Engineered Probiotics: Applications and Biological Containment

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Abstract

Bioengineered probiotics represent the next generation of whole cell–mediated biotherapeutics. Advances in synthetic biology, genome engineering, and DNA sequencing and synthesis have enabled scientists to design and develop probiotics with increased stress tolerance and the ability to target specific pathogens and their associated toxins, as well as to mediate targeted delivery of vaccines, drugs, and immunomodulators directly to host cells. Herein, we review the most significant advances in the development of this field. We discuss the critical issue of biological containment and consider the role of synthetic biology in the design and construction of the probiotics of the future.

INTRODUCTION

The human body, once characterized as a single organism, is now widely considered a diverse assortment of organisms intertwined in delicate symbiotic relationships (Sleator 2010a). Decades of studies have led to the identification of many of the microorganisms that colonize the human body (the microbiota), particularly the gastrointestinal tract (Culligan et al. 2014, Sleator et al. 2008b). Several positive functions of the microbiota in human health have been established, including the metabolism of food, synthesis of vitamins, protection against pathogens (Cummings & Macfarlane 1997), and immunomodulation to allow tolerance of environmental antigens (Sekirov et al. 2010). More recently, communication between the microbiota and the central nervous system (CNS) has been observed, stimulating further research into the role of these microbes in brain development, neurological function, and psychological disorders (Dinan & Cryan 2015, Petra et al. 2015).

The idea that the microbiota could be altered to promote health was first proposed by Elie Metchnikoff, who suggested that eastern European peasants lived long lives owing to regular consumption of fermented yogurt (Metchnikoff 1907). Probiotics, defined by the World Health Organization (WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001), have been a focus of attention during the past two decades as potential biotherapeutics. The benefits of probiotics both in health and in fighting a multitude of diseases have been well documented (Butel 2014, Sleator 2015a, Vandenplas et al. 2015). However, studies show that the efficacy of probiotic treatment varies from person to person, a phenomenon that may be explained by individual intestinal microbial composition and strain variation (Barzegari & Saei 2012).

Bioengineering of probiotic organisms facilitates a more targeted approach to the prevention and treatment of certain diseases (Sleator & Hill 2008d). More recently, bioengineering of probiotic organisms has driven the development of probiotics as versatile delivery vehicles, an approach that broadens their applications to include drug administration, vaccine delivery, and immunomodulation (Culligan et al. 2009; Sleator 2010d, 2015a,b; Sleator & Hill 2008b). The successes of such bioengineered probiotics coupled with the emergence of psychobiotics suggest that both natural and genetically modified probiotics capable of producing psychoactive compounds may be employed in the future as biotherapeutic agents for the treatment of mild psychological conditions (Dinan et al. 2013). This review focuses on current information on the development of more robust, efficient probiotics and their potential as biotherapeutics for specific conditions. The importance of ensuring the safety of bioengineered probiotics using innovative biological containment strategies is also considered.

STRESS TOLERANCE

The physiological robustness of a bacterial strain is important in its ability to function as a probiotic (Sleator 2010c; Sleator & Hill 2006, 2007, 2008d, 2009). The most commonly used probiotic microorganisms, bifidobacteria and lactobacilli, are anaerobic fastidious bacteria that are generally sensitive to different forms of physiological stress (Cronin et al. 2008, Sheehan et al. 2006, Watson et al. 2008). Any potential probiotic strain must remain viable after the industrial processes associated with producing and storing the organism, involving differing levels of water activity (a_w), pH, temperature, pressure, and oxygen content (Considine et al. 2008, Forssten et al. 2011, Gueimonde & Sánchez 2012, Sleator 2010d). Furthermore, the organism is required to remain stable in the delivery matrix, for example, yogurt, for the duration of its shelf life and, following ingestion, must be capable of surviving transit through the gastrointestinal tract. Enhancing the stress tolerance of probiotic strains is thus a key first step in the development of bioengineered probiotics.

Several studies have shown that it is possible to improve the stress tolerance of probiotic strains (Sheehan et al. 2007; Sleator & Hill 2008c, 2009; Watson et al. 2008). This adaptation is generally conferred through one of two ways: prolonged sublethal exposure of the strains to specific stresses to generate stress-tolerant mutants or by the introduction/modification of genes involved in enhancing stress tolerance. The former has been applied successfully to *Bifidobacterium* and *Lactobacillus* strains, with several studies showing the adaptive evolution in response to acid stress (Broadbent et al. 2010, Jiang et al. 2015), oxygen content (Li et al. 2010b), and temperature (Aakko et al. 2014, Desmond et al. 2001).

