# Synthetic biology and engineered live biotherapeutics: towards increasing system complexity

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**Abstract**

Recent advances in synthetic biology and biological system engineering have allowed the design and construction of engineered live biotherapeutics targeting a range of human clinical applications. In this review, we outline how systems approaches have been used to move from simple constitutive systems, where a single therapeutic molecule is expressed, to systems that incorporate sensing of the *in vivo* environment, feedback, computation and biocontainment. We outline examples where each of these capabilities are achieved in different human disorders including cancer, inflammation and metabolic disease in a number of environments including the gastrointestinal tract, the liver and the oral cavity. Throughout we highlight the challenges of developing microbial therapeutics that are both sensitive and specific. Finally we discuss how these systems are leading to the realisation of engineered live biotherapeutics in the clinic.

# Introduction

Systemically administered drugs can often lead to significant off-target delivery, giving rise to high doses in unintended locations and toxic side-effects (Bae & Park 2011). The goal of targeted drug delivery is to maximise drug accumulation within a target area and minimise off-target effects. This requires four key components; a delivery vehicle, sufficient stability to reach the target site, retention within the intended site, and timely drug release for the effective function of the drug (Bae & Park 2011). Recent advances in biotechnology have enabled the use of nanoparticles and biopolymers for targeted delivery and remote activation (Farokhzad & Langer 2009, Timko et al. 2011), but these also face significant challenges (Kwon et al. 2012). The human microbiota refers to the ecosystem of microorganisms living on or within the human body, and is increasingly being implicated as a regulator of health and disease (Li et al. 2016, Wu et al. 2010, Vijay-Kumar et al. 2010, Wang et al. 2011). This community consists of a wide variety of well-tolerated microorganisms such as bacteria, yeast and phages that occupy a large and interactive buffer zone between the host and the external environment. The habitability of niches in the body to such microorganisms can in principle allow for their use in the delivery of therapeutics in a targeted and controllable fashion. There are three broad approaches to achieve this; application of natural organisms that have therapeutic properties (live biotherapeutics), artificially assembled microbial consortia, and genetically engineered commensal microbes (herein referred to as engineered live biotherapeutics).

A live biotherapeutic is a product that contains a live microorganism and is applicable to the prevention, treatment or cure of human diseases (Olle 2013). The discovery of commensal relationships between microbes and humans led to the clinical investigation of the therapeutic effects of ingestion of natural microbial probiotic strains such as *E. coli* Nissle 1917 (Henker et al. 2007) and *Lactobacillus* (Grüber et al. 2007, Drouault-Holowacz et al. 2008). The development of live biotherapeutic products (LBPs) is rapidly evolving. Historically, exploration of these strains was confined to the fields of microbiology and nutritional supplementation. The most commonly used microorganisms were those closely linked to food processing such as *Lactobacillus* spp. *Bifidobacterium* spp. and *Saccharomyces*. The increasingly fine-detailed characterisation of the human microbiota is leading to the identification of many other strains for therapeutic interventions including *Bacillus* spp., *Weissella* spp. and *Escherichia coli* (O'Toole et al. 2017). Another development is in the use of synbiotics, a combination of a probiotic

and a prebiotic, with a recent study demonstrating that *Lactobacillus plantarum* plus fructooligosaccharide can prevent infant sepsis in a randomized placebo controlled trial (Panigrahi et al. 2017).

It is becoming increasingly apparent that the host-microbiota interaction constitutes a complex ecosystem between the microbes and the immune system. The potential benefit of communities of probiotic bacteria has been recognised for some time, although their effectiveness has yet to be conclusively demonstrated (Chapman et al. 2011). A classic example is the VL#3 formulation of eight strains of *Bifidobacterium*, *Lactobacillus* and *Streptococcus*, which is prescribed for inflammatory bowel disease. More recently, the clinical potential of wild-type bacterial communities was demonstrated through the use of faecal microbiota transplantation (FMT) in combating recurrent *C. difficile* infections (van Nood et al. 2013). Leading on from these findings, Seres Therapeutics (MA, USA) is running human clinical trials with an oral drug cocktail mimicking the wild-type bacterial composition of these faecal transplants (Khanna et al. 2016).

The third approach - and the main focus of this review article - is to leverage the tools of synthetic biology to engineer microbes and their communities to perform targeted therapeutic delivery with much greater control of location and timing. This approach has many advantages over the other two including the ability to direct therapeutics to particular niches, ultimately allowing for higher doses with lower systemic effects (Claesen & Fischbach 2014). In addition, the reciprocal interactions between the microbiota, immune system, diet and genome makes it extremely difficult to distinguish cause and effect in several pathologies (Cerf-Bensussan & Gaboriau-Routhiau 2010, Serino et al. 2012, Blekhman et al. 2015). Engineered microbes can be used to explore these interactions and modulate their effects, opening up many avenues to new biology and, ultimately, new therapeutic strategies.

In this review, we begin by outlining foundational work in the therapeutic use of engineered bacteria then discuss the latest synthetic and systems biology approaches taken with engineered live biotherapeutics for human health. We discuss the complexity of these methods and future directions in which cutting edge synthetic biology can enable the design of robust and multi-layered system tools to effectively engineer the human microbiome for therapeutics.

