NEW TECHNOLOGIES: METHODS AND APPLICATIONS

Engineering bacteria for diagnostic and therapeutic applications

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Abstract | Our ability to generate bacterial strains with unique and increasingly complex functions has rapidly expanded in recent times. The capacity for DNA synthesis is increasing and costing less; new tools are being developed for fast, large-scale genetic manipulation; and more tested genetic parts are available for use, as is the knowledge of how to use them effectively. These advances promise to unlock an exciting array of 'smart' bacteria for clinical use but will also challenge scientists to better optimize preclinical testing regimes for early identification and validation of promising strains and strategies. Here, we review recent advances in the development and testing of engineered bacterial diagnostics and therapeutics. We highlight new technologies that will assist the development of more complex, robust and reliable engineered bacteria for future clinical applications, and we discuss approaches to more efficiently evaluate engineered strains throughout their preclinical development.

Prodrug
An inactive form of a drug that requires activation, often by enzymatic cleavage, before adopting its therapeutic form.

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doi:10.1038/nrmicro.2017.172
Published online 5 Feb 2018

Although the use of bacteria as therapeutics dates back more than a century[1–3], recent advances in synthetic biology are making the clinical use of genetically engineered bacteria as 'smart' therapeutics and diagnostics a tangible reality. Bacteria interact intimately with their niche in the human body, respond to a range of diseases and are well tuned by evolution towards detecting and producing physiological levels of biomolecules of interest. It is the combination of these features with the abilities of living systems, such as chemotaxis and biomolecule secretion, that could allow engineered bacterial systems to one day outperform traditional diagnostics and therapeutics.

Applications of bacteria as engineered therapeutics have targeted diseases as disparate as diabetes mellitus[4], inflammatory bowel disease[5], HIV infection[6] and cancer[7]. Examples include bacteria engineered to deliver therapies that otherwise degrade in the stomach or bloodstream[8–10], to achieve effective treatment with reduced systemic drug exposure[8,11–14], to activate the immune system in novel ways, including DNA-based and protein-based vaccination[15–17], and to record transient signals, such as reactive inflammatory metabolites, for noninvasive testing[18,19].

Despite this sky-is-the-limit potential, the number of engineered therapeutic bacteria that are tested in clinical trials remains limited[5,20–26] (see Supplementary information S1 (table) for details, including not yet published trials). It is thus timely to pause and reassess whether our approaches to preclinical development are sufficient to make good on the promise of engineered bacteria. This is particularly pertinent given the speed of technological development, including an ever-increasing ability to rapidly engineer bacterial strains, which outpaces the ability to test them[27,28]. Therefore, it would be highly desirable to establish strategies for evaluating promising technologies for their likelihood of future success in early phases of development.

In this Review, we summarize recent examples of engineered bacteria that have been preclinically or clinically tested and technological advances that will allow the development of more complex, safe and successful clinical applications. We focus on recent examples of rational gain-of-function genetic engineering and examples that have been tested in complex environments. We also propose areas that deserve particular attention when assessing preclinical engineered bacteria, particularly more rigorous testing and modelling of thresholds for successful therapeutic delivery and robustness of engineered functions when delivered to patients.

Clinical and preclinical applications

There are far too many examples of preclinically tested engineered bacteria to mention them all in this Review. Several broad categories of approach have been explored (FIG. 1; Supplementary information S2 (table) for specific examples): expression of a drug or molecule to act directly on the host; expression of an enzyme to locally cleave a prodrug; expression of an antimicrobial peptide or microbial toxin-binding protein; and activation of the immune system for immunization or to induce

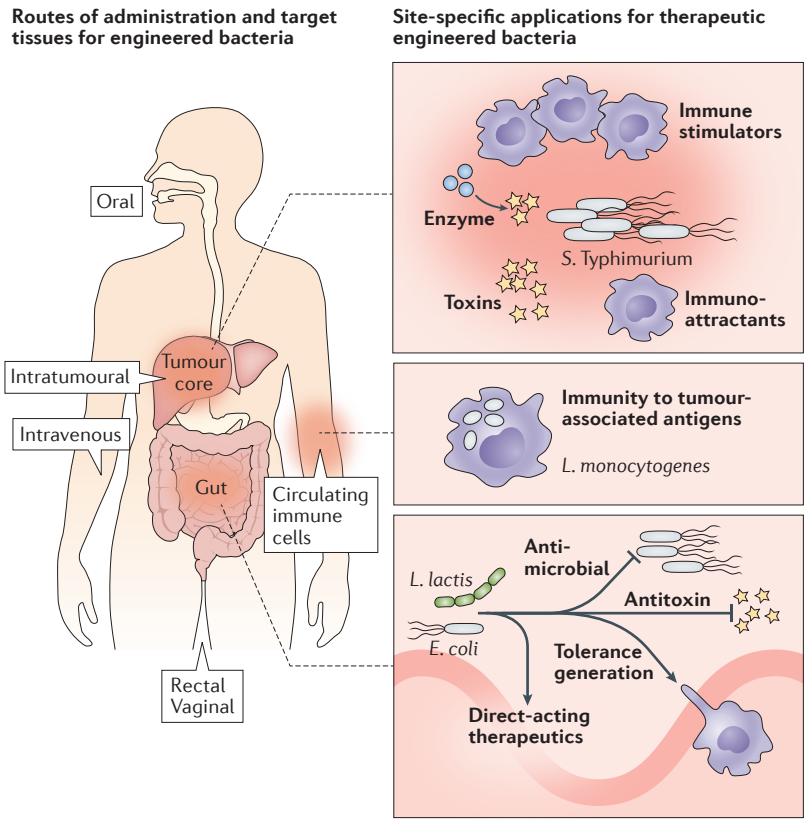

Fig. 1 | **Examples of strategies for bacterial therapeutic delivery.** Engineered bacteria have been administered in preclinical animal studies (Supplementary information S2 (table)) and human clinical trials (Supplementary information S1 (table)) through a range of routes to target different areas of the body, including the gut, circulating immune cells and solid tumours. *Salmonella enterica* subsp. *enterica* serovar Typhimurium, which can target the gut mucosa or, more commonly, hypoxic tissue at the core of tumours, has been engineered to release toxins[36], prodrug-cleaving enzymes[31] and immune stimulators and attractants[30] in attempts to clear cancer cells. *Listeria monocytogenes* has been used to immunize circulating immune cells against common cancer antigens[32]. In the gut, bacterial species such as *Lactococcus lactis* and *Escherichia coli* have been engineered to release therapeutics directly within the gut or throughout the body[46], to prime the immune system to tolerate autoantigens[47] or to protect their host from microbial attacks and toxins[51,53].

tolerance. Bacteria have also been engineered to deliver DNA or RNA to host cells with the same aims as those mentioned above[16,29]. The choice of bacterial strain (BOX 1) and genetic circuits (BOX 2; FIG. 2) depends on the targeted process and the site of action.

