Receptors Determine Lytic Phage Host Range in Pseudomonas aeruginosa

Authors:

Dea M Müller[1]*, Julie D Pourtois[1], Minyoung Kevin Kim[1], Bobby Targ, Robert McBride[2] and Paul L. Bollyky[1]*

Affiliations:

[1]Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University, Stanford, CA, USA.

[2]Felix Biotechnology, San Francisco, CA, USA.

[*]**Correspondence:** Dea M Müller (deam@stanford.edu) and Paul L. Bollyky (pbollyky@stanford.edu).

Abstract

As antimicrobial-resistant pathogens, particularly the rapidly mutating and adapting *Pseudomonas aeruginosa*, claim millions of lives worldwide each year, the development of novel therapeutic approaches becomes increasingly important. Bacteriophages (phages), viral predators of bacteria, represent a promising solution to fight bacterial infections. However, persistent challenges hinder the widespread application of phage therapy. One of these challenges is the narrow host range of bacteriophages, which are highly specific and only target a limited range of bacterial strains. This specificity poses difficulties in treating polymicrobial infections and limits broader application. Extensive research has been conducted to study host range, however, a definitive answer to the phage or bacterial factors determining phage host range remains elusive. In this work, we combined a transposon mutagenesis library and a Genome-Wide Association Study to identify bacterial genes associated with resistance or susceptibility to the lytic phages OMKO1 and LPS-5. We find that the flagellum is crucial for infection by phage OMKO1 and that lipopolysaccharide biosynthetic genes were significantly associated with LPS-5 phage susceptibility. These results suggest that these structures serve as receptors, which we confirmed through knockout swimming and LPS/O-antigen assays, respectively. Surprisingly, the presence of bacterial defense mechanisms was not a significant predictor of lytic phage infection but was for lysogenic phage infection. Together, these data indicate that receptor structures are important for phage infection. Conversely, they do not support existing paradigms regarding the central role of phage defense mechanisms in determining host range for lytic phages.

Introduction

The emergence of antimicrobial-resistant (AMR) pathogens has become a critical global health concern, necessitating urgent efforts for the development of innovative therapeutic strategies[1]. The gram-negative bacterium *Pseudomonas aeruginosa* stands out among these resistant pathogens; *Pseudomonas aeruginosa* is one of the most frequently-detected nosocomial pathogen and accounts for roughly 10% of all hospital-acquired infections[2], including chronic infections such as wounds of diabetic ulcers and lung infections in individuals with cystic fibrosis[3]. The morbidity and mortality rates associated with both acute and chronic *Pseudomonas aeruginosa* infections can reach as high as 40% [4].

There has been considerable interest in exploring bacteriophages as agents in the fight against AMR bacteria to address the urgent need for novel therapeutic approaches. Bacteriophages are viral predators of bacteria that use the bacterial cell to replicate, resulting in the death of that cell.

There are lytic and temperate phages[5,6]. Lytic phages follow a cycle where they adsorb and infect host bacteria via cell receptors, replicate using cellular machinery, and cause host cell lysis to release new phage particles. This rapid release enables them to initiate new infection cycles in nearby cells. In contrast, temperate phages can enter a lysogenic cycle, where their DNA integrates into the host chromosome or exists as a plasmid-based prophage. Prophages replicate alongside the host cell without immediate lysis, persisting until conditions trigger their transition to the lytic cycle. Bacterial strains have developed mechanisms to resist these processes, sparking an ongoing evolutionary arm race[7]. In response, phages evolve counteractive strategies, such as attachment to essential bacterial structures, enhancing their specificity [8].

Phage therapy has been successfully used in the past, particularly in compassionate use cases[9–14]. There is a growing interest to apply phage therapy in a broader context, with 30,000 papers and literature on phages published during the period between 1965 and 2010, indicating substantial interest and investment in the field[15]. Despite promising results reported in human case reports and studies, therapeutic failure has been documented in other studies, including clinical trials[16,17]. These challenges underscore the importance of further investigation in the field.

One primary impediment is the narrow host range exhibited by many phages[18]. Each phage typically targets a specific bacterial host, a characteristic that also contributes to the safety of phage therapy. The specificity of phages ensures that they exclusively target and eliminate bacteria without causing harm to human cells[19]. However, this very specificity also creates a significant challenge for the development and application of phage therapy. The identification of bacterial receptors, which are important for host range determination, remains a major hurdle, as the majority of these receptors remain unknown. The lack of comprehensive knowledge about bacterial receptors hinders our ability to predict and engineer phages for effective therapies[18].

