system composed of one or more components which performs a specific well-defined task with inputs and outputs; this can include sensors, genetic circuits, or actuators.

**Diagnostic gut bacteria:** Bacteria that sense one or more biomarkers, compute that those biomarkers are present in a combination indicative of disease, and produce a reporter which can be externally measured by a clinician.

**Drug factory probiotic:** Bacteria engineered to constitutively produce a therapeutic molecule within the body.

**Genetic circuit:** A network of interacting regulatory molecules, such as transcription factors and their target promoters, that perform computations such as multi-input logic or memory to convert a chemical or physical input into a biological output.

**One-component system (OCS):** Cytoplasmic transcription factors that directly interact with and are allosterically modulated by chemical and physical inputs.

**Retroactivity:** The effect of downstream circuit component activity on the performance of upstream circuit components.

**Sensor:** A genetically encoded molecule, often an RNA or protein, that converts a chemical or physical input into a change in a biological signal such as kinase activity or transcription rate.

**Smart probiotic:** Bacteria that sense one or more biomarkers, compute that those biomarkers are present in a combination indicative of disease, and respond by delivering a precise dose of one or more appropriate therapeutics at the diseased tissue.

**Two-component system (TCS):** A bacterial sensing network composed of a membrane-bound or cytoplasmic sensor histidine kinase which senses a chemical or physical input and regulates a cytoplasmic response regulator that controls promoter activity.

## ACKNOWLEDGMENTS

B.L. is supported by the Department of Defense, Air Force Office of Scientific Research, National Defense Science and Engineering Graduate (NDSEG) Fellowship, 32 CFR 168a. Smart probiotic research in the Tabor laboratory is supported by the Office of Naval Research Young Investigator (N00014-14-1-0487) and NSF CAREER (1553317) programs and the Welch Foundation (C-1856).

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