## Constitutive Therapeutic Delivery Systems

A fundamental approach in engineering microorganisms is the constitutive expression of an effector protein. Upon ingestion and colonisation with an engineered commensal strain, the protein produced directly acts upon the host or the resident microbial population to elicit a physiological response (Figure **1**). This method has been used to target several different conditions such as obesity (Chen et al. 2014), diabetes (Duan et al. 2008), colitis (Steidler et al. 2000), cancer (Yoon et al. 2017, Forbes 2010), and infection by pathogenic bacteria (Focareta et al. 2006, Liu et al. 2016) (Table 1). A recent example includes the engineering of *S. typhimurium* to express the *Vibrio vulnificus* flagellin protein to act as a toll-like receptor ligand, triggering an immunogenic anti-cancer response from the host (Zheng et al. 2017). A further example of this approach involved the use of engineered *E. coli* Nissle to disrupt the quorum sensing pathways of *V. cholerae* (Duan & March 2010). This pathogen is known to control its population density and the amount of cholera toxin being produced via quorum sensing molecules cholera autoinducer 1 (CAI-1) and autoinducer 2 (AI-2). At high concentrations of both CAI-1 and AI-2, virulence factors such as cholera toxin are no longer expressed. To reduce virulence, *E. coli* Nissle was transformed with a plasmid that constitutively expresses *CqsA*, the gene for CAI-1. In a mouse model, the ingestion of this engineered strain before *V. cholerae* administration significantly reduced cholera toxin production and mortality (Duan & March 2010). Another recent study, demonstrated *E. coli* Nissle harbouring a modular expression system that could produce seven different antimicrobial proteins to target several gastrointestinal pathogens in the intestines (Geldart et al. 2016). An exciting approach to colorectal-cancer chemoprevention was recently detailed in which *E. coli* Nissle was engineered to express histone-like protein A (HlpA) on the cell surface, allowing it to bind to cancer cells with upregulated heparan sulfate proteoglycan (HSPG) on their cell surface (Ho et al. 2018). At the same time, the engineered bacteria secreted an enzyme, myrosinase, which converts dietary glucosinolate from cruciferous vegetables into the known anti-cancer molecule sulphoraphane. This system was capable of killing colorectal cancer cell lines *in vitro* and preventing tumour progression in a murine colorectal cancer model (Ho et al. 2018).

In these constitutive expression systems, the amount of therapeutic delivered depends on several factors, such as the population size of the engineered microorganism. The parameter that is perhaps

most under the control of the system designer is the strength of the promoter. A number of different constitutive promoters have been used, such as the lac (Paton et al. 2005), tac (Chaudhari et al. 2017), fliC (Duan & March 2010), slpA (Duan et al. 2015), and Anderson promoters (Ho et al. 2018). Rarely is any reason for promoter choice given and often it seems to be due to ease of cloning rather than consideration for promoter strength. It has been shown that using a weaker promoter, with a lower metabolic burden, can lead to an increased therapeutic yield (Din et al. 2016) and as such, greater consideration of promoter strength should be intrinsic to system design.

The export of active therapeutics from the engineered microorganism into the environment has been approached in two ways: by lysing the producing cells to release the intra-cellular therapeutic or by using secretion machinery to transport the therapeutic out of the cell. Lysis is generally not used in simpler systems as there needs to be a mechanism for ensuring lysis only occurs once enough protein has been produced within a cell or once a desired population density has been reached (Din et al. 2016). Several secretion mechanisms have been used and often those already present in the bacterial strain are commandeered, such as ABC transporters (Choi et al. 2012, Geldart et al. 2016), Sec systems (Zheng et al. 2017), and the flageller secretion apparatus (Duan et al. 2008, Gupta et al. 2013). However, it should be noted that the efficiency of secretion of active folded proteins is not 100%, with the flageller system secreting as little as 5% of total protein produced (Gupta et al. 2013). As such, consideration needs to be taken as to whether pushing cells to express as much of a therapeutic protein as possible is sensible if it cannot be exported in to the environment.

A synthetic circuit, whether a simple constitutive expression system or a more complex system, will place a burden on the host microorganism. The greater the burden, the greater the selective pressure to remove the system or to develop mutations that reduce its function (Sleight & Sauro 2013). Tools to help us understand how designed synthetic circuits will interact with their bacterial hosts (Weiße et al. 2015, Liao et al. 2017) and methods to identify less burdensome designs (Ceroni et al. 2015) are being developed. A system has been developed which attempts to reduce the metabolic burden on the host by limiting the production of a protein until the host has grown enough and there is sufficient substrate in the environment (Lo et al. 2016). However, this approach subsequently changes the dynamics of gene expression and, therefore, may not be appropriate for more complex systems with tighter parameters.

While these approaches are developing alongside our understanding of disease biology, they solely capitalise on the capability of the engineered strains to transiently occupy the relevant niche, such as the intestines or the hypoxic microenviroment of a tumour, and recombinantly express a protein. However, bacteria also occupy niches other than those desired. When administered intravenously, bacteria can be found in a range of organs including the spleen, kidneys, liver, brain, lungs and heart (Forbes et al. 2003, Zheng et al. 2017). If an LBP was engineered to constitutively express an anticancer drug, low level accumulation in untargeted healthy tissue would lead to systemic toxicity (Forbes et al. 2003, Forbes 2010). An example in which a constitutive expression system showed off-target effects was in the delivery of a therapeutic gene to epithelial cells in a mouse model of colitis (Castagliuolo et al. 2005). In this case, a system was developed where a non-pathogenic *E. coli* strain could invade epithelial cells and deliver functional DNA. However, when the gene was expressed constitutively, transcripts of the therapeutic gene were discovered in other tissues. Replacing the constitutive promoter with an inflammation-inducible promoter led to the mRNA transcripts only being detectable within the inflamed tissue. In line with this, there has been a subsequent effort to design systems that can detect and respond to environmental factors for regulated expression. These systems could enable the production of therapeutic proteins to be targeted to specific locations through the sensing of environmental stimuli, and to regulate expression using feedback to prevent overproduction.