A second impediment is the emergence of phage resistance by host bacteria. Modifications in receptors and bacterial defense mechanisms, such as the CRISPR system and the Modification and Restriction system, are believed to play a role in resistance development[20–26]. The ongoing arms race between phages and bacteria necessitates a comprehensive understanding of both phage susceptibility and resistance. Much effort is also dedicated to engineering phages, offering potential solutions to the challenges encountered in phage therapy[27].

In this context, the identification of genes involved in phage infection is a fundamental research focus. To study the narrow host range, phage resistance and advance the field of phage therapy, it is important to examine the complex mechanisms including both phage susceptibility and resistance. In this work, we combined a transposon mutagenesis library and a Genome-Wide Association Study to identify bacterial genes associated with resistance or susceptibility to the lytic phages OMKO1 and LPS-5. Our findings can support the development of more effective phage therapies that address the challenges of antibiotic-resistant bacterial infections.

Results

Flagella-associated genes are significantly associated with OMKO1 phage infection

We conducted a transposon mutagenesis (Tn) assay to identify genes crucial for phage infection (Figure 1a). Out of 49 identified genes that were significantly associated with OMKO1 phage susceptibility (Fisher's test, p-value < 0.05), 28 genes were involved in flagellum structure and function (Figure 1b). The complete list of significant genes can be found in Supplementary Figure 1. We then used the enrichKEGG package to explore which functional pathways were significantly enriched. We found that significant functional categories are linked to flagella assembly (enrichKEGG, p-value < 0.05) (Figure 1c). For a more detailed examination, we conducted a functional enrichment analysis using PANTHER (Figure 1d). Here, we were able to identify the particular flagella associated pathways linked to OMKO1 phage infection which include flagellum-dependent cell motility and flagellum assembly (PANTHER, p-value < 0.05). In conclusion, we found OMKO1 infection to be associated with many flagella genes that encode proteins across the flagellum structure (Figure 1e). Similar results were obtained at MOI 20 (Supplementary Figure 2).

In a second approach, a lytic plaque assay was conducted to determine which clinical *Pa* strains are susceptible to specific phages (Figure 2a). This phenotypic data, combined with the genomic information of the clinical isolates, was used in a Genome-Wide Association Study (GWAS) to identify genes associated with either phage susceptibility or resistance. Given the numerous genes identified by the GWAS in association with OMKO1 phage infection, a functional enrichment analysis was conducted to pinpoint enriched pathways. We found that biofilm formation and flagellar assembly were both significantly enriched in genes relevant to phage susceptibility (Figure 2b). We then differentiated between genes from these pathways associated with phage susceptibility and those associated with phage resistance. Notably, genes FliY and FliP in the flagellar assembly group are highlighted as important for OMKO1 phage susceptibility (Figure 2c). These genes were mostly located on the intracellular portion of the flagellum (Figure 2d).

To validate the involvement of flagella as a receptor in OMKO1 infection, we conducted a suppression assay assessing phage infection of OMKO1 across various clinical isolates (Figure 3a). The functionality of the flagella in these clinical isolates was also evaluated using a swimming assay (Figure 3b, c). We observed that bacterial strains resistant to the OMKO1 phage exhibited impaired flagella function, resulting in reduced swimming ability like the ΔfliC strain. On the other hand, susceptible strains demonstrate functional flagella, as evidenced by their swimming area being comparable to the WT strain.

Various bacterial sequences and mutations were identified to be associated with OMKO1 phage infection. The largest differences were observed in the genes FliC, FliD, FlgK, and FlgL. Sequence similarity for these genes were clustered according to percent identity within resistant and susceptible, and consensus sequences for both resistant and susceptible strains were generated (Supplementary Figure 3). The pairwise identity of the consensus sequences was assessed (FliC=52.7%, FliD=only resistant strains had a blast output, FlgK=83.4%, FlgL=72.6%). Additionally, single mutations were identified in other flagella genes, however, with the current sample size, no associations to phage infection could be identified (Figure 3d).

LPS-associated genes are associated with LPS-5 phage infection

In the Tn assay, 4 out of 29 significant genes were associated with LPS biosynthesis (Fisher's test, p-value < 0.05, Figure 4a). The complete list of significant genes can be found in Supplementary Figure 4. We then used the enrichKEGG package to explore which functional pathways were significantly enriched. Mismatch repair was the only significant LPS-5 infection associated group (Figure 4b). For a more detailed examination, we conducted a functional enrichment analysis using PANTHER, which showed some categories associated with LPS function for the phage LPS-5 (Figure 4c). In conclusion, some lipopolysaccharide-associated genes were identified in the Tn assay, which are important for LPS-5 phage infection (Figure 4d). Similar results were obtained at MOI 20 (Supplementary Figure 5).