The use of genetic engineering to increase stress tolerance may involve the overexpression of certain genes already present in an organism, as was shown by Desmond et al. (2004). Using plasmids containing the *groESL* operon and a nisin-controlled expression (NICE) system, the authors showed that the overexpression of *groESL* (a molecular chaperone) in *Lactobacillus paracasei* and *Lactococcus lactis* improved the survival of the strain in common industrial processes, such as spray-drying and freeze-drying, while also generating moderate improvements in thermotolerance and osmotolerance (Desmond et al. 2004). Other studies involve heterologous gene expression; for example, expression of trehalose biosynthetic genes, *otsBA*, from *Escherichia coli* in *L. lactis* led to the accumulation of trehalose (a cellular osmoprotectant) within the cells and resulted in almost 100% cell viability after freeze-drying (Termont et al. 2006). Additionally, the freeze-dried *L. lactis* showed increased resistance to bile and gastric acid. A study by Sheehan et al. (2006) demonstrated an improved stress tolerance in *Lactobacillus salivarius* UCC118 when BetL, a listerial betaine uptake system, was expressed in the strain. This heterologous expression resulted in elevated osmo-, cryo-, baro-, and chill tolerance. Further work in our laboratory with *Bifidobacterium breve* UCC2003 produced similar increases in stress tolerance (Sheehan et al. 2007). In addition, increased gastrointestinal persistence and protection against *Listeria monocytogenes* infection were also observed for *B. breve* 2003-BetL^{+}.

Overall, heterologous expression of stress resistance genes in probiotic strains provides a means to increase their processing and host-associated stress tolerance, potentially increasing cell numbers reaching the intestine, increasing persistence time, and ultimately boosting probiotic efficacy.

ADHESION AND LIGAND-RECEPTOR INTERACTIONS

Our understanding of the pathogenesis of certain enteric infections, combined with our knowledge of the mechanisms through which naturally occurring probiotics are of benefit to the host, opens a wide range of opportunities to bioengineer highly effective and specific probiotics. It is well established that many pathogenic bacteria, including *Helicobacter pylori* and certain *Neisseria* strains, express mimics of host oligosaccharides in the core regions of their lipopolysaccharides or lipooligosaccharides (Preston et al. 1996). These ligand-receptor interactions are contributing factors to the invasiveness and survivability of such organisms. Probiotics capable of expressing host receptor mimics have been developed to exploit this, whereas others express toxin receptor mimics (Sleator & Hill 2008b).

Koo et al. (2012) showed that *L. monocytogenes* adhesion and invasion could be disrupted by a recombinant probiotic *L. paracasei* expressing *Listeria* adhesion protein (LAP). A separate study demonstrated the ability of a recombinant *L. lactis* strain expressing the *Bacillus cereus* CH flagellin gene to inhibit the adhesion of *E. coli* LMG2092 and *Salmonella enterica* sp. *enterica* LMG15860 to mucin (Sánchez et al. 2011). Probiotics expressing receptor mimics have also been developed against a number of bacterial toxins, including Shiga toxin (Stx). Shiga toxin–associated infections such as *E. coli* O157:H7 and *Shigella dysenteriae* often involve life-threatening complications (Pacheco & Sperandio 2012, Spinale et al. 2013). A recombinant nonpathogenic *E. coli* (CWG308)

expressing a Stx receptor mimic has been shown to neutralize the toxin effectively (Paton et al. 2000). When challenged with a 100%-fatal dose of Shiga toxigenic *E. coli* (STEC), all mice that received the recombinant bacterium survived. Furthermore, the expression of globotriose and globotetraose has been shown to neutralize Shiga toxins in humans and pigs, respectively (Paton et al. 2001). Similar recombinant *E. coli* CWG308 strains expressing lacto-N-neotetraose and the ganglioside GM_1 receptor [successfully targeting the labile toxin (LT) of enterotoxigenic *E. coli* (ETEC) (Paton et al. 2005) and cholera toxin (Ctx), respectively (Focareta et al. 2006)] have also been shown to have biotherapeutic potential.

The ability to neutralize toxin binding to host cells has the potential to reduce disease symptoms and allow the immune system to overcome the pathogen. Furthermore, this approach does not apply a selective pressure on the pathogen, making it less likely for the toxin to develop reduced affinity to the receptor mimics. In any case, modification of the toxin sequence is likely to change its binding affinity for its intended target of receptors on the host cell surface.

PRODUCTION OF ANTIMICROBIAL COMPOUNDS

The beneficial impact of some probiotics has been ascribed to their ability to produce antimicrobial compounds in the host that target and kill invading pathogens (Corr et al. 2007). Chen et al. (2010) describe the construction of a recombinant *Lactobacillus casei* capable of producing and releasing human lactoferrin (hLF). Histopathological analyses of the small intestine following oral administration revealed decreased intestinal injury and increased villi length in mice fed with the hLF-expressing recombinant strain compared with the control group, following challenge with a pathogenic *E. coli* strain ATCC25922 (Chen et al. 2010). Additionally, Saeidi and coworkers described a novel system that enables a bioengineered *E. coli* strain to sense and kill *Pseudomonas aeruginosa*. Designed to detect the presence of *P. aeruginosa* through intercellular quorum communication, pyocin S5 (a colicin-like bacteriocin) is synthesized intracellularly and released by E7-mediated cell lysis, exposing *P. aeruginosa* to the toxic bacteriocin (Saeidi et al. 2011). Recombinant strains have also been designed to target Gram-positive pathogens, such as the intracellular foodborne pathogen *L. monocytogenes*. Field et al. (2010) described the construction of a *L. lactis* designed to express Nisin V, a novel and more potent derivative of Nisin A. The heterologously expressed Nisin V exhibited a twofold enhanced potency in comparison to Nisin A against two *L. monocytogenes* strains, indicating its potential as a biotherapeutic or food additive in the future (Field et al. 2010). Another bioengineered probiotic exhibiting antilisterial activity is an enterocin A (EntA)-expressing *L. casei* (Jiménez et al. 2015).

