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In situ biomolecule production by bacteria; A synthetic biology approach to medicine

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ABSTRACT

The ability to modify existing microbiota at different sites presents enormous potential for local or indirect management of various diseases. Because bacteria can be maintained for lengthy periods in various regions of the body, they represent a platform with enormous potential for targeted production of biomolecules, which offer tremendous promise for therapeutic and diagnostic approaches for various diseases. While biological medicines are currently limited in the clinic to patient administration of exogenously produced biomolecules from engineered cells, *in situ* production of biomolecules presents enormous scope in medicine and beyond.

The slow pace and high expense of traditional research approaches has particularly hampered the development of biological medicines. It may be argued that bacterial-based medicine has been 'waiting' for the advent of enabling technology. We propose that this technology is Synthetic Biology, and that the wait is over. Synthetic Biology facilitates a systematic approach to programming living entities and/or their products, using an approach to Research and Development (R&D) that facilitates rapid, cheap, accessible, yet sophisticated product development. Full engagement with the Synthetic Biology approach to R&D can unlock the potential for bacteria as medicines for cancer and other indications.

In this review, we describe how by employing Synthetic Biology, designer bugs can be used as drugs, drug-production factories or diagnostic devices, using oncology as an exemplar for the concept of *in situ* biomolecule production in medicine.

Keywords: Microbiome, engineering, gene therapy, tumour, drug delivery

1 INTRODUCTION

1.1 BACTERIAL-PRODUCED ANTI-DISEASE AGENTS

In '*ex vivo*' settings (industrial fermentation), engineered bacteria have long been used to produce recombinant proteins, such as insulin, human growth hormone and others [1, 2]. More recently, precedents have been set for bacterial production of small molecules and chemical entities for pharmaceutical uses [3, 4]. The commercial production of semi-synthetic artemisinin is frequently held up as the first demonstration of the potential of synthetic biology for the development and production of pharmaceutical agents [3]. *E. coli* has been the bacterium of choice for the majority of agent production systems to date, although the range of bacterial genera is recently increasing with advances in engineering technology, and the capacity of different genera to provide more optimal agent production depending on the agent [5]. Given that *E. coli* and other bacteria can naturally, or be induced to, colonise different parts of the body, we ask if there is potential to 'skip the middle man', where the producing bacteria themselves may represent the final 'drug' product for administration to patients. In this context, the bacteria act as *in situ* drug producing 'biofactories', with the intervention focused at the site of pathology.

1.2 BACTERIA AS REGION-SPECIFIC COLONISERS

The microbiome research field has exploded in recent years, and while originally primarily focussed on bacterial colonisation of the gastrointestinal tract (GIT), research has expanded to various regions of the body, with characterisations of the microbiota of humans, animals, insects and non-living locations. 'Tract' regions of the human body, such as the vaginal and oral tract, feature distinct microbiota [6-8], and the microbiome of the skin, the largest organ of the body, is increasingly characterised [9]. The growing body of evidence supporting associations between the human microbiome and our health has

drawn significant attention. The ability to modify existing microbiota at different sites presents enormous potential for local or indirect management of various diseases.

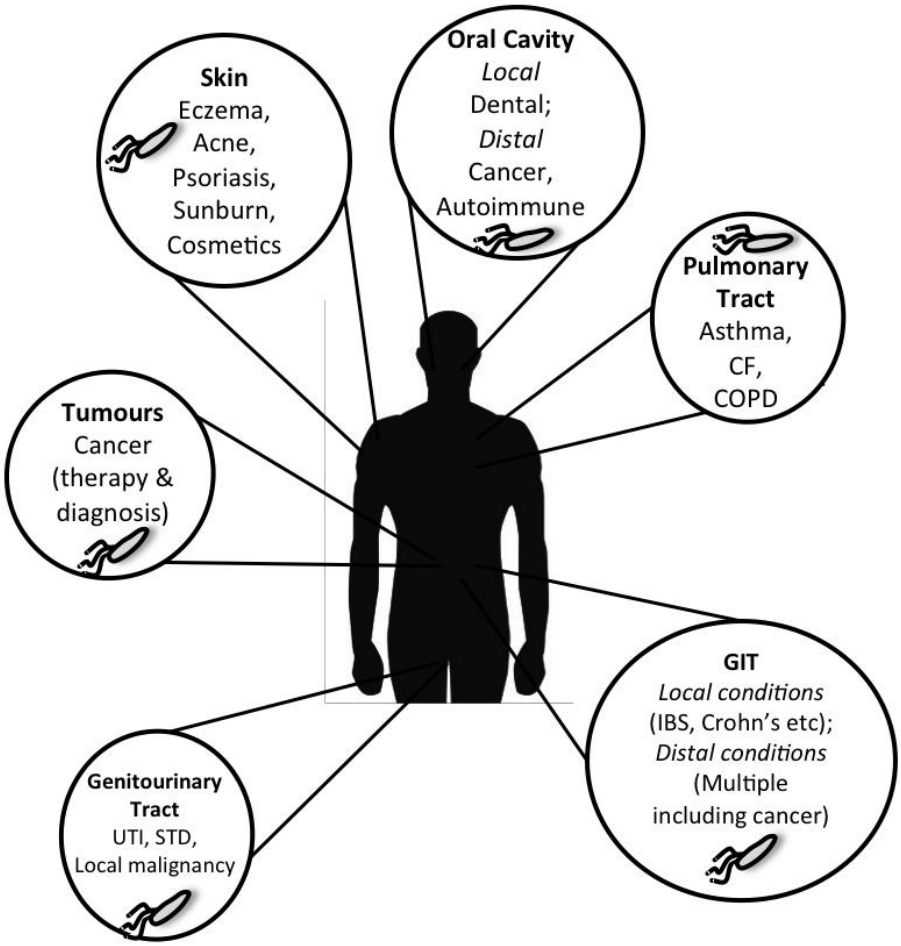


Figure 1 ***Example regions of the body where bacteria can be induced to colonise.*** *Sample conditions representing treatment targets for local bacteria are indicated for each location.*

While the ability to induce growth of different bacteria in the GIT (via oral administration of probiotics) is widely known, there are precedents for artificial inoculation of other body sites. Table 1 shows a selection of examples of biomolecule production from bacteria at different body sites, examples of these are represented in Figure 2. In addition to supplementing the microbiome of more well described tract regions with engineered bacteria, targeting of solid tumours by this strategy is also

under development. Various studies have shown that tumours support the growth of different bacterial species, and many clinical and preclinical studies are underway to effect tumour-specific therapies through administration of engineered bacteria (see later). In addition to these directly-acting therapies, associations between the nature of cancer patients' gut microbiota and tumour progression have been established [10]. For example, recent research in experimental cancer models has revealed that gut bacteria may influence the outcome of chemotherapy or immunotherapy indirectly via influencing the immune system [11, 12].