Given the numerous genes identified by the GWAS in association with LPS-5 phage infection, a functional enrichment analysis was conducted to pinpoint enriched pathways. We found different metabolic groups as well as mismatch repair pathways significantly enriched and relevant to phage infection (Figure 5a). We then differentiated between genes from these pathways associated with phage susceptibility and those associated with phage resistance (Figure 5b).

To validate the involvement of LPS as a receptor in LPS-5 infection, we conducted a suppression assay across various clinical isolates (Figure 6a). The function of the O-Antigen in these clinical isolates was also assessed using a rugae O-AG/LPS assay.

We observed that some bacterial strains resistant to the LPS-5 phage did not form rugae suggesting mutations in their O-antigen within the LPS (Figure 6b). Conversely, some susceptible cells exhibited a rugae phenotype, indicative of LPS containing the O-antigen.

Mutations in LPS biosynthetic genes in these strains were studied alongside with LPS-5 phage infection. The most mutations were observed in the genes MigA and wzy, with some mutations also found in the genes ssg and wapH. With the current sample size, no associations between single mutations and phage infection could be identified (Figure 6c).

The presence of bacterial defense systems is not associated with lytic phage susceptibility

Bacterial defense systems were not identified in the GWAS, indicating that defense systems may not be involved in initial phage infection. To confirm the potential involvement of bacterial defense systems in phage infection, we first screened the bacterial genomes for defense systems, assessed the individual relationship between each system and phage susceptibility using a Wilcoxon test and then included significant predictors of phage susceptibility in a generalized linear model (GLM) to consider the combined effect of these defense mechanisms on phage susceptibility (Figure 7a). The genomes of the 80 clinical isolates were examined for defense systems, resulting in the identification of 123 systems in total (Figure 7b). Each defense system was tested using a Wilcoxon test (eg. RM type I, Figure 7c). No defense system was significantly associated with the phenotype of phage susceptibility or resistance (Wilcoxon test, p-value > 0.05) (Figure 7d). The defense systems depicted in Figure 7d were included in a Generalized Linear Model. This analysis reaffirms the findings of the Wilcoxon analysis, demonstrating that none of the systems are significantly associated with LPS-5 susceptibility or OMKO1 susceptibility (glm, p-value > 0.05) (Figure 7e).

The presence of bacterial defense systems is associated with temperate phages

Bacterial genomes were screened for temperate phages. We first assessed the individual relationship between each system and temperate phages using a Wilcoxon test. We then included significant predictors in a GLM to consider the combined effect of these defense mechanisms on number of temperate phages.

The presence of defense systems PDC-S02, PDC-M61, Helicase-DUF2290 and cas type I-F1 was significantly associated with the number of temperate phages (Wilcoxon test, p-value < 0.05 for all). Two of the significant defense systems (PDC-202 and Helicase-DUF2290) exhibit a positive correlation with the number of phages, while the other two systems (PDC-M61 and Cas type I-F1) show a negative correlation.

The significant defense systems were included in a Generalized Linear Model. This analysis reaffirmed the findings of the Wilcoxon analysis, demonstrating that defense systems were significantly associated with temperate phages (GLM, p-value < 0.05 for all, Figure 7f).

Discussion

Phages offer a promising approach to combat the immense challenge of AMR infections. However, persistent challenges such as the narrow host range of phages hinder the widespread application of phage therapy. Extensive research has been conducted to study host range, however, a definitive answer to the phage or bacterial factors determining phage host range remains elusive. In this work, we study bacterial genes associated with resistance or susceptibility to the lytic phages OMKO1 and LPS-5.

Flagella as receptor and mechanism for OMKO1 phage infection

Our data demonstrates that the phage OMKO1 utilizes the flagellum as a receptor for host infection, which has previously been shown for other phages.[28–33] It is likely that the phage binds to a specific region of the flagellum, serving as a receptor, and then uses the flagellar motion to approach the bacterial surface. Upon reaching the bacterial body, the phage might interact with the entry receptor (OprM) and subsequently infect the bacterium, which has previously been shown for other phages.[28] We hypothesize that OMKO1 uses the outer region of the flagellum, notably FliC, FliD, FlgK, and/or FlgL, as binding region. The high diversity observed in these genes indicates that this region may be under selective pressure, likely due to its involvement in the binding process between the phage and the flagellum. These regions are known to be involved in host-phage interaction for other phages.[28] Phages that utilize the flagellum for infection present a unique opportunity in phage therapy due to the evolutionary tradeoff they impose on their host bacteria. By targeting the flagellum, these phages force the bacterium to downregulate or eliminate motility to evade infection, potentially leading to a reduction in bacterial virulence.[33] In this study, the absence of a transposon insertion in the OprM gene made it impossible to draw any conclusions from the Tn assay regarding the involvement of the OprM gene in phage infection.