These strategies are being used in attempts to treat a range of diseases, including cancer[7,29–37], inflammatory disease[11–13,38–43], oral mucositis[44], hyperammonaemia (Supplementary information S1 (table)), dental caries[45], diabetes mellitus[4,46–50], gastrointestinal infections[9,10,51–53], HIV infection[6,54,55], obesity[56], allergies[57] and hypertension[58]. Human clinical trials are under way or have been completed for the first five of these diseases (Supplementary information S1 (table)).

Here, we focus on those studies that illustrate the particular advantages of using engineered bacteria for diagnosis and therapy, such as reaching remote sites of the body, low systemic exposure owing to local delivery and sensing of otherwise undetectable biomarkers.

Targeting therapeutic delivery. Bacteria have been used to deliver therapeutics to the human body. In particular, bacteria can deliver drugs that would otherwise be rapidly degraded in the bloodstream or during transit of the upper gastrointestinal tract[59]. Another advantage is the delivery of therapeutics to sites of the body that bacteria can live within but that are hard to reach by oral or parenteral drug delivery, such as the colon or the centre of tumours[15,30].

Bacterial delivery of glucagon-like peptide 1 (GLP1) to the gut exemplifies these benefits. GLP1 is a peptide hormone that has a range of actions; for example, its active form GLP1(7–37) increases insulin secretion of pancreatic cells[60]. The full-length protein GLP1(1–37), however, can reprogramme intestinal epithelial cells to respond to glucose and to produce insulin[61]. *Lactobacillus gasseri* that secreted GLP1(1–37) induced the differentiation of rat epithelial cells into functional glucose-responsive insulin-producing cells, and this improved glucose control in a rat model of diabetes mellitus[46]. Bacterial delivery was more effective at converting epithelial cells than previous efforts with injected peptide[61], likely due to the short half-life of the peptide in the bloodstream[60]. The insulinogenic epithelial cells are naturally extruded from the epithelium over the course of days[62]; thus the therapeutic effects of such a treatment would not be permanent.

Other treatment options for type 1 diabetes mellitus take advantage of the tolerogenic environment of the gut, which promotes immune tolerance to antigens presented by bacteria (reviewed in REF. 63). Recognition of several autoantigens, including proinsulin and glutamate decarboxylase 65 (GAD65; also known as GAD2), is associated with the destruction of pancreatic β-cells[64,65]. Generating tolerance to these antigens may protect from disease. Oral administration of *Lactococcus lactis* that secreted either proinsulin[4,47] or GAD65 (REF. 48) in combination with the tolerance-promoting cytokine interleukin-10 (IL-10)[66] prevented, and even reversed, β-cell destruction in mouse models of type 1 diabetes mellitus. Such bacteria have been prepared using clinical grade processes and tested in mice, which sets them up for future clinical tests[4].

Clinically, the most often used bacterial function is stimulation of dendritic cells by engineered *Listeria monocytogenes* (see Supplementary information S1 (table) for clinical trial details). *L. monocytogenes* engineered to express tumour-associated antigens activate both innate and adaptive immunity against tumours[67]. Attenuation of the bacteria makes them noninfectious and thus safe for delivery to the bloodstream. *L. monocytogenes* vaccines expressing the human papilloma virus (HPV) serotype 16 E7 oncoprotein (axalimogene filolisbac; ADXS11-001) or human mesothelin (CRS-207), which are known to be overexpressed and exposed on the surface of HPV-derived tumours or mesothelioma and ovarian and pancreatic adenocarcinoma, respectively, have reached phase III and II clinical trials, respectively (see Supplementary information S1 (table) for clinical trial numbers). HER2 (also known as ERBB2) and prostate-specific antigen (PSA)-expressing

Box 1 | **Factors governing the choice of a bacterial species for use in clinical applications**

The majority of engineered probiotics uses a small set of bacterial species (see Supplementary information S2 (table) for specific engineering examples). *Lactococcus lactis*[15], historically used in dairy product fermentation and bioindustrial processes, and *Escherichia coli*, a human gut resident bacterium and laboratory workhorse[51], are commonly used for oral delivery to the gut. Attenuated versions of *Salmonella enterica* subsp. *enterica* serovar Typhimurium, a pathogen capable of protein expression and immune stimulation in the human body, target the hypoxic tumour environment when administered systemically and the gut mucosa when provided orally[30,31]. Attenuated *Listeria monocytogenes*, another pathogen, activates the immune system through growth within circulating immune cells to elicit antitumour responses[32]. Besides these species, several species of *Lactobacillus* have also been used for gut, mouth and vaginal targeting[6,45,46] and *Clostridia* spp. for tumours[33].

Several properties must be taken into account when identifying the ideal host species for a clinical application. All species that have successfully progressed to clinical trials (Supplementary information S1 (table)), or late-stage preclinical testing, share an ease of genetic manipulation and rapid growth in the laboratory. The environmental niches a strain can grow in, for example, the gut, mouth, tumour core or macrophages, also determine choice of strains.

The bacteria used in the clinic to date either have short or no colonization capacity in humans (*L. lactis* and *E. coli)* or are capable of being cleared by routine antibiotic administration (*S.* Typhimurium and *L. monocytogenes*)[100]. *L. lactis*, for example, despite being metabolically active in the gut[136], does not colonize and thus is eliminated from humans in approximately 3 days[137]. *L. lactis* strains approved for human consumption have expressed their therapeutic genes, such as interleukin-10, from the native *thyA* locus[38]. This strategy forces dependence on thymine, a molecule found within the gut but not present once the bacteria are excreted. Following excretion from the pig gut, these bacteria underwent a 6-log reduction in viability over 60 hours, with live bacteria no longer detectable after 72 hours[38]. *S.* Typhimurium has been attenuated to prevent growth in the bloodstream while permitting growth in tumours. Examples of attenuation strategies include interrupting transport of lipids, purine and/or metabolites[30,138–140]. Similarly, *L. monocytogenes* strains administered intravenously or intratumourally in clinical trials have been attenuated to reduce growth outside of the tumour environment by removing virulence factors such as actA and internalin B[140,141].

Genetic engineering tools have advanced considerably since the earliest examples of engineered bacteria, as has the knowledge of how to culture many human commensal bacterial strains that were previously thought to be uncultivable[142]. These factors should allow a wider set of bacterial species, with a correspondingly broad set of attributes, to be used as therapeutics or diagnostics. Factors such as abundance, degree of colonization and interaction with the immune system[143] vary widely between strains and could be critical for the successful implementation of a given application. The continued development of tools for engineering more species, alongside data describing their natural physiological attributes, therefore remains an important area for research. *Bacteroides* strains have received recent attention as part of these efforts[144–146], as they are able to grow in high abundance in the human gut and thus may be suited to a range of otherwise impossible diagnostic and therapeutic applications. Abundance, however, is just one factor to be taken into account and may be not desirable for all applications.