Table 1 Examples of in situ bacterial products in development for various diseases by body site

Company/ Product	Technology	Target Indication	Stage of develop-ment	Source
GIT				
ActoBiotics	*Lactococcus lactis in situ* production of cytokines, enzymes, hormones, and monoclonal antibodies	Allergic diseases, type 2 diabetes, autoimmune disorders (celiac disease; type 1 diabetes)	Clinical & preclinical	https://www.dna.com/Technologies/ActoBiotics
Synthetic Biologics (Ribaxamase)	*Lactococcus lactis in situ* production of therapeutic protein	*C. difficile* infection and antibiotic-associated diarrhoea	Clinical (Phase 2)	http://www.syntheticbiologics.com
Synlogic	Programming of the local microbiome metabolism: Probiotic bacteria with circuits that sense the patient's GIT environment regulate metabolic pathways	Inflammation, metabolism, oncology	Preclinical	http://www.synlogictx.com
Advaxis	*Listeria monocytogenes* delivery of Tumour-Associated Antigens to mucosal immune cells	Cancer (Cervical, Prostate, Breast)	Clinical (Phase 3; Phase 2)	http://www.advaxis.com
Oral cavity				
ActoBiotics AG013	*Lactococcus lactis in situ* production of TreFoil Factor-1	Oral mucositis	Clinical (Phase 1b)	[13]
Skin				
AOBiome	Ammonia-oxidizing bacteria (*Nitrosomonas*)	Acne, Eczema, Wound healing, Thermo regulation, Hypertension	Clinical (Phase 2; Phase 1). Preclinical	http://www.aobiome.com

TopgeniX	Platform technology for enduring application of natural compounds by skin microbiome	Sun protection, skin health, cosmetics	Preclinical	http://www.topgenix.com
Tumours				
Multiple (see this review)	Tumour-selective bacterial production of biomolecules	Any solid tumour	Clinical (Phase 2). Preclinical	[14]

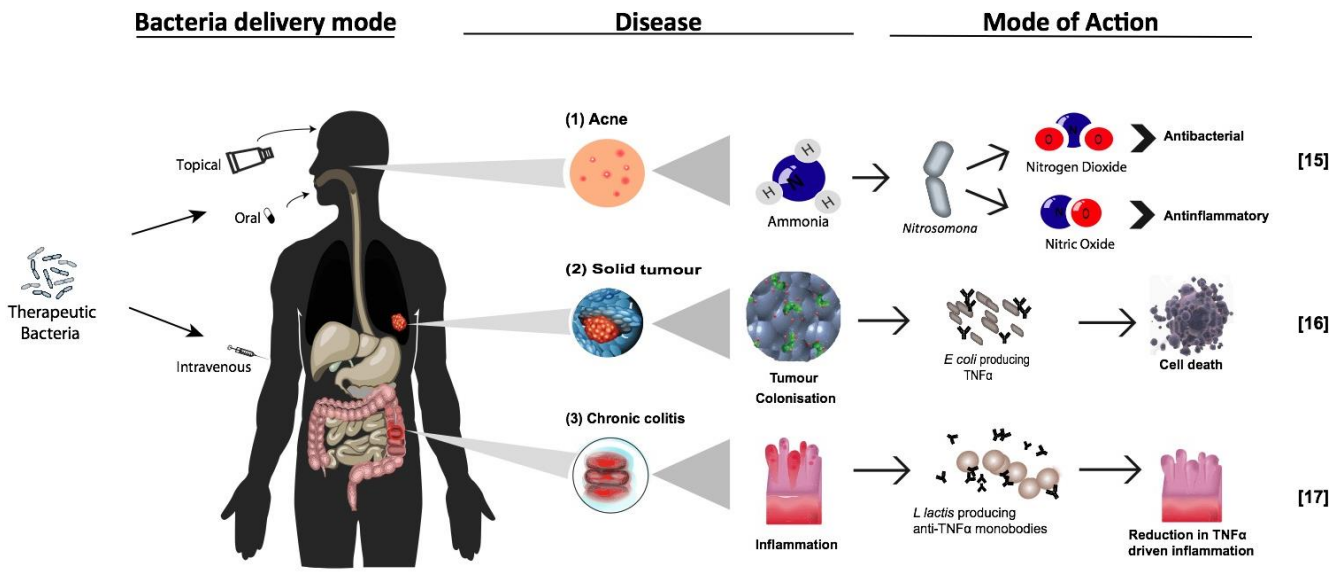


Figure 2 ***Illustration of in situ bacterial products*** *(1) Topical application of Nitrosomona eutropha oxidises ammonia into nitrogen dioxide (antibacterial) and nitric oxide (anti-inflammatory), preventing and treating acne [15]; (2) Intravenously administered E. coli colonises solid tumours and locally produces TNFα, impeding tumour growth [16]; (3) Orally administered Lactococcus lactis produces anti-TNFα monobodies in the colon, reducing inflammation in a chronic colitis model [17].*

2 SYNTHETIC BIOLOGY AS A TECHNOLOGY

Synthetic Biology is an evolving discipline focused on engineering biological systems for global needs, representing an umbrella term that covers many approaches aimed at bestowing biological entities with novel functions or replicating biological functions outside a cell [18]. Synthetic biology aims at the rational design of biological systems by integrating engineering principles (standardisation, modularity, abstraction) and technologies (*in silico* modelling systems, repositories of standard biological parts) [19, 20]. This engineering approach featuring a model-based rational-design, was first proven successful with the publication of the first genetic switches, the repressilator and the toggle switch [21,

22], which laid the foundation of a promising field whose potential applications fostered the creation of dedicated programmes advancing its key enabling technologies and expanding its applications by creating a well-knit community, yielding remarkable breakthroughs and potentiating our ability to engineering biological systems. The 'tipping point' for broad, market-meaningful adoption of Synthetic Biology came with the arrival of dramatically cheaper high-throughput DNA synthesis and sequencing, easily-employed biodesign tools and the availability of public repositories [23].The rapid adoption of these technologies by the expanding Synthetic Biology community provided evidence of a growing market, encouraging competition and further innovation targeting the creation of user-friendly toolkits and services accessible for all kinds of end-users. Consequently, the scope for synthetic biology has transcended from an emerging discipline to a foundational technological framework adopted widely in research and industry [24].

Now, Synthetic Biology is applicable to many areas; general bioengineering, editing of genomes of organisms in order to improve human health, transforming microorganisms to factories for producing certain drugs, creating cell-free systems capable of mimicking a cell's machinery or constructing unnatural molecular biology with non-canonical molecules and interactions to be used in diagnostics [18]. The engineering potential for bacteria using Synthetic Biology is immense and innovations are almost limitless. Using Synthetic Biology, it is possible to transform bacteria into production vehicles for biomolecules, to design biomolecules to our specifications, and to control the behaviour of the vehicle and the biomolecule production. For example, we can exploit bacteria as biochemical factories by creating new enzymes to produce desired chemicals [25-28]; bacterial genomes can be edited to render the chassis-cell compatible with a given strategy [29]; the cell's environmental sensing may be influenced, and much more [30]. Synthetic Biology is now finally delivering the early promise of bacteria & cancer therapy.