As they are not restricted to treating bacterial infections, commensal bacteria have also been bioengineered to produce antiviral peptides. Several constructs have been developed to target HIV infections at the mucosal layers of the cervico-vaginal and gastrointestinal tracts, where viral transmission predominantly occurs. Chang et al. (2003) describe how a common vaginal isolate of *Lactobacillus jensenii* was bioengineered to secrete CD4 proteins, significantly reducing viral infectivity of HeLa cells. Even more dramatic results were reported by Rao et al. (2005) using a probiotic *E. coli* strain Nissle 1917 capable of forming a protective biofilm on mucosal surfaces as well as secreting an HIV fusion inhibitor peptide.

VACCINE AND DRUG DELIVERY

The development of recombinant probiotics as delivery vehicles unveils exciting new possibilities for their use not only as biotherapeutics but also as effective prophylactic agents in enteric infections (Sleator 2015a). Although vaccine development to date has focused on the use of attenuated pathogens, the attendant risk of such constructs reverting to a virulent phenotype remains a very

real possibility. Employing live vectors, particularly lactic acid bacteria (LAB), as vaccine delivery vehicles has the advantage of oral administration and mucosal immune stimulation (Johnston et al. 2013, 2014). The gut-associated lymphoid tissue (GALT) is exploited to induce humoral and cellular immune responses (Johnston et al. 2010). Additionally, the native tolerance of the intestinal immune system to many LAB represents a significant advantage, as there is little risk of hypersensitivity following repeated administration (Wells & Mercenier 2008).

Vaccinations using recombinant probiotics against *Yersinia pseudotuberculosis* (Daniel et al. 2009), *S. enterica* (Kajikawa et al. 2012), ETEC (Wu & Chung 2007), and *Streptococcus pneumoniae* (Hernani et al. 2011) have elicited specific immune responses in murine models. Furthermore, Mohamadzadeh et al. (2009, 2010) described recombinant probiotic-delivered vaccines targeted against *Bacillus anthracis* by using *Lactobacillus acidophilus* and *Lactobacillus gasseri* to deliver the *B. anthracis* protective antigen (PA). In both studies, anti-PA antibody and T-cell-mediated responses were observed. An expression system based on the aggregation-promoting factor (*apf*) gene has also been designed to facilitate the delivery of antibody fragments to the gastrointestinal tract by lactobacilli (Martín et al. 2011). The recombinant *L. paracasei* strain displayed protective properties against rotavirus in a murine model of infection.

Additionally, several recombinant lactococcal delivery strains have been modified for improved host colonization/cellular invasion and, by extension, improved payload delivery. For example, Guimarãesa et al. (2005) have developed a *L. lactis* strain expressing *inlA* (a listerial derived internalin that mediates intracellular spread) to function as a DNA vaccine delivery vector. In this study, a functional *gfp* gene was successfully transformed into Caco-2 cells by the *inlA*-expressing *L. lactis*, resulting in the GFP protein being expressed and detected in 1% of the cells. Furthermore, a recombinant *L. lactis* strain expressing *Staphylococcus aureus* fibronectin-binding protein A (FbpA) has been shown to efficiently deliver DNA to human epithelial cells, with comparable internalization rates to *L. lactis inlA*$^+$ (Innocentin et al. 2009).

In addition to whole cell–mediated biotherapeutics, the natural robustness of bacterial spores has led to their emergence as potential vaccine delivery vehicles. Duc et al. (2003) bioengineered recombinant *B. subtilis* spores expressing the tetanus toxin fragment C (TTFC) antigen, which after oral administration were shown to elicit an immune response in a murine model. The mice produced sufficient levels of IgG tetanus antitoxin to survive an otherwise lethal dose of tetanus toxin. Similar results have been reported in studies using *B. subtilis* as a delivery vector for a vaccine against rotavirus (Lee et al. 2010), *Clostridium difficile* (Permpoonpattana et al. 2011), and tuberculosis (Sibley et al. 2014).

The high costs associated with protein drug manufacturing have made the development of cheaper and more robust probiotics capable of delivering recombinant protein an area of growing interest in biotechnology (Sleator 2015a). Furthermore, conventional modes of protein drug delivery, such as intravenous and intramuscular administration, are inherently invasive but necessary, owing to the low bioavailability of such drugs when administered orally. The feasibility of protein drug delivery via recombinant probiotics has been investigated using β-lactamase as a model protein (Kaushal & Shao 2006). In this study, a recombinant *L. lactis* strain efficiently delivered β-lactamase in rats, increasing the oral bioavailability two- to threefold in comparison to the free solution form of administration. Additionally, a linear relationship between the *L. lactis* dose and the β-lactamase absorption has been described, highlighting the potential of this method for the delivery of long-term therapeutics such as insulin and growth hormone (Kaushal & Shao 2009).

