Acid-Stable Prophage-Derived Phages and Endolysins Against Antibiotic-Resistant *Helicobacter pylori*: A Genome-Mined Precision Eradication Strategy

Funding mechanism: NIDDK · R21 (Exploratory/Developmental Research Grant)

Project Summary / Abstract

Helicobacter pylori colonizes roughly half the world's population, is a WHO Group I (definite) human carcinogen, and causes peptic ulcer disease, gastric MALT lymphoma, and gastric adenocarcinoma. Eradication is the single most effective way to prevent these outcomes, yet first-line triple and quadruple regimens are failing at rising rates because of surging resistance to clarithromycin, metronidazole, and levofloxacin — leading the WHO to designate clarithromycin-resistant *H. pylori* a high-priority pathogen for new antibacterials. Bacteriophages are mechanistically attractive precisely where antibiotics fail: they kill in a strain-specific, microbiome-sparing way, self-amplify at the infection site, and act independently of the targets that drive antibiotic resistance. The field's central obstacle is concrete and well-documented: almost no strictly virulent (obligately lytic) *H. pylori* phages have been isolated, and the temperate phages that do exist are largely incomplete — among 1,011 complete clinical genomes, 29.5% carried prophages but only ~32% of those were intact (Vale et al., 2024).

This R21 turns that constraint into a strategy. We will (1) genome-mine and induce intact prophages from a panel of antibiotic-resistant clinical strains and profile their host range, acid stability, and gastric-digestion survival; (2) computationally identify and recombinantly produce phage-derived endolysins, screen them against resistant strains and biofilms, and test phage/endolysin–antibiotic combinations for resensitization; and (3) assemble candidate cocktails and obtain proof-of-concept suppression data in established in vitro gastric/biofilm models, with a *Galleria mellonella* invertebrate screen as a non-mammalian efficacy and tolerability gate. Acid-stability (pH 3–11 plus a simulated gastric-digestion model) is a primary screening axis from the outset, anchored to the induced podovirus prophage HPy1R, which remained stable from pH 3 to 11 for 24 h and suppressed *H.*

pylori for up to 24 h post-infection (Ferreira et al., 2022). The scope is deliberately matched to the field's true preclinical status — no completed or registered human trials and no approved product. Deliverables are a characterized prophage/endolysin panel, defined cocktail candidates, and the integrated host-range, stability, and in vitro/invertebrate efficacy dataset needed to justify a subsequent R01 and an eventual FDA investigational pathway. The work advances NIDDK's digestive-diseases mission and intersects gastric-cancer prevention (NCI) and antimicrobial-resistance (NIAID) priorities.

Specific Aims

Antibiotic-resistant *H. pylori* is a high-priority, carcinogenic pathogen for which conventional eradication is increasingly failing, yet no validated non-antibiotic precision therapy exists. Whole-phage therapy is constrained by the near-absence of lytic *H. pylori* phages and by the fact that most temperate prophages are incomplete (Vale et al., 2024). The realistic near-term path is therefore to mine the temperate-prophage reservoir of resistant clinical strains and pivot to engineered, phage-derived agents that survive the acidic gastric niche (Li et al., 2026). This R21 will generate the foundational reagents and proof-of-concept efficacy data for that strategy.

Aim 1 — Genome-mine, induce, and characterize prophages from antibiotic-resistant clinical *H. pylori*. Across a curated panel of clarithromycin-, metronidazole-, and levofloxacin-resistant isolates, we will catalog intact, inducible prophages by PCR and whole-genome sequencing, recover virions by UV induction (with mitomycin C as a standard alternative), and confirm morphology by TEM. Each recovered phage will be profiled for host range across the resistant panel and for stability across pH 3–11 and a simulated gastric-digestion model. This extends the established PCR/in-silico prophage-screening workflow that recovered 12 intact prophages and raised complete-prophage detection to 54.1% in clinical strains (Ferreira et al., 2024), and the HPy1R acid-stability characterization (Ferreira et al., 2022). *Milestone:* ≥3 sequenced, morphologically confirmed, pH 3–11–stable phage candidates.