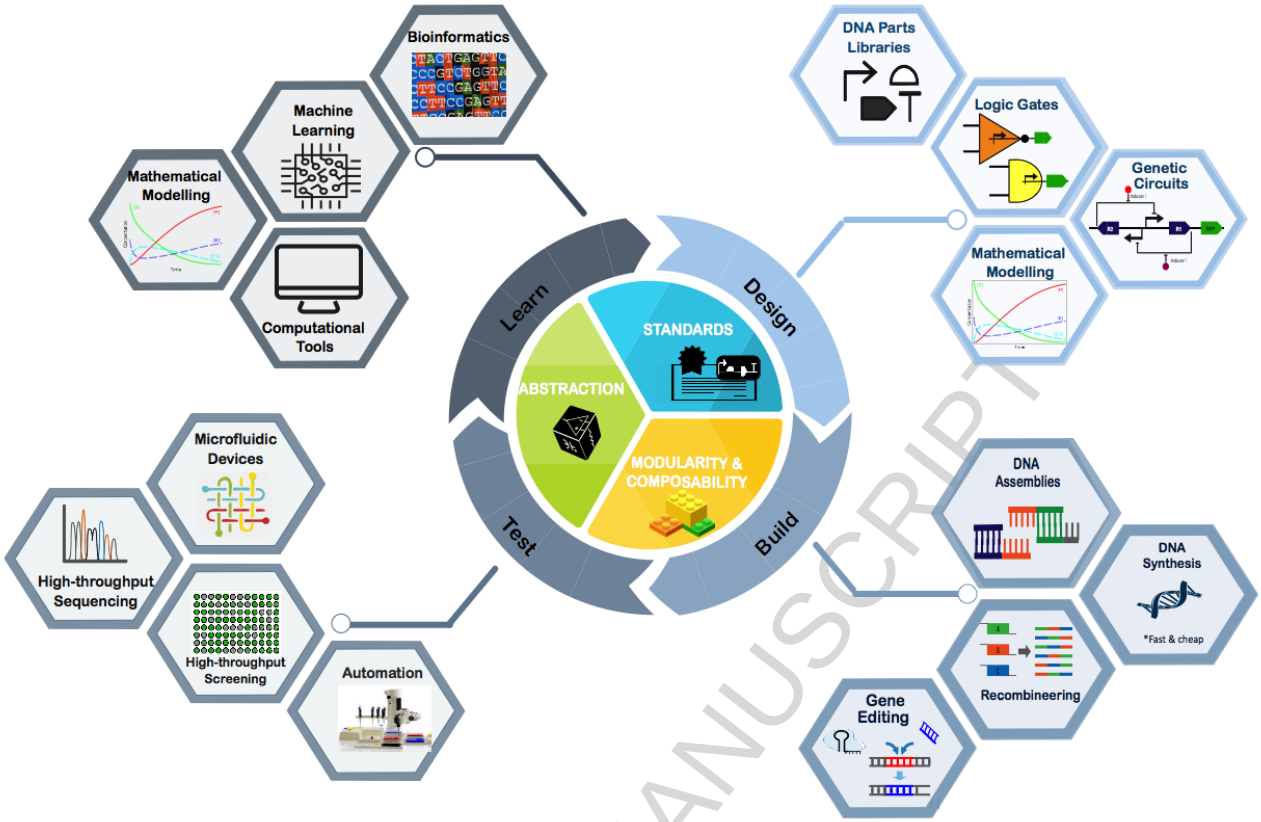


Figure 3 ***Synthetic Biology's design, build, test & learn (DBTL) cycle.*** *The foundation of synthetic biology lies in the introduction of engineering principles (see section 2.2) that enables the DBTL cycle [20]. Also portrayed are the different technologies developed by the synthetic biology community for the advance of the DBTL cycle (see section 2.2.1) [31-33].*

2.1 PATH TO MARKET

Full engagement with the Synthetic Biology approach goes beyond the scientific aspects of a technology, and incorporates all stages of R&D required to achieve an appropriate product. The SB process embraces, from the idea stage, multiple actors/stakeholders along the product development chain. The Design-Build-Test approach (see later) and rapid prototyping capacity of Synthetic Biology facilitates incorporation of design/redesign input to address multiple needs, at earlier, cheaper stages of R&D, before it is too late. The power to bestow sophisticated properties on bacterial chassis, devices

and biomolecules permits early addressing/pre-empting of aspects of safety, efficacy in the field, scale-up etc., in addition to reducing the duration of the product development path for a product, thereby cost & risk of medicine development and therefore the final cost of the actual product.

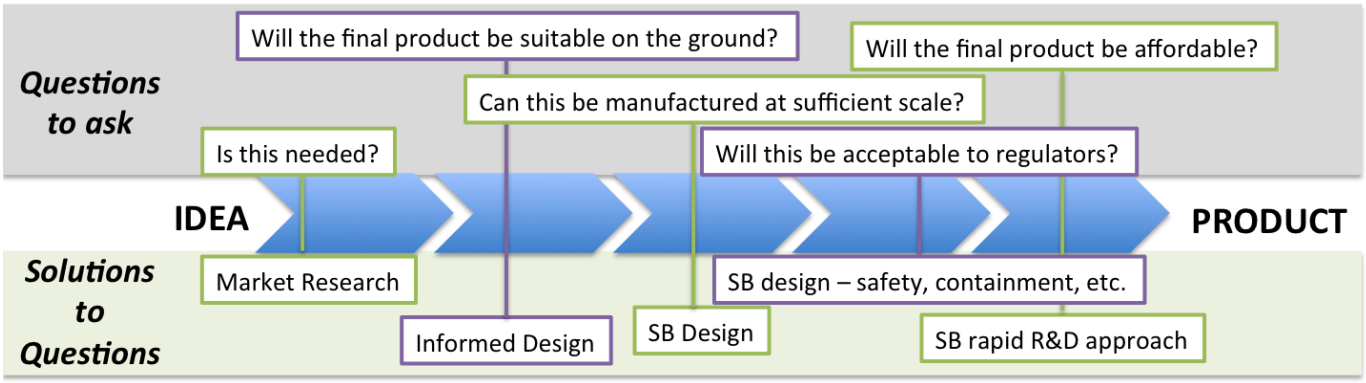


Figure 4 ***Synthetic Biology 'Built-In' Market-driven R&D Considerations*** *(SB – Synthetic Biology)*

2.2 THE SYNTHETIC BIOLOGY APPROACH TO BACTERIAL ENGINEERING

Synthetic biology borrows ideas, concepts and lingo from the engineering world and applies them to biology. In nature, complex systems comprise highly interconnected entities performing synchronized functions. However, synthetic biology, applies engineering principles (modularity, composability, abstraction, and standardisation) to redefine them into a modular and composable way. Through this framework, the elementary unit of a system is a thoroughly characterised and standardised 'part' – a motif (DNA sequence or genetically encoded product) with a defined task in a coding region. These motifs are the building blocks of a 'Lego like' scheme, where they are mix-matched to build fully functional genetic 'devices', capable of performing a defined function and an established input/out relationship. Devices are integrated into a chassis (e.g. a bacterial cell), to build a 'system', capable of producing a targeted biomolecule or behaviour (Figures 3, 5) [20, 34-36].

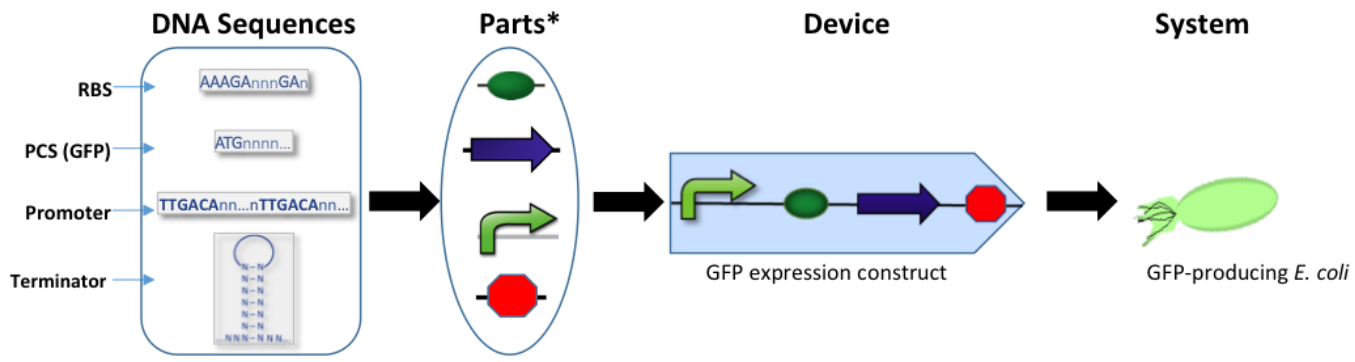


Figure 5 ***Schematic representation of an abstraction hierarchy.*** *Here, a genetic component, (a gene, transcription factor or a promoter) is defined as a 'part'; a collection of parts that together have a defined function = a 'device'; a collection of devices integrate to create 'systems'. (RBS: Ribosome binding site; PCS: Protein coding sequence).*

Furthermore, in order to achieve a logical form of cellular control through rational design, synthetic biologists apply electrical circuit analogies to describe genetic networks and biological pathways. In this context, a 'circuit' is a network-like composition of parts and/or devices, perform logical operations, that can be modelled, e.g. 'if' X condition is met, 'then' provide Y output [30].