IMMUNOMODULATION

Probiotics have also been bioengineered to modulate the immune response. The delivery of anti-inflammatory compounds to the gut by such probiotics may be exploited in the treatment of

inflammatory bowel disease (IBD). Anti-inflammatory cytokine interleukin-10 (IL-10) has been proposed as a possible treatment for IBD, although systemic administration of IL-10 has, to date, been disappointing in clinical trials (Asadullah et al. 2003). *L. lactis* strains secreting IL-10 have been shown to have a protective effect against colitis in murine models (Martín et al. 2014). The feasibility of this mode of IL-10 delivery in humans was demonstrated in a Phase I human clinical trial, the first of its kind for a bioengineered probiotic (Braat et al. 2006). This approach aims to increase the mucosal bioavailability of IL-10 through production at the site of inflammation in the intestine and circumvents unfavorably high levels of IL-10 following systemic administration (Marlow et al. 2013). The results revealed that the bioengineered *L. lactis* was safe, well tolerated, and biologically contained (see below for an overview of biological containment), and reduced disease symptoms. Although it must be taken into consideration that the trial was small ($n = 10$) and without a control group (common in Phase I trials), the results are nevertheless promising and warrant future, larger, placebo-controlled studies (Braat et al. 2006).

A number of bacteria have also been bioengineered to produce immune molecules that successfully target colitis. *L. lactis* secreting interleukin-27 (IL-27) (Hanson et al. 2014), anti-TNF (tumor necrosis factor) (Vandenbroucke et al. 2010), and a serine protease inhibitor, Elafin, have been shown to prevent or reduce colitis in murine models. Furthermore, a *Bacteroides ovatus* strain engineered to produce human keratinocyte growth factor-2 (KGF-2) or transforming growth factor beta (TGF-β1) in response to xylan has also been shown to prevent dextran sodium sulfate–induced colitis in murine models (Hamady 2013). Additionally, immunomodulatory probiotic strains bioengineered to produce catalase or superoxidase dismutase demonstrated increased anti-inflammatory properties compared to their wild-type variants (Carmen et al. 2014). In clinical trials, recombinant *L. lactis* producing mucosal protectant Trefoil Factor 1 (TFF1) has been shown to reduce ulcerative oral mucositis induction in chemotherapy patients (Limaye et al. 2013).

Allergic responses have also been a target of recombinant probiotic therapy. The modulation of acute allergic airway inflammation using IL-10-expressing *L. lactis* was demonstrated in a murine model (Marinho et al. 2010). Treated mice showed a significant reduction in pulmonary inflammation. The immunomodulatory effect of recombinant *L. lactis* expressing bovine β-lactoglobulin has been described (Adel-Patient et al. 2005). A specific Th1 response was stimulated, inhibiting a further Th2 response. Moreover, a significant decrease in specific IgE response was observed in conjunction with increased IgG2 and IFNγ levels. The use of recombinant *Lactobacillus plantarum* and *L. lactis* to induce a specific IgA response to pollen allergen Bet v1 has also been described (Daniel et al. 2006). As a prophylactic, these secretions may function as blocking antibodies at the sites of antigen exposure, potentially preventing type-1 hypersensitivity. Huibregtse et al. (2007) showed that tolerance to ovalbumin could be induced by ovalbumin-secreting *L. lactis*. Additionally, the APC-mediated, OVA-specific, T-cell proliferation that was observed through LL-OVA secretion could not be replicated with a 2,000-fold ingestion of OVA, suggesting that *L. lactis* must play a role in influencing this response.

The numerous examples listed above demonstrate the versatility of bacteria successfully producing various recombinant proteins with positive effects in murine models. However, translational studies from mouse to human are ultimately required and need to go beyond the limited, yet promising, number of Phase I human clinical trials performed to date.

CANCER TREATMENT AND PREVENTION

The incidence of cancer is increasing as a result of the growth and aging of the population, as well as an increasing prevalence of established risk factors (Torre et al. 2015). Colorectal cancer is the second and third most commonly diagnosed cancer in males and females, respectively, with