L. monocytogenes are also being tested in ongoing phase I and II trials (see Supplementary information S1 (table) for clinical trial details). Although phase II testing of CRS-207 did not increase survival of patients with pancreatic cancer[26], more advanced therapies that target polyvalent combinations of tumour antigens are in various stages of phase I clinical trials and may be more efficacious (see Supplementary information S1 (table) for examples). Generally, owing to good biocontainment and safety profiles, these therapies could be tested in healthier and earlier-stage patients than these initial trials, which increases the chance of a good immune response and successful reversion of tumour growth. Certain bacterial species are well suited to live directly within and act upon the hypoxic tumour microenvironment[1–3]. This complements standard chemotherapy, in which it is difficult to access and effectively kill cells in the tumour core. For example, *Salmonella enterica* subsp. *enterica* serovar Typhimurium can grow successfully in tumours, with attenuated variants preventing off-target growth and toxic effects in other tissues and the bloodstream (BOX 1). Although no significant tumour regression was noted over placebo controls in phase I trials of attenuated bacteria alone[68], optimism remains for strategies that use *S.* Typhimurium to deliver anticancer agents to the tumour core. *S.* Typhimurium engineered to express cytosine deaminase, which converts the nontoxic prodrug 5-fluorocytosine into the anticancer agent 5-fluorouracil, was tested in a pilot study of three patients[69]; however, a proposed phase I clinical trial was ultimately abandoned due to slow patient enrolment[70].

Nevertheless, many preclinical studies have pursued this line of inquiry[7,30,31,34–37]. One recent example of note is an attenuated *S.* Typhimurium strain engineered to express the *Vibrio vulnificus* flagellin B protein, which is a Toll-like receptor (TLR) 5 agonist[7]. This leads to the synergistic activation of TLR5 and TLR4, which recognizes lipopolysaccharide on the *S.* Typhimurium surface. Therefore, the bacteria reduced tumour growth more effectively than either TLR4 or TLR5 agonists alone. These bacteria suppressed metastasis when tested in a mouse model of intestinal tumours, which suggests that future therapies will benefit from similar synergistic approaches[7].

Efficiency of therapeutic delivery. One of the greatest benefits of bacterial delivery is local targeting and thus reducing systemic exposure to therapeutics. Whereas direct comparisons between administration methods are difficult in clinical settings, several preclinical studies that involve anti-inflammatory cytokines in mice have looked at the question. One high profile example is secretion of IL-10 by orally delivered

Toll-like receptor
(TLR). A class of membrane receptors used by the innate immune system to recognize microbial molecules.

Metastasis
The spread of cancer cells from the original tumour to secondary sites around the body.

Colitis
Inflammation of the colon.

Trefoil factors
(TFFs). A family of peptides that are expressed at mucous membranes, including the gastrointestinal mucosa, and may have a protective role.

Recombinases
Enzymes that catalyse the excision, insertion, inversion or translocation of DNA between sites of specific DNA sequence.

Memory circuit
A genetic circuit that is designed to encode an extended response, or memory, following a transient cellular event.

Burden
The combined resources required by a cell to operate a given synthetic genetic pathway.

L. lactis, which reduced inflammation in mouse colitis models[11]. Clinical trials of systemic administration of recombinant IL-10 protein had been inefficacious and, although generally safe[71,72], one study found potentially harmful induction of the pro-inflammatory cytokine interferon-γ (IFNγ) at high IL-10 levels[73]. In mice, *L. lactis* achieved equivalent results as a systemically delivered recombinant protein with an estimated >10,000-fold lower IL-10 exposure, which raises hopes for more efficacy and less side effects of bacterial delivery than of direct protein administration in humans[11]. A clinically approved version of the strain, AG011[38], proved safe in a phase I clinical trial[5] and a phase II follow-up trial (Supplementary information S1 (table)). However, the therapy showed no significant benefit over placebo during the latter trial[74] and is no longer pursued by the company that developed it.

L. lactis strains that produced trefoil factors (TFFs) were also more effective at reducing gut inflammation than oral or rectal delivery of 1,200-fold higher levels of purified recombinant TFFs[13]. A clinically approved strain recently underwent phase Ib clinical testing as a mouthwash to treat oral mucositis[20].

Similarly, *L. lactis* that secreted anti-tumour necrosis factor (TNF) nanobodies (certolizumab)[14] or IL-27 (REF. 8) reduced inflammation in the mouse gut more than systemically administered proteins. IL-27 promotes expression of IL-10 from a subset of T cells[75,76]. Interestingly, systemically delivered recombinant IL-27 was unable to induce IL-10 production in the mouse colon, whereas *L. lactis* producing IL-27 elicited a strong response in this tissue[8]. Thus, this study provides an example of bacterial delivery that has local effects with low systemic exposure and of a new functionality that is unavailable for traditionally delivered therapeutics[8].

Interestingly, *L. lactis* producing IL-10 entered the inflamed, but not healthy, lamina propria through the paracellular route[38,39]. The increased permeability of the gut during inflammation could mean that healing reduces therapeutic delivery and effectiveness. However, there are examples of successful systemic delivery even through an intact, noninflamed mucosal layer. For example, *L. lactis* delivered GLP1(7–37) systemically more efficiently than a direct oral administration of recombinant control[49]. The development of specific gut absorption models will be crucial to maximize the potential for tailored engineering of future bacterial therapeutics. Because orally delivered drugs are largely absorbed in the small intestine, our current understanding of uptake in the colon is less developed[77], and there is a particular need to model uptake in the colon.

Sensors for diagnosis and therapeutic regulation. Bacterial sensing circuits are generally one-component or two-component systems (FIG. 3) and can respond to molecules that are relevant for health and disease, including cytokines such as IL-1β, TNF and IFNγ[78–80], hormones such as adrenaline and γ-aminobutyric acid[81,82], physiological stimuli such as temperature[83,84] and metabolites, such as fucose, which is expressed on small intestine epithelial cells during infection[85], or tetrathionate, which is produced during inflammation[86].

Bacteria can be designed as diagnostics for sensing and reporting on human diseases, either directly in the body[9,18,19] or after being exposed to clinical samples ex vivo[87]. Engineering bacteria to sense transient molecules that are degraded, modified or absorbed before exiting the gut, and thus cannot be easily captured and quantified by traditional noninvasive tests, is an exciting prospect for measuring novel biomarkers. Furthermore, diagnostic bacteria can also be equipped with additional functions, such as recording of measurements and therapeutic delivery, which hints at great future potential of this technology.

A commensal mouse *Escherichia coli* strain was recently engineered to sense exposure to tetrathionate[18], which is formed transiently in the gut during inflammation[86], and to record this exposure using a memory circuit[88] (FIG. 1a). The study demonstrated the benefit of engineered bacteria as noninvasive reporters for transient events in the body. The combined use of a commensal bacterial strain with circuits that are limited in their total output so as not to place undue stress, that is, burden, on the bacteria allowed monitoring of inflammation in the mouse gut for over 6 months. Moreover, the memory circuit integrated the signal over a number of days, which makes the bacteria more reliable sensors of fluctuating inflammation, for example, during chronic inflammation, which can flare and wane. This permitted detection of the inflammatory state even after the original tetrathionate signal had disappeared[18].