2.2.1 Advancing the design-build-test cycle

The expansion of open-access catalogues of thoroughly characterised biological parts in computer readable formats, has advanced the rational *design* of biological systems [37]. Advances increasing our capabilities for DNA synthesis and assembly [38], and genome-scale engineering [39], and their translation into automated, high-throughput systems have potentiated our *building* capabilities, and increased their standardisation, efficiency and reproducibility. The thorough characterisation and measurement of a system's functionality *(test)* in 'real-time', is now made possible through high-throughput quantitative analysis tools that provide feed-back, facilitating the parameterisation of predictive models (See Figure 3) [37]. Altogether, these advances accelerated the pace of *design-build-test* cycle, and allowed the construction of highly sophisticated systems, built from multiple

components and implying multiple layers of cellular regulation [40]. The arrival of systems with higher complexity, brought along a new level in the abstraction hierarchy: biological 'modules'. These are subsystems made from a collection of discrete and defined devices with interconnected functions that together perform a complex task, as part of a higher wholesome system. Such operate as pathways resembling integrated circuits [34, 35, 41].

In this context, intelligent and tuneable systems or circuits, are made possible by integrating parts with a thoroughly characterised function. Parts catalogues, now supply a vast number of parts (sensors, regulators, actuators). These are constantly enriched with *de novo* parts harvested from nature, or *variants* created by predictive modelling (iterative rational design) or directed evolution [36]. Expansion that paved the way for the creation of regulatory elements (devices, modules) capable of manipulating different biological processes, simultaneously. Beyond transcription, synthetic systems now include modules regulating translation [42], post-translational modifications [43], and epigenomics [44, 45]. Novel parts advancing a multi-layered control include: CRISPRi [46], recombinases [47] invertases [48] feed-back and feed-forward loops [34, 49-51] for transcription; ribozymes and riboregulators [52-54] for post-transcriptional processes; and novel receptors [55], secretion tags, degradation tags, protein-binding tags for post-translational processes [36]. These provided the building blocks for building regulatory devices with logic behaviour, such as: switches [47, 56], logic gates [57, 58], stable oscillators [59], Riboswitches [60], and diverted scaffolds [61-64]. Similarly, these devices have now been applied to develop systems integrating logic to create permanent memory or produce complex calculations [65, 66], wire circuits through quorum-sensing [67, 68], building genetic edge detection programmes [69], controlling multicellular migration pattern and population growth [70], and building layered logic programmes enabling the construction of large integrated circuits in a cell [65]. There is an abundance of literature demonstrating the diversity and potential of these systems [30, 37, 71].

Applying synthetic biology principles for i*n situ* biomolecule production by bacteria now offers controllable strategies to externally controlled or self-regulated (intelligent) chassis cell and device behaviour (see later). Since much of the foundational work on Synthetic Biology was carried out on microbes including *E. coli*, the technical knowhow for sophisticated modifications for heterologous agent production, controlled expression, and safety-attenuation is readily available for deployment in the setting of *in situ* therapeutic production [72]. Synthetic Biology can improve this technology at all levels; i) the vehicle; ii) the production of the biomolecule carried by the vehicle; and iii) the biomolecule's activity.

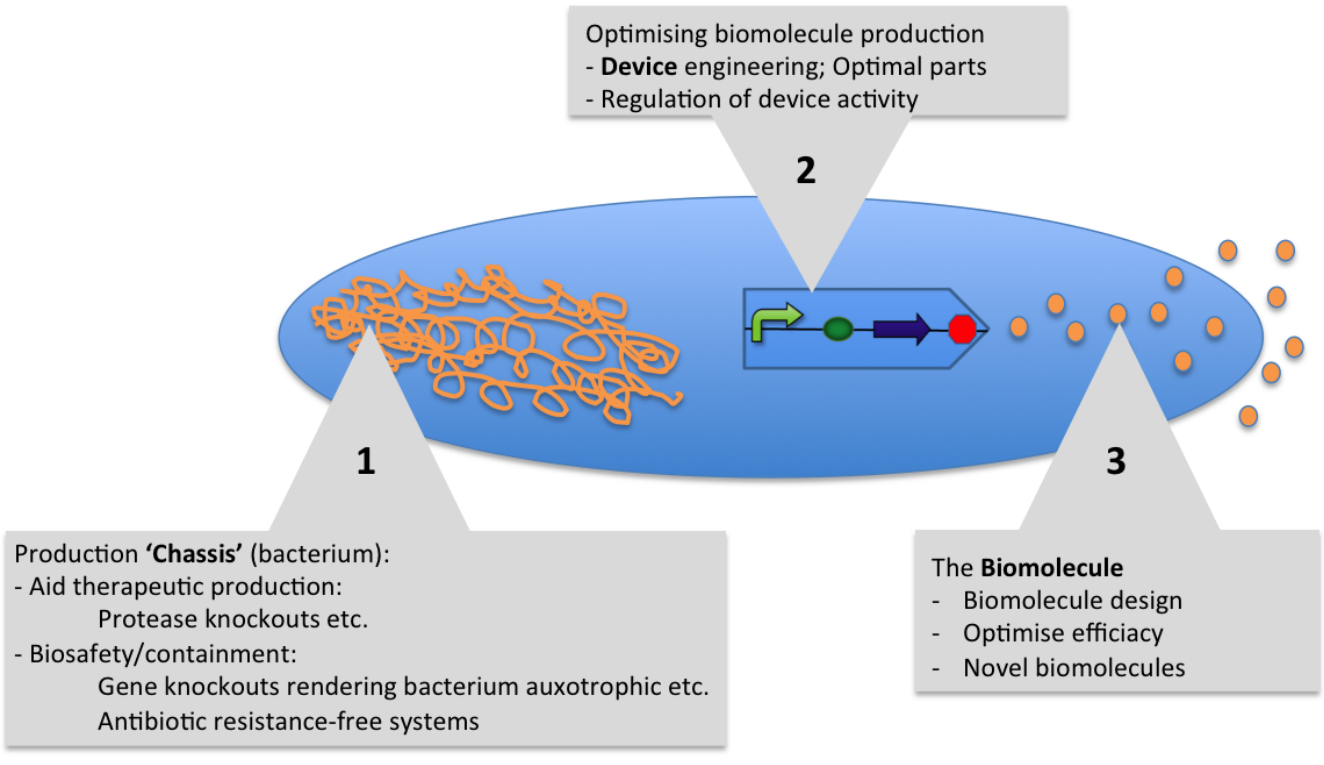


Figure 6 ***Synthetic Biology improves the technology at all levels.*** *1. The chassis cell (through bacterial genome engineering); 2. The production of the biomolecule by the system (through device engineering (including regulation of device activity)); 3. The biomolecule (e.g. modelling to obtain the optimal final biomolecule).*

3 CANCER AS AN EXAMPLE INDICATION

In the cancer context, bacteria are being investigated for biomolecule production/delivery both locally (direct therapy), and distally to tumours (within the GIT; immunotherapy) [12, 73-75].