## Systems approaches with increased functionality

Through the integration of synthetic biology tools and circuit design principles, researchers have been able to devise and implement systems approaches with greater complexity (Table 2, Figure **2**). An underlying thread of these efforts is the use of robust biological components and system designs to create reproducible and consistent outcomes. Insulation of promoters (Davis et al. 2011) and ribosome binding sites (Lou et al. 2012) reduce the contextual effects of the surrounding DNA sequences, allowing for greater interchangeability of parts. Furthermore, the development of standardised transcription and translation elements (Mutalik et al. 2013), and terminators (Chen et al. 2013), has improved our ability to produce circuits that behave predictably. The use of

computational methods to determine the optimal strengths of components or optimal circuit designs to produce desired behaviours (Otero-Muras & Banga 2017, Leon et al. 2016, Woods et al. 2016), in conjunction with these more reliable parts, has allowed the pace of development and attainable complexity to greatly increase.

## Systems that can sense and respond

The next step up in complexity from the simple design of an engineered strain expressing a recombinant effector protein is a system that can detect a stimulus and produce a designated response (Figure **2**). The stimuli can be detectable molecules or conditions in the host environment, or externally provided signals. This approach enables a more precisely localised or timed effect on the host. Such spatiotemporal control is a vital facet in the design of a therapeutic product.

A system capable of detecting native, therapeutically relevant stimuli was demonstrated using *Lactococcus lactis* to treat *E. faecium* infection (Borrero et al. 2015). The *L. lactis* strain was engineered to produce an antimicrobial bacteriocin when it detected the cCF10 enterococcal sex pheromone produced by *E. faecium*. Similar systems have been developed to sense and destroy *Pseudomonas aeruginosa* using engineered *E. coli* (Saeidi et al. 2011, Gupta et al. 2013). These both involved production of an antimicrobial bacteriocin upon detection of a quorum sensing molecule from *P. aeruginosa*, followed by release of the bacteriocin, either through lysis (Saeidi et al. 2011) or the use of a secretion tag (Gupta et al. 2013). The system of Saeidi et al. has been expanded to include the rewiring of the CheZ chemotaxis pathway to respond to the quorum sensing molecules produced by *P. aeruginosa* (Hwang et al. 2013), plus the incorporation of the anti-biofilm dispersin B protein (Hwang et al. 2017).

The collection of available sensors to detect therapeutically relevant information is expanding. Sensors for markers of gut inflammation such as nitric oxide (Archer et al. 2012), and thiosulfate and tetrathionate (Daeffler et al. 2017) have been developed. A thermo-sensitive expression system has also been demonstrated to be able to detect a fever in a mouse model (Piraner et al. 2017).

The rapid advancement of bioinformatics tools and genome sequencing has enabled the discovery of novel sensors but other approaches are also possible. A series of modular transcription factors have been

developed in which ligand binding domains, which recognise different sugars, can be attached to a DNA binding domain to produce novel repressors (Shis et al. 2014). A more general modular approach, using single-domain antibodies coupled to a DNA binding domain, has recently been described that aims to vastly increase the repertoire of ligands that can be detected (Chang et al. 2017). Tools such as feedback-regulated evolution of phenotype (FREP) (Chou & Keasling 2013) or compartmentalised partnered replication (CPR) (Ellefson et al. 2014) can be used to evolve more responsive sensors.

In addition to the detection of native molecules and conditions, a number of systems have been developed that allow for deliberate, external stimulation. This was demonstrated in mice with *E. coli* Nissle where a luminescent reporter circuit was controlled with the ingestion of synthetic inducers in drinking water (Loessner et al. 2009). In an alternative approach, a synthetic circuit in *E. coli* Nissle enabled the strain to sense an *in vivo* liver metastasis signal and report on the finding with an easily detectable reporter in urine (Danino et al. 2015). In addition to being able to sense host fever, the thermo-sensitive expression system mentioned previously was shown to be capable of being induced externally through ultrasound (Piraner et al. 2017).

Instead of transiently occupying a destined niche after ingestion or intravenous injection, live biotherapeutics can also be engineered to invade the surrounding host cells in response to environmental signals. Engineered invasiveness paves the way for new drug delivery strategies with a more precise targeting capability. Using the invasin coding *inv* gene from *Yersinia pseudotuberculosis*, several groups have shown the ability of engineered bacteria to invade host cells. The *inv* invasion system was shown to allow engineered *E. coli* to invade intestinal mucosal cells after colonisation (Critchley et al. 2004) and even deliver a therapeutic gene under an inflammation-inducible promoter (Castagliuolo et al. 2005). A further approach was shown that incorporated the *inv* gene into a quorum sensing circuit to create a live biotherapeutic that only invaded tumours after a certain population density was reached (Anderson et al. 2006).

Species from the *Bacteroides* genus are now known to be much more abundant and stable in the human intestinal microbiota than model strains such as *E. coli* and *S. typhimurium*, which are only capable of occupying the niche transiently (Consortium 2012). However, *Bacteroides* do not possess a rich library of catalogued genetic parts like those of the model strains (Mutalik et al. 2013) and

therefore pose a greater challenge to engineer. In light of their relative stature in the intestines, the therapeutic functionality of *Bacteroides* strains with complex synthetic circuits has the potential to be much wider than that of model strains. In a demonstration of the expanding toolbox for engineering the *Bacteroides*, a series of orthogonal inducible promoters using rhamnose, chondroitin sulfate, arabinogalactan and isopropyl β-D-1-thiogalactopyranoside (IPTG) were designed and characterised (Mimee et al. 2015). Furthermore, the rhamnose inducible system was coupled with an integrase element to create an inducible memory switch that detected and recorded *in vivo* the addition of the sugar into the diet of the colonised mice. In a more recent example, a finely tuned anhydrotetracycline (aTc) promoter system with a 9,000-fold dynamic range was characterised in *Bacteroides* (Lim et al. 2017). Due to aTc being a synthetic inducer not found naturally in the mouse diet or microbiota, it is possible to use this system to precisely investigate dynamic host-microbe interactions. In this case, the inducible system was used in *Bacteroides* to investigate the effect of sialidase expression on the liberation of host mucosal sialic acid, a nutrient linked to pathogens such as antibiotic-associated *Clostridium difficile* (Ng et al. 2013).