Box 2 | **Examples of commonly used genetic circuits**

The building blocks for synthetic biology and genetic engineering are modules of DNA that encode functional proteins (the therapeutic molecules themselves) and modulate transcription and/or translation (repressors, activators and promoters). To date, bacterial therapeutics have generally expressed single proteins constitutively or under simple inducible systems (see Supplementary information S2 (table) for examples of each). Recent decades, however, have seen the development of far more complex synthetic genetic circuits, which are able to intricately control gene expression responses (FIG. 1). Applying these advances in circuit design should present opportunities for new functionalities, safety features and control mechanisms for engineered probiotics.

Memory circuits and counters can record cellular events by causing changes to protein expression that are maintained, and reported on, by feedback loops[88,147,148] or permanent changes to the DNA made by recombinases[148–151] (FIG. 1a). These circuits can be used to engineer diagnostics[18,144] and could also control sustained therapeutic expression following a given signal, or a given number of signals in the case of counters. Genetic logic gates, analogous to the Boolean logic gates used in electronics, turn on expression of a controlled element only in the presence of a determined combination of inputs[152] (FIG. 1b). For example, AND gates permit expression only when two or more specific signals are present. These circuits could integrate information from several sources to obtain an extra level of information for diagnostics or specificity for on-demand therapeutics. Several advances have recently been made in the design and complexity of available logic circuits[28,153]. Recording the combined experience and order of events that a bacterial population is exposed to is even possible using state machines, in whichoutcomes depend on not just the combination of inputs but also their order of exposure[154].

Other core circuits such as genetic oscillators[155,156] and cell-to-cell communication pathways[92] have also been successfully optimized and used in vitro and in vivo[37,157]. These circuits could be used in combination with each other to generate even more complex outcomes. Variable expression therapeutics, amplifiers and synthetic functional consortia that exploit features of several host bacterial species are just the start of what should theoretically be possible.

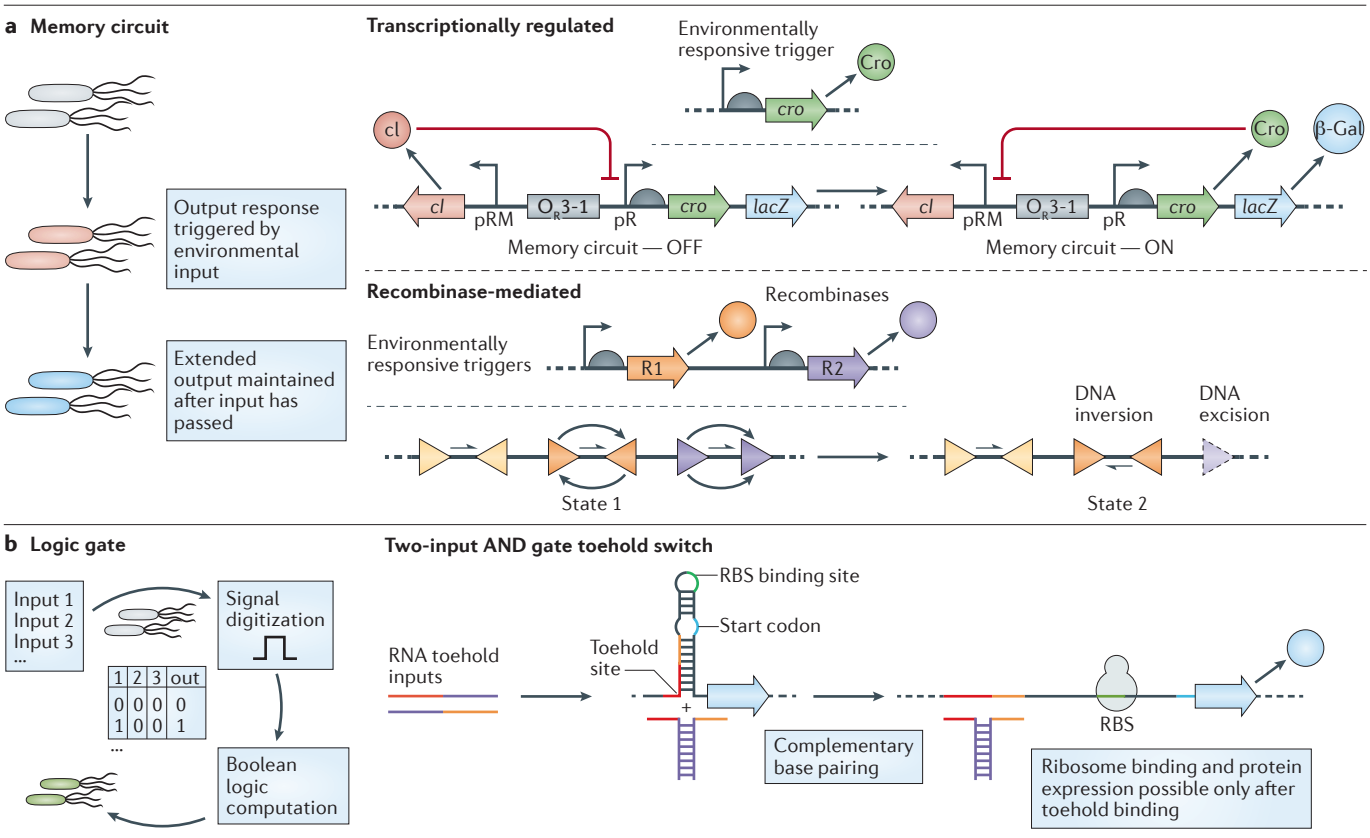

Fig. 2 | **Examples of recently developed synthetic circuits.** Synthetic biology uses basic DNA and RNA building blocks to create complex circuits that provide cells with new capabilities. Two circuits that have received attention for their potential application in bacterial diagnostics and therapeutics are memory circuits and genetic logic gates. **a** | Memory circuits can convert a temporary signal into a longer-term cellular response. Two examples have been tested in the context of preclinical bacterial diagnostics: (top) a transcriptionally regulated memory circuit derived from λ-phage[18,88] that maintains stable OFF and ON states by expression of repressors to the opposite state and (bottom) recombinase-mediated memory that uses the ability of these enzymes to invert or excise DNA, which creates permanent recordings of temporary cellular events. The latter was created as an array of 12 invertible reporter sequences that can be read by PCR[144,150]. **b** | Genetic Boolean logic gates allow cellular computation by allowing a given output only under a defined combination of inputs. One recent example is the development of RNA toehold switches able to compute logic[153]. The example of a two-input AND logic gate is shown: sequences upstream of a gene of interest cause a hairpin loop to form, which blocks downstream protein expression. Binding between complementary sections of the two-input RNAs creates a toehold trigger, which can bind to complementary sequences in the toehold switch to allow ribosome binding and translation to occur. In this way, reporter gene expression is activated only when the expression systems for input RNA 1 AND input RNA 2 are active. cI, repressor cI; Cro, regulatory protein; β-Gal, β-galactosidase; O_R3-1, operator right; pR, promoter, pRM, promoter; RBS, ribosomal binding site.