3.1 BACTERIAL GROWTH IN TUMOURS

Various studies have shown that tumours support the growth of different bacterial species. A tumour microbiome has been described by different laboratories [76-79]. Separately, both in clinical and pre-clinical studies, different bacteria have been shown to preferentially colonise and proliferate within tumours following systemic administration [73, 75, 80]. It is believed that bacteria in the bloodstream leak from the abnormal vasculature within tumours and lodge locally where they are protected from the immune system due to the immune-suppressed microenvironment of tumours. The 'targeting' process therefore is more of a passive phenomenon of selective growth, without the involvement of chemo-attractants and relates to the tumour environment being permissive to bacterial survival and replication, unlike most healthy tissue. Chemotaxis may play a role post tumour targeting, influencing the manner in which certain bacteria distribute within the tumour [81]. Further parameters that distinguish tumour from healthy tissue include nutrient availability to bacteria (from tumour cell turnover in necrotic regions) and regions of low oxygen potential (where anaerobes and facultative anaerobes can grow optimally) [74, 80].

Bacterial tumour-targeting technology is based on the bacterium to selectively survive and replicate within solid tumours, growing to high concentrations, where they can 'pump out' therapeutics or locally activate agents. Depending on the strategy, the bacterium itself (the chassis) may possess intrinsic oncolytic properties (often the case with pathogens), or may have no effect on tumour growth

unless engineered to produce an agent. This platform technology is applicable to a wide range of therapeutic or diagnostic strategies. Clinical trials have demonstrated the safe use of live engineered bacteria in cancer patients, and preclinical studies using modified bacteria as tumour-selective agents have demonstrated the high potential for bacterial-mediated cancer therapy via *in situ* biomolecule production [14, 73, 80].

3.2 NON-TUMOUR TARGETS FOR CANCER THERAPY

The vaccination using live microbes field is, by comparison to the above, a mature area of research with significant commercial interest that employs different types of microbial vehicles including modified viruses or bacteria which confer immunological responses against infectious diseases or cancer. The goal of cancer vaccines is to break tolerance of the immune system to specific antigens known to be expressed mainly or exclusively by particular tumour cells - tumour-associated antigens (TAA). Bacteria are advantageous as antigen delivery vehicles due to their ease of bioengineering and diverse collateral effects on the immune system. As part of their natural life cycle, infectious bacteria, following entry to the body, are internalized by phagocytes, followed by MHC presentation of their antigens to the rest of the immune system. Through addition of synthetic antigens to a bacterial system, the process can be hijacked to mount a host immune response to a desired antigen (e.g. tumour-associated). Used in this setting, the chassis delivers an antigen to antigen presenting cells (APC), such as M cells in the gut mucosa, and does not involve growth in tumours. The bacterium is safety attenuated to render it non-infectious, and equipped with a device to produce specific tumour antigens (either genes or proteins). The vehicle itself also induces a desirable immune response in the vaccine context (similar to an adjuvant). Following administration (per oral, intramuscular, or intravenous), the bacterium is taken up by the patient's antigen presenting cells. The bacterium releases genetic material or antigens into the immune cells that then initiate a systemic immune response specific to the target

antigen. There are multiple safety-attenuated strains under study as vehicles for vaccination; *Listeria monocytogenes, Salmonella, E. coli* and strains of *Shigella, Lactobacillus* and *Yersinia* of which *L. monocytogenes* (Lm) and *Salmonella* are being studied clinically [82, 83]. Significant, highly promising therapeutic outcomes are being realised from these vaccine platforms in multiple Phase II and III trials with patients of disparate cancer indications [83]; (https://clinicaltrials.gov/ct2/show/NCT02853604).

4 SYNTHETIC BIOLOGY APPROACHES TO IMPROVEMENT OF BACTERIAL AGENTS AND TREATMENT STRATEGIES

4.1 THE CHASSIS CELL

4.1.1 Safety attenuation

Employment of bacterial strains with a natural ability to survive and grow within human tissues (i.e. pathogens) is attractive from an efficacy standpoint, but obviously undesirable from a safety perspective due to off-target growth within healthy organs, coupled with recognition by the patient's immune system as a disease-causing agent. Strain attenuation can be used to limit capacity to survive in non-target healthy tissues e.g. liver, or to reduce pro-inflammatory reactions. Traditionally, attenuation was achieved by random mutagenesis of a wild type strain and selection for certain favourable phenotypes e.g. tumour invasion, proliferation etc. Purpose-designed systems are preferable, involving editing of genes that are known to be involved in pathogenesis. For example, *msbB* and *purI* are two genes that have been eliminated from the genome of *Salmonella* in order to create VNP20009 [84] the first *Salmonella* clinical trial agent. Another attenuated *S.* Typhimurium defective in guanosine 5′-diphosphate-3′-diphosphate (ppGpp) synthesis (a molecule responsible for regulating salmonella pathogenesis [85]) was also generated by genomic editing. Similar editing can also reduce unwanted

host responses to non-pathogenic bacteria; e.g. the probiotic *E. coli* Nissle 1917, which is part of our natural gut microbiome, has been attenuated via an *msbB* deletion which reduced pro-inflammatory cytokine stimulation compared with wild type [86].

4.1.2 Cell targeting

Although bacteria do not actively home to tumours, it is possible to improve their specificity to the tumour environment and limit their ability to proliferate in healthy tissue through exploitation of unique tumour traits to guide the design of more tumour-selective bacteria. For example, Yu *et al* [87] restricted the growth of bacteria to hypoxic regions, a phenotype found only within tumours inside the body. An essential gene for cell wall synthesis, *asd*, was placed under a hypoxia-inducible promoter (*PpepT*) which allowed expression to take place only under hypoxic or anoxic conditions. In parallel, a second device expressed the antisense of *asd* under an aerobic promoter (*PsodA*). This device inhibited growth under normoxic conditions. Integrating both devices into a module, enabled a logic gate restricting replication to areas with low oxygen concentration, such as those found inside the tumour. Such a circuit would eliminate the capacity of bacteria to grow in healthy tissue, thus adding another layer of safety. 'Trapping' bacteria within tumours can also be achieved via addition of tumour cell ligands. Using a sophisticated surface display system, the peptide RGD was surface tethered to *Salmonella* in order to improve its targeting capabilities towards specific integrin expressing cancer cells [88]. Similar strategies could be used in other systems to target bacteria to specific cells/tissues. Such levels of bioengineering sophistication can upgrade chassis cells in both efficacy and safety.

4.1.3 Bacterial vs viral chassis

Both, bacteria and viruses are effective delivery vehicles for different cargoes. Here we outline the characteristics that will determine their feasibility under different scenarios.

Table 2 Bacterial v Viral vectors.

Pro-Bacterium	Pro-Viral vector
Bacterial chassis = final biofactory. Multiple components of the biofactory cell genome can be engineered *in vitro (see Figure 4 part 1)*	
Bacteria can generally carry more devices	
Bacteria can produce biomolecules independent of / external to host cells	
	If biomolecule must be delivered internally to host cell, viral vector transduction efficiency is much higher than bactofection
Viral vectors must be invasive => safety concerns	
	Viral vector better as *in situ* host cell (biofactory) editor
Antibiotic sensitivity can act as safety 'Off switch'	
Bacterial manufacture cheaper	
Bacterial biomolecule type may be nucleic acid, protein or small molecule, while viral vector biomolecules are restricted to nucleic acid	
	Bacterial expression of eukaryotic gene sequences may not be as efficient as with viral vectors
Bacteria may naturally colonise and replicate in specific tissue/location, more so than viruses	
Bacteria more transient than viral vector (safer is some circumstances)	Bacteria more transient than viral vector (viral vector better for integrating device in host cell genome)

4.2 BIOMOLECULE DELIVERY AND PRODUCTION

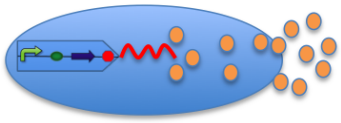
There are two broad ways to deliver a biomolecule in the bacterial context – i) at the tissue level, normally external to target cells, or ii) internal to target cells. The delivery modality must be matched with the biomolecule's therapeutic modality. For several therapeutic strategies, simply 'flooding' the environment with bacterial-produced protein is sufficient, and non-invasive chassis are suitable, and from a safety perspective, desirable.

