SlpA-Guided, CRISPR-Cas3-Armed Lytic Phage Cocktails as a Microbiome-Sparing Adjunct for Recurrent *Clostridioides difficile* Infection

Project Summary / Abstract

Recurrent *Clostridioides difficile* infection (rCDI) is a NIAID/CDC priority threat sustained by a self-reinforcing cycle: the antibiotics used to treat it (vancomycin, fidaxomicin) collapse the protective commensal gut microbiota, so roughly 20–30% of patients relapse after a first episode and the risk of further recurrence compounds with each relapse. FDA-approved microbiota restoration therapies (REBYOTA, 2022; VOWST, 2023) and fecal microbiota transplant reduce recurrence by rebuilding the community but do not directly eliminate the pathogen or its spores. Bacteriophages offer a complementary, mechanistically distinct tool: strain-specific predators that lyse *C. difficile* while largely sparing the surrounding flora that antibiotics destroy. We propose to develop a rationally assembled, engineered phage cocktail as a **microbiome-sparing adjunct** that reduces the vegetative, toxin-producing pathogen burden so that restored commensals can outcompete germinating spores.

The central obstacle is biological: no strictly lytic *C. difficile* phage has been isolated; all characterized phages are temperate, and in epidemic strains such as R20291 (ribotype 027) prophage carriage can modulate toxin expression. We will therefore (1) map the SlpA/S-layer cassette type (SLCT) basis of host range across representative U.S. ribotypes to guide cocktail composition; (2) engineer cocktail phages to redirect the bacterium's endogenous type I-B CRISPR-Cas3 nuclease against its own chromosome and to disrupt lysogeny-control modules; and (3) test cocktail efficacy, microbiome sparing, and microbiota-restoration sequencing in established CDI models. The work builds directly on multi-phage cocktails that achieved complete *in vitro* lysis, suppressed resistant/lysogenic outgrowth, reduced gut colonization, and delayed disease onset in the hamster model (Nale et al., 2016), and on a phage-delivered CRISPR-Cas3 antimicrobial that outperformed its wild-type parent phage in a mouse model of CDI (Selle et al., 2020). The deliverable is a defined, engineered cocktail and an IND-enabling preclinical evidence package for a precision antimicrobial that helps break the recurrence cycle without further microbiome damage.

Specific Aims

rCDI persists in part because its mainstay treatments perpetuate the microbiome collapse that drives it. Phages can complement these treatments by killing *C. difficile* with species-level precision — but every characterized *C. difficile* phage is temperate, risks lysogeny, and (in strains such as R20291) can be associated with altered toxin expression, and host range is narrow because phage adsorption depends on the strain's surface-layer protein A (SlpA) cassette type (Royer et al., 2023). **Central hypothesis:** an SLCT-informed, CRISPR-Cas3-armed, lysogeny-attenuated phage cocktail can reduce vegetative toxigenic *C. difficile* burden while sparing commensals, enabling more durable microbiome recovery when paired with microbiota restoration.

Aim 1. Define the SlpA/SLCT-determined host range of a candidate cocktail across representative ribotypes. *C. difficile* phages adsorb to SlpA, and the SLCT a strain carries governs which phages can infect it (Royer et al., 2023). We will phenotype adsorption and lysis against a panel of clinically relevant U.S. ribotypes (e.g., 027/R20291, 014/020, 078, 106), genotype their SLCTs, and build a strain-by-phage susceptibility matrix to assemble a cocktail whose coverage across SLCTs is rationally maximized. *Quantitative milestone (end Y2 [ILLUSTRATIVE]):* a ≤4-phage cocktail covering ≥80% of panel isolates with documented suppression of resistant/lysogenic outgrowth in co-culture.

Aim 2. Engineer cocktail phages for genome-targeting killing and attenuated lysogeny. We will arm cocktail members with a self-targeting CRISPR array that redirects the endogenous type I-B CRISPR-Cas3 nuclease against conserved essential chromosomal loci — adding genome degradation to holin/endolysin lysis (Selle et al., 2020) — and disrupt lysogeny-control modules (integrase/repressor) to reduce stable lysogen formation. We will apply SlpA structural-determinant data (PLOS Pathogens, 2025) to guide receptor-binding-protein changes that extend coverage to under-served SLCTs. *Quantitative milestone (end Y3 [ILLUSTRATIVE]):* ≥2 engineered leads showing Cas3-dependent killing, ≥10-fold reduced lysogen formation versus parent, and no detectable increase in toxin output in R20291-type strains.