LPS-5 exploits the O-antigen of lipopolysaccharide as a receptor

Our findings suggest that the phage LPS-5 uses the lipopolysaccharide as a receptor for infecting its host. Specifically, given that three genes identified in the transposon mutagenesis are linked to the biosynthesis machinery of the O-antigen, we propose that LPS-5 binds to the O-antigen of the lipopolysaccharide. It is known for several phages to bind lipopolysaccharides, specifically the O-antigen as their receptor.[34,35] LPS-5 is among the phages employed in the CF Bacteriophage Study at Yale. The researchers hypothesized that their isolated phage LPS-5 binds to lipopolysaccharide, however, the data supporting this hypothesis was not presented in the study.[36] Our O-antigen experiments partly validated the hypothesis that the O-antigen serves as the receptor for phage LPS-5. Observations of bacterial strains resistant to the LPS-5 phage forming colonies without rugae suggests mutations in their O-antigen within the LPS structure and are consistent with phage LPS-5 using wild-type O-antigen as a receptor. However, only some susceptible cells displayed a rugae phenotype (wt O-antigen) and three susceptible strains exhibited a phenotype lacking rugae. It is possible that in these strains, mutations in other O-antigen genes do not affect phage susceptibility but influence colony morphology. Additionally, other genes controlling rugae formation, such as those involved in quorum sensing, may also play a role in influencing rugae formation.[37] Further work will be necessary to confirm the role of the O-antigen as the receptor for LPS-5.

Metabolic genes associated with phage infection

Experiments for both phages showed enrichment of numerous metabolic pathways in the GWAS and Tn assay, including fatty acid metabolism, amino acid metabolism, pyrimidine biosynthetic and pyrimidine metabolic processes, amino sugar and nucleotide sugar metabolism, glycolysis, citrate cycle and others. Previous studies have highlighted the relationship between phage infection and metabolic changes in host bacteria. [38–40] In one study, multi-phage resistance was found to be associated with reduced fitness in a PAO1 population, but not in clinical isolates. The authors hypothesized that clinical strains, having co-evolved with phages, controlled phage infection not solely through direct modification of genes responsible for surface receptor biosynthesis, but rather through global regulation of cell metabolism. [38] Another study has shown that different Pa phages can not only

deplete metabolites but also completely reorganize the metabolism in a phage- and infection-specific manner, eliciting an increase in pyrimidine and nucleotide sugar metabolism. [39] Thus, phage pressure and infection exert considerable stress on bacteria, which can lead to alterations in their metabolic profiles. This response may manifest in two ways: bacteria may experience metabolic reduction as a result of the stress, or they may adapt their metabolic pathways to compensate for the pressure imposed by phage infection or attempt to develop resistance through metabolic changes.

In our screens, pathways for mismatch repair, particularly involving the protein MutL, and nucleotide excision repair were identified. Some viruses employ non-homologous end joining (NHEJ) for viral genome circularization, and the loss of NHEJ could potentially serve as an anti-viral strategy for the host bacteria.[41] Another study also identified mutations in genes unrelated to the corresponding phage receptor molecules but rather involving deletions in the DNA mismatch repair protein MutL.[40]

Furthermore, in the case of LPS-5, genes linked to the RND multidrug efflux complex were identified. Previous research has demonstrated that the outer membrane porins of multidrug efflux systems may serve as receptor-binding sites for phages. This phenomenon can result in an evolutionary trade-off, wherein phage pressure and bacterial resistance development to phages via alterations in the efflux mechanism may render bacteria more sensitive to various antibiotic classes. [42]

Bacterial defense systems do not explain lytic phage susceptibility

Our investigation into bacterial defense systems yielded unexpected results, as we found no evidence linking these systems to lytic phage susceptibility or resistance. Our findings are surprising given previous studies indicating the opposite, where defense mechanisms were shown to protect from phage infection [26,43,44], or a recent study indicating an accumulation of defense systems in phage-resistant Pa strains [45].