the highest incidence rates occurring in Australia, New Zealand, Europe, and North America (Torre et al. 2015). As a result, cancer treatment and prevention strategies remain a significant focus of clinical and biological research, with recombinant probiotic bacteria representing a new and emerging class of biotherapeutic agents for this disease. The hypoxic environment associated with tumor growth has been identified as a possible therapeutic target (Harris 2002). Based on this, Sasaki et al. (2006) developed an enzyme prodrug therapy involving the systemic coadministration of cytosine deaminase-secreting *Bifidobacterium longum* and 5-fluorocytosine (5-FC). As predicted, the *Bifidobacterium* localized to, and selectively grew in, the hypoxic regions of the tumors. Once established in the tumor mass, conversion of 5-FC to 5-fluorouracil (5-FU) (an anticancer chemotherapeutic drug) was catalyzed by cytosine deaminase produced by the bacterium, resulting in targeted tumor regression, without the collateral damage associated with conventional therapeutic approaches. A similar approach was employed for the delivery of endostatin, an antiangiogenic agent, using *Bifidobacterium adolescentis* (Li et al. 2003). Work from our own group describes an oral administration strategy for drug delivery to system tumors using a modified *B. breve* strain (Cronin et al. 2010). *B. breve* UCC2003 expressing the *lux* operon migrated from the gastrointestinal tract to organs and tumors via systemic circulation in a murine model, demonstrating the feasibility of this approach. Indeed, bifidobacteria are not the only tumor-targeting probiotics in development; a catalase-producing *L. lactis* has been shown to prevent tumor appearance in 1,2-dimethylhydrazine (DMH)-induced colon cancer in mice (de Moreno de LeBlanc et al. 2008), and *L. lactis* and *L. plantarum* strains expressing the E7 antigen from human papillomavirus type-16 have been developed as a means of vaccination against cervical cancer (Cortes-Perez et al. 2005). In addition to functioning as potential biotherapeutics, probiotics are also being developed as improved and minimally invasive cancer diagnostic agents. Danino and colleagues, for example, have developed a method for detecting hepatic cancer metastases using orally administered recombinant *E. coli* Nissle 1917 (EcN) expressing LacZ. When administered orally with a conjugate of luciferin and galactose, LuGal, the EcN strain targets hepatic metastases; the bacterially expressed LacZ then cleaves LuGal, allowing for luciferin detection in the urine (Danino et al. 2015).

Given a number of rare, yet widely publicized, infections due to probiotics (Besselink et al. 2008, Mackay et al. 1999, Rautio et al. 1999), diagnostics rather than biotherapeutics remain the most likely short- to medium-term application of probiotics in cancer biology. Not restricted to cancer diagnostics, *lux*-labeled *B. breve* has been used for in vivo tracking of probiotic colonization in real time, facilitating detailed anatomical and physiological exploration (Cronin et al. 2008). Indeed, using this approach, we identified a possible role for the appendix as a potential reservoir of bifidobacteria in the body (Sleator et al. 2008a).

PSYCHOBIOTICS

In addition to physical well-being, probiotics might also find applications in improving patients' mental health. Indeed, recent research suggests that gut microbiota may play a role in modulating psychological conditions such as anxiety and depression (Collins et al. 2012). Thus, a novel class of probiotics termed psychobiotics, live organisms that produce health benefits in patients suffering from psychiatric illness, has been described (Dinan et al. 2013). A number of natural, commensal, and probiotic bacterial strains can produce and secrete psychoactive molecules such as γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the mammalian central nervous system (Komatsuzaki et al. 2008, Li et al. 2010a). GABA concentrations in the central nervous system, which are abnormally low in depression (Price et al. 2009) and anxiety (Möhler 2012), may be influenced to some extent by the gut microbiota. Other neurochemicals such as norepinephrine, serotonin, and dopamine have been isolated from a diverse range of genera,

including *Saccharomyces*, *Streptococcus*, and *Bacillus*, respectively (Lyte 2011). Further studies are needed to determine precisely how the microbiota (or their metabolites) may influence the brain (Dinan & Cryan 2016).

Oral administration of *Lactobacillus rhamnosus* has been shown to alter GABA receptor expression in the prefrontal cortex, amygdala, and hippocampus of the brain while decreasing the levels of stress-induced corticosterone in mice, effects that were abolished following vagotomy (Bravo et al. 2011). Moreover, anxiety- and depression-related behaviors were reduced. *Bifidobacterium infantis* has been reported to display antidepressant properties in rats subjected to forced swim tests (Desbonnet et al. 2008). Pro-inflammatory immune responses were attenuated and levels of tryptophan, a precursor of serotonin, were increased following bifidobacterial treatment. Low serotonin levels have been linked with a lowered mood state and cognitive impairments. Supplementation with tryptophan in this state has positive effects on memory and attention (Jenkins et al. 2016). A recent study by Hoban et al. (2016) suggested that the gut microbiota plays an important role in cortical myelination and may be a therapeutic target in myelination diseases such as multiple sclerosis (MS) to promote remyelination.

Although some human trials with psychobiotics have taken place (reviewed by Romijn & Rucklidge 2015), there is a need for more mechanistic studies and larger, placebo-controlled trials to establish efficacy; however, as noted by Dinan & Cryan (2016), this is likely to require a significant cultural change among the probiotic fraternity to commit the necessary level of funding for such trials. Further research into the action of psychobiotics would improve our understanding of their specific modes of action and present an opportunity to produce genetically modified variants, which may be employed in the future as biotherapeutic agents in the treatment of psychological disorders.

