# Containment Considerations for Safe Bacteria Based Drug Delivery

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## Introduction

Synthetic biology is an emerging field of research propelled forward by rapid technological advancements at the intersection between science and engineering. The complementation of our improving capacity to design artificial biological systems and our current open-minded innovative zeitgeist has permitted the potential application of synthetic biology to human health. However, the use of synthetic biology tools as therapeutics or diagnostics in human populations presents a series set of risks. Care must be taken to ensure that synthetic biologists do not exceed the limitations of available safeguards for preventing dangers associated with genetic engineering, unsafe practices, or related public health hazards. Reflecting specifically on bacteria-mediated therapies for their intimate interactions with prospective human hosts, this guideline review identifies three potential ethical and health concerns associated with bacterial drug delivery systems: lateral gene transfer, bacterial overgrowth, and chassis failure. These areas of risk are explored in the context of probable underlying cellular and molecular mechanisms and form the basis of our design recommendations to mitigate the risk of a synthetic biology-related catastrophe. In doing so, the McMaster iGEM Team 2 established a set of key Human Practices principles which then informed the rational design of its own self-regulating bacterial drug delivery system.

## *In vivo* chassis delivery design considerations

At the crux of protecting against potential health hazards is the selection of a proper-functioning, yet also safe, stable, and optimally symbiotic bacterial chassis. While a commensal vehicle may appear to be the most intuitive choice, idiosyncratic health risks could emerge if the commensal or probiotic chassis invades a niche other than its intended target.[1] Even within an organ system such as the gut, microbial composition can differ between regions, highlighting the intimate relationship between *in vivo* microenvironments and the ecology of endogenous bacteria. To give a historical example, early attempts at bacteria-mediated oncolysis were associated with acute autoimmunity and mortality in various mice and rat models as they were injected directly into the systemic circulation.[2] [2b] [3] Due to the inherent risk that lipopolysaccharide and other foreign proteins associated with gram-negative bacteria will be

recognized by the body's innate immune system, systemic *Salmonella* infection has been shown to mount septic shock and mortality in clinical studies.[4] In this regard, bacterial chassis and route of administration must both be taken into consideration to ensure that a given bacteria-delivered drug therapy is neither intrinsically incompatible with its intended niche nor capable of eliciting off-target adverse effects.

To ensure that the chassis arrives at the intended location, researchers designing synthetic biology treatments must carefully consider the bacteria's route of administration. When specifically localizing a therapy to a cancerous lesion, it is important to note that certain bacteria, such as *Salmonella typhymurium*, are significantly more effective at suppressing tumour growth when injected directly into the tumour itself, rather than intravenously.[5] Because tumors are immune-compromised, intratumoral injection could also minimize the risk of triggering a systemic inflammatory response to bacteria-mediated therapy.[6] In addition, survival of *S. typhymurium* is not compromised by the characteristically hypoxic conditions of the tumor micro-environment.[7] Meanwhile, less of a difference may be expected for strains that are known to almost exclusively accumulate in cancerous tissue, as with *C. novyi*.[8] By comparison, oral administration remains the most widely accepted drug-delivery route; it is generally heralded as a simple, versatile, non-invasive alternative to systemic or intratumoral injection. Nevertheless, a prominent concern associated with this route of administration is whether the bacteria will desirably interact with or integrate into the gastrointestinal microbiota, and effectively cross the intestinal epithelial barrier to reach peripheral niches.[1]

A final point of consideration is the differential propensity of select bacteria to succumb to metabolic overload *in vivo*. One may envision, as if the bacterial chassis were a mere membranous chamber storing plasmids or drugs for delivery, that the settings of a particular *in vivo* niche might result in influx, efflux, or disruption of metabolic processes in the cell cytoplasm. Whether endotoxins can leak through the bacteria's own physical defenses, or whether this is a liposaccharide envelope or peptidoglycan coat are specific examples.[9] Further downstream, these homeostatic imbalances could pose both an indirect risk to the sustainability of the chosen chassis and a direct risk to the delivery of the treatment molecule, particularly if foreign conditions induce unintended chassis-circuit interactions.[10] In this regard, synthetic

biologists must remain conscious that a bacterial chassis, while it may be purposed for therapeutic delivery, is subject to subpar performance if its intrinsic biological needs are unmet or incompatible with its target environment. Otherwise, there remains the persistent risk that the number and fitness of bacteria, while highly predictable *in vitro*, will become dysregulated and secondary to pathogenesis in a clinical setting.