On the other hand, other studies have also shown that bacterial defense mechanisms do not play a role in *Pa* resistance to lytic phages[21,46,47]. One explanation is provided by the diverse and sophisticated strategies phages have developed to counteract bacterial defense systems.[8,23] The findings presented are highly specific to the phages under investigation. The distinction between lytic and lysogenic phages is critical. We show that defense systems indeed don't explain lytic phage susceptibility, however, defense systems are associated with the number of temperate phages in the bacterial genome. Lytic phages almost immediately lyse their hosts, while lysogenic phages integrate into the host genome, allowing the bacterial host time to recognize and eliminate the intruder, as well as to potentially establish an immune memory for further encounters. It is interesting to note that in the temperate phage analysis two of the significant defense systems (PDC-202 and Helicase-DUF2290) exhibit a positive correlation with the number of phages, while the other two systems (PDC-M61 and Cas type I-F1) show a negative correlation. This suggests the presence of two distinct mechanisms. Defense mechanisms are often encoded in prophages, which could confer a competitive advantage to bacteria harboring temperate phages, thus explaining the positive correlation.[48] Conversely, other defense mechanisms may provide protection against temperate phages, accounting for the negative correlation. Indeed, CRISPR systems are known to confer resistance against lysogenic insertion, supporting this hypothesis.[49]

Additionally, the findings presented here are specific to the experimental design and phages studied. The results are tailored to the employed experiments, including Tn mutagenesis, GWAS, experimental validation and the approach using Padloc. Our experiments demonstrate that bacterial defense systems do not influence initial lytic phage infection, which determines host range. Different approaches or experiments exploring the relationship and evolution between phages and hosts over an extended period, particularly those focusing on the development of phage resistance, may yield different outcomes, shown by a recent study suggested an accumulation of defense systems in phage-resistant Pa strains. [45]

Receptors determine phage host range

In conclusion, we identified genes and structural proteins crucial for phage susceptibility and resistance. Specifically, we found that flagella plays a pivotal role in OMKO1 phage infection, while lipopolysaccharide was identified as important for LPS-5 phage infection. Subsequent knockout swimming assays confirmed these structures as receptors for their respective phages.

Surprisingly, our investigation into bacterial defense systems yielded unexpected results, as we found no evidence linking these systems to phage susceptibility or resistance in the context of lytic phages. Screening bacterial genomes using Padloc and correlating with phenotypes from the lytic plaque assay revealed no significant relationship between bacterial defense systems and lytic phage infection. However, our findings suggest that defense systems may play a role in temperate phage infection.

Thus, we show that receptor structures are important for phage infection. Conversely, our data does not support existing paradigms regarding the central role of phage defense mechanisms in determining host range for lytic phages (Figure 7g).

Possibilities and limitations of the applied experimental techniques

Transposon mutagenesis is a powerful, high-throughput technique that enables the study of diverse biological questions. While Tn mutagenesis assays can identify genes linked to phage susceptibility, they do not reveal genes associated with phage resistance, such as resistance mechanisms. A transposon insertion into such a gene (loss-of-function) would not confer protection against phage, making it impossible to study a connection with phage infection. Alternatively, gain-of-function assays, for example Dub-seq assays [50], or cloning genes associated with phage resistance into bacterial cells and subsequently assessing phage infection mechanisms could be considered for further investigation.[47]

When comparing the Tn assay and GWAS methods, several differences emerge in their ability to identify genes associated with phage susceptibility. In the case of flagella identified as a receptor for OMKO1, for example, the Tn assay revealed almost all structural genes involved in flagella structure, while the GWAS identified only a few. These inequalities are due to the distinct principles underlying each method. While knocking out a flagella gene in the Tn assay can demonstrate its impact as a receptor for phage infection, the GWAS may reveal subtle genetic changes associated with phage susceptibility, making it a potent tool for identifying single mutations that may confer bacterial resistance.

Padloc represents a robust tool for identifying bacterial defense systems within bacterial strains, being one of the most updated platforms.[51] However, it's important to acknowledge that the mere presence of a gene in the genome does not guarantee its expression and functionality as well as that the tool may not encompass all known defense systems, let alone undiscovered ones. Moreover, defense systems across bacterial species and strains are highly diverse.[52] This diversity along with their potential interactions or analogies may reduce the power to detect individual systems and their correlation with phage susceptibility. Nonetheless, Padloc remains the best choice for comprehensive screening, given the impracticality of manual identification. Further studies, including the cloning of systems into bacterial strains, would be necessary for comprehensive validation and understanding.

Conclusion

Together, these data indicate that receptor structures are important for phage infection. Conversely, they do not support existing paradigms regarding the central role of phage defense mechanisms in determining host range. Through our investigation, we successfully identified genetic determinants influencing susceptibility and resistance to *Pa* phages, highlighting the significance of receptors such as LPS for the phage LPS-5 and flagella for OMKO1. These results contribute to our understanding of the constraints of phage infection and could facilitate the development of tailored interventions such as phage cocktails or the targeted engineering of phages to enhance efficacy.