Aim 2 — Identify, produce, and test phage-derived endolysins and combinations against resistant strains and biofilms. Guided by Aim 1 sequences and *H. pylori* Genome Project lytic-module/regulatory genomics (Vale et al., 2024), we will computationally select candidate endolysins, recombinantly express a prioritized set, and quantify bactericidal (time-kill) and anti-biofilm activity against the resistant panel across gastric-relevant pH. We will then test endolysin- and phage-plus-sub-inhibitory-antibiotic combinations to detect synergy and resensitization, consistent with the field's pivot toward enzymatic and engineered agents as the likeliest near-term clinical entry (Li et al., 2026). *Milestone:* ≥2 expressed, active endolysins with ≥2-log kill at gastric-relevant pH, and ≥1

combination that lowers the effective antibiotic concentration.

Aim 3 — Assemble candidate cocktails and obtain proof-of-concept efficacy in gastric and invertebrate models. We will rationally combine top phages and endolysins to broaden coverage and limit escape, evaluate suppression of resistant *H. pylori* in established in vitro mucus/gastric-mimic and biofilm systems (benchmarked to the ~24-h suppression reference point; Ferreira et al., 2022), and run a *Galleria mellonella* infection screen as a non-mammalian efficacy/tolerability gate. Pre-specified go/no-go thresholds define candidates worth advancing. *Milestone:* ≥1 acid-stable cocktail meeting all go criteria (coverage, log-reduction, *Galleria* survival benefit).

Impact. Success yields a defined, acid-stable, microbiome-sparing reagent set and an integrated host-range/stability/efficacy dataset for engineered anti–*H. pylori* phage strategies, de-risking a future R01 and a clinical-translation pathway toward precision eradication of resistant strains.

Significance

H. pylori is among the most consequential chronic bacterial infections in humans: it colonizes about half the global population, is classified as a definite (Group I) carcinogen, and is causally linked to peptic ulcer disease, gastric MALT lymphoma, and gastric adenocarcinoma. Eradication is the central lever for preventing these outcomes, yet the standard of care is eroding — clarithromycin, metronidazole, and levofloxacin resistance is rising globally, eradication rates with legacy triple/quadruple therapy are declining, and the WHO has formally listed clarithromycin-resistant *H. pylori* as a high-priority pathogen for new antibacterial development. This sits squarely within NIDDK's digestive-diseases mission; the gastric-cancer connection aligns with NCI and the antimicrobial-resistance dimension with NIAID.

Phages offer a mechanistically distinct option where antibiotics fail. They recognize surface receptors on specific strains and, for lytic phages, replicate and lyse the host; this strain specificity spares the surrounding gastric and gut microbiota, and phage killing is independent of the resistance mechanisms compromising current drugs (Li et al., 2026). The honest constraint is twofold: almost no strictly virulent *H. pylori* phages have been isolated, and the temperate reservoir is largely incomplete — in 1,011 complete clinical genomes, 29.5% (298) carried prophages, of which only 32.2% (96) were intact, and prophage prevalence tracked geography and ancestry rather than disease status (Vale et al., 2024). That is exactly why the credible near-term strategy is to mine the rare *intact* prophages of resistant strains and develop engineered, phage-derived agents, rather than wait for natural lytic isolates. By delivering characterized, acid-stable reagents and proof-of-concept efficacy data, this project addresses the principal translational bottleneck and creates the empirical basis for a precision

alternative or adjunct for multidrug-resistant *H. pylori*.