Sensors for tetrathionate and its precursor thiosulfate were also used as bacterial diagnostics in another recent study, this time using flow cytometry to measure a fluorescent protein readout[19]. *E. coli* Nissle 1917 that were engineered to carry the sensors detected increased thiosulfate, but not tetrathionate, during chemically induced colitis in mice[19]. Without direct in vivo metabolite measurements, it is not possible to determine whether tetrathionate was not detected because of low sensitivity of its sensor or because tetrathionate was not produced in this colitis model[19]. An important next step is to understand the relationship of tetrathionate and thiosulfate to disease in humans, for example, through mass spectrometry analysis of gut biopsy samples.

Preliminary studies have also investigated the possibility of bacteria to detect and report on the presence of tumours. One study tested the ability of an orally administered probiotic *E. coli* Nissle 1917 strain to cross the gut in mice and preferentially grow within metastatic liver tumours[89]. The bacteria were engineered to express an enzyme that could cleave a systemically administered substrate, which leads to a colour change that was detected in the urine[89]. Although translocation of *E. coli* across the gut has been detected in humans, it occurs in only a small percentage of healthy individuals, which limits the use of *E. coli* as a systemic diagnostic[90]. Another study engineered attenuated *S.* Typhimurium to secrete a fluorescent reporter, ZsGreen, during growth within a tumour[91]. Modelling based on tests in a tumour-on-a-chip device suggested that detection of tumours >2,500 times smaller than are currently detectable by tomographic techniques could be possible[91]. Technologies such as these could substantially improve the early detection and treatment of cancer and metastasis.

Other diagnostic approaches have also been explored in preclinical studies. Several systems have been designed to detect infection through quorum sensing (FIG. 4), a mechanism that many bacteria use to sense nearby bacteria (reviewed in REF. 92). Bacteria that detect native quorum signals from *Pseudomonas aeruginosa*[93–95] and *Enterococcus faecalis*[96] have been tested in vitro. The most comprehensive testing of a quorum signal-responsive engineered bacterial strain to date has been a sense-and-respond therapeutic using *E. coli* Nissle 1917 (FIG. 4b). The bacteria detected the *P. aeruginosa* quorum signalling *N*-acyl homoserine lactone molecule, $3OC_{12}HSL$, and responded by producing an antibiofilm factor, dispersin B, an antimicrobial peptide, s5 pyocin, and a lysin, E7 (REF. 9). Pre-colonization of mice or post-colonization of *Caenorhabditis elegans* with the engineered bacteria reduced gut infection with *P. aeruginosa*. We expect that many more sense-and-respond bacterial systems will be developed in the coming years.

Quorum sensing has also been used to control the expression of engineered functions and to restrict expression to relevant body sites. For example, quorum sensing was used to induce the production of GFP in systemically administered *S.* Typhimurium[97] (FIG. 4a). High-level induction was restricted to tumours, in which the bacteria accumulate, and expression was prevented in the liver, in which only low-density off-target bacterial growth occurs[97]. This system promises to reduce off-target effects when delivering cytotoxic drugs and therefore is an important step towards limiting side effects of treatment. A second example involved density-dependent expression of several different factors, including antitumour agents and a gene that induced bacterial lysis[37]. The quorum sensing circuit led to synchronized lysis of a large percentage of the engineered *S.* Typhimurium population in a mouse tumour. Lysis not only delivered antitumour therapeutics but also helped to maintain relatively low overall colonization levels in the body, a factor that may further reduce unwanted side effects that accompany bacterial therapies. Further methods for restricting the location and timing of therapeutic expression include induction by the hypoxic conditions at the core of a tumour[36], control by externally administered compounds[98] or signals such as ultrasonagraphy[83].

Challenges and new technologies

The regulatory frameworks that govern the approval and use of engineered bacterial therapeutics and diagnostics in humans are still evolving. The European Medicines Agency Committee for Advanced Therapies regulates many engineered bacteria for clinical applications as gene therapy medicinal products[99]. The Center for Biologics Evaluation and Research at the US Food and Drug Administration (FDA) is most likely to cover the same bacteria. The recently updated FDA Recommendations for Microbial Vectors used for Gene Therapy, although not necessarily directly applicable for all purposes, provide valuable insight into the general requirements for progressing an engineered bacterial strain into the clinic[100].

Quorum sensing
A common mechanism by which bacteria naturally sense the local population density of their own or other bacterial species to enable density-dependent cellular responses. Bacteria produce and sense a specific quorum sensing molecule. Constant secretion ensures that concentrations only reach threshold levels and change downstream transcriptional profiles when many bacteria are present in the population.

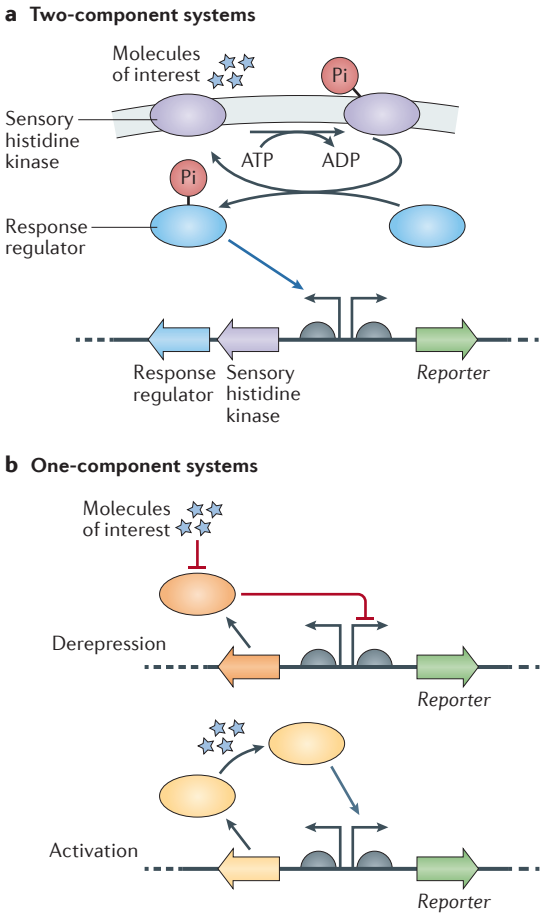

Fig. 3 | **Bacterial sensing through one-component and two-component systems.** Bacteria can function as biosensors, using natural or synthetically constructed pathways to sense their surroundings. Bacterial sensing and signalling pathways are usually either one-component or two-component systems. **a** | Two-component systems comprise a sensory histidine kinase, which is often membrane bound, and a response regulator. Following ligand binding to the sensory histidine kinase, it phosphorylates the response regulator, which in turn activates gene expression in its phosphorylated state. **b** | One-component systems, by comparison, involve transcription factors that bind to both the ligand of interest and the regulatory DNA sequence. Ligand binding by these proteins can either derepress (top) or activate (bottom) downstream gene expression. Pi, phosphate.