BIOLOGICAL CONTAINMENT

The safety and biological containment of all bioengineered probiotic products must first be assessed before being deemed suitable for widespread use. We should not assume that probiotic organisms are innately safe. Conditions in the human gastrointestinal tract favor horizontal gene transfer between members of the microbiota (Sleator 2011, 2013a); therefore, phenotypic characterization in conjunction with genome screening should be performed on any potential probiotic to determine the presence of undesirable virulence factors or mobile genetic elements (Amalaradjou & Bhunia 2013, Sleator 2010b). Additionally, antibiotic resistance must be taken into account in the assessment of the safety of probiotic organisms (Sanders et al. 2010). Furthermore, essential control measures must be enforced, to prevent the escape of bioengineered probiotics into the external environment, by incorporating suitable biological containment systems.

Biological containment systems may be active or passive. Passive systems are based mainly on the complementation of an auxotrophy or other gene defect by supplementation with either the intact gene or the essential metabolite (Lee 2010). A thymidylate synthase–deficient *L. lactis* strain bioengineered by Steidler et al. (2003) loses its viability when deprived of thymine, thus preventing its accumulation in the environment. However, metabolic auxotrophy as a method of biological containment can be bypassed by an availability of the metabolite in the environment or by cross-feeding. The development of synthetic auxotrophic systems, whereby the survival of the organism is dependent on non-natural compounds, circumvents this. Rovner et al. (2015) described a genetically recoded *E. coli* that is dependent on synthetic amino acids to remain viable. The TAG stop codon was converted to a sense codon for the synthetic amino acids through an orthogonal translation auxotrophy. Similar systems using synthetic amino acids have been designed in other

studies (Mandell et al. 2015). A method to develop synthetic auxotrophs for benzothiazole by bioengineering ligand dependence in essential genes has also been described (Lopez & Anderson 2015). Alternatively, active biological containment systems are based on controlling the expression of a lethal function via sensory systems that recognize physical or chemical signals in the surrounding environment (Molina et al. 1998). Torres et al. (2003) devised an example of active biological containment using colicin E3 in *E. coli*. An *E.coli* strain was bioengineered to contain a suicide system that expresses a nuclease gene from *Serratia marcescens* in the presence of arabinose (Li & Wu 2009).

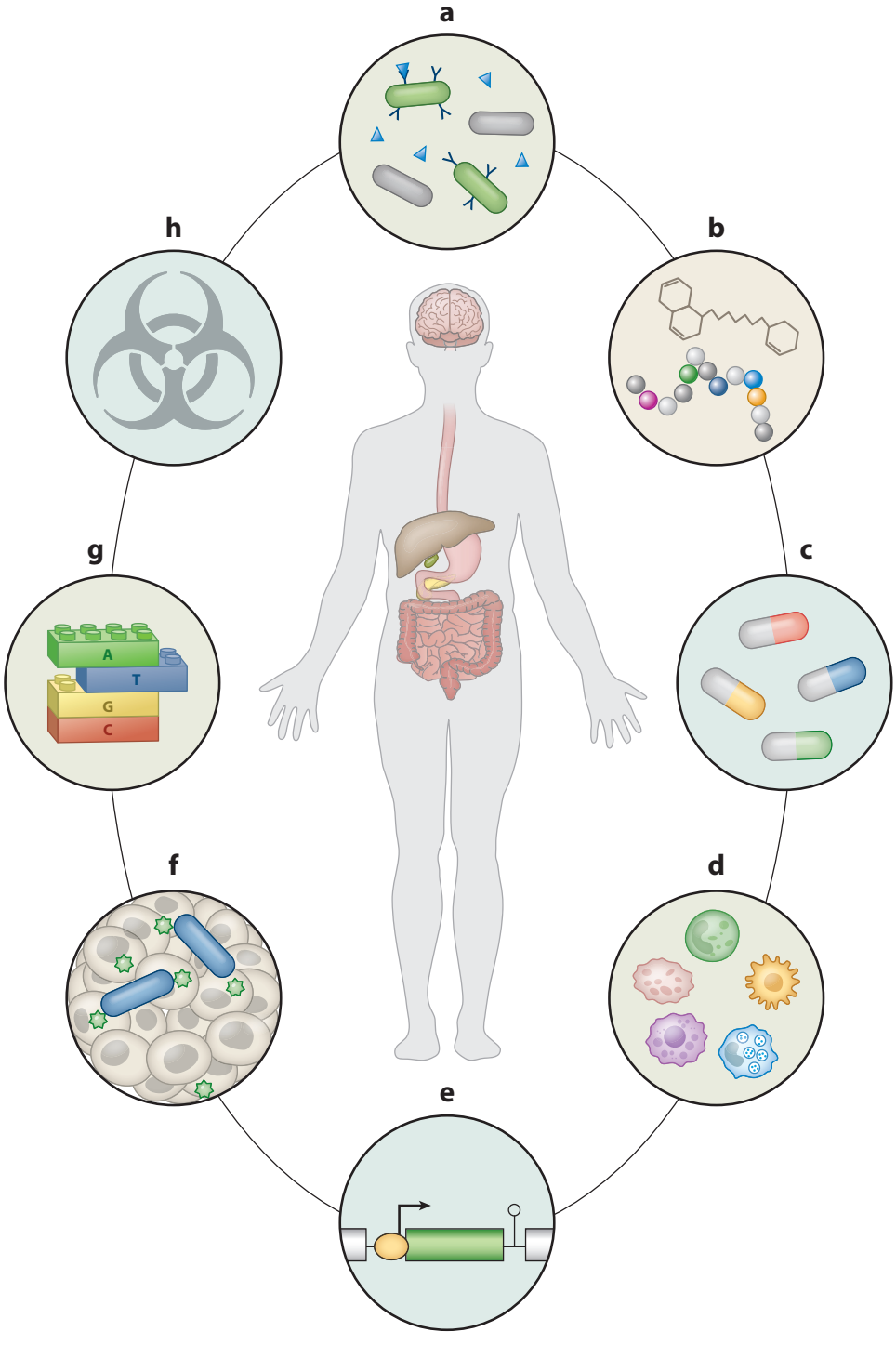
Recently, Chan et al. (2016) described the Deadman and Passcode kill switches as methods of biological containment. The Deadman circuit uses a genetic toggle switch whereby the organism is in a survival or death state depending on the presence or absence of a TetR inhibitor, anhydrotetracycline (ATc). In the absence of ATc, the death state is triggered, which involves the derepression of toxin genes and results in cell death. A variant of this kill switch was also described in which, upon activation, *mf*-Lon protease degradation of essential proteins is induced, resulting in cell death. A combination of both strategies resulted in more efficient biological containment than either system alone. Furthermore, the architecture of the Passcode kill switch requires the presence of two molecules in order to maintain cell viability. A lack of either or both molecules results in toxin expression and cell death. The modularity of these systems presents opportunities to customize both the chemical input signals and the output modules to adapt to a wide range of organisms and environments. The Deadman and Passcode kill switches include a fail-safe mechanism whereby the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) results in toxin expression and ultimately cell death, regardless of the circuit state.