In choosing an appropriate bacterial chassis, interactions between the circuit and chassis, the possibility for metabolic overload, cell envelope robustness, and chassis microenvironmental interactions must be considered from a holistic standpoint, as illustrated in the following diagram.

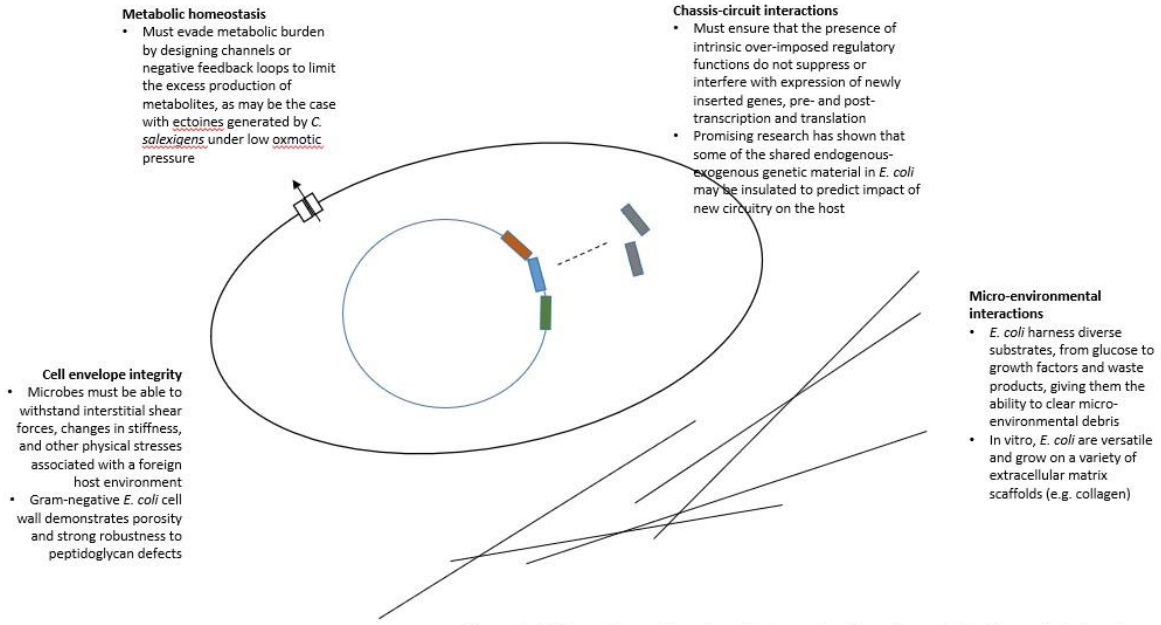


**Figure 1.** Points of considerations in the selection of a suitable bacterial chassis

## Plasmid-based dysregulation considerations

### Mechanisms of Plasmid-based dysregulation

*Plasmid-based delivery systems are inherently unstable in vivo.* Environmental selection is a critical part of plasmid-based genetic engineering. Without selection, there is no advantage

for the bacteria to carry a specific gene, and it can be lost over generations.[11] As the concept of bacteria delivery to human tissues is still a novel concept, the exact rate at which this loss occurs has not been fully documented. In one study, only 10% of *Salmonella typhimurium* designed to target tumours were found to contain the original bio-engineered plasmids after 24 hours within a mouse model.[12] Furthermore, it was shown that the rate of plasmid loss was directly correlated to bacterial turnover rate and indirectly to the rate of bacteria growth. Some factors that have been found to affect plasmid loss in *in vivo* systems are: (1) the inherent growth capacity of the bacteria strain and (2) Spatio-temporal position of the bacteria within the body, as areas of high nutritional availability like the periphery of a tumour are highly conductive towards bacterial growth and turnover.[12]