In this section, we explore the challenges, new technologies and opportunities for engineered bacteria in the clinic. Three factors are key for the success of an engineered probiotic — its functional stability, clinical potency and safety. Although satisfaction of all these aspects is not essential for preclinical success, we believe that an appreciation for these factors is beneficial when approaching, designing and evaluating any new probiotic or technology.

Circuit burden: stability, mutation and loss of function. Functional stability is important for all clinical applications of engineered bacteria. The burden placed upon engineered cells by the costs of expressing synthetic

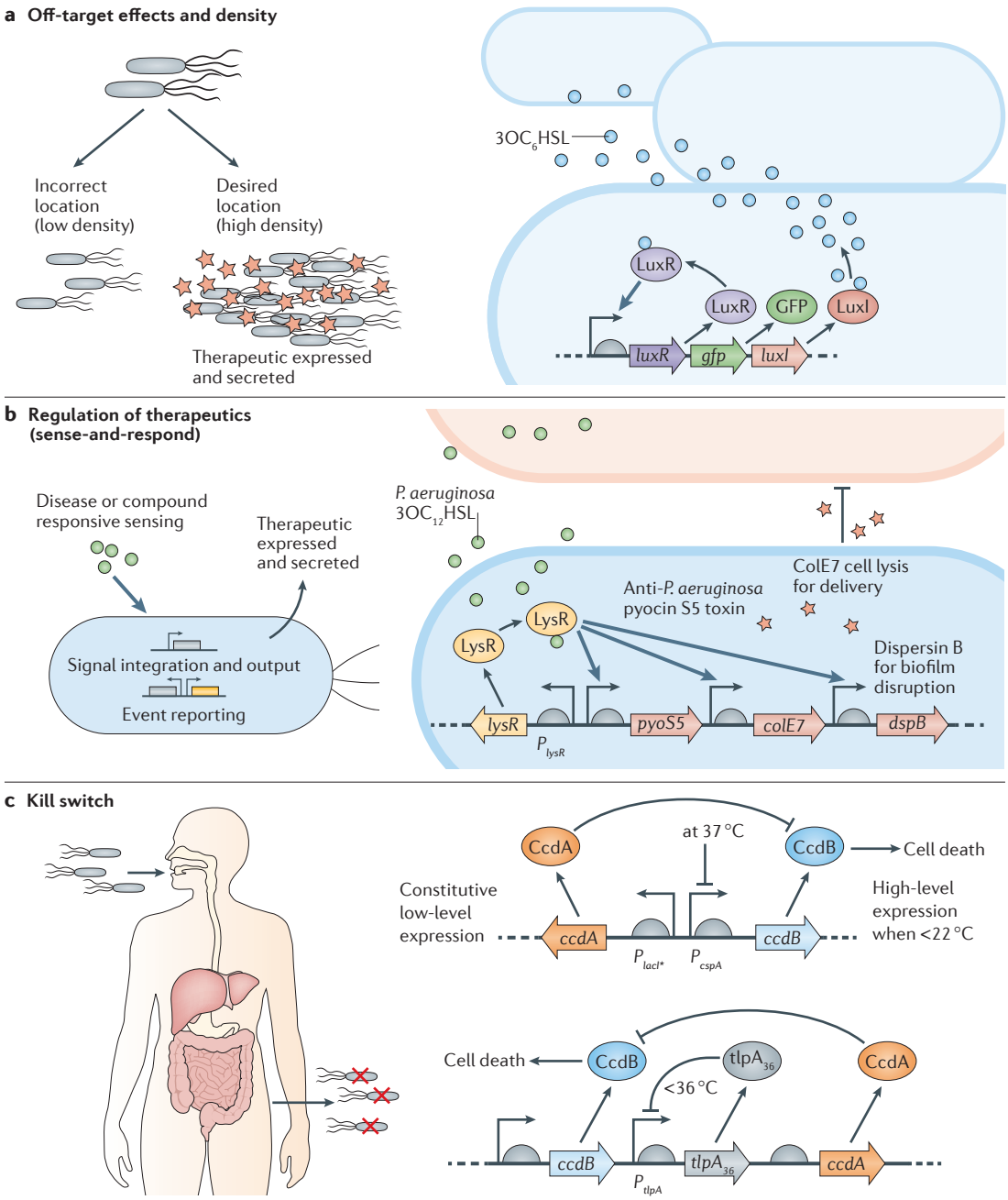

Fig. 4 | **Control, biosafety and biocontainment strategies for therapeutic bacteria.** The regulation of the function and viability of engineered bacteria is important for biosafety and biocontainment. **a** | One strategy for reducing potential off-target toxicity is restricting therapeutic expression to sites of disease, for example, the tumour core. A recent study used quorum sensing to couple *Salmonella enterica* subsp. *Enterica* serovar Typhimurium cell density to expression[97]. Expression was turned on only in tumours, as density was high there, and remained off in off-target locations, such as the liver, in which bacterial cell density is low. Other strategies have also been used to achieve this goal, for example, using the hypoxic environment of the tumour core to control expression[36]. **b** | Sense-and-respond systems use disease-specific signals for on-demand therapeutic release. Quorum signalling through the *N*-acyl homoserine lactone $3OC_{12}HSL$ in *Pseudomonas aeruginosa* has been used to induce expression of a group of genes for the delivery of an antibacterial toxin and a biofilm dispersion factor[9]. **c** | Kill switches are designed to provide biocontainment by preventing bacterial growth following excretion from the body, which reduces the chance of escape into natural bacterial populations or transfer between individuals. Two recent studies have designed kill switches based on the temperature shift that bacteria experience upon exit from the gut. Both studies engineered systems to induce expression of the ccdB toxin at low temperatures, killing the host bacteria[83,84]. In one example (top), low temperatures caused derepression of the toxin gene, which overwhelmed the low levels of ccdA antitoxin present in the cell[84]. In the other example (bottom), reduced temperatures caused $tlpA_{36}$ to repress expression of the ccdA antitoxin, permitting the toxic action of ccdB. $3OC_6HSL$, *N*(3-oxohexanoyl) homoserine lactone; LuxI, acyl-homoserine lactone synthase; LuxR, transcriptional activator protein.

circuits[101] can reduce the growth rate and total molecular output of a cell[102]. In turn, this could provide a selective pressure for loss-of-function escape by point mutation, large-scale insertions or deletions, or entire loss of plasmid-based circuits. The selective pressure and the rate of mutations are likely highest in stressful and competitive environments, such as those encountered within the body, and the risk of loss of function increases with the duration of administration and colonization.