## Systems with feedback

The overproduction of certain therapeutic molecules can cause harm. If an engineered strain is designed to express a therapeutic protein, either constitutively or through induction, without consideration for the concentration of that protein currently in the environment, there is a risk of overproduction. Designing a system with feedback, in which the system senses its effect on the environment and uses that information to modulate its expression, prevents overexpression.

An example of a system in which feedback could be used is a circuit that responds to the production of butyrate in the gut. The short chain fatty acid butyrate is beneficial for gut health (Hamer et al. 2009, Furusawa et al. 2013) and therapeutic approaches have been demonstrated with butyrate producing strains (Geirnaert et al. 2017). Furthermore, synthetic systems have been used to produce butyrate in *E. coli* (Saini et al. 2014). However, there is also evidence that high levels of butyrate have an adverse effect on the intestines (Hamer et al. 2008). In this example, the use of a butyrate sensor in the system could be used to provide feedback on the level of butyrate in the

environment and turn off butyrate production when a threshold is met.

In addition to the problems caused by protein overproduction, overgrowth of the engineered bacterial strains themselves can set off a systemic inflammatory response in the host. Feedback can be used to control population density and prevent population overgrowth. An example of this form of population feedback and control was demonstrated with a clinically relevant strain of *S. typhimurium* (Din et al. 2016). Using a quorum sensing system, previously developed to produce synchronised population oscillations (Danino et al. 2010, Prindle et al. 2011, 2012), a synchronised lysis circuit was developed that enabled the population to repeatedly grow and lyse in a synchronous fashion once a population threshold was reached. This system was used to deliver three cancer therapeutics in a mouse hepatic colorectal metastases model while the population density feedback prevented overgrowth of the bacterial population (Din et al. 2016).

## Systems that compute

To mimic the complexity of some natural responses to external stimuli, researchers are incorporating novel biological components into synthetic circuits that allow strains to integrate multiple signals and perform higher order functionalities (Figure **2**). Through the rational design and coupling of genetic components, a plethora of examples exist demonstrating a wide spectrum of functionalities. The importance of being able to integrate multiple signals to control therapeutic delivery can be seen in a system developed to target obesity. An *E. coli* Nissle strain engineered to secrete *N* -acylphosphatidylethanolamines (NAPEs) was able to reduce the obesity of mice fed a high fat diet when added to drinking water (Chen et al. 2014). However, it has been shown that elevated levels of *N* -acylethanolamide (NAE), of which NAPEs are a precursor, in plasma and the brain led to decreased energy expenditure (Brown et al. 2012). By using multiple external stimuli, such as pH or levels of bile acids, to determine the location of the live biotherapeutic, future systems could limit NAPE production to key areas of the colon.

The most notable recent leap in the realization of complex circuits has been the automated design of transcriptional networks, as was demonstrated with Cello, which utilizes a specification language based on Verilog for implementing design of gene circuits that perform Boolean logic

functions (Nielsen et al. 2016). Using this system, the authors were able to demonstrate a functioning three-input consensus circuit, involving 55 biological components. Circuits such as these allow for the integration of several stimuli which in turn allows for greater control over therapeutic delivery. In addition to transcriptional logic gates, translational logic circuits are being developed (Rodrigo et al. 2012) with ever increasing complexity (Green et al. 2017). With the addition of modules that provide functionality such as memory (Yang et al. 2014), oscillation (Stricker et al. 2008), counting (Friedland et al. 2009), and orthogonal communication (Scott & Hasty 2016), the diversity of types of computation that are theoretically possible is continually expanding (Table 3).

The incorporation of a phage-lambda-based memory circuit was used to show that an engineered *E. coli* strain could detect, record and report on intestinal inflammation *in vivo* over 6 months (Riglar et al. 2017). Recombinase based memory was used to create a simple finite state machine (Roquet et al. 2016), though returning to prior states does not yet seem to be possible using this method. New memory devices are being developed that allow for the recording of information over long periods of time on genomic "tape" using either CRISPR (Sheth et al. 2017) or recombinases (Farzadfard & Lu 2014).

Integrating memory with logic gates allows for the construction of sequential logic circuits rather than solely the combinational logic that Cello enables. A conditioning system was constructed using a bistable toggle switch as a memory module that allowed for a Pavlovian-like association to be built between two unrelated inducible inputs such as salicylate ("food") and arabinose ("ring"), and a specific fluorescent output ("salivation") (Zhang et al. 2014). By taking advantage of a heterogeneous population response, the Pavlovian-like response to the "ring" stimulus was only reinforced by rounds of simultaneous training with the "food" input.

The sensitivity of a live biotherapeutic is crucial to its function. The addition of an integrase switch element into a sensor circuit was shown to digitise and significantly improve the signal to noise ratio in the detection of pathological nitrate by an *E. coli* biosensor (Courbet et al. 2015). Using quorum sensing communication systems to link logic gates together in different bacteria allows for the distribution of parts of larger logic circuits across a population (Tamsir et al. 2011).