*Horizontal gene transfer (HGT) leads to an increase of pathogenic bacteria.* In HGT, bacteria in close proximity can share genetic material through mobile genetic elements like plasmids, insertion sequences, transposons, and introns.[13] The human body is composed of an entire microbiome of bacteria, containing potentially pathogenic species. Each bacteria fills a niche in order to colonize, and transferred genes may be kept if it increases the overall fitness. HGE can occur through three different mechanisms: Transformation, transduction, and conjugation.[14] Transformation involves the uptake of DNA by competent cells, or bacteria with cell walls that allow DNA to through easily.[14] The genetic material must first be released from the original host cell, commonly achieved through cell lysis after apoptosis. This is particularly relevant in human systems, which contains numerous pathogenic and competent bacteria such as *Campylobacter, Haemophilus, Helicobacter, Neisseria, Pseudomonas, Staphylococcus* and *Streptococcus*.[14] Transduction is achieved virally. Bacteriophages can be incorporated into host genomes and facilitate HGT. While they can carry dangerous virulence factors, bacteriophages have a limited host range. Lastly, conjugation is a method of HGT that is achieved primarily through plasmids. It is the most likely mechanism behind the transfer of antibiotic resistance genes. It is mediated by cell to cell contact, and then the transfer of plasmids through conjugative pili. The genes are sent through episomes called F-plasmids. These can integrate into the genome of the target bacteria through homologous recombination. Each of the plasmids must contain its own origin of replication (oriV) and origin of transfer (oriT).[14]

Reasons for concern

*The instability of plasmids leads to unpredictable dosages administered.* The dosage administered to patients when using bacteria-based drug delivery is dependent on logic gates present within its genetic circuit that use environmental stimuli to calculate drug release. However, due to the high number of factors affecting plasmid loss within the bacteria, the actual amount *in vivo* can greatly differ from case to case.[10] The volatile nature of this treatment increases the risk profile significantly, reducing the likelihood of safety agency authorization. Deleterious effects can arise from extreme dosage amounts. Additionally, if the plasmid is lost before the necessary compounds are delivered, then the effectiveness of the treatment could become compromised.[14]

*HGT can lead to virulence factors being transferred bi-directionally between bio-engineered bacteria and pathogenic bacteria.* Through the three mechanisms mentioned above, HGT can lead to virulence factors being transferred from pathogenic bacteria to the introduced bacteria. Through this mechanism, two important genes can be transferred: antibiotic-resistance genes and virulence factors. The susceptibility of bio-engineered bacteria to antibiotics is an important containment measure, ensuring that antibiotic administration can manage resultant unintended symptoms if they are found. However, due to prior use of antibiotics, the human microbiome has been characterized to harbour plasmids containing antibiotic-resistance genes . Formed by random mutations, antibiotic-resistance genes are continuously transferred throughout the system through selection processes in an antibiotic rich environment.[13] Additionally, pathogenic bacteria can develop virulence factors that promote their fitness, but may induce host disease They are known as colonization factors due to their ability to increase their own colonization rates within the host. Common virulence factors include: (1) Adhesins, which promote adhesion of the bacteria to tissue surface. They bind to the carbohydrate moieties on glycoproteins and glycolipids (2) Invasins, which promote invasion into epithelial cells. This active process can occur through either micropinocytosis or phagocytosis. (3) Evasins, which prevent clearance by the host immune system via processes such as phagocytosis, the complement system, or antibodies. (4) Siderophores, which extract iron from iron complexes in

the host. Iron is an essential mineral used by bacteria to synthesize cytochromes and other proteins.[15]

HGT can also occur in the opposite direction, where genes are transferred from the bioengineered bacteria into bacteria native to the human microbiome. This would all depend on what is added into the plasmids, so can vary on a case by case basis. Virulence can be induced in even non-pathogenic bacteria if the wrong gene is introduced. A simple example involves antibiotic resistance. If a bacteria is grown in a selective antibiotic medium -- as convention in the bacterial genetic engineering process-- and thus contains an antibiotic resistance gene of its own, then this can be transferred to pathogenic bacteria directly, or indirectly through other bacteria in the human microbiome.