Aim 3. Test microbiome-sparing efficacy and microbiota-restoration sequencing in vivo. Using an *in vitro* gut fermenter and the hamster CDI model (Nale et al., 2016), we will quantify *C. difficile* burden, toxin, commensal community composition, and clinical outcome, compare prophylactic versus remedial dosing, and test the cocktail administered before or after a microbiota-restoration therapy. *Galleria mellonella* will pre-screen engineered variants. *Quantitative milestone (Y5 [ILLUSTRATIVE]):* significant reduction in vegetative burden and toxin with preserved commensal diversity versus vehicle, and a defined dosing/sequencing schedule for IND-enabling study.

Impact. Success yields a defined, engineered phage cocktail and the preclinical data package needed to pursue an investigational phage IND for rCDI — a precision, microbiome-sparing complement to

antibiotics and microbiota restoration.

Significance

rCDI is among the most consequential healthcare-associated infections and a recognized NIAID/CDC priority. The core problem is mechanistic, not merely logistical: vancomycin and fidaxomicin are themselves disruptors of the protective gut community, so clearing an acute episode leaves patients in a state of microbiome collapse that licenses recurrence in ~20–30% of cases, with risk escalating at each relapse. Microbiota restoration therapies — FMT and the FDA-approved live biotherapeutics REBYOTA (2022) and VOWST (2023) — reduce recurrence by rebuilding the community but do not directly eliminate *C. difficile* or its spores, leaving a reservoir from which toxigenic vegetative cells can re-emerge. There is therefore an unmet need for a therapy that *directly reduces* pathogen burden without inflicting the collateral microbiome damage that perpetuates the disease.

Phages address this gap. Their defining property — strain-specific predation — lets them lyse *C. difficile* while largely sparing commensals, the opposite of broad-spectrum antibiotics. This makes phages a rational partner for microbiota restoration: clearing vegetative pathogen so restored commensals can suppress germinating spores. Foundational preclinical work supports feasibility. Nale et al. (2016) showed that optimized three- to four-phage combinations (e.g., phiCDHM1/2/5/6) produced complete lysis *in vitro*, prevented the emergence of resistant/lysogenic clones, reduced *C. difficile* colonization in the hamster gut at 36 h, and **delayed the onset of clinical signs by ~33 h** relative to untreated animals. These data establish proof of concept for cocktail-based control while leaving clear engineering problems — temperance, narrow host range, durability — that this proposal is designed to solve. By defining and de-risking a microbiome-sparing precision antimicrobial, the project targets a priority pathogen at the mechanistic root of its recurrence.

Innovation

This proposal advances four innovations, each anchored to the cited evidence base and aimed at a specific field-limiting obstacle.

- **Receptor-guided cocktail design.** Rather than empirical screening alone, we exploit the finding that SlpA is a general *C. difficile* phage receptor and that the SLCT a strain carries dictates susceptibility (Royer et al., 2023), using an SLCT-indexed susceptibility matrix to maximize coverage — conceptually analogous to matching a vaccine to circulating strains.
- **Dual-mechanism, genome-targeting killing.** We build on phage-delivered CRISPR-Cas3 antimicrobials (Selle et al., 2020), which redirect the endogenous type I-B system against the chromosome and outperformed the wild-type parent phage in a mouse model, layering

genome degradation onto lytic killing to raise the genetic barrier to escape.

- **Confronting temperance directly.** Because all characterized *C. difficile* phages are temperate and prophage carriage in strains such as R20291 can affect toxin expression, we pair cocktail design with targeted disruption of lysogeny-control modules — engaging the field's central liability rather than ignoring it.
- **Positioning as a microbiome-sparing adjunct** explicitly sequenced with microbiota restoration, targeting the vegetative toxin-producing state while treating spores as a phage-resistant reservoir best managed by restored commensals.

Approach

Rigor, reproducibility, and biological variables. All quantitative experiments will be powered a priori, use pre-registered analysis plans, randomize and blind in vivo readouts, include vehicle and wild-type-phage comparators, and report findings across two independent systems (fermenter and animal model). Animal studies will include **both sexes** and analyze sex as a biological variable. Phage stocks will be endotoxin-controlled and authenticated by whole-genome sequencing. Key reagents, strains, and SLCT genotypes will be deposited and documented for reproducibility.