Innovation

- **Embraces the field's real constraint instead of fighting it.** Rather than an unproductive hunt for rare lytic phages, the project treats the temperate-prophage reservoir as starting material and pairs it with phage-derived enzymes — the approach the most recent review identifies as the likeliest first clinical win, alongside genome-based mining, endolysin discovery, and host-range engineering (Li et al., 2026).
- **Integrated pipeline, not isolated parts.** Genome mining, induction, computational endolysin selection, and combination testing are unified into a single discovery-to-candidate workflow rather than studied separately.
- **Gastric-niche survivability as a first-class screening axis.** Acid stability (pH 3–11) and performance through a simulated gastric-digestion model are primary screens from the outset, building on the induced podovirus HPy1R, which remained stable from pH 3 to 11 for 24 h, showed only a small decrease in the gastric phase of an in vitro digestion model, and suppressed *H. pylori* for up to 24 h post-infection (Ferreira et al., 2022).
- **Resistant isolates and resensitization, by design.** The pipeline targets antibiotic-resistant clinical isolates and tests phage/endolysin–antibiotic combinations for resensitization, positioning the output as an adjunct that could lower antibiotic exposure for stubborn, biofilm-associated strains.

This is, to our knowledge, the first effort to combine intact-prophage mining from resistant clinical *H. pylori* with computationally guided endolysin development under an explicit acid-stability gate.

Approach

Aim 1 — Genome-mine, induce, and characterize prophages from antibiotic-resistant clinical *H. pylori*

Rationale. Because lytic *H. pylori* phages are essentially unavailable and most prophages are incomplete (only ~32% intact among prophage-carrying genomes; Vale et al., 2024), the rare *intact, inducible* prophages of resistant strains are the most practical source of whole-virion candidates and the necessary first step toward any cocktail.

Experimental design. We will assemble a panel of well-characterized clinical isolates with defined resistance to clarithromycin, metronidazole, and/or levofloxacin, sourced from both male and female patients and authenticated by sequencing. Each isolate will be screened for intact, potentially inducible prophages by PCR and whole-genome sequencing using the established in-silico workflow that recovered 12 intact prophages and raised complete-prophage detection to 54.1% (Ferreira et al., 2024). Candidate lysogens will be induced (UV; mitomycin C as a standard alternative), virions recovered and purified, and morphology confirmed by TEM. Recovered phages will be tested for host range by spot and efficiency-of-plating assays across the full resistant panel, and for stability across pH 3–11 and through a simulated gastric-digestion model, mirroring the HPy1R characterization (Ferreira et al., 2022).

Expected outcomes. A curated, sequenced intact-prophage catalog from resistant isolates; several recovered, morphologically confirmed phages; and a host-range × acid-stability matrix identifying the most promising whole-virion candidates for Aim 3.

Potential pitfalls & alternatives. Induction may yield few infectious particles, or recovered phages may remain strictly temperate. This is anticipated, not fatal: if virion yield or lytic activity is inadequate, we shift emphasis to phage-derived enzymes (Aim 2), which do not require productive infection, and use prophage sequences purely as an endolysin-gene source — the intended hedge for a field with scarce lytic isolates.

Aim 2 — Identify, produce, and test phage-derived endolysins and combinations

Rationale. Endolysins degrade peptidoglycan directly and sidestep the lytic-phage shortage, which is why the field is pivoting toward enzymatic and engineered approaches as the likeliest near-term clinical entry (Li et al., 2026). Prophage regulatory and lytic-module genomics from the *H. pylori* Genome Project provide a principled basis for locating lysin genes (Vale et al., 2024).

Experimental design. From Aim 1 sequences and published *H. pylori* prophage genomics, we will computationally identify candidate endolysin domains, prioritize a tractable set, and produce them recombinantly. Purified enzymes will be assayed for bactericidal (time-kill) and anti-biofilm activity against the resistant panel across gastric-relevant pH. We will then test combinations — endolysin plus sub-inhibitory clarithromycin/metronidazole/levofloxacin, and endolysin plus candidate phage — to quantify synergy and resensitization (e.g., fractional inhibitory concentration, change in effective antibiotic concentration).

Expected outcomes. A small set of expressed, active anti–*H. pylori* endolysins; quantitative potency and anti-biofilm data; and identification of combinations that resensitize resistant strains or reduce effective antibiotic concentrations.

Potential pitfalls & alternatives. Some endolysins may express poorly or lose activity at low pH. We will screen multiple candidates and constructs, prioritize acid-tolerant variants, and where needed test formulation buffering. Computational selection is hypothesis-generating; every hit is confirmed empirically before advancing.