Necessary Safety Considerations

*Bioengineered bacteria can be designed to prevent its genetic material from being transferred.* For all three mechanisms of HGT mentioned, genetic "checks" can be put in place to ensure that genes are not lost. In conjugation, plasmids require both an origin of replication and an origin of transfer. As such, plasmids introduced into the bioengineered bacteria should have no origin of transfer. Origins of replication cannot be removed as they are vital for the expression of plasmid genes. In transduction, bacteriophages have very narrow host ranges. Therefore, the strain of bacteria must be chosen very carefully, with special consideration afforded to those that do not associate with bacteriophages. In transformation, naked DNA cannot be allowed outside of the bacteria. Therefore, the method of programmed cell death must be tightly controlled. For example, if lysis is the mechanism used, the DNA can freely be exposed to the extracellular space, to be taken up by other bacteria via transformation. There are many pathways in which DNA degradation can be activated, such as the Apoptosis inducing factor 2 (Aif-2) pathway endogenous to *E. coli.*[16] Other pathways can be activated as well, such as the exogenously activated T4 exonuclease dependent pathways.[17]

*Precise modelling of plasmid loss dynamics must be conducted on a case by case basis.* To prevent complications involved with unpredictable plasmid loss, plasmid degradation within

specific *in vivo* scenarios must be carefully modelled. All relevant spatio-temporal factors must be considered, as bacteria growth is highly sensitive to the outside environment. The strain of bacteria is also particularly important, as growth rates can be intrinsically derived.[12] It is recommended that a similar model to the ordinary differential equation (ODE) described by Danino et al is pursued for each case.[12] Computational models can be used to make predictions on dosage, straing growth rate, and plasmid loss rate to create transient expression profiles. *In vivo* experimentation would need to ensue to confirm the computational predictions, due to how dynamic and unpredictable human systems can be. These experimental models would require testing in humans, due to the significant difference in bacteria translocation between humans and model organisms like mice.[18] Other considerations can be made to ensure that plasmid loss is reduced to ensure drug delivery, such as decreasing the size of the inserts or increasing the number of copy numbers in the plasmid backbone.[19]

*Genome editing allows for plasmid-free systems.* While plasmids are the simplest and most cost-efficient way to bioengineer a bacteria, genome editing techniques can accomplish the same tasks but in the chromosome of the bacteria itself. For example, the CRISPR Cas9 method of editing can be used to add the required sequences similar to how they appear in a plasmid. As horizontal gene transfer requires mobile genetic elements such as plasmids, integration directly into the genome prevents genetic transfer to pathogenic bacteria and the lost of plasmids.

*Bacteria ghosts allow for DNA-free systems.* DNA-free bacteria, called bacteria ghosts, can be used as a mechanism  for drug delivery. It lacks the ability to colonize on organs, but can still be regulated via customization of its inner and outer surfaces. It keeps the surface structures as well as its immunomodulation abilities. Furthermore, without internal mechanisms for gene expression, no forms of horizontal gene transfer can affect it. It still takes advantage of two properties of bacteria: (1) Propulsion: Bacteria can use their flagella for propulsion through both liquid and semi-solid environments. They are able to move in different ways including swimming, swarming, twitching, gliding, and sliding. As such, they are more efficient than man-made propellers, and are highly desirable as physical carriers. *(2)* Bacteriotaxis: The spacio-temporal locating abilities of the bacteria can still be maintained within these bacteria ghosts. They still contain all surface antigens as a normal bacteria that help with bacteriotaxis, or the

movement along an environmental gradient. Different forms of bacteriotaxis include aerotaxis, phototaxis, chemotaxis, pH-taxis, and thermotaxis.[1]

The process to make a bacteria ghost first involves controlled expression of lysis gene E. A transmembrane tunnel structure is formed on the outer membrane, allowing for outflow of all cytoplasmic content. Once the bacteria is emptied, a pre-made drug is encapsulated within and attached to the inner cell membrane.[1]