## Systems that modify native gene expression

More recently, live biotherapeutics have been developed that are capable of interfering with gene expression within the host genome or microbiome. This approach can allow the engineering of host or microbiota biology on a sequence specific level. Initial attempts have consisted of *E. coli* or *S. typhimurium* expressing short hairpin RNAs (shRNAs) for trans-kingdom RNA interference in mucosal tissues after intestinal colonisation (Xiang et al. 2006, Guo et al. 2011). The demonstrations with *E. coli* showed that the system could be used in the intestinal epithelium to knockdown the expression of *CTNNB1*, an oncogene implicit in the initiation of colorectal cancer (Xiang et al. 2006)

With recent advancements in the use of CRISPR-Cas technologies, it has also been possible to devise live biotherapeutic approaches to engineer the microbiome on a sequence specific level. Through the use of phages that can inject DNA directly into bacterial cells, sequence-specific RNA guided nucleases (RGNs) can be expressed that precisely target the host genome. This method was used to specifically differentiate virulent and avirulent *Staphylococcus aureus*, and to function in an *in vivo* mouse skin colonisation model after topical administration of engineered phage lysates (Bikard et al. 2014). In another recent example, engineered phages were used to target antibiotic resistance harbouring *Enterobacteriaceae* (Citorik et al. 2014). In this example, it was also shown that *E. coli* could be engineered with conjugative plasmids to deliver RGNs to the surrounding bacterial population and subsequently control the compostion of a synthetic consortium based on the presence of a single gene. With the capability of targeting specific genetic signatures, this approach provides the power to eventually engineer and remodel complex bacterial populations such as the intestinal microbiome with an incredible level of resolution and precision.

## Systems for biocontainment

As with any therapeutic, safety concerns must be addressed when constructing live biotherapeutics. Several approaches have been developed to tackle the challenge of biocontainment; ensuring that any engineered strain is not allowed to escape from a well-defined environment (Figure **3**A). The most often explored method is the use of an environmental input for the survival of the engineered strain. This is primarily achieved through the use of auxotrophic strains (Steidler et al. 2003), however metabolic cross-feeding in environments such as the gut must be taken into consideration when

designing the auxotroph (Germerodt et al. 2016). As such, auxotrophs that require supplementation with non-standard or synthetic amino acids for production of essential proteins have been developed that show greater robustness to escape (Mandell et al. 2015, Rovner et al. 2015). The Deadman and Passcode systems, although not auxotrophs, also require environmental input to enable survival (Chan et al. 2016). These work through the two-layered transcriptional repression of a toxic gene and the targeted degradation of an essential gene. This system, as with the auxotrophic systems, requires the delivery of a molecule into the environment. Another approach is to use an environmental input already present in the target environment but not present outside of it. The temperature dependent production of an antitoxin, active against a constitutively produced toxin was shown to reduce survival of engineered bacteria unless they were incubated at 37C, even after having passed through the mouse gut (Piraner et al. 2017).

Preventing the escape of engineered bacteria into the environment is not the only biocontainment concern when designing live biotherapeutics. The transfer of genes from the engineered strain to wild strains present in the environment is a further challenge that must be addressed. This is particularly problematic in the inflamed gut due to increased horizontal gene transfer (Stecher et al. 2012). The GeneGuard system takes a three-pronged approach to prevent the escape of engineered plasmids into wild species by designing a strain and plasmid that could not survive without each other (Wright et al. 2014). They designed an auxotrophic strain that could not survive without an essential gene encoded on the plasmid. This prevents the genetically modified strain ejecting the plasmid and outcompeting the plasmid-carrying strain. Secondly, the plasmid produces a toxin which is nullified by a genomically produced antitoxin, preventing the survival of wild strains that acquire the plasmid (Figure **3**B). Finally, the plasmid cannot replicate without a genomically encoded protein. This prevents the amplification of the engineered DNA if a wild strain manages to acquire the plasmid and not be susceptible to the toxin (Figure **3**C).

## Conclusions

The examples discussed here demonstrate that as our understanding of host biology and circuit designs

evolve, we can integrate a variety of approaches to devise live biotherapeutics that act in a systematic, precise and robust manner. The growing repertoire of genetically tractable strains, genetic parts and system design tools will enable us to target human pathologies such as cancer and metabolic conditions to which there is still an urgent unmet need. Increasing the complexity of live biotherapeutics can allow us to, in theory, bypass and overcome traditional hurdles to effective pharmaceutical therapeutics such as cost, dosing, side-effects and efficient delivery. The ability to precisely control a mixed population of bacteria could also allow us to use engineered strains with communication modules that enable a tunable division of labour *in vivo*. However, it is also apparent that increasing the complexity of these systems could potentially hinder the therapeutic efficacy of engineered LBPs, particularly in respect to host metabolic burden and genetic circuit stability. The method of genetic engineering used is also critical in the clinical translation of LBPs. A number of different approaches have reached human clinical trials, including Intrexon's (VA, USA) engineered *L. lactis* strain with chromosomal integration (Limaye et al. 2013) and Marina Biotech's (CA, USA) engineered *E. coli* strain incorporating plasmid based systems (Xiang et al. 2006). A topic of discussion is whether the use of plasmid based approaches are safe considering the risk of horizontal gene transfer (Stecher et al. 2012) and the chance of mutations disrupting plasmid copy number and subsequently effecting the dose of the therapeutic. These clinical trials demonstrate that live biotherapeutics show great promise, emphasised by increased recent investment in this area of biotechnology (Olle 2013, Maxmen 2017). Although there are still regulatory challenges to be addressed, history is rife with examples of new technologies that faced similar issues, such as *in vitro* fertilisation (IVF), which then became widely accepted once the potential dangers were studied and understood. Continued research into live biotherapeutics can not only provide clear biomedical advances in areas such as the treatment of cancer, obesity and type 2 diabetes, but also allow the development of precision tools to facilitate experimental investigation. These tools will further elucidate the convoluted and multifaceted relationship between the human host, metabolome and microbiota.

