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Review

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Synthetic Microbes As Drug Delivery Systems

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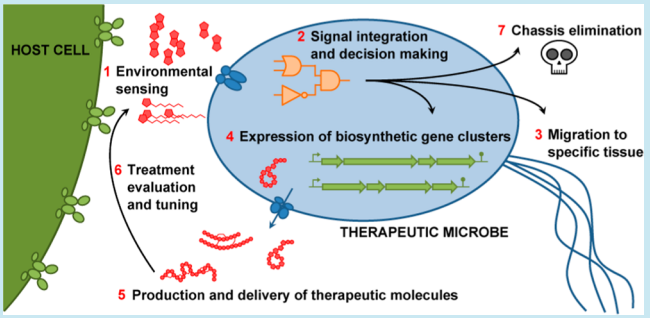
ABSTRACT: Synthetic cell therapy is a field that has broad potential for future applications in human disease treatment. Next generation therapies will consist of engineered bacterial strains capable of diagnosing disease, producing and delivering therapeutics, and controlling their numbers to meet containment and safety concerns. A thorough understanding of the microbial ecology of the human body and the interaction of the microbes with the immune system will benefit the choice of an appropriate chassis that engrafts stably and interacts productively with the resident community in specific body niches.



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Synthetic biology strives to develop novel genetic circuits that can be employed in a variety of applications. The construction of cell therapy systems for use in human medicine is an important emerging subfield within this context. Several chassis have been used for synthetic drug delivery, including bacteriophage,[1,2] bacteria, bacteria-derived lipid vesicles,[3] and eukaryotic cells.[4] In this review, we focus on the current state of the art in applications with bacterial chassis as we believe this holds much promise for the development of microbiota-derived therapeutics.

In vivo synthesis and delivery via cell therapy has several important advantages over systemic treatment. First, the required dosage of the therapeutic agent is reduced by several orders of magnitude to achieve a comparable therapeutic effect.[5,6] This helps in reducing undesired side effects, both at the site of delivery and elsewhere in the body. Second, the route of administration is less invasive than intravenous or subcutaneous injection. For example, certain diseases of the gastrointestinal tract could be treated, by oral administration of a synthetic bacterium that can traverse to the desired location, engraft, and start delivering a drug. This is especially useful for proteinaceous compounds that would not naturally survive passage through the acidic stomach environment. Third, multiple therapeutic agents can be produced by the same cell simultaneously as a combination therapy. Finally, *in vivo* production and delivery by a synthetic chassis provides a more cost-effective treatment as it obviates the need for purification and formulation of the active compound(s). Despite the many advantages, synthetic cell therapy raises issues surrounding safety, containment, and the public opinion on using genetically modified organisms in medicine.

To cause minimal undesired impact upon introduction of a synthetic strain, it is important to understand the dynamics of the microbial ecosystem already present at the body niche of interest as well as the potential effect on the immune system. An emerging theme is that some diseases are related to an imbalance (termed "dysbiosis") in microbe-host interactions,[7] but the precise disease mechanisms and the small molecules that mediate interactions under normal, healthy conditions are still largely unknown. One example of such a microbiome-related disease is *Clostridium difficile* associated diarrhea (CDAD). This chronic intestinal infectious disease is highly contagious, especially in hospital settings where patients receive antibiotic treaments, and difficult to cure with classic approaches such as the antibiotics vancomycin, metronidazole, or fidaxomicin. Recent studies have shown very promising results as the condition can be treated by fecal transplant from a healthy donor,[8] or in a simplified format as a cocktail containing six different gut bacterial strains that aid in the re-establishment of health-associated commensals and thereby displace *C. difficile* in a mouse model.[9] Despite these promising treatment options, the disease state remains poorly understood on a molecular level, and this would be a requirement if a more directed approach with synthetic bacteria expressing small molecule natural products is to be offered as an alternative.