4.2.1 Bactofection

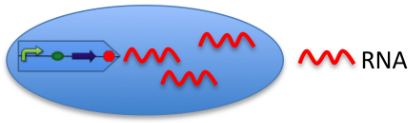
Delivery of biomolecule internal to cells involves chassis lysis after which its contents are released to the cytoplasm of the target cell. In this context, the biomolecule may be protein, RNA or DNA depending on the strategy employed. This strategy is often referred to as bactofection (bacterial transfection). Bactofection can be 'active', involving an invasive bacterium mediating its entry to a cell, or 'passive', as is the case with phagocytic immune cells [89]. 'Smart' target cell entry may be achieved through Synthetic Biology approaches, using devices that sense different inputs leading to an invasive output. Host cell invasion by *E. coli* was achieved by expressing the protein *inv* gene from *Yersinia pseudotuberculosis* which was triggered by hypoxia, cell density or an exogenous inducer [90]. Once the bacterial cells came into proximity with the host cell membrane and reached a certain density, the circuit became activated leading to the production of *inv* gene resulting in tumour cell invasion. Some strategies utilise occurrences post-invasion, for example van Pijkeren *et al* [91] devised a system by which a lysin was expressed only following host cell internalisation, in order to induce a cascade of bacterial lysis.

4.2.2 Types of biomolecule 'payloads' and optimal production

There is a large and diverse collection of biomolecules which have been investigated in studies with bacteria to date, and may be peptide-based, RNA or DNA in nature (Figure 7).



Biomolecule Type	Target Cell: External to- Internal to-	Examples			
		Delivery Modality	**Biomolecule example**	**Target cells**	**Strategy**
Biomolecule	Either	Local Secretion	Peptide	Host cells	Anti-inflammatory
			Small Molecule	Cancer cells	Toxicity Induction
				Immune cells	Vaccination
				Local microbes	Anti-Pathogen



		Delivery Modality	**Biomolecule example**	**Target cells**	**Strategy**
RNA	Internal to	Bactofection	miR	Phagocytic cells	Cell Programming
			shRNA		

		Delivery Modality	**Biomolecule example**	**Target cells**	**Strategy**
DNA	Internal to	Bactofection	Plasmid DNA	Cancer cells	Toxicity induction
				Phagocytic cell	Cell Programming

Figure 7 Example types of biomolecules and relevant medical strategies

4.2.3 Controllable and intelligent systems

Currently, a tight regulation of multi-module circuits is more easily achieved, increasing the predictability of desired phenotypes. This applies to biomolecule production, which kinetics benefit from a sophisticated control in gene expression. By applying a rational design, different layers of control over biomolecule production and/or the vehicle can be applied, when appropriate for a chosen strategy. Such a system can incorporate a sensing module able to respond to numerous stimuli. A rich-repertoire of now available, characterised sensory parts, enables the creation of systems capable of responding to a variety of physical or chemical inputs, such as oxygen concentrations, acidity, cell density, drugs, molecules, radiation. Sensors are often built upon promoters whose activity can be regulated by environmentally responsive DNA binding protein [36, 92, 93]. Here, regulation is mediated by the binding of the protein into a transcription activator or repressor site in the promoter sequence. This generates a conditional 'switch (ON/OFF)' behaviour – nominated positive or negative

feedback loops. A combination of these parts can be used to create AND/OR/NOR gates [30]. Depending on requirements, designs can range in flexibility and sophistication. The design of complex systems, whose multi-components' interaction rely on multiple factors, (e.g. DNA-protein binding/dissociation constants, kinetics and other biophysics), is now made possible by *in silico* analysis. These tools enable the prediction of such level of regulation by applying mathematical based, known biophysical constants and coefficients to model biological processes [94, 95].

An early example of a controllable system in this context involved an engineered *Clostridium* [96]. These authors created a switch turned on by radiation that could trigger the production of a protein with therapeutic properties (e.g. TNFα, cytosine deaminase (CD)) and induce a cytotoxic response in preclinical models. In an analogous manner [97] used a device switched on by the sugar arabinose to give a toxic output in order to treat colon carcinoma. More recently, circuitry was taken to the next level. A circuit was designed whose input is cell density but leads to several outputs regulated by a common part. The circuit is composed of an activator, a reporter, a therapeutic and a therapeutic gene delivery device [98]. The circuit is based on the quorum sensing system *lux*. *LuxI* catalyses the synthesis of N-3-oxohexanoyl-L-homoserine lactone (AHL) which freely diffuses and accumulates in the surrounding proportional to cell density. AHL activates the transcriptional activator *LuxR* to activate genes that have a downstream *luxI* promoter. The *LuxI* promoter itself was inserted in front of *luxI* gene in order to create a positive feedback regulation to support the integrity of the circuit. GFP was used to give a light signal output. The bacteriophage lysis gene (ϕX174 E) was used to aid bacterial lysis and deliver the cytotoxic payload, and finally, the payload itself was the cytolysin, a pore forming protein. In an analogous circuit, two parallel devices where employed to deliver a cytotoxic payload to tumours in mice [99]. Therapeutic protein production was controlled by salicylate and lysis of bacterial cells was controlled by tetracycline. Such a system first allows bacteria to target to tumours without putting a metabolic burden on them. Production begins only after bacteria reach optimum

numbers within the tumour, and lysis serves to deliver the therapeutic protein to the surroundings in the most efficient manner. More recently, the Hasty group engineered an elegant 'synchronized lysis circuit' in *S.* Typhimurium to induce lysis at a threshold population density (through quorum sensing) and release its therapeutic cargo [68].

