Expanding the genetic toolbox for engineering commensal clostridia as live bio-therapeutics

Scientific and Societal Impact

The research presented in this thesis promotes advancements in the field of live bio-therapeutics, particularly focusing on the genetic engineering of commensal *Clostridium* species. Live bio-therapeutics, which include living mammalian cells and microbes, represent a new approach in medical treatments, providing a platform for novel therapeutic strategies that go beyond the capabilities of traditional small molecules and biologics. By leveraging the inherent properties of living organisms, live bio-therapeutics can modulate host physiological processes in more dynamic and responsive ways.

Scientific Impact

A major scientific contribution of this thesis is the expansion of the genetic toolkit for *Clostridium* species, particularly *C. sporogenes* and *C. butyricum*. These tools include:

1. **Molecular Vectors and Gene Regulation:** The development of new molecular vectors and promoter libraries, including strong synthetic promoters, enhances our ability to regulate gene expression rationally. The thesis also reports the optimization of tetracycline-inducible systems and the identification of effective non-canonical start codons in clostridia. These advancements facilitate the controlled expression of therapeutic genes, which is vital for tailoring the therapeutic functions of engineered clostridia and ensuring efficacy in therapeutic applications.
2. **Genome Engineering:** The introduction and refinement of CRISPR-Cas9 and CRISPR-Cas12a systems tailored for clostridia allow for efficient and precise genome manipulations. This includes large-gene deletions, integrations, and the development of multi-gene constructs, which are essential for creating sophisticated genetic circuits. The use of native-cryptic plasmids provides an antibiotic-free method for stable overexpression of gene products in *Clostridium* applications.

The expanded genetic toolkit enables the stable production and delivery of therapeutic proteins, which has several implications:

1. **Cancer Therapy:** Engineered *Clostridium* strains, such as *C. sporogenes* expressing two pro-inflammatory cytokines, interleukin-2 and granulocyte macrophage-colony stimulating factor, and pro-drug converting enzymes, hold promise for targeted cancer therapies. The

ability of these bacteria to selectively germinate in hypoxic tumor environments can be harnessed to deliver therapeutic agents directly to tumors, potentially enhancing treatment efficacy and reducing side effects.

2. **Vaccination:** The potential development of *Clostridium* oral-spore vaccination systems represents a novel approach to vaccination. The ability to produce and deliver recombinant antigen proteins via intestinal germanation of *Clostridium* spores opens new avenues for creating effective and stable oral vaccines.
3. **Probiotic Treatments:** The engineering of probiotic *C. butyricum* for stable overexpression of therapeutic proteins, such as an anti-inflammatory cytokine, interleukin-10, demonstrates potential for enhancing probiotic-mediated therapies. These treatments could play a significant role in managing intestinal diseases, such as inflammatory bowel disease and pathogen infections.

Societal Impact

The advancements in *Clostridium* bio-therapeutics outlined in this thesis have the potential to transform medical treatments for a wide range of conditions:

1. **Personalized Medicine:** The ability to engineer commensal clostridia that can be tailored to individual patients' needs aligns with the growing trend towards personalized medicine. Such treatments can be customized based on the patient's specific genetic and microbiome profiles, leading to more effective and targeted therapies.
2. **Improved Cancer Treatment:** The use of oncolytic clostridia for cancer therapy offers a novel approach that could complement existing treatments. By targeting the hypoxic regions of tumors, these engineered bacteria can deliver therapeutic agents directly to cancer cells, potentially improving treatment outcomes and reducing the burden of chemotherapy and radiation therapy.
3. **Enhanced Vaccination Strategies:** *Clostridium* oral-spore vaccination systems could could change vaccine administration, particularly in regions with limited healthcare infrastructure. These systems are stable, do not require specially evironmental storage (e.g., refrigerator and

freezer), and can be easily administered, making them ideal for mass vaccination campaigns in developing countries.

The development of *Clostridium* bio-therapeutics may also raise important ethical and regulatory considerations:

1. **Biosafety and Containment:** Ensuring the safety of engineered clostridia is paramount. The incorporation of biocontainment mechanisms and visible reporters, as highlighted in this thesis, addresses some of these concerns by facilitating the tracking and containment of live bio-therapeutics within the host.
2. **Regulatory Frameworks:** As live bio-therapeutics move closer to clinical application, robust regulatory frameworks will be needed to oversee development, testing, and deployment of *Clostridium* bio-therapeutics. This includes guidelines for genetic modifications, safety testing, and long-term monitoring of therapeutic efficacy and potential side effects.

In conclusion, the expansion of genetic tools for *Clostridium* species not only paves the way for innovative treatments but also promises to make these treatments more accessible, cost-effective, and tailored to individual patient needs. As research progresses, live bio-therapeutics have the potential to significantly impact healthcare worldwide.