Several examples of these mutations and losses have been documented in preclinical studies following delivery of engineered therapeutic bacteria to a host or even during in vitro growth. During transit of the rat gut, 40–65% of *L. paracasei* BL23 cells lost their engineered plasmids[50]. Similar loss of luminescence gene-carrying plasmids occurred in *S.* Typhimurium during 60 hours of growth in a mouse tumour model[103]. Similar plasmids with added retention mechanisms remained in 90% of bacteria during 72 hours of in-tumour growth[89]. However, another recent application of engineered *S.* Typhimurium carrying plasmids with the same retention mechanisms showed evidence of functional escape, possibly through plasmid loss or mutation[37]. Components that encode cell death mechanisms, such as kill switches[83,84,104] (see below), are under particularly strong negative selection. Indeed, large-scale deleterious insertions and point mutations were found in a recent example[104].

By contrast, plasmids encoding the HPV-16 E7 gene were retained using a D-alanine complementation-based retention mechanism in a high percentage of *L. monocytogenes* cells up to 120 hours after systemic administration in mice[105]. *Lactobacillus jensenii* maintained its ability to express the anti-HIV cyanovirin-N protein during 6 weeks of vaginal colonization in a rhesus macaque model[6], although no individual colony testing or genetic mutation analysis was performed to rule out low levels of mutation. The functional and genetic maintenance of synthetic diagnostic circuits in bacteria that colonized the mouse gut for over 6 months was also recently demonstrated[18]. These latter studies show that, at least under specific conditions, a burden can be small enough to avoid negative selection, even over extended growth within the host.

Whereas the outcomes of burden are hard to rationally predict and protect against through design[106], there are computational[107] and experimental[102] methods to predict and estimate levels of burden. Tuning total and relative expression levels can reduce the impact of a burden[108,109]. Most recently, a CRISPR–dCas9-based system has been developed that automatically restricts expression of a synthetic circuit when cells initiate natural responses to burden[110]. This resulted in both higher growth rates and higher total circuit output as measured by fluorescent protein expression compared to unregulated controls[110]. Tools such as these could revolutionize both the stability and therapeutic capacity of bacteria in vivo.

Estimating therapeutic potency. The rapid expansion of genetic engineering techniques and their increasing speed and ease-of-use for large-scale strain generation necessitate screening and selecting promising variants with some level of certainty before testing individually in an animal model. A realistic in vitro estimate for the in vivo output of an engineered circuit, usually the total amount and concentration of an expressed biologic, is important when predicting its clinical efficacy. This value should be compared with disease-specific estimates for the therapeutic or diagnostic requirements.

However, expression is usually measured during in vitro growth in an ideal medium — an environment far from the realities of the competitive and often oxygen-poor and nutrient-poor mammalian environment. In the body, expression levels from a strain may be suboptimal, and the number of bacterial cells varies between body sites. These factors add to the already complex landscape of pharmacokinetics, pharmacodynamics and bioavailability that any drug faces. Development of convenient and realistic in vitro testing environments will be important for achieving better accuracy. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) fermenter system[111], organ-on-chip microfluidics[112] or ex vivo organ growth techniques[113] are all examples of systems that could one day allow more accurate quantification and estimation of circuit output. However, the throughput of each of these systems is currently limited by complex setup and testing.

Biocontainment and biosafety. Administration of clinical probiotics to patients inherently involves release of those bacteria into the environment. Biocontainment and biosafety are therefore key factors for the approval of therapeutics for clinical testing and use[100]. Factors such as prevention of transfer between individuals, control of growth and therapeutic expression, and prevention of transfer of genes into and out of the engineered strain are all important (FIG. 4).

The choice of the bacterial strain and how it is attenuated (BOX 1) are important for its biocontainment and biosafety profiles. So far, serious adverse events have been rare in clinical trials. Two patients contracted listeriosis from CRS-207, which has led to revisions in administration protocols for ongoing trials; however, these cases represent a very small percentage of the patients who have received this therapy[114]. We expect that the use of longer-term colonizing bacteria will become increasingly attractive and feasible, even in humans. To this end, isolating a mouse commensal *E. coli*[88] has allowed testing of extended gut colonization in mouse models[18].

Far greater control over these safety factors will likely be necessary for any long-term applications of engineered diagnostics and therapeutics. To this end, several promising approaches have recently been tested in preliminary studies, particularly kill switches[83,84,104] (FIG. 4c) and genetic firewalls[115–120]. These systems aim to prevent growth of engineered bacteria if they escape from the desired clinical environment. By their nature, however, there is strong selective pressure for mutation of any circuit that encodes for the death of an organism.

Kill switches that respond to temperature changes were recently developed[83,84]. Excretion of *E. coli* from mice and the following temperature-dependent expression of ccdB toxin resulted in a 3–4 (REF. 83) or 5–6 (REF. 84) log reduction in growth. While yet to be tested

Kill switches
Circuits used as safety mechanisms to prevent incorrect activity of an engineered bacterial strain, usually by attempting to kill it or to prevent engineered functions.

Probiotics
Live microorganisms that are beneficial to health.

Genetic firewalls
Changes to the underlying genetic code of an organism in an attempt to prevent the possibility of effective genetic exchange between the engineered strain and other bacteria in the environment.

Log reduction
Measure of reduced bacterial growth based on the logarithm (base 10). Every additional log reduction therefore corresponds to tenfold lower growth or survival.

Box 3 | **Important factors for assessment of preclinical bacterial diagnostics and therapeutics**

- Choice of host bacterium: is it a wild strain, a laboratory strain or an even more attenuated cloning strain? What is the relevance and reasonable application to the model it is being tested in or the proposed application?
- Choice of expression systems: are the circuits on plasmids (with or without mechanisms for maintenance) or chromosomally integrated? If they are on a plasmid, what is the plasmid copy number? Could the copy number or expression level be incompatible with the proposed application?
- Control of engineered circuits: is the control logical for the application (for example, if provision of an exogenous ligand is required, is the ligand available in vivo and safe to give to an animal or human)?
- Stability of the system: what is the burden of the system on the bacteria? Have the relative growth rates of the strains been tested and stated? If a plasmid is used, has the plasmid loss rate been tested? Have functional and/or genetic mutation of the circuit been tested? Was the testing performed in a relevant growth condition (for example, nutrient rich or poor, anaerobic or aerobic), ideally in vivo and for a relevant time period for the application? Has the stability of the system been measured?
- Translatability: is the clinical reasoning sound? Are there good reasons to believe the sensitivities of the application (for example, input molecule for diagnostics or efficacy thresholds for therapeutics) are achievable by the bacterial system? Have they been defined in relevant conditions? Have they been tested and stated?
- Models for testing: has the system been rigorously tested for functionality in a strong model system? Are multiple conditions and hosts tested for general applicability of circuit claims? Has it been tested in relevant in vitro growth conditions? Has it been tested in a complex in vitro mimetic system or an animal model? If so, is the model a strong one, and does it provide further application or stability-related information (or is it a 'toy system')?
- Study design: is the study otherwise sound? Are there sufficient numbers in all experiments, including animal and in vitro mimetic systems experiments, properly executed statistics and well-designed controls?
- Reporting: if submitted for publication, have all synthetic DNA constructs been adequately reported for future replication?[158]

in vivo, another study also recently engineered two types of kill switches that achieved as high as 6–8 log reduction in growth[104]. Each circuit was controlled by exogenous signal molecules. The PASSCODE system responded specifically to any combination of three exogenous factors, isopropyl-β-D-thiogalactoside (IPTG), galactose and cellobiose[104]. Conversely, the DEADMAN switch responded to the removal of anhydrotetracycline[104]. However, both kill switches were highly susceptible to mutation, even without repeated exposure to death-inducing signals[104].