**Moderating Growth Considerations**

Engineered bacterial cancer therapies aim to provide controllable and targeted treatment of tumors. These features contrast the major issues arising with current cancer treatments. Common concerns with bacterial therapies surround safety and the risk of systemic infection. One way of mitigating the risks involved with such therapies is through the development of regulations, which prevent the unmodulated proliferation of bacteria. Current advancements in synthetic biology have allowed for controllability of bacterial systems through the introduction of logic gates that regulate growth and promoters that are sensitive to environmental stimuli, for example, specific to the tumour microenvironment. The goal of these features is to allow for the development of a bacterial niche in a desired location. This would avoid risks associated with systemic contamination by bacteria. In the general public, this is a prominent concern. There has consistently been fear associated with the idea of introducing bacteria into the body as a form of treatment. The goal of growth modulation of to mitigate this concerns in order to allow for the development of a low risk and effective cancer therapy.

A significant advantage to the use of biohybrid drug delivery systems is the ability for greater spatio-temporal control either through active or passive means. Genetic manipulation of bacteria enables fine control over the exhibition of therapeutic effects. Active mediation includes the addition of chemical inducers or the incorporation of a magnetic based control system, for instance, while passive control relies on environmental stimuli. These mechanisms allow for greater control than what is possible for standard drug delivery systems, which often rely on

passive diffusion to the target site and consistent administration of doses to maintain an adequate concentration of the drug.

As previously mentioned, one method of containing bacterial proliferation to the target region is through the implementation of logic gates in the engineered genetic circuit. Logic gates help to ensure that the survival of bacteria is only possible under certain conditions, which are specific to the target region. Some common environmental stimuli harnessed for bacterial cancer therapies include pH and oxygen levels as well as temperature and glucose gradients.[1] By developing a circuit that includes promoters sensitive to multiple stimuli specific to the tumour microenvironment in combination with logic gates that require the presence of these stimuli to allow for bacterial growth, containment can be achieved. For example, Anderson et al., first demonstrated the use of an AND gate in bacterial therapy to integrate the sensing of both acyl-homoserine lactone (AHL) and Mg+.[20] This system made use of quorum sensing to monitor bacterial density. Quorum sensing further enables regulation by limiting bacterial growth once population density increases past a certain threshold. This is another method of preventing the proliferation of bacteria to toxic levels. This was demonstrated by You et al. through the development of an autonomous population control circuit which regulates bacterial population density based on the signals broadcasted through quorum sensing. The steady state in terms of cell density and gene expression can be set and is easily adjustable by altering the stability of the quorum sensing molecule.[21]

As knowledge surrounding characteristics of the tumour microenvironment and biomarkers increases, more complex and specific logic circuits also increases, allowing for more comprehensive and safer bacterial cancer therapies. Future challenges involve the demonstration of the safety of bacterial therapies to both the general public as well as law granting agencies. Potential strategies to accomplish this include the development of a standardized proof of concept experimental test to explicitly demonstrate the survival and proliferation of the chassis organism only within the target niche.[10] This will allow for the assessment of potential therapies in a uniform manner prior to approval. It will also help to ensure the safety of any therapies granted approval for further testing. In addition, the design of increasingly complex genetic

circuits is a consistent aim among researchers, in order to develop an even more specific form of treatment.[22]

## Conclusion

Along with the numerous advantageous treatments made possible by synthetic biology and genetic engineering research, also comes many risks. In order to harness the benefits of such therapies, a necessary first step is the implementation of safety protocols and regulations to prevent the transference of hazardous practices to the public. Some considerations that must be undertaken prior to the implementation of any treatments include the prevention of lateral gene transfer, the careful selection of a chassis, and a means of moderating bacterial growth in the body. This paper has discussed each of these challenges and outlines possible routes for future work. The goal of future research therefore, must be directed not only toward the improvement of such bacterial therapies, but also toward the development of safeguards and means of regulation so that these therapies can progress to further stages of testing. Synthetic biology has the potential to be utilized in the future as a means treatment for diseases such as cancer, for which current therapeutics have multiple issues that bacterial therapies have the ability to overcome, but in order to do this, the possible safety and health concerns must first be addressed.

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