We envision that the next generation of bacterial cell therapy systems will be autonomous microbial "physicians", integrating the capacities to diagnose human disease, make decisions on the appropriate treatment and bring it into effect, and self-eliminate from the human host when the condition is alleviated (Figure 1). In this review, we will highlight current examples of modules that are employed in each of these functions in a synthetic system, as well as speculate about future directions for their implementation.

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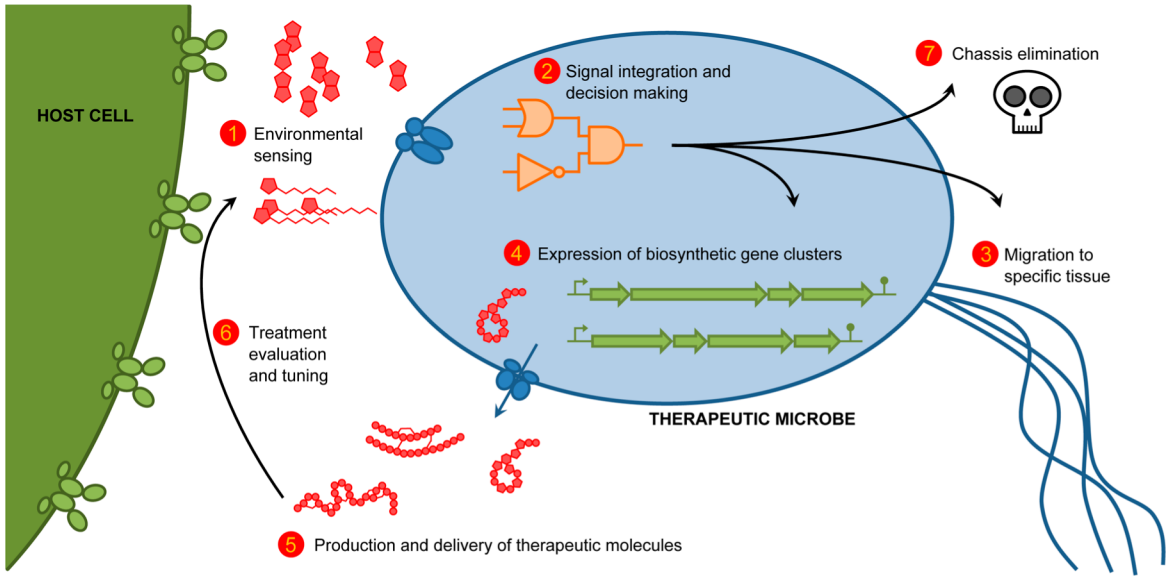

Figure 1. Features of an autonomous synthetic bacterial "physician".

DETECTION OF MOLECULAR INPUTS

An important capability of synthetic bacterial therapies will be the detection of small molecules or cellular markers associated with a certain human disease (Figure 1). Desirable characteristics of such a module are a high degree of molecular specificity, sensitivity, and dose-dependency. When detection systems are available for the desired molecules or condition, they can be implemented in the construction of diagnostic sensing modules. On the other hand, if no known sensor system is available, one could be engineered via protein design, directed evolution, site-directed mutagenesis, or domain swapping.[10]

Bacteria monitor changes in their surroundings by sensing a variety of small molecules, for example, through transcriptional regulators, extracytoplasmic function sigma factors, and two-component signal transduction systems.[11,12] Many of these parts are already available for the synthetic biologist, each one capable of sensing a different specific molecule or cue. An added advantage is that they can be rewired to generate new signaling pathways.[12,13] Two-component systems function in sensing a wide variety of environmental signals. A typical two-component signaling system consists of a histidine kinase and a response regulator. Upon binding of a small molecule inducer, the histidine kinase autophosphorylates and transfers the phosphate group to the response regulator, thereby activating it to transcribe genes under the control of a dedicated promoter sequence. A different mode of bacterial signaling is quorum sensing (QS).[14,15] In QS, bacteria synthesize and secrete a strain-specific autoinducer molecule, for example an acyl homoserine lactone or a small peptide. When a certain density or "quorum" of extracellular autoinducer molecules is reached, the autoinducer binds to a dedicated transcriptional regulator, thereby activating transcription of specific genes. A QS system allows for bacteria to sense their population density based on nearby autoinducer concentration and in response synchronize their gene expression.