## Declaration of Interests

The authors declare no competing interests.

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## Figure Legends

**Figure 1.** The gastro-intestinal tract is an example of an environment in which live biotherapeutics can provide targeted benefit. Engineered strains have been designed to target (a) pathogenic microbes, (b) molecules that may lead to pathological condition, and (c) specific disease states.

**Figure 2.** Increasing complexity of synthetic systems allows for improved levels of control and robustness. Constitutive production of a therapeutic from a host is the simplest form of engineered live biotherapeutic. Linking expression of the therapeutic to an exogenous signal allows for more spatially or temporally targeted delivery. Logic circuits allow for the integration of several exogenous signals, enabling greater control and robustness. Incorporating memory and clock systems further expands the computational capabilities of engineered strains. Similarly, incorporating signalling systems, allowing communication allows for synchronised behaviour. With several orthogonal communication systems, complex heterogeneous engineered populations can be built that allow for distribution of function.

**Figure 3.** Biocontainment of engineered strains and recombinant DNA is a significant challenge. (A) Several efforts have been made to limit the environment under which engineered strains are viable through auxotrophy, temperature range, and pH level. Even if an engineered strain is no longer viable the engineered DNA can prove to be a risk through horizontal gene transfer. (B) Producing a toxin from

the engineered plasmid prevents survival of transformed wild bacteria. (C) Requiring specific proteins for replication of the engineered plasmid prevents the amplification of engineered DNA outside of specific strains.

**Table 1.** Constitutive therapeutic delivery systems

| **Target** | **Location** | **Model Organism** | **Chassis** | **Mechanism** | **Ref.** |
|---|---|---|---|---|---|
| Cancer | GI tract | Mouse | *B. longum* | Production of Tumstatin, inhibiting proliferation and inducing apoptosis of tumorous vascular endothelial cells | (Wei et al. 2016) |
| Cancer | GI tract | Mouse | *E. coli* | Production of tumour-specific modular synthetic adhesins to enhance targeting | (Piero-Lambea et al. 2015) |
| Cancer | GI tract | Mouse | *E. coli* Nissle 1917 | Expression of HlpA to enable specific binding to cancer cell and secretion of myrosinase to convert dietary glucosinolates in to sulphoraphane | (Ho et al. 2018) |
| Cancer | Mammaries | Rat and Guinea pig | *B. longum* | Production of an enzyme which converts the pro-drug 5-FC to the toxic 5-FU within tumours | (Sasaki et al. 2006) |
| Cancer | Subcutaneous | Mouse | *S. typhimurium* | Production of the interferon-gamma cytokine to enhance tumour death after invading | (Yoon et al. 2017) |

| | | | | | |
|---|---|---|---|---|---|
| | | | | melanoma cells | |
| Cholesterol | GI tract | Human | *L. reuteri* | Reduction of absorption of non-cholesterol sterols by bile salt hydrolase-active capsules | (Jones et al. 2012) |
| Colitis | GI tract | Mouse | *L. lactis* | Secretion of immunosuppressive cytokine interleukin (IL)-27 | (Hanson et al. 2014) |
| Colitis | GI tract | Mouse | *L. lactis* | Treatment of Crohn's disease with expression synthetic human IL-10, with effective containment strategy | Steidler et al. 2000) |
| Diabetes | GI tract | Rat | *L. gasseri* | Secretion of GLP-1(1-37) to stimulate conversion of intestinal epithelial cells in to insulin-secreting cells | (Duan et al. 2015) |
| *E. coli* | GI tract | Mouse | *L. casei* | Expression of human lactoferrin (hLF) to protect the host against bacterial infection | (Chen et al. 2010) |
| Enterotoxigenic *E. coli* | GI tract | Rabbit | *E. coli* | Production of chimeric lipopolysaccharide capable of binding heat-labile enterotoxin | (Paton et al. 2005) |
| *H. pylori* | GI tract | Mouse | *B. subtilis* | Display of *H. pylori* urease B protein on spore coat | (Zhou et al. 2015) |
| HIV | Vaginal tract | Mouse | *E. coli* Nissle 1917 | Production of an antiviral peptide (HIV-gp41-hemolysin A) that block HIV entry | (Rao et al. 2005) |
| HIV | Vaginal tract | - | *Streptococcus* | Expression of a antiviral protein to block entry of HIV | (Giomarelli et al. 2002) |

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| | | | | into the vaginal mucosa | |
| Liver disease | GI tract | Rat | *E. coli* Nissle 1917 | Secretion of PQQ (an antioxidant) to prevent EtOH induced oxidative damage in liver and other tissues | (Singh et al. 2014) |
| Lyme disease | GI tract | Mouse | *S. typhimurium* | Expression of a major surface protein, OspA, to enable the production of anti-OspA antibodies | (Dunne et al. 1995) |
| Mucosal injuries | GI tract | - | *E. coli* Nissle 1917 | Production of human EGF and lipase ABC transporter recognition domain to enhance wound healing | (Choi et al. 2012) |
| Obesity | GI tract | Mouse | *E. coli* Nissle 1917 | Expression of NAPE, a lipid hormone which is released in response to food in the intestines | (Chen et al. 2014) |
| *S. enteritidis* | GI tract | Turkey | *E. coli* Nissle 1917 | Secretion of the bacteriocin microcin J25 | (Forkus et al. 2017) |
| *S. typhimurium* | GI tract | Mouse | *B. longum* | Display of Salmonella-antigen protects mice from lethal challenge of *S. typhimurium* in a murine typhoid fever model | (Yamamoto et al. 2010) |
| Streptococcal | Oral cavity | Rat | *Lactobacillus* | Expression of an antibody fragment which recognizes a streptococcal antigen | (Krüger et al. 2002) |
| Tetanus | GI tract and Nasal | Mouse | *L. plantarum* | Engineering Lactobacillus to produce a 50,000MW | (Shaw et al. 2000) |

