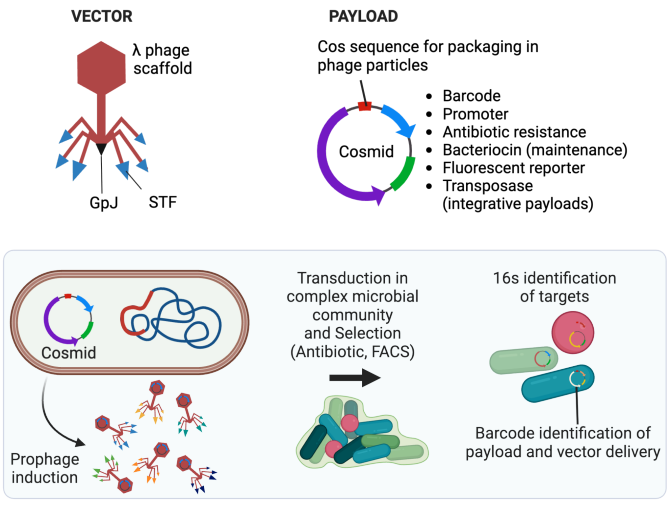
High-Throughput modulation of Phage Host Recognition for *in Situ* Genetic Engineering of Non-Model Gut Bacteria

Research Context and State of the Art. The vast majority of bacterial species remain uncharacterized due to their resistance to traditional genetic manipulation techniques and our inability to reach and modify these bacteria directly in their natural communities. Indeed, the disproportionate focus on a handful of model organisms leaves much of the microbial world unexplored: 74% of bacterial species are not featured in any individual papers, while 50% of papers focus on just 10 species (Callaway 2025). Recent advances in microbiome engineering, such as the Metagenomic Alteration of Gut microbiome by In situ Conjugation (MAGIC) platform (Ronda et al. 2019), enable the genetic modification of diverse gut microbiota through broad-host-range bacterial conjugation. This method employs a donor strain equipped with a conjugative plasmid carrying a selectable genetic payload, which is transferred to recipients in the gut microbiome. By using fluorescence-activated cell sorting (FACS) and sequencing, successful genetic delivery can be identified in various bacterial species. However, MAGIC's reliance on conjugation inherently limits its specificity, as donor bacteria must be introduced into the system and conjugation occurs broadly across bacterial populations rather than being restricted to a specific target organism. Our project aims to overcome these limitations by leveraging bacteriophages as precise DNA delivery vectors, capitalizing on their natural efficiency and our capacity to adapt their host specificity to match hitherto untapped microbiome species.

This research will be conducted under the supervision of Ariel Lindner at Sorbonne Université (SU) and the co-supervision of Raphaël Laurenceau at Institut Pasteur (IP). The project will combine expertise in synthetic biology, notably synthetic phages (SU) and microbiome manipulation through phage-mediated gene delivery (IP), with the high-throughput capabilities of the Paris Biofoundry (SU). By integrating these cutting-edge approaches, the PhD student will establish new methods for precise microbiome engineering and apply them to perform an *in situ* CRISPRi screen in a target bacterium of interest.

AIM1 | Assembling a Systematic Array of Phage Delivery Vectors at the Paris Biofoundry.
Vector engineering. The first objective of this research is to develop a robust and scalable framework for phage-based delivery of genetic payloads into non-model gut bacteria. To this end, we will deploy two parallel approaches: (i) Modulation of protein-protein mediated phage entry, using bacteriophage lambda as delivery scaffold, with modifications to its receptor-binding proteins to expand its host-range (Brödel et al. 2024). Specifically, we will engineer a collection of broad-host-range GpJ variants that recognize major porins such as OmpF and OmpC, combined with a panel of STF (side tail fiber) (See figure). (ii) Modulation of protein-carbohydrate mediated phage entry by using all *in vitro* T7 phage synthesis in cell-free transcription-translation mix (TXTL; Levrier et al 2024), incorporating T7 tail fiber mutant library and cargo (Levrier et al. 2024). Using *in vitro* synthetic T7 facilitates the use of phage mutants that cannot propagate *in vivo* and are thus used only for delivery.
Payload engineering. We will use the Paris Biofoundry high-throughput capacity to design, build, and test combinatorial assemblies of sets of regulatory sequences, markers, transposase, or origins of replications, drawing from existing resources such as the Possum toolkit (22 origins of replications; 20 selectable markers) (Gilbert et al. 2023) and the MAGIC toolkit (Ronda et al. 2019) both available through Addgene. Genetic payloads from these toolkits will be barcoded and modularly assembled individually using Golden Gate assembly workflow and fused onto a cosmid backbone compatible with phage λ packaging and delivery or T7 *in vitro* assembly. The ability to generate and test these constructs at scale will provide unprecedented insights into the host range and effectiveness of different delivery strategies.