The attractive characteristics of QS have led to its integration as a versatile module for synthetic systems. Integration of the QS sensor device from the pathogen *Pseudomonas aeruginosa* into an *Escherichia coli* chassis enables *E. coli* to detect the presence of *P. aeruginosa* via its autoinducer molecule and mount a killing response.[13] In a different study, a squamous cancer cell line of the head and neck was targeted with "nanofactories" consisting of a fusion protein between an antibody and autoinducer-2 (AI-2) synthase.[16] These nanofactories bind to the epidermal growth factor receptor (which is more densely expressed on the cancer cells), where they catalyze the synthesis of *E. coli* AI-2, a chemoattractant that can recruit engineered therapeutic bacteria to the site.

Sensing of hypoxic environments in the human body is used as a cue for the presence of tumor tissue. *E. coli* formate dehydrogenase (*fdhF*) is strongly induced after transition from aerobic to anaerobic growth, and its promoter has been used to drive expression of the *Yersinia pseudotuberculosis* invasin adhesion protein.[17] This allows for β-1 integrin receptor-mediated uptake of *E. coli* into several cancer-derived cell lines; the same bacteria are not invasive under aerobic conditions. In a different report, *E. coli* expressing the invasin protein was shown to invade xenografted human colon cancer cells in an *in vivo* mouse experiment.[18] A study with the naturally tumor-targeting *Salmonella enterica* serovar typhimurium chassis employs the anaerobic-inducible *nirB* promoter for expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in a mouse model of melanoma.[19] Other examples of specific environmental sensing devices include a powerful multi-input miRNA-based cell classifier circuit that can distinguish between different cancer cell types[4] and the use of the *E. coli* nitric oxide (NO) sensing system NorR to detect this important marker for gut inflammation in inflammatory bowel disease (IBD).[20]

Dietary supplements can be administered to exert control over synthetic delivery systems. Delivery of keratinocyte growth factor-2 (kgf-2) by an engineered strain of *Bacteroides ovatus* has been put under the control of dietary xylan.[5] This system provided significant improvement in mice with dextran sodium sulfate-induced colitis. In a different study, a *S. enterica* based system was developed to allow for acetylsalicylate- or salicylate-induced gene expression and this was used to drive expression of the *E. coli* cytosine deaminase.[21] This deaminase converts the prodrug 5-fluorocytosine into the cytotoxic compound 5-

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fluorouracil. A significant reduction in tumor growth was reported when this system was introduced in an *in vivo* mouse model.[21]

Most of the current sensing systems are specific for a small molecule. We envision that the next generation of sensing modules will be able to identify target human cell types based on the receptors on their surface. In addition, the number of small molecule metabolites that microbes can detect with specific receptors is likely to increase, enabling the construction of bacteria engineered to monitor multiple molecular components of their environment.

INTRACELLULAR SIGNAL INTEGRATION

Bacterial therapeutic devises are expected to increase in complexity, and this will require the integration of multiple sensory signals in order to generate an appropriate response to a specific disease. To achieve specificity in this response with minimal impact on healthy cells or resident microflora, multiple environmental cues will have to be assessed during the diagnosis stage, bringing into account the disease state, magnitude, and location in the host. From this information, the synthetic bacterium will have to decide on an appropriate response and relay the signal to different outputs such as migration, initiation of invasion, and production of a therapeutic at a level governed by feedback loops (Figure 1). Genetic logic circuits can be built to integrate the signals and to generate a tailored response.[4,22] Complex decision-making and effector programs can be generated for example by using networks of logic AND, OR, and NOT gates.[23−25] Decisions have to be made regarding the required promoter strength for a specific application and how promoters can be tuned to result in simultaneous or individual expression of multiple target genes.[26] While the application of signal integration systems for therapeutic microbes is still in its infancy, some successful examples are discussed below.