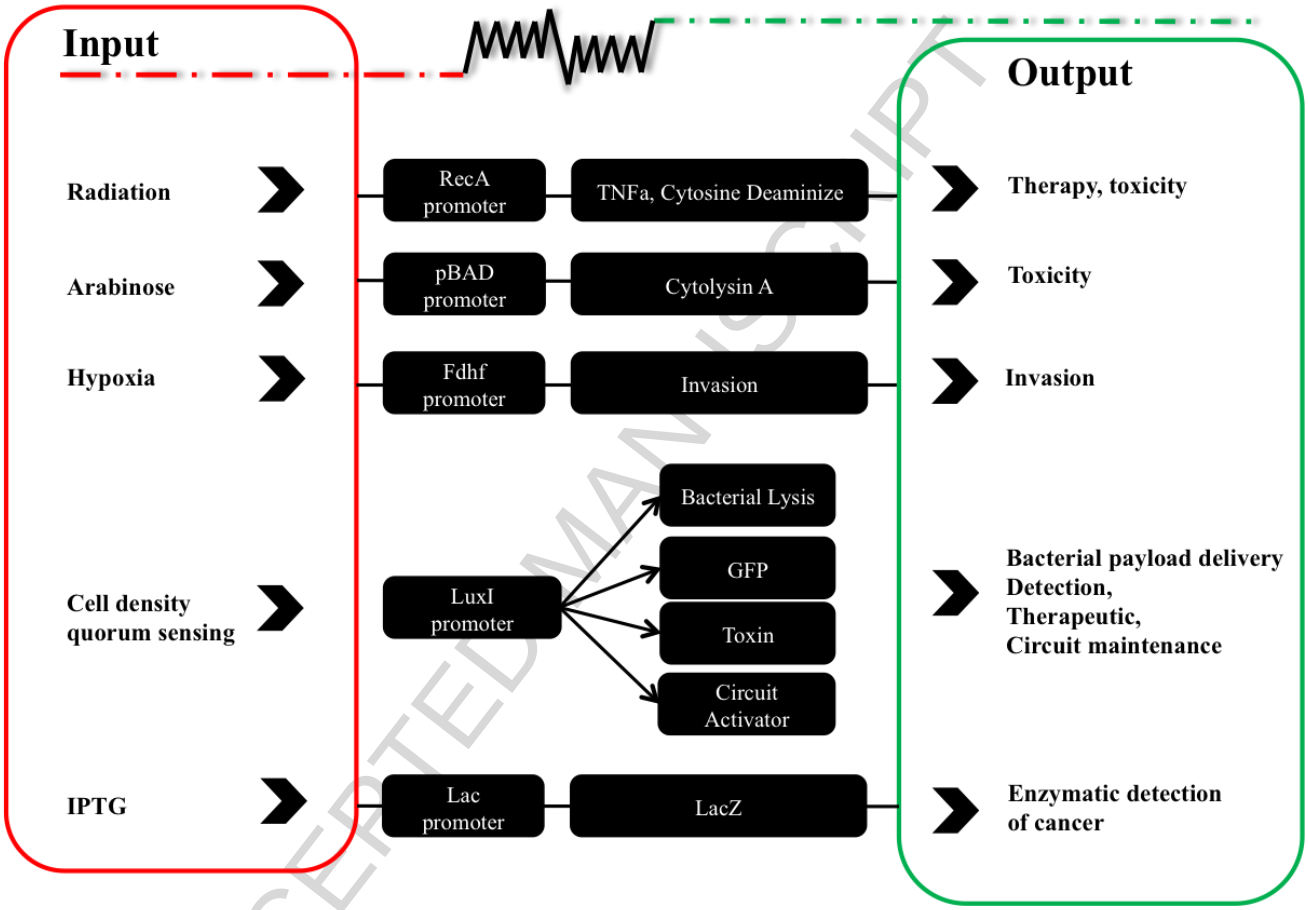


Figure 8 ***Examples of controllable/intelligent bacterial systems in oncology studies***

5 TREATMENT STRATEGIES

As Synthetic Biology became more sophisticated, new possibilities became realized. Strategies could now be re-designed to deliver maximum efficacy. We now have the capacity to deliver protein, RNA, DNA and to activate small drug molecules specifically at bacterial-specified sites. Production of

biomolecules can now occur in bacteria and/or host cells and parameters such as the kinetics, location and level of production and the function of the product itself can be controlled.

5.1 THERAPEUTIC PRODUCTION

5.1.1 Peptide production

Bacterial production of peptides is well described in several research domains, and is commonly used industrially for recombinant protein production [100, 101]. In *in vivo* strategies, non-invasive/apathogenic bacteria are primarily employed in this context. As an example, Lactic Acid Bacterial chassis producing *in situ* antiproteases and antioxidant enzymes have been tested successfully for their prophylactic and therapeutic effects in murine models of colitis [102]. Numerous intratumoural bacterial production of various peptides at both clinical and pre-clinical stages have been described [14, 103]. A wealth of technology has been developed for optimisation of protein production by bacteria [104]. Bacterial cells are enveloped by sophisticated membranes that regulate what enters and exits the cell. In Gram-negative bacteria, for example, the cytoplasmic interior is separated from the exterior by a thick outer membrane, a periplasmic space and an inner membrane. Depending on the nature of the therapeutic biomolecule used, cytoplasmic expression may hinder its activity. A number of systems exist that place biomolecules of interest in different compartments of the bacterial cell or secrete them to the exterior. Secretion to the surrounding environment is frequently desirable, and a number of systems are available for different bacterial genera employing signal sequence 'parts' in devices to promote appropriate secretion [104, 105]. Surface display parts can direct proteins to the outer membrane of bacteria [88, 106]. Recombinant proteins commonly surfaced exposed are antigens and antibodies [107].

5.1.2 RNA production

Small interfering RNA and microRNA has generated much interest in recent years in both basic and applied biology. For example, *S.* Typhimurium has been utilised in various preclinical cancer studies as a chassis to deliver small hairpin RNA (shRNA) against GFP, STAT3 or bcl-2 [108, 109].

5.1.3 Small molecule activation

In order to address the problem of target specificity of small drug chemotherapy, researchers have been using synthetic biology to enable bacterial-colonised tumours to act as the final stage of toxic drug 'synthesis'. Here, enzymes are produced by bacteria at the tumour site, while the chemical reactants (prodrugs) are administered later. The active drug generation takes place at the tumour site, mediated by the bacteria which enzymatically activate the actual chemotherapeutic (reviewed in [110]). Recent work suggests that multiple drugs can be activated concurrently [111] and opens doors to new ideas such as having devices that can concurrently activate *in situ*, multiple drugs with diverse mechanisms of action in order to overcome drug resistance.

5.2 HOST CELL MODIFICATION

In some circumstances, it may be desirable to induce the host cell to produce the biomolecule itself. Invasive chassis deliver devices or biomolecules to mammalian cells by bactofection. Such devices feature parts that are compatible with eukaryotic environments, and therefore switched on post-delivery. Usually, the specificity in such systems comes from bacteria and the devices themselves have a constitutively active switch (a eukaryotic promoter such as CMV) which fires upon delivery to any host cell. However in other cases, another layer of regulation can be introduced at the device itself, by using a switch that is only turned on by cancer cells (though use of a tumour-selective promoter [112]) therefore providing an extra level of specificity as well as therapeutic potency. Delivery of eukaryotic

devices is not limited to cancer cells, or invasive bacteria. Byrne *et al* used a non-invasive *E. coli* to infect phagocytic cells (Tumour Associated Macrophages (TAM)) and deliver DNA modules that produced light as an output [89]. In this case, the specificity towards the phagocytes was brought by the 'non-invasiveness' of the bacteria. Vaccine strategies employ a similar strategy.

5.3 DIAGNOSTICS

Co-localisation of a bacterial agent with a specific site/cell type presents opportunities for diagnostic strategies. For example, in the context of oncology, tumour detection can either be direct, for example by intratumoural bacterial imaging, or indirect by biological fluid analysis of biomarkers (liquid biopsy). In this context, representing a prototype Point of Care test, a prototype system has been developed to detect cancer by urine sampling. *E. coli* expressing a regulated *LacZ* was constructed in order to detect murine liver tumours [113]. Following bacterial colonization of hepatic tumours in mice, bacteria express *LacZ* enzyme following induction by IPTG. Subsequently, a derivative of luciferin is administered which is cleaved by *LacZ* to pure luciferin and cleared through the urine. Luciferin is then measured by emission of light directly from the urine sample offering quick non-invasive tumour detection. Similar to the above, [114] created an inducible reporter/biomarker module that can be detected in blood samples by antibodies in an ELISA type assay. The biomarker, ZsGreen expressed by *Salmonella,* was shown to be suitable for detection of colon carcinoma in mice.