Aim 1 — SlpA/SLCT-resolved host-range mapping and rational cocktail assembly

Rationale. Narrow host range is a defining constraint of *C. difficile* phages and is explained by SlpA-mediated adsorption: the SLCT a strain carries governs susceptibility, and loss of SlpA renders cells resistant (Royer et al., 2023). Mapping this receptor–phage relationship across circulating ribotypes is the prerequisite for a cocktail with predictable coverage.

Experimental design. We will assemble a panel of clinically relevant U.S. *C. difficile* ribotypes (including toxigenic 027/R20291, 014/020, 078, 106). For each isolate we will (i) genotype the *slpA*/SLCT locus; (ii) quantify adsorption and lytic activity (efficiency of plaquing; growth-kinetic suppression in anaerobic culture) for a library of candidate phages; and (iii) build a strain-by-phage susceptibility matrix indexed to SLCT. Using this matrix we will select a minimal phage set (informed by the three- to four-phage combinations effective in Nale et al., 2016) that maximizes SLCT coverage and combines phages with complementary receptor requirements to constrain resistant escape, validated in mixed-culture outgrowth assays.

Expected outcomes. A validated SLCT-to-susceptibility map and a defined candidate cocktail covering the major circulating SLCTs, with documented suppression of resistant/lysogenic outgrowth in co-culture.

Potential pitfalls & alternatives. Some SLCTs may lack covering phages in our library; we will expand isolation and, in Aim 2, apply SlpA structural determinants (PLOS Pathogens, 2025) to engineer broadened adsorption. If adsorption does not predict productive lysis (e.g., abortive infection or restriction-modification), we will weight selection toward functional lysis over binding alone and sequence escape mutants to identify the limiting step.

Aim 2 — Engineering for genome-targeting killing and attenuated lysogeny

Rationale. No strictly lytic *C. difficile* phage exists; temperance risks lysogeny and, in R20291-type strains, altered toxin expression. Selle et al. (2020) showed that a phage-delivered self-targeting CRISPR array can redirect the endogenous type I-B CRISPR-Cas3 nuclease against the chromosome, killing *C. difficile* more effectively than the wild-type parent phage in vitro and in a mouse model — a lethal route that does not depend solely on lytic replication.

Experimental design. We will engineer cocktail-member phages to carry self-targeting CRISPR arrays directed at conserved, essential chromosomal loci, confirming Cas3 dependence (loss of killing in *cas3*-inactivated hosts) and dual-route lethality (genome degradation plus holin/endolysin lysis) against the Aim 1 panel. To attenuate temperance, we will disrupt or delete lysogeny-control modules (integrase/repressor) and screen variants for reduced stable lysogen formation and for absence of any increase in toxin output in R20291-type strains (toxin ELISA/cytotoxicity). We will use SlpA structural-determinant data (PLOS Pathogens, 2025) to guide receptor-binding-protein modifications extending coverage to under-served SLCTs from Aim 1.

Expected outcomes. Engineered phages with Cas3-dependent, genome-targeting killing, ≥10-fold reduced lysogen formation versus parent, no detectable lysogeny-associated increase in toxin, and broadened SLCT coverage, integrated into an optimized cocktail.

Potential pitfalls & alternatives. Genetic manipulation of *C. difficile* phages is technically demanding; if direct engineering of a given phage fails, we will transfer validated CRISPR-Cas3 payloads into more tractable cocktail members or deploy them as standalone components. If lysogeny modules cannot be disabled without crippling the phage, we will rely on cocktail-level suppression of lysogenic escape (Aim 1) plus CRISPR-mediated killing as the primary lethal route, and restrict therapeutic claims to the vegetative state.

Aim 3 — Microbiome-sparing efficacy and microbiota-restoration sequencing in vivo

Rationale. Demonstrating that the engineered cocktail reduces *C. difficile* while sparing commensals — and integrates with microbiota restoration — is the translational crux. Spores remain a phage-resistant reservoir, motivating a vegetative-targeting, restoration-paired strategy.

Experimental design. We will use complementary preclinical systems consistent with the cited evidence base: an *in vitro* gut fermenter and the hamster CDI model used by Nale et al. (2016). Endpoints include *C. difficile* burden (CFU and qPCR), toxin levels, 16S/shotgun-metagenomic profiling of the commensal community (microbiome-sparing readout), and clinical/colonization outcome. We will directly compare **prophylactic versus remedial dosing** (a comparison not resolved by prior work and a key open question), evaluate antibiotic sequencing (e.g., vancomycin then phage) as an exploratory arm, and test the cocktail administered before or after a microbiota-restoration therapy to assess whether reducing vegetative cells enables restored commensals to suppress spore-germination–driven relapse. *Galleria mellonella* will serve as an early triage screen for engineered variants before mammalian studies.