An example of a synthetic system with a product-dependent feedback loop is the mammalian circuit engineered to decrease and stabilize blood urate concentration.[27] The closed-loop device senses urate levels via the HucR transcriptional repressor from *Deinococcus radiodurans* R1. In the presence of urate, HucR dissociates from its *hucO* recognition motif and in turn allows for the expression of the *Aspergillus flavus*-derived urate oxidase, resulting in a drop in extracellular urate. This circuit could be used to treat patients with hyperuricemia and has been tested in a mouse model, where it was shown to control urate levels and shuts down when physiologic concentrations are attained.[27]

Two studies have explored the use of synthetic systems to store an environmental signal as genetic memory. A FimE recombinase-based DNA switch was designed for the NO sensing *E. coli* chassis.[20] Upon activation, this switch remains in a permanent on-state that is inherited by the progeny after cell division. The concept of environmental signal sensing followed by genetic memory recording was expanded in a recent study that describes the integration of a tetracycline-responsive memory circuit in *E. coli*.[28] The memory element was derived from the phage λ cI/Cro switch and proved stable over many generations and after passage through the mouse gut.

As logic circuits grow more complex, the need arises for the identification and characterization of additional orthogonal regulators that can be used as parts in their construction. Factors that could contribute to this increased complexity include the integration of multiple feedback loops from compound production as well as a constant monitoring of the therapeutic effect on the alleviation of the disease. Moreover, as we gain a better insight in the molecular environment of pathogens or malignant cells, more signals from the presence or absence of certain metabolites can be integrated to ensure specificity of the bacterial therapeutic.

PRODUCTION AND DELIVERY OF A SMALL MOLECULE THERAPEUTIC

An important module in a synthetic therapeutic system is the synthesis and delivery of the active compound. Production usually involves heterologous expression of a gene encoding a therapeutic protein, a gene cluster encoding the biosynthesis of a small molecule or knockdown of eukaryotic gene expression by bacterially delivered small RNAs. Many different aspects of cellular function and metabolite synthesis are encoded by gene clusters and provide convenient building blocks for synthetic biology.[29] However, recurring problems in heterologous expression of these clusters are low production efficiencies and unpredictable gene expression levels, often due to cryptic regulatory elements. An attractive solution to the unpredictability of native gene clusters is to refactor them by reconstructing them from known parts.[30] This bottom-up approach reduces gene cluster complexity without changing functionality and removes any form of regulation that might cause unexpected outcomes when applied in an *in vivo* setting. While refactoring at present constitutes a large effort, a better understanding of the regulatory and biochemical mechanisms behind the biosynthesis of key compounds will expedite future endeavors.

In the near future, synthetic bacterial therapy systems will likely involve the production of therapeutic proteins or peptides. Production and delivery of small molecules that are currently in use as antibiotics (e.g., polyketides, nonribosomal peptides, and aminoglycosides) will be a lot more challenging, but as an intermediate step, ribosomally synthesized and post-translationally modified peptides such as lantibiotics and thiopeptides are good candidate compounds. These often have potent activity and stability, and their biosynthesis is encoded by relatively small biosynthetic gene clusters.[31] An added advantage of using lantibiotics, such as the food-grade compound nisin, is that their regulation is well studied and that they have an associated two-component system that allows for QS, based on the actual lantibiotic itself.[32] This enables control not only of the level of producer cell density but also of the level of antibiotic produced, allowing for fine-tuning production specific to the needs of the environment. Nisin-based gene expression in *Lactococcus lactis* has many desirable characteristics to implement in a synthetic system, such as the ability to detect extracellular nisin, no detectable leaky expression, and high level of control because of a linear induction with dose-responsiveness to over a 1000-fold.[33] A nisin-inducible signaling system has been engineered in a *L. lactis* NZ9000 chassis to control the delivery of bioactive single-chain insulin in the small intestine.[34]