Existing biological containment systems can achieve current NIH (National Institutes of Health) benchmarks for escape of fewer than 1 in 10^8 recombinant or synthetic nucleic acid molecules via either survival of the organism or transmission to another organism(s) (NIH 2016). Nevertheless, each method retains risk and could lead to a reduction in containment efficiency or completely circumvent the system through (*a*) multiple random mutations in important sequences such as regulatory elements or toxin genes, (*b*) natural availability of an essential metabolite in the environment or generation via species cross-feeding complementing auxotrophic strains, and/or (*c*) leaky expression (Gallagher et al. 2015, Kong et al. 2008, Ritger et al. 2011).

Suicide circuits whereby multiple copies of a toxin gene are employed have been designed to decrease the impact of such mutations (García & Díaz 2014). More robust systems incorporating multilayered approaches of both active and passive biological containment have also been described (Gallagher et al. 2015). Auxotrophy, riboregulation of essential genes, and bioengineered addiction in *E. coli* were implemented independently and in combination and demonstrated reduced escape frequency in multilayered, modular systems, even when one layer of containment was compromised. By combining a number of safeguards, escape frequencies were reduced to approximately 1 in 10^{12} (Gallagher et al. 2015). Furthermore, genome reduction experiments of wild-type probiotic strains produced leaner genomes that contained only the genes essential for growth and survival, increasing the likelihood of multiple mutations that lead to cell death (Dewall & Cheng 2011). An overview of the applications of bioengineered probiotics is presented in **Figure 1**.

CONCLUSION AND FUTURE DIRECTIONS

The feasibility of employing genetically modified probiotics as biotherapeutics has been the focus of numerous studies in recent years (Sleator 2010c; Sleator & Hill 2008a,d). Understanding the specific mechanisms underlying the beneficial effects of wild-type probiotics and the pathogenicity in enteric diseases has formed the basis of bioengineered probiotics (Sleator & Hill 2006,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2007). Improving the stress tolerance of probiotics has led to the development of more robust recombinant strains capable of withstanding the challenges of the manufacturing process and, subsequently, the innate defenses of the gastrointestinal tract (Sheehan et al. 2006, 2007; Watson et al. 2008). The improvements in probiotic specificity combined with their development as molecular delivery vehicles could substantially increase their potential as biotherapeutics for infections, chronic inflammatory diseases, cancer, and possibly even psychological illnesses (Sleator 2010d, 2015a,b).

The majority of bioengineered probiotics to date have focused on well-characterized species, such as *L. lactis*, *E. coli*, and members of the lactobacilli, for which extensive genetic modification tools have been developed. Advances in genetic engineering have expanded the repertoire of species available for modification to include dominant members of the gut microbiota, for example, *Bacteroides thetaiotaomicron* (Mimee et al. 2015). A suite of promoters, ribosome binding sites (RBS), and inducible expression systems using recombinases and CRISPRi (CRISPR interference) was developed for *B. thetaiotaomicron*. The authors propose such developments will enable noninvasive monitoring and surveillance of the gastrointestinal tract in vivo, mapping the effects of different genes and pathways on colonization potential, and controlled and focused delivery of biotherapeutics. Integration of strict biological containment and/or suicide systems in such expert gut colonizers (and opportunistic pathogens) is essential. The risk for displacement of natural *B. thetaiotaomicron* populations (which can account for 30% of culturable anaerobic bacteria in the intestine) (Salyers 1984) with their bioengineered counterparts would be higher compared to, for example, *L. lactis* or *E. coli*. Nevertheless, use of similar approaches to other species will add to the catalog of species available for bioengineering applications in the future.