Method and Materials

Bacteriophages and Pseudomonas aeruginosa

The phages investigated in this study include the Myoviridae phages LPS-5 and OMKO1. LPS-5 is used in the Cystic Fibrosis Bacteriophage Study at Yale (CYPHY) (NCT04684641) [53], while OMKO1 (outer-membrane-porin M knockout dependent phage 1) was isolated at Yale University [42] (Table 1, Supplementary Figure 6).

PAO1 and the clinical *Pa* isolates used in this study were obtained from our research group (Table 2).

Table 1: Bacteriophages

Name	Family	Host	Origin
OMKO1 (outer-membrane-porin M knockout dependent phage 1)	Myoviridae	*Pseudomonas aeruginosa* PAO1	B. K. Chan et al., Yale University [42]
LPS-5	Myoviridae	*Pseudomonas aeruginosa* PAO1	B. K. Chan et al., Yale University [53]

Table 2: Bacterial strains

Strain	Species	Application
PAO1	*Pseudomonas aeruginosa*	Tn assay, experimental validation
80 clinical isolates	*Pseudomonas aeruginosa*	lytic plaque assay

Transposon mutagenesis assay

Experiments

The creation of a transposon mutagenesis library was previously described by Larry Gallagher et al. in the Manoil group at the University of Washington. [54] Tn mutagenesis was performed via conjugation of the E. coli SM10λpir/pIT2, a Tn5-derived transposon, followed by selection for *Pa* derivatives carrying the transposon tetracycline-resistance element. This results in a different transposon insertion and gene disruption in each mutant strain. The composition of transposon mutant pools was characterized using the Tn-seq circle method (35).

The *Pa* library, along with the phages LPS-5 and OMKO1, underwent expansion and titration. For the assay, a bacterial concentration at least 1000 times greater than the number of unique mutants ($1x10^5$) was utilized, resulting in a colony-forming unit (cfu) of $1x10^8$ in 25 ml of LB medium. The phages were added at two multiplicities of infection (MOI), 20 and 0.1, corresponding to plaque-forming units (pfu) of $2x10^9$ and $1x10^7$, respectively. Bacterial cultures were grown to an optical density (OD) of 0.2-0.4, ranging from 30 minutes to 3 hours depending on the MOI, which is equivalent to 1-3 phage propagation cycles. Subsequently, the bacterial cultures were harvested and prepared for genomic DNA (gDNA) extraction. Transposon region amplification of the gDNA using the C-tailing method was conducted, a PCR reaction with one primer targeting a C-tail region near the 3' end of the transposon and the second primer complementary to bacterial genomic DNA. Following PCR Tn amplification, quantification ensures equal DNA amounts for subsequent Tn-Seq protocol steps. The following library preparation adds adapter sequences necessary for Illumina sequencing. Denaturation separates double-stranded DNA molecules into single strands, ensuring only the template strand is sequenced during Illumina sequencing. The sequencing of transposons encompassed distinct timepoints and conditions: before phage administration (T0), after killing assay with phage (T1P sequence condition at timepoint 1), and without phage (T1 condition). Reads are mapped to the bacterial genome and quantified. Abundant transposon counts indicated bacterial survival, suggesting that the disrupted gene played a protective role against phage infection. This allowed the identification

of genes crucial for phage infection. Conversely, minimal or absent transposon counts signified bacterial death despite the non-functioning gene and that the disrupted gene was not essential for phage infection.

Analysis

We first normalized the read counts, dividing the count of a particular gene under a specific condition (timepoint and phage condition) by the sum of all the reads for all the genes at that same timepoint and condition. Following normalization, the gene fitness score was computed by taking the log2 of the normalized gene count at timepoint T1 under the specific phage condition, divided by the count of that gene at timepoint T0 (Equation (1)).

$$\text{gene fitness score} = log2\left(\frac{count_{gene,\ \ phage\ condition\ T1}}{count_{gene,\ \ T0}}\right) \tag{1}$$

We then performed a Fisher's test to identify genes significantly enriched in the Tn assay. The Fisher's exact test calculates the significance of the deviation from the null hypothesis from a contingency table of two categorical variables [55], in this case phage condition and time.

A functional enrichment analysis was performed using the enrichKEGG package [56] to explore these genes further and discover pathways significantly enriched (Table 3). Significant hits are assigned to classes of genes and pathways that are over-represented in the gene set are identified, likely associated with the phage infection phenotype. For a more detailed examination of pathways, functional enrichment analysis using the PANTHER [57] program was then conducted. These two analytical methods use different databases where enrichKEGG focuses on pathway enrichment analysis using the KEGG database and PANTHER offers a broader spectrum of functional analysis tools, including pathway enrichment along with gene ontology and protein interaction analysis.