In most current case studies of bacterial cell therapy, the compounds that are produced are proteinaceous. For example, *L. lactis* has been engineered for delivery of the cytokine IL-10 in the treatment of murine colitis.[6] This system is the only example of a bacterial therapeutic to date that made it to tests involving human patients.[35] A similar approach was taken for the secretion of anti-tumor necrosis factor α (anti-TNF-α) nanobodies by *L. lactis*, resulting in a reduction of inflammation

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when tested in a mouse model for colitis.[36] The probiotic strain *E. coli* Nissle 1917 has been used as a chassis for treatment of diabetes. Extracellular glucose concentration is sensed via a glucose-responsive promoter element and the bacteria respond by secreting insulinotropic proteins that are fused to a cell-penetrating peptide to facilitate rapid uptake by the epithelial cells.[37] The engineered strain stimulated Caco-2 intestinal epithelial cells *in vitro* to secrete insulin up to 1 ng/mL. This current system could be expanded by integrating a negative feedback loop to decrease secretion of the insulinotropic proteins after glucose levels drop again.

Synthetic bacterial antigen delivery systems will be cost-effective treatments that could replace traditional purified antigen formulations. *Lactobacillus acidophilus* has been engineered to express a *Bacillus anthracis* protective antigen fused to a dendritic cell-targeting peptide. Upon administration as an oral vaccine, the synthetic strain provided the same protection against infection as pure antigen.[38] Synthetic bacteria can also be used to prevent viral infections. A *S. typhimurium* DNA vaccine delivery platform for immunization against influenza induced complete protection when tested in a mouse model.[39] This vaccine system has the added advantage of rapid adaptability to emerging influenza strains by introducing different combinations of hemagglutinin and neuraminidase. A second example of protection against pathogenic viruses involves the vaginal commensal *Lactobacillus jensenii*, which has been engineered to express the HIV-1 entry inhibitor protein cyanovirin-N. Macaques colonized with this strain were repeatedly challenged with simian HIV and showed a 63% reduction of acquisition and a 6-fold lower viral load was observed in macaques with breakthrough infection.[40]

Effector molecules could also serve as decoys to trick bacterial pathogens. Since QS small molecule inducers are strain-specific, they can be hijacked to target pathogens that naturally use them to regulate their virulence. *Vibrio cholerae* produces the autoinducer molecule CAI-1 ((*S*)-3-hydroxydecan-4-one), which is sensed by the CqsS sensor kinase. A LuxO response regulator downregulates expression of virulence factors (cholera toxin and toxin coregulated pilus) when a high level of CAI-1 is reached. When tested as a preventive treatment in an infant mouse model, a CAI-1 producing *E. coli* strain increased survival after *V. cholera* ingestion by 77−92%.[41] Additional engineering of this system for eradication of *V. cholerae* will be required as it now mainly serves as a preventive measure that attenuates virulence. In a different approach to combat *V. cholerae*, an *E. coli* strain expressing a ganglioside GM mimic (the target of cholera toxin) successfully reduced virulence after prior challenge.[42]

Once small molecules are produced, they need to be delivered outside the bacterial cell. Depending on the type of molecule, this can be done in different ways. Gene clusters encoding small molecules often contain dedicated transporter proteins to secrete the products into the extracellular environment. In order to secrete heterologously expressed proteins on the other hand, a bacterial transport signal sequence has to be added for recognition by the Sec or Tat secretion machinery.[43] Secretion of the proteinaceous product can be additionally optimized by engineering this signal sequence. More complex transporters such as the *Salmonella* type III secretion system could be rewired for efficient export,[44] or to inject protein directly into eukaryotic cells.[45] An alternative protein delivery method involves intracellular production of the active compound and subsequent lysis of the bacterial chassis. The aforementioned *P. aeruginosa* killing *E. coli* system functions in this manner by expressing two toxic proteins.[13] When the presence of *P. aeruginosa* is sensed, production of the bacteriocin pyocin S5 is induced. This antibacterial protein accumulates inside *E. coli* until a second toxic protein (E7 lysis protein) is expressed and causes the producer cell to lyse. The released pyocin S5 can in turn kill *P. aeruginosa*. This strategy could be applied to the delivery of different types of small molecules since no specific transport system is required.