AIM2 | Broad Delivery and Screening of New Bacteria from Microbiome Communities. Synthetic phage libraries from AIM1 will be used to screen for successful genetic uptake across diverse gut microbiome communities harvested from

mouse and human fecal samples. The IP lab possesses an anaerobic chamber and expertise in growing microbiome samples *in vitro* while maintaining a high community diversity. Using high-throughput 16S and payload marker sequencing will allow for the rapid identification and quantification of successful transductions and the determination of the host range of engineered phages.
A key outcome of this phase of the project will be the isolation and characterization of new genetically modifiable bacterial species, which will then be deposited in the Institut Pasteur Bacterial Collection (CIP). By systematically identifying and cataloging new host organisms, we will significantly expand the range of genetically tractable bacteria available for further research. This will enhance our ability to study microbial interactions within the gut and pave the way for future microbiome engineering applications. An integrated payload within these novel strains could be later used as landing pads for further genetic modulation.

AIM3 | Targeted *In Situ* Microbiome Delivery and CRISPRi in a Non-Model Bacterium. The ability to selectively introduce genetic modifications directly into target bacteria within the gut environment will represent a major advance in microbiome research. By leveraging a phage vector and a target species identified in AIM1&2 and building upon existing proof-of-concept CRISPRi gut microbiome studies in the IP lab (Maire et al., in preparation), the PhD student will aim to demonstrate *in situ* phage-mediated delivery of dCas9 and guide RNA libraries directly into gut bacteria in mice. This will provide a direct comparison of the efficiency and specificity of phage-based delivery versus conjugation-based methods.

Risk Assessment and Mitigation. One of the primary challenges of this project is ensuring the broad host range of the engineered phage vectors. To address this, we will derisk the project by using both the phage λ scaffold which has already shown its capacity for host-range extension, and allows including positive control tail fibers and bacterial targets to benchmark the engineering pipeline, as well as T7 phage for which extensive knowledge of host specificity modulation was recently demonstrated by the SU team. Scalability and reproducibility will also be carefully considered throughout the project. The Paris Biofoundry's high-throughput automation capabilities will be leveraged to ensure consistent and systematic assembly and testing of all constructs. This will allow for the rapid optimization of vector and payload design, minimizing variability and improving efficiency.
Another key challenge is overcoming bacterial immune defenses, which may hinder the maintenance of the payload. This will be partially mitigated by incorporating payloads inside our screens, which have already shown some capacity for overcoming some host defense mechanisms. Other members of the IP lab are currently working on design tools to bypass restriction-modification systems.
It is important to note at this stage that successful phage-mediated delivery to even a handful of distantly related bacteria will be considered a success, providing a proof of concept suitable for publishing the results. We expect that this platform will require several design iterations before reaching the ambitious goal of targeting a large fraction of underexplored bacterial species, likely necessitating a panel of different phage scaffolds.
Notably, AIM3 could be started without AIM1 and 2 being completed by implementing CRISPRi using an already identified phage vector and target bacteria (Brodel et al., 2024).

Conclusion. By integrating advanced synthetic biology approaches with high-throughput automation, this project aims to establish a novel and highly effective method for genetically engineering previously intractable bacterial species. The combination of engineered phage vectors, scalable biofoundry workflows, and in situ microbiome modification strategies will unlock new possibilities for microbiome research and therapeutic applications. This project represents a major step toward the systematic and targeted engineering of gut bacteria, with broad implications for both fundamental science and clinical interventions.

References.

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