6 REGULATORY AGENCY ASPECTS

The expanding scope for and adoption of biological engineering applications potentially presents the need for our current ethics and governance to evolve also [23]. If Synthetic Biology actors approach this aspect correctly, regulatory concerns can be overcome. Currently, Bacillus Calmette-Guerin (BCG), a local treatment for bladder cancer, is the only live bacterium in clinical use, and is not

genetically modified. However, precedents for licencing of live GM microbes have been set, with viral-based chassis. 2012 yielded the first licensing of a gene-based therapy in the western world, with the EU EMA licencing of Glybera - an AAV chassis engineered to express lipoprotein lipase in the muscle of deficient patients [115]. Talimogene laherparepvec (also known as T-Vec) was approved by the FDA in 2015, with the brand name Imlygic, for the treatment of advanced inoperable melanoma. In 2016, it was approved in Europe. It is an oncolytic virus and consists of a genetically modified Herpes Simplex Virus (HSV) chassis carrying a device producing *in situ* a cytokine (GM-CSF) that helps to induce immune responses following intralesional injection. [116]

Engineered bacteria for vaccine use have advanced to late stage clinical trials and therefore the safety/regulatory aspects of live GM bacteria are also being tested concomitantly. Clinical candidates have medical and environmental safety requirements which can only be met by the use of bioengineering, involving biological containment of both the vehicle and any 'non-natural' DNA elements. For example, Aduro Biotech has been developing a *Listerial monocytogenes* agent for use in patients [117]. An attenuated form was created by deleting two genes critical to pathogenicity – *internalin* B and *act* A, while antigen gene cassettes are inserted in the bacterial genome therefore obviating antibiotic use [118]. To maximize agent production, it may be desirable to maintain the antigen gene on an episomal plasmid in order to increase gene copy number. While plasmid maintenance in the lab environment employs antibiotic resistance modules, this is not acceptable for a market product from a regulatory aspect. Alternative plasmid maintenance systems have been created, based on modifications of both chassis and plasmid. Conditional or Balanced Lethal Systems involve genes required for bacterial survival being deleted from the genome of a chassis and transferred to a plasmid into which the device is also inserted. Bacteria produce the biomolecule as long as the plasmid is retained [119] and die in the event of a plasmid loss. There are many more examples demonstrating

that Synthetic Biology offers realistic solutions for the development of bacterial systems in order to meet clinical requirements.

7 CONCLUSIONS

Synthetic Biology is a burgeoning field that is driving the progression of bacterial agents in the health industry. The application of Synthetic Biology to improve bacterial agents for use in the strategies described is key to fulfilling earlier promises. Unlike before, intelligent precision engineering will permit the generation of effective agents. Further new developments pertaining to the regulation of bacterial safety will also be attractive to market stakeholders, paving the way for state of the art bacterial therapeutics. Perhaps the most valuable aspect overall, is the Synthetic Biology all-stakeholder-inclusive approach to R&D from idea to product. Thanks to Synthetic Biology, the time for developing successful bacterial-based disease treatments has finally arrived.

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Figure 1 Example regions of the body where bacteria can be induced to colonise. Sample conditions representing treatment targets for local bacteria are indicated for each location.

Figure 2 Illustration of in situ bacterial products (1) Topical application of Nitrosomona eutropha oxidises ammonia into nitrogen dioxide (antibacterial) and nitric oxide (anti-inflammatory), preventing and treating acne [15]; (2) Intravenously administered E. coli colonises solid tumours and locally produces TNFα, impeding tumour growth [16]; (3) Orally administered Lactococcus lactis produces anti-TNFα monobodies in the colon, reducing inflammation in a chronic colitis model [17].

Figure 3 Synthetic Biology's design, build, test & learn (DBTL) cycle. The foundation of synthetic biology lies in the introduction of engineering principles (see section 2.2) that enables the DBTL cycle [20]. Also portrayed are the different technologies developed by the synthetic biology community for the advance of the DBTL cycle (see section 2.2.1) [31-33].

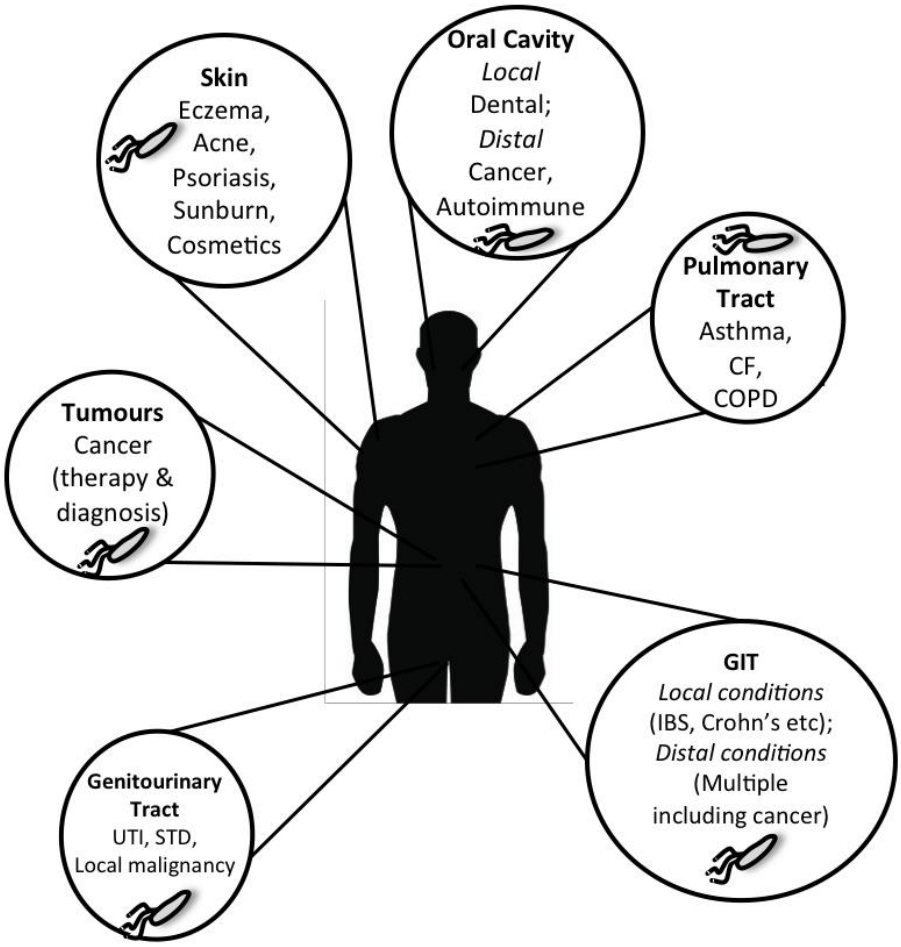
Figure 4 Synthetic Biology 'Built-In' Market-driven R&D Considerations (SB – Synthetic Biology)

Figure 5 Schematic representation of an abstraction hierarchy. Here, a genetic component, (a gene, transcription factor or a promoter) is defined as a 'part'; a collection of parts that together have a defined function = a 'device'; a collection of devices integrate to create 'systems'. (RBS: Ribosome binding site; PCS: Protein coding sequence).

Figure 6 Synthetic Biology improves the technology at all levels. 1. The chassis cell (through bacterial genome engineering); 2. The production of the biomolecule by the system (through device engineering (including regulation of device activity)); 3. The biomolecule (e.g. modelling to obtain the optimal final biomolecule).

Figure 7 Example types of biomolecules and relevant medical strategies

Figure 8 **Examples of controllable/intelligent bacterial systems in oncology studies**



Graphical Abstract

Example regions of the body where bacteria can be induced to colonise. *Sample conditions representing treatment targets for local bacteria are indicated for each location.*