Optimization of heterologous expression systems will benefit the variety of small molecules that can be produced by a synthetic microbe. An important consideration regarding the choice of active compound is its activity spectrum. Narrow-spectrum compounds are ideal candidates to target a specific pathogen with minimal disturbance of the resident microflora. Intensive studies of substrate promiscuity in bacterial transporters and secretion systems will allow for the determination of a set of rules to which a transported compound needs to adhere. This will enable the most appropriate transport system to be used as delivery device for a specific small molecule.

CONTAINMENT AND SAFETY CONCERNS

A major concern regarding the use of genetically modified organisms is their containment. Bacterial strains used for clinical trials must be plasmid-free to prevent horizontal gene transfer. Plasmid-encoded systems could also display a different dynamic behavior because of *in vivo* instability over time, which, when not controlled, could lead to undesired dosage effects.[46] Therefore, integration of the synthetic systems into genomic DNA with the removal of integrase genes is advisable.

To prevent the synthetic strains from colonizing undesired niches inside the human body, a bacterial chassis can be used that does not normally colonize the host. The downside of this approach is that therapeutic bacteria will have to be administered on a regular basis to continue treatment. Moreover, dosages and treatment continuity might not be as easily controlled compared to the use of a strain that is capable of colonization and continuously monitors perturbations in its environment and adapts its behavior accordingly.

Alternatively, an additional containment module can be built into the synthetic therapeutic system to "switch off" or eliminate the chassis either when the strain is no longer required after the treatment is completed or when the chassis leaves its desired location inside the body. An example of a containment mechanism has been engineered into *L. lactis* by deleting the thymidylate synthase gene from its genome. When exogenous thymine or thymidine is no longer supplemented, the auxotrophic strain will be eliminated by thymine-less cell death.[47] In another study, *S. typhimurium* has been engineered to initiate programmed bacterial cell lysis after colonization of host lymphoid tissue by placing the expression of two essential genes under the control of arabinose.[48] This lysis system was shown to be functional upon colonization of arabinose-free host tissue, and it is expected that the synthetic strain would not survive outside the host because environmental arabinose levels are too low.

The future challenges regarding safety modules lie in devising standardized test experiments that demonstrate that the chassis does not survive in the environment and ideally remains restricted to its target body niche within the host. It will be critically important to obtain well-founded proof of containment control over bacterial therapeutics in order to convince

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Table 1. Current Developments and Future Prospects in Synthetic Bacterial Cell Therapy

Current Developments	Future Prospects
Diagnosis	
Quorum sensing-based chassis recruitment or pathogen detection	Extending the repertoire of detectable molecules by exploration of the ligand landscape for bacterial receptors and hybrid engineering
Detection of hypoxic tumor environments	Identification of interactors with specific receptors expressed on target human cell types
Sensing of the inflammatory marker nitric oxide	
Signal Integration	
Construction of logic circuits using genetic AND, OR, and NOT gates	Further characterization of orthogonal regulators as logic gates to engineer increasingly complex circuits
Recording of memory using irreversible genetic switches	Integration of several feedback signals that evaluate a disease state and the therapy effectiveness
Production and Delivery	
Heterologous production of mostly proteinaceous compounds	Regulatory and biochemical studies of key biosynthetic gene clusters will aid in their refactoring
Refactoring of biosynthetic gene clusters allows for tight and predictable regulatory control	Expansion of therapeutic arsenal with small molecules produced by more complicated biosynthetic machinery
	Intensive study of bacterial secretion systems as delivery devices
Safety	
Chassis self-elimination by engineered programmed cell death	Development of systems to prevent horizontal gene transfer of synthetic parts or entire systems
Controlled lysis for compound delivery	Tests to measure the efficacy of chassis containment in its intended niche and to ensure it does not spread to the environment
Choice of Chassis	
E. coli, *Lactobacillus*, *Bacteroides* mostly for applications in the gut	Focus on development of genetic systems for key members of the human microbiota at various body sites
Tumor-targeting *Salmonella*	Engineering of therapeutic T cells rather than bacteria for bloodborne applications

government agencies and the public opinion to allow for their use in medicine.