So far, the only successful strategy to prevent detectable escape from containment measures has been synthetic auxotrophy. Two recent studies built upon an *E. coli* strain in which the genome was modified so that one codon was recoded to match a non-native tRNA synthetase corresponding to an exogenously provided non-native amino acid[115]. By engineering several essential proteins to structurally depend on the non-native amino acid for activity, the bacteria were forced to grow only in the presence of the non-native amino acid[117,119]. This strategy prevented growth of the bacteria in the absence of non-native amino acid below the detection limits of 10^{11} and 10^{12}, respectively[117,119]. For this approach to be clinically applicable, a sufficient amount of the non-native substrate needs to be administered, which might be difficult in humans. Otherwise, engineered bacteria will not be capable of competitive growth in the complex environment of the body. Future in vivo testing of these recoded organisms is thus of great importance to assess their potential for human administration.

Building upon these recoded organisms, there are several examples of more elaborately genetically recoded bacteria that promise to increase biocontainment and reduce the interaction between native and engineered microbiota[116,118,120]. Most recently, it was demonstrated that replacement of 200 kb, which corresponds to over 4% of the *S.* Typhimurium genome, with recoded, synthesized DNA resulted in a strain without growth defects compared to the original native strain[116]. These studies demonstrate it likely will be possible in the near future to construct whole bacterial genomes with at least one, if not several[120], re-assigned codons, and this could feasibly result in organisms that are capable of competitive growth in the human body[116,118,120]. Although further research and development is necessary to realize the full potential of these technologies, the progress to date gives us optimism that recoded organisms will one day contribute to the safety of clinically used engineered bacteria.

Opportunities and outlook

Adding functionality: the integration of synthetic biology. The future potential for synthetic biology to contribute to clinical diagnostics and therapeutics is both profound and exciting. Therapeutic bacteria, especially those tested in clinical trials, have just started to scratch the surface with respect to the complexity of engineering used. Integration of complex synthetic control circuits (BOX 2; FIG. 1), such as memory circuits (FIG. 1a), logic gates (FIG. 1b) and state machines, which can link the expression of a therapeutic to a specific environmental context, promise far greater control and biological reach for the next generation of clinical strains.

At the same time, synthetic biology has advanced our understanding and control over protein expression and degradation[121,122]. It has led to the rapid automation of construct design, building and testing[27] and to the development of methods for fast construction and variation of large DNA circuits[123–125]. These tools allow engineering of synthetic DNA constructs up to hundreds of kilobases long, which makes it possible to engineer therapeutic

Logic gates
Circuits that use Boolean logic to activate an output only when a given combination of inputs is present.

State machines
Devices able to exist in one ofseveral unique states depending on the history of its inputs.

bacteria with complex biosynthetic pathways[126]. Rapid mutational testing, directed protein evolution and the potential to test and deliver peptides designed in silico[127] provide opportunities to move beyond natural therapeutic designs towards novel therapeutic functions.

The identification of disease-responsive inducible promoters is an area of need. Whereas there are numerous preclinically tested engineered bacterial therapeutics, the sensing arm of 'smart' therapeutics is currently lagging. Disease-relevant inducible promoters, particularly virulence factors from pathogens, were traditionally investigated using standard molecular methods, such as fluorescent, luminescent and colorimetric assays, but more recently have been screened through transcriptomics and promoter-trap experiments[128–131]. However, the integration of identified disease-responsive promoters with other synthetic circuits can be complex. Continued efforts to allow predictable circuit function across different genetic contexts[121] and to more effectively screen promoters in functional synthetic circuits may be beneficial.

Synthetic biology also provides opportunities to use novel inputs, such as light[132,133], to control circuits. Tuning of sensor properties[19,83] and the development of hybrid or orthogonal transcription systems[134] provide further opportunities to expand this repertoire. These inputs are not limited to proteins as sensors; for example, the presence of specific endogenous RNA expression can be sensed through induction of the expression of a reporter gene[91].

A disciplined approach. It is easy to get carried away imagining the exciting future of bacteria in the clinic and forget the rigorous steps necessary to realize advances that will truly benefit humankind. Thus, our message is as much one of caution and discipline as of excitement and imagination, adding to similar sentiments previously expressed regarding drug discovery more broadly[135]. Accordingly, we recommend a set of criteria (BOX 3) to help judge the potential for the true impact of preclinical research.

Projects should be embarked on with a strong clinical rationale and some knowledge of the input and output levels that are needed for therapeutic and diagnostic success. In vitro testing should then be undertaken with these in vivo targets in mind. It will be important to develop more relevant in vitro testing systems and to optimize pharmacodynamics models specifically for engineered bacteria. For both efficacy and safety considerations, we should also embrace testing the burdens that circuits place on a system; any resulting mutation rates and/or growth defects that circuits cause should be measured and reported at publication. Testing in vivo should be done with rigour and with carefully chosen animal models rather than systems in which little is learned beyond what can be learned in an in vitro test.

Many factors continue to complicate the engineering of bacteria to create clinically robust therapeutics and diagnostics. Nevertheless, living organisms have unique abilities: they can integrate and respond to new signals and produce novel therapies; they can access parts of the body and interface with molecules inaccessible to standard technology; and they can produce and deliver biological therapeutics directly to the site of disease. By capitalizing on these features, bacterial therapeutics and diagnostics have the potential to radically change how we care for patients.

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Acknowledgements
The authors thank A. Naydich for critical review of the manuscript. D.T.R. is supported by a Human Frontier Science Program Long-Term Fellowship and a National Health and Medical Research Council (NHMRC) RG Menzies Early Career Fellowship from the Menzies Foundation through the Australian NHMRC.

Author contributions
D.T.R. researched data for the article and wrote the article. D.T.R. and P.A.S. substantially contributed to the discussion of content and reviewed and edited the manuscript before submission.

Competing interests statement
The authors declare no competing interests.

Reviewer information
Nature Reviews Microbiology thanks Matthew Chang, Neil Forbes and Ting Lu for their contribution to the peer review of this work.

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