Table 3: Statistical and analytical tools

Tool	Origin	Application
Fisher's Exact Test for Count Data	R. A. Fisher [55]	Tn assay
enrichKEGG	G. Yu et al. [56]	Functional enrichment analysis
PANTHER	H. Mi et al. [57]	Functional enrichment analysis
Genome-Wide Association Study (GWAS) / De Bruijn Genome-Wide Association Study (DBGWAS)	M. Jaillard et al. [58]	GWAS with lytic plaque assay
GraphPad Prism Version 7	GraphPad Prism	Suppression Ratio calculation
Blast	NCBI [59]	Mutations in flagella and LPS associated genes
Mafft	Katoh K. et al. [60]	Sequence alignments
Cons, EMBOSS	Rice P. et al. [61]	Consensus sequences
Prokaryotic Antiviral Defence LOCator (Padloc)	L. J Payne et al. [51]	Bacterial defense systems
Wilcoxon signed-rank test	Kurt Hornik [62]	Bacterial defense systems, single system analysis

Generalized Linear Model (glm)	Simon Davies [63]	Bacterial defense systems, all systems
R Version 4.3 and RStudio	R and RStudio	Analysis, graphs
BioRender	BioRender [64]	Schematics

GWAS with lytic plaque assay

A lytic plaque assay was conducted to determine which clinical *Pa* strains were susceptible to the phages OMKO1 and LPS-5. This phenotypic data, combined with the genomic information of the clinical isolates, was used in a Genome-Wide Association Study (GWAS) to identify genes associated with either phage susceptibility or resistance.

Experiments

In the lytic plaque assay, 80 clinical *Pa* strains were plated on rectangular LB agar plates, followed by the addition of drops of distinct phages in different dilutions (dilution factors: 0, 1, 3, 5, 7). Each experiment was repeated three times and the phages tested were OMKO1 and LPS-5. The plates were then incubated to allow phage infection and plaque formation.

Analysis

The assessment of plaques involved several parameters (Equation (2)), including single plaque status (SPS), plaque score of the sample (PS) and reference (RS), and inhibition score (IS). SPS was assigned a value of 1 if single plaque resolution was observed at any phage dilution, and 0 otherwise. When SPS was 0, PS was also assigned 0, however, if SPS was 1, its value was determined by the dilution factor with single plaque resolution. RS represented the dilution factor of the reference strain when SPS was 1, assuming a nonzero plaque score for the reference strain. In cases where SPS was 0, IS was set to the highest dilution factor with signs of bacterial growth inhibition. These parameters collectively contributed to the derived score for each clinical isolate, ranging from 0 to 2. Scores between 0 and 1 indicate phage resistance or inhibition of bacterial growth, while scores between 1 and 2 indicate phage susceptibility, representing the phenotypic data. This phenotypic data, combined with the genome sequences of the clinical isolates as genotypic data, was analyzed in the GWAS to identify genes associated with either phage susceptibility or resistance.

$$\boldsymbol{Score = SPS + SPS * (1 - (RS - PS) * 0.1) + \frac{IS}{RS}}$$

SPS: Single plaque status (0 or 1)

PS: Plaque score (0,1,3,5 or 7)

RS: Plaque score for reference strain (0,1,3,5 or 7)

IS: Inhibition score (0,1,3,5 or 7)

(2)

A GWAS, more specifically a De Bruijn Genome-Wide Association Study (DBGWAS), consists of three main steps. A DBGWAS requires genome assemblies and phenotypic data for a panel of bacterial strains as input and outputs phenotype-associated genetic events and an interactive interface featuring regions of the DBG surrounding statistically significant nodes.

Given the numerous genes identified by the GWAS in association with phage infection, a functional enrichment analysis was conducted to identify enriched pathways using the enrichKEGG package. Significant hits are assigned to classes of genes and entire pathways that are over-represented in the gene set are identified, likely associated with the phage infection phenotype.

Experimental validation of flagella and LPS genes

Suppression Assay
Cultures from frozen stock were initially incubated overnight at 37°C with shaking at 250 rpm. Following this, overnight cultures of bacterial strains were diluted 1:200 with LB medium (Table 4) and regrown for 2-3 hours until reaching an OD600 of approximately 0.1-0.2. Subsequently, this culture was diluted 1:10 into the wells of a 96-well plate, and phage was added to achieve a MOI of 100, resulting in a total volume of 150 µl per well. If necessary, an appropriate antibiotic was included in each well to achieve the desired concentration. The 96-well plate was then incubated at 37°C with shaking for 30 hours, during which the OD600 was measured every 20 minutes using an automated spectrophotometer (Biotek microplate reader). All growth curves were adjusted by subtracting the background curve (LB medium with phage buffer at a concentration of 1 mM MgSO4, 4 mM CaCl2, 50 mM Tris-HCl (pH=7.8), 6 g/L NaCl, and 1 g/L gelatin). The Suppression Ratio, expressed as a percentage, was calculated using GraphPad Prism Version 7.0 software. This ratio was defined as the area under the curve (AUC) for the non-phage-treated condition minus the AUC for the phage-treated condition, divided by the AUC of the non-treated condition.