CHOOSING AN APPROPRIATE CHASSIS BASED ON THE TARGET BODY NICHE

Most synthetic systems developed to date were built in an *E. coli* chassis, an attractive system because of its ease of manipulation. Although this is valuable as a proof of principle, commensal bacteria might be more suitable chassis for *in vivo* applications, since they are harmless under normal circumstances, already capable of colonizing specific niches in the human body and interacting productively with the host immune system. Another consideration that has to be taken into account is that it might beneficial for public and FDA acceptance to use strains that are already used as probiotics.

Currently developed systems are mostly for applications in the gut and tumor cells, but the oral cavity and skin will emerge as targets in future systems. We will briefly discuss organisms that are promising candidates to further develop as chassis for each of these body niches. For applications in the gut, *Bacteroides* is a good candidate because it is a prominent commensal. Its association with the mucus layer and ability to produce outer membrane vesicles can be harnessed for targeted compound delivery. *L. lactis* is another attractive chassis because of its current use as a probiotic and metabolic activity in all compartments of the intestinal tract.[6] While it is true that *E. coli* is a gut commensal, it is not as prominently present *Bacteroides* for example. For intracellular tumor-targeting applications, attenuated strains of *S. typhimurium* that naturally colonize tumors have been used. They have an intrinsic specificity for colonization of tumor compared to normal tissue of 1000−10000:1 and can remain in the hypoxic regions for a long time.[19,21] Since the occurrence of bacteria in the bloodstream could lead to undesired complications, the synthetic biologist could instead resort to engineering T cells for therapeutic purposes in order to better mimic natural conditions in the host.[49] A potentially promising chassis for the oral cavity is *Streptococcus salivarius* K12, a natural colonizer of the oral mucosal surfaces that has been used as a probiotic strain for over 10 years.[50] Another candidate for this niche is *L. lactis*, which has already been developed as a mouth rinse for treatment of oral mucosistis.[51] For use as a topical therapeutic on the skin, a chassis is required that can survive this rather harsh body niche. Commensal *Corynebacterium* species would be a suitable candidate, as would *Staphylococcus epidermidis*.

OUTLOOK

Synthetic bacterial cell therapy is a research field that is still in its infancy. While several proof of concept systems have been engineered to date, the next generation is expected to be significantly more complex, incorporating modules for diagnosis, signal integration and decision making, compound production and delivery, and safety. Including several of these modules into the same therapeutic bacterial strain will contribute to achieving a greater level of control and the possibility to fine-tune the therapy to the specific needs of the patient. An overview of current developments and future prospects for each module, as discussed in this review, is summarized in Table 1.

Essential to the development of synthetic bacterial "physicians" is a good understanding of the underlying molecular basis for human diseases and the ecology of healthy

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bacterial communities. Not only will these insights benefit the design of specific therapeutic systems, synthetic biology could lend a hand in these fundamental investigations, for example by designing sensor bacteria that can measure the levels of certain small molecules or markers *in vivo* and report this acquired information back to the researcher.

We predict that there will be a change in the chassis used for bacterial cell therapy applications. Where several currently developed systems use *E. coli* for proof of principle experiments, a gradual shift will take place toward the use of commensal and probiotic bacteria that can naturally colonize the target niches.

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Notes

The authors declare the following competing financial interest(s): M.A.F. is on the scientific advisory boards of NGM Biopharmaceuticals and Warp Drive Bio.

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