Aim 3 — Assemble candidate cocktails and obtain proof-of-concept efficacy in gastric and invertebrate models

Rationale. *H. pylori*'s extreme genetic diversity means single agents will not cover clinical populations, motivating rationally designed cocktails; and any candidate must function in the acidic, mucus-covered gastric niche and against biofilm (Li et al., 2026).

Experimental design. Guided by Aim 1–2 host-range and potency data, we will combine complementary phages and/or endolysins to maximize coverage and minimize escape. Cocktails will be evaluated for *H. pylori* suppression in established in vitro mucus/gastric-mimic and biofilm systems, benchmarked against the ~24-h suppression reference point (Ferreira et al., 2022), and in a *Galleria mellonella* infection screen for non-mammalian efficacy and tolerability. Pre-specified go/no-go thresholds (below) define candidates worth advancing.

Expected outcomes. One or more defined cocktail candidates with broadened coverage, acid-stable in vitro suppression, anti-biofilm activity, and invertebrate proof-of-concept — the integrated dataset required to justify an R01 with mammalian in vivo studies.

Potential pitfalls & alternatives. Bacterial escape and narrow coverage are likely; we counter with multi-agent cocktails and phage–antibiotic combinations, and treat *Galleria* as a screening gate rather than a definitive efficacy model, explicitly deferring mammalian studies to future work.

Quantitative go/no-go criteria

Decision point	Go criterion (illustrative)	If not met
Aim 1 → Aim 3 (whole virions)	≥3 phages, ≥2-log host-range coverage of panel, stable pH 3–11 + gastric-digestion model	Redirect to endolysin-only path (Aim 2)
Aim 2 → Aim 3 (endolysins)	≥2 endolysins, ≥2-log kill at gastric pH, ≥1 resensitizing combination	Broaden candidate set / engineer acid tolerance
Aim 3 → R01	≥1 cocktail: ≥3-log suppression, ≥80% panel coverage, anti-biofilm activity, *Galleria* survival benefit vs. control	Re-design cocktail

Decision point	Go criterion (illustrative)	If not met
		composition; defer in vivo

Rigor, Reproducibility & Relevant Biological Variables

Strains will be authenticated by whole-genome sequencing, with resistance genotype/phenotype confirmed by standardized antimicrobial susceptibility testing. All assays use defined controls (untreated, vehicle, reference antibiotic) with biological and technical replicates and pre-specified statistical analysis; effect sizes drive go/no-go calls. To address relevant biological variables, the clinical-isolate panel will include strains from both male and female patients and span the major resistance phenotypes; sex of the source patient and geographic/ancestral origin (which influence prophage prevalence; Vale et al., 2024) will be recorded and reported. Phage and endolysin sequences, genomes, and assay datasets will be deposited in public repositories (e.g., NCBI/GenBank), and protocols and analysis code will be shared per NIH data-sharing policy to support reproducibility.

Timeline

Months 1–9 [ILLUSTRATIVE]: Aim 1 — strain-panel assembly and authentication, PCR/WGS prophage screening, induction, host-range and acid-stability characterization. **Months 6–18 [ILLUSTRATIVE]:** Aim 2 — endolysin selection, expression, potency/anti-biofilm and combination testing (overlapping Aim 1). **Months 15–24 [ILLUSTRATIVE]:** Aim 3 — cocktail assembly, gastric-mimic/biofilm efficacy, *Galleria* screen, go/no-go analysis and R01 planning. **Total project period: 24 months [ILLUSTRATIVE]**, consistent with the R21 mechanism.