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| | | | | fragment of tetanus toxin to immunise mice | |
| *V. cholerae* | GI tract | Mouse | *E. coli* | Production of chimeric lipopolysaccharide capable of binding cholera toxin | (Focareta et al. 2006) |
| *V. cholerae* | GI tract | Mouse | *E. coli* Nissle 1917 | Expression of cholera autoinducer 1 (CAI-1) to prevent *V. cholerae* virulence gene expression | (Duan & March 2010) |

**Table 2.** Complex live biotherapeutic systems

| **Target** | **Location** | **Model Organism** | **Chassis** | **Mechanism** | **Ref.** |
|---|---|---|---|---|---|
| Cancer | Liver | Mouse | *E. coli* Nissle 1917 | Engineered strain secretes an enzyme to cleave a substrate which can be detected in urine. | (Danino et al. 2015) |
| Cancer | Subcutaneous and Liver | Mouse | *S. typhimurium* | Synchronised population lysis to release triple combination of cancer therapeutics. | (Din et al. 2016) |
| Cancer | Liver | Mouse | *Salmonella* | Quorum sensing to only produce protein when population threshold has been reached, reducing off-target therapeutic delivery. | (Swofford et al. 2015) |
| Cancer | Subcutaneous | Mouse | *S. typhimurium* | Inducible expression of flagellin B (FlaB) in tumour tissue to stimulate an immune response. | (Zheng et al. 2017) |
| Colitis | GI tract | Mouse | *E. coli* | Use *inv* to invade intestinal mucosal cells and deliver therapeutic under control of inflammation-inducible promoter. | (Castagliuolo et al. 2005) |
| Enterohemorrhagic *E. coli* | - | *G. mellonella* | Bacteriophage | Delivery of CRISPR-Cas9 based, targeted antimicrobial. | (Citorik et al. 2014) |
| Fever | Subcutaneous and GI tract | Mouse | *E. coli* | Use thermo-sensitive promoters to detect fever and remote-control gene expression using ultrasound. | (Piraner et al. 2017) |

| | | | | | |
|---|---|---|---|---|---|
| Inflammation | GI tract | Mouse | *E. coli* | phage-lambda-based memory circuit to record markers of inflammation, stable for 200 days *in vivo*. | (Riglar et al. 2017) |
| Inflammation | GI tract | Mouse | *E. coli* Nissle 1917 | Detection of inflammation using tetrothionate and thiosulfate sensors. | (Daeffler et al. 2017) |
| Inflammation and glycosuria | - | - | *E. coli* | Thresholding, digitising and amplifying circuit for the sensitive detection of nitrogen oxides and glucose in pathological samples. | (Courbet et al. 2015) |
| *P. aeruginosa* | GI tract | *C. elegans* and Mouse | *L. casei* | Sense quorum molecule and produce bacteriocin and dispersin B for lysing. | (Hwang et al. 2017) |
| *S. aureus* | Skin | Mouse | Bacteriophage | Delivery of CRISPR-Cas9 based, targeted antimicrobial. | (Bikard et al. 2014) |

**Table 3.** Modules available for building computational synthetic circuits

| **Module function** | **Mechanism** |
|---|---|
| Boolean logic | Transcriptional (Nielsen et al. 2016), translational (Green et al. 2017), recombinase (Siuti et al. 2013) |
| Memory | Recombinase/integrase (Yang et al. 2014, Mimee et al. 2015), toggle switch (Zhang et al. 2014), CRISPR (Sheth et al. 2017) |
| Oscillator | Single cell (Stricker et al. 2008, Potvin-Trottier et al. 2016), population (Danino et al. 2010) |
| Amplifier | Recombinase/integrase (Courbet et al. 2015, Bonnet et al. 2013), transcriptional (*hrp*) (Wang et al. 2014) |
| Counter | Ribo-regulated transcriptional or recombinase cascade (Friedland et al. 2009) |
| Digitiser | Recombinase/integrase (Courbet et al. 2015, Rubens et al. 2016) |
| Filter | Spatial bandpass (Kong et al. 2017), recombinase-based bandpass (Rubens et al. 2016) |