Furthermore, Kotula and colleagues (2014) created an *E. coli* strain with a genetic memory switch, which could sense, record, and report exposure to tetracycline during passage through the murine gastrointestinal tract. The authors propose that such cell-based reporters could be further developed and used to respond to chemical biomarkers indicative of inflammation, cancer, infection, and toxins in the intestine or on the skin, or to produce a biotherapeutic or antibiotic compound as a real-time response to environmental stimuli (Kotula et al. 2014). Indeed, Hwang et al. (2014) used a modular bioengineering approach for *E. coli* to seek and destroy *P. aeruginosa* planktonic cells and biofilms. The three-pronged approach involved production of a bacteriocin (microcin S) and a nuclease (DNase I) in response to quorum-sensing molecules produced by *P. aeruginosa*, while the bioengineered strain also actively moved toward its target via reprogramming the chemotaxis protein CheZ in response to the same quorum-sensing molecule [*N*-acyl homoserine lactone (AHL)] (Hwang et al. 2014).

Figure 1

Overview of bioengineered probiotic applications. (*a*) Probiotics have been bioengineered to express toxin and ligand-receptor mimics, targeting pathogenic microorganisms and their associated toxins. (*b*) Production of antimicrobials (such as antibiotics and bacteriocins) to kill specific pathogens. (*c*) Use of probiotics as delivery vehicles for vaccines and biotherapeutic drugs. (*d*) Production of a variety of compounds for stimulation and modulation of immune cells. (*e*) Creation of probiotics with enhanced stress tolerance via expression of heterologous genes and design and creation of genetic circuitry with precise transcriptional and translational control modules. (*f*) Probiotics to target cancer cells through production of chemotherapeutic compounds in situ and real-time tracking via bioluminescence monitoring within tumors. (*g*) The application of synthetic biology to create the next generation of probiotics; designing and synthesizing genomes and genetic circuits with a specific set of genes tailored for a specific purpose. (*h*) Finally, all such bioengineered probiotics must have strict, built-in biological containment systems that prevent their escape into the environment and/or transfer of genetic modifications to other microorganisms.

An excellent review by Smanski and coworkers (2016) discusses the role of synthetic biology in the design and synthesis of natural products. Significant advances, coupled with ever-reducing costs in DNA sequencing and synthesis technologies, will drive the production of novel natural products such as antibiotics, antivirals, anticancer and immunosuppressive compounds, and enzyme inhibitors. A staggering number of such products remain undiscovered; the genus *Streptomyces* alone is estimated to produce 150,000 natural compounds, of which fewer than 5% have been described to date (Smanski et al. 2016, Watve et al. 2001). Such untapped resources will undoubtedly yield novel candidate antibiotics, which are urgently required to combat the increasing rates of antibiotic resistance among bacteria (O'Neill 2014).

Utilizing advances in synthetic biology enables precise regulation of gene expression and allows modular assembly of large operons with built-in synthetic transcriptional and translational control for each module, which can ultimately result in increased expression and product yield and the ability to heterologously express such gene clusters in different hosts. Such developments have applications not only in human biotherapeutics but also in crop protection and production of biomaterials, biocontrol agents, and biosensors (Smanski et al. 2016). Furthermore, recently in the United States, the White House announced the National Microbiome Initiative (NMI) (Alivisatos et al. 2015), pledging $121 million in federal funding for microbiome research. In addition, more than 100 external institutions have also pledged funding to support the initiative. For example, Novartis has teamed up with the University of California, San Francisco, and the Broad Institute at the Massachusetts Institute of Technology to leverage bioinformatics, synthetic biology, and analytical chemistry to mine microbiomes for novel natural products and new classes of pharmaceuticals. The full list of projects can be found at the following link: **https://www.whitehouse.gov/sites/whitehouse.gov/files/documents/OSTP%20National%20Microbiome%20Initiative%20Fact%20Sheet.pdf**.

In the longer term, synthetic biology is likely to play a key role in the further development of the field (Sleator 2014b). The creation of the first cell with a synthetic genome (Gibson et al. 2010) and more recently the creation of a minimal bacterial genome (Hutchison et al. 2016, Sleator 2016) have laid the foundation for the design and creation of whole new bacterial cells, tailored for a specific purpose and with a specific set of genes. In just 20 years, the field has advanced from sequencing the first bacterial genome to the design, synthesis, and assembly of a synthetic genome (Sleator 2016) and associated biomolecules (Sleator 2013b). Further advances, including automated genetic circuit design (Nielsen et al. 2016), whole-cell computational models (Sleator 2012), DNA-mediated big data storage (O'Driscoll & Sleator 2013), and an expanding genetic alphabet (Sleator 2014a), will ultimately lead to the next generation of true designer probiotics.

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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