Budget Justification (R21 modular)

Requested as an R21 within the standard exploratory limits: **no more than $275,000 in direct costs over the two-year project period, and no more than $200,000 in any single year [ILLUSTRATIVE]. Direct costs (illustrative):** $125,000 (Year 1) + $150,000 (Year 2) = $275,000 total [ILLUSTRATIVE], at or under the R21 two-year direct-cost cap. **Personnel:** PI (2.4 calendar months/year [ILLUSTRATIVE]); co-investigator/phage biologist (1.2 months/year

[ILLUSTRATIVE]); one postdoctoral microbiologist (≈9–12 months/year, scaled to budget [ILLUSTRATIVE]); ~25% bioinformatics-analyst effort [ILLUSTRATIVE]. **Supplies/services:** clinical-isolate acquisition and antimicrobial susceptibility testing, whole-genome sequencing, recombinant expression/purification reagents, TEM, biofilm and gastric-mimic assays, and *Galleria mellonella* (proportionally scaled within the cap [ILLUSTRATIVE]). **Other:** publication and BSL-2 consumables [ILLUSTRATIVE]. No major equipment is requested; existing institutional cores are assumed. Final figures will follow institutional rates and the applicable R21 limits.

Vertebrate Animals

Not applicable. No vertebrate animal work is proposed. *Galleria mellonella* (an invertebrate) is used solely as a non-mammalian infection screen and is not subject to vertebrate-animal regulations. Mammalian in vivo efficacy is explicitly deferred to a future R01, at which point full Vertebrate Animals documentation and IACUC approval will be provided.

Human Subjects / Clinical Trial

Not applicable as proposed; this R21 is non-clinical. Bacterial isolates will be obtained as de-identified clinical strains or from established repositories; any acquisition involving residual patient specimens will undergo IRB review or exemption determination at the performing institution. No human phage administration is proposed. For forward planning only: investigational phage or phage-derived products administered to patients in the United States require FDA oversight, and early access for individual patients has historically proceeded via the FDA emergency/expanded-access investigational new drug (eIND) route under IRB supervision. Consistent with the field's status — no completed or registered human trials and no approved product — such steps are outside this exploratory project and would be pursued only after the proof-of-concept and biosafety data generated here.

Team & Environment

Principal Investigator [NAME, INSTITUTION] — microbiologist/translational lead with antimicrobial-resistance expertise; overall direction and Aim 3. **Co-Investigator, Phage Biology [NAME, INSTITUTION]** — prophage induction, characterization, and endolysin production (Aims

1–2). **Co-Investigator/Consultant, Computational Genomics [NAME, INSTITUTION]** — prophage mining and endolysin selection (Aims 1–2). **Clinical Collaborator, Gastroenterology [NAME, INSTITUTION]** — resistant clinical-isolate sourcing and susceptibility data. **Postdoctoral Fellow and Bioinformatics Analyst [NAMES]** — day-to-day execution.

This proposal builds on published methods and reagents from the international *H. pylori* phage and prophage-genomics community; the team will pursue appropriate material-transfer agreements and collaborations to access reference strains and characterized prophages, and will confirm any named collaboration in the assembled application. **Environment:** BSL-2 microbiology, microbial genomics/sequencing, recombinant-protein, TEM, and biofilm-assay capabilities via institutional cores [INSTITUTION].

References

1. Li Z, Chen L, Huang S, et al. *Helicobacter pylori* phages: resource landscape, translational challenges, and engineered antibacterial strategies. *Archives of Microbiology*. 2026;208(9):441. https://doi.org/10.1007/s00203-026-04996-2
2. Ferreira R, Sousa C, Gonçalves RFS, et al. Characterization and Genomic Analysis of a New Phage Infecting *Helicobacter pylori*. *International Journal of Molecular Sciences*. 2022;23(14):7885. https://doi.org/10.3390/ijms23147885
3. Ferreira R, Pinto G, Presa E, et al. Screening and in silico characterization of prophages in *Helicobacter pylori* clinical strains. *Microbes and Infection*. 2024;27(3):105429. https://doi.org/10.1016/j.micinf.2024.105429
4. Vale FF, Roberts RJ, Kobayashi I, Camargo MC, Rabkin CS. Gene content, phage cycle regulation model and prophage inactivation disclosed by prophage genomics in the *Helicobacter pylori* Genome Project. *Gut Microbes*. 2024;16(1):2379440. https://doi.org/10.1080/19490976.2024.2379440