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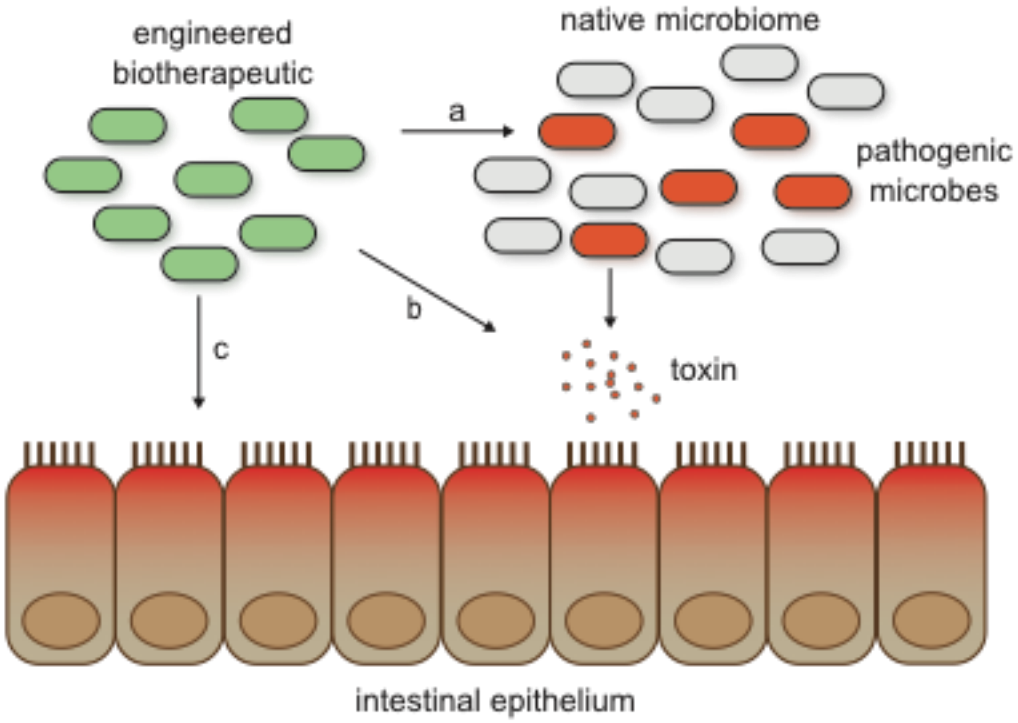
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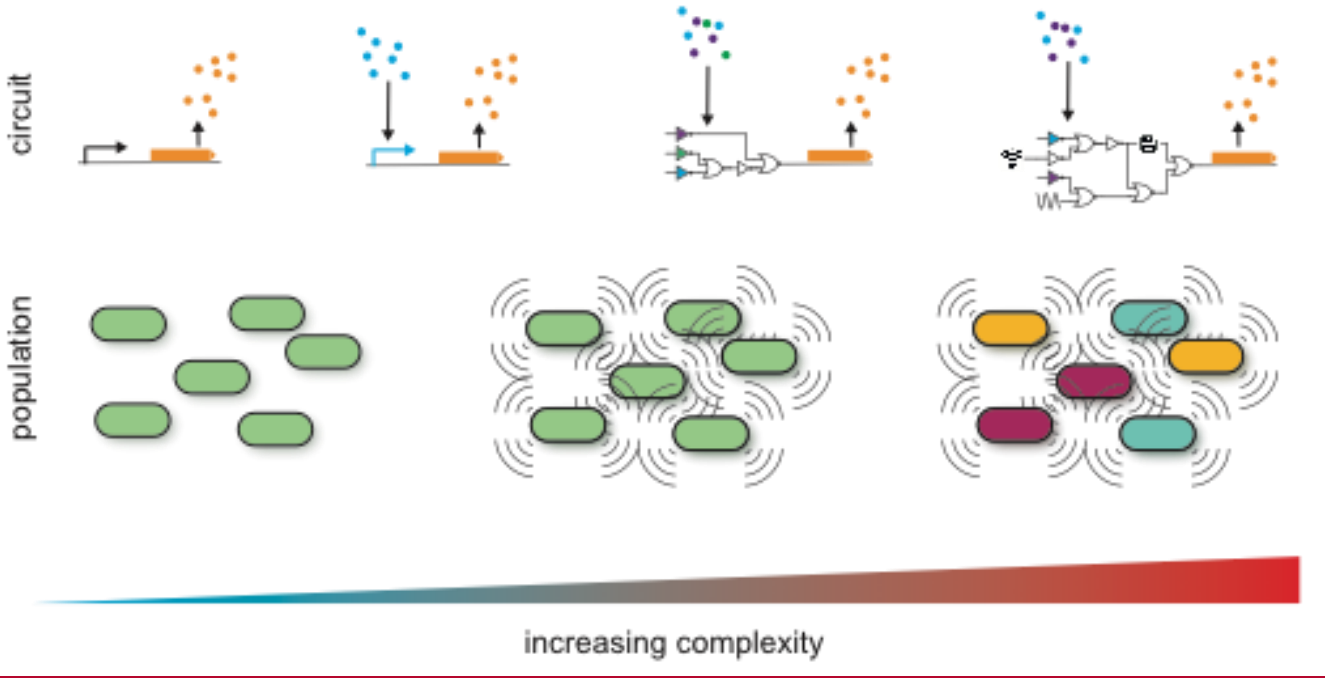
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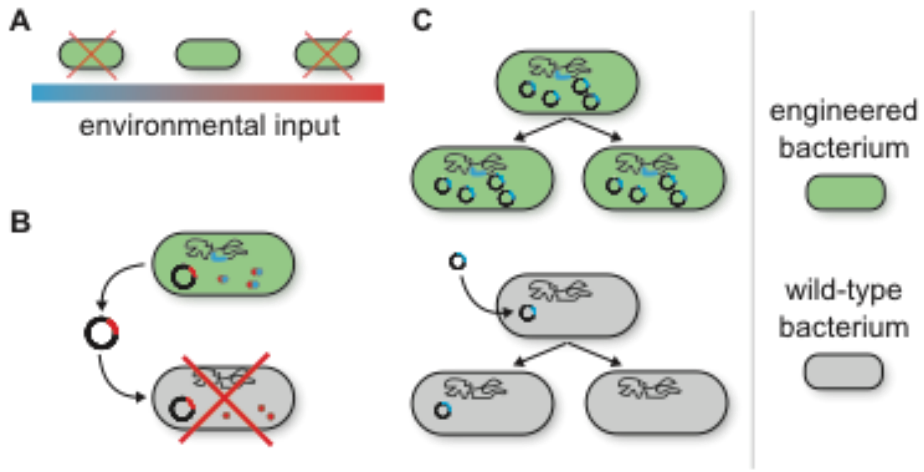
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