Expected outcomes. Evidence that the engineered cocktail reduces vegetative *C. difficile* burden and toxin while preserving commensal diversity, a defined determination of whether prophylactic or remedial dosing is superior, and an assessment of whether pairing with microbiota restoration improves durability versus either alone.

Potential pitfalls & alternatives. Phage will not clear spores; we frame success as vegetative reduction plus restoration-enabled spore suppression, not sterilization. If hamster pharmacokinetics limit gut phage persistence, we will optimize dosing/formulation and lean on fermenter data for mechanism. Model-specific microbiota differences will be interpreted cautiously and cross-checked across systems.

Timeline

[ILLUSTRATIVE] 5-year project. Years 1–2 [ILLUSTRATIVE]: Aim 1 host-range mapping and cocktail assembly; begin Aim 2 engineering. Years 2–4 [ILLUSTRATIVE]: complete Aim 2 engineering and validation; initiate Aim 3 fermenter and *Galleria* screens. Years 3–5 [ILLUSTRATIVE]: Aim 3 hamster efficacy and microbiota-restoration sequencing; assemble IND-enabling data package. Go/no-go milestones gate progression: coverage-validated cocktail (end Y2 [ILLUSTRATIVE]) and engineered lead variants meeting the Aim 2 quantitative criteria (end Y3 [ILLUSTRATIVE]).

Budget Justification (modular R01-style sketch)

[ILLUSTRATIVE] Modular budget of [ILLUSTRATIVE] $250,000 direct costs/year for [ILLUSTRATIVE] 5 years. **Personnel:** PI (microbiology/phage biology) [ILLUSTRATIVE] 2.4 calendar months; Co-I (CRISPR/phage engineering) [ILLUSTRATIVE] 1.8 months; Co-I (gnotobiotic/animal models) [ILLUSTRATIVE] 1.2 months; [ILLUSTRATIVE] 2 postdocs and

[ILLUSTRATIVE] 1 technician for anaerobic culture, engineering, sequencing, and *in vivo* work. **Other:** anaerobic culture and BSL-2 consumables, phage engineering reagents, NGS for SLCT genotyping and microbiome profiling, fermenter operation, and per-diem animal costs. Subaward [ILLUSTRATIVE] supports a partnering engineering lab. All figures are placeholders pending institutional rates.

Vertebrate Animals

Animal work is proposed. The hamster CDI model (consistent with Nale et al., 2016) and *Galleria mellonella* invertebrate screens will assess colonization, toxin, microbiome composition, and clinical outcome. Procedures, humane endpoints, anesthesia/analgesia where applicable, euthanasia methods, and IACUC approval will be detailed per NIH Vertebrate Animals Section requirements; [ILLUSTRATIVE] group sizes will be justified by power analysis and will include both sexes. *Galleria* is invertebrate and not subject to vertebrate oversight. The minimum number of animals needed for statistical rigor will be used, with the fermenter system reducing mammalian use.

Human Subjects / Clinical Trial

Not applicable to the proposed preclinical aims; no human subjects research or clinical trial is included. For context on translation: investigational phage may be administered to individual patients via the FDA emergency/expanded-access IND (eIND) route under IRB oversight, and this project is designed to generate the IND-enabling preclinical package (host-range, engineering, safety/efficacy) that would precede any such use or a formal IND-based trial.

Team & Environment

[Template — fill with real names/institutions.] **Contact PI:** [Name, Institution] — *C. difficile* phage biology and cocktail development. **Co-Investigator (Phage Engineering / CRISPR-Cas3):** [Name] — type I-B CRISPR-Cas3 payload design and phage genome editing. **Co-Investigator (SlpA Receptor Biology):** [Name] — S-layer/SLCT genotyping and receptor-binding-protein engineering. **Co-Investigator (Cocktail Design / In Vivo Models):** [Name] — anaerobic cocktail formulation and hamster CDI efficacy. **Environment:** anaerobic microbiology, BSL-2, phage genetic engineering, NGS, gut fermenter, and approved animal facilities. An industry collaboration with a phage-engineering platform developer [ILLUSTRATIVE] may support IND-enabling translation. (Investigator names and biosketches to be supplied; expertise descriptions above define the roles each Co-I must fill.)

References

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