Table 4: Media, Buffers, Chemicals

Media, Buffer, Chemical	Ingredients	Concentration
LB Agar for Swimming Assay	LB Agar Tryptone medium	 0.03% 1%
LB Agar for LPS/O-AG Assay	LB Agar TSB medium Congo red Coomassie brilliant blue	 1% 1% 40 mg/L 20mg/L
Phage buffer	MgSO4 CaCl2 Tris-HCl (pH=7.8) NaCl Gelatin	1 mM 4 mM 50 mM 6 g/L 1 g/L

Swimming assay
Following a standardized protocol [65], overnight cultures were initially grown and subsequently diluted before regrowth to an OD of 0.1-0.2. The bacterial suspension was then transferred onto agar plates via puncturing with a pipette tip. These agar plates were prepared using 1% tryptone medium supplemented with 0.03% agar. After incubation for 24 hours at 37°C, the growth zones were measured. The experiment was conducted with a minimum of 5 repetitions.

LPS/O-AG assay
Following an established protocol[37], 1ul of overnight culture of clinical isolates were spotted onto agar plates containing 1% TSB medium fortified with 40 mg/L Congo red and 20 mg/L Coomassie brilliant blue dyes and solidified with 1% agar. Colony biofilms were grown for 4 days at 25°C before images were taken and the morphology analysed.

Finding mutations
Flagella genes and LPS biosynthetic gene sequences (Supplementary Figure 7) were downloaded from the Pseudomonas genome database.[66] These sequences were blasted against the genomes of the clinical isolates using the ncbi-blast software.[59] Hits were then aligned using mafft. [60] To identify non-synonymous mutations, aligned sequences over a specific length (>20% of the total sequence, typically around >400 bp) were translated. Subsequently, each amino acid was compared to the reference sequence used for the blast analysis. Changes in amino acids were documented, including the amino acid position and the specific alteration. For sequences exhibiting high diversity (sequence

similarity < 90%), consensus sequences for both resistant and susceptible strains were generated. Consensus sequences for each variant were derived using the cons function from the European Molecular Biology Open Software Suite (EMBOSS [61]).

Bacterial defense systems and lytic phage infection

The genomes of the clinical isolates were screened for such systems using the Prokaryotic Antiviral Defence LOCator (Padloc).[51] We assessed the contribution of each system using a Generalized Linear Model (GLM), which was only fitted to systems that were significantly associated with phage resistance or susceptiblity, as determined by a Wilcoxon signed-rank test. [67] P-values from the Wilcoxon test were adjusted for multiple testing using a Bonferroni correction (each p-value was multiplied by the total number of tests conducted), corresponding to the number of defense systems evaluated. Any corrected p-value exceeding 1 was set to 1. Since no defense systems showed significance in the Wilcoxon test, the systems that exhibited significance prior to p-value correction were selected for inclusion in the GLM [63]. The GLM was fitted with a binomial distribution, and the AIC criterion [68] was employed to compare the fit of different distributions and determine the optimal number of defense systems to include. Additionally, residual diagnostics were performed using the DHARMa package [69] to confirm the goodness of fit of the model.

Bacterial defense systems and temperate phages

We followed the steps described above using the number of temperate phages in the bacterial genome as a response variable. Bacterial genomes underwent screening for temperate phages using VIBRANT and blast and were then correlated with the presence of bacterial defense systems identified by Padloc. The relationship between each system and temperate phages was individually analysed using a Wilcoxon test. Subsequently, significant predictors were included in a generalized linear model with a Poisson distribution to evaluate the collective impact of these defense mechanisms on the number of temperate phages.

Data and code availability

Data availability: All bacterial strains, bacteriophages, materials, and experimental data are available upon request.

Code availability: All detailed codes used for analysis are available upon request.

Acknowledgments
We extend our heartfelt gratitude to all members of the Bollyky Laboratory for insightful discussions and support. We thank Rob McBride (Co-Founder & CEO of Felix Biotechnology) and the members of Felix Biotechnology, Inc (San Francisco, CA) for the data from the Transposon mutagenesis experiment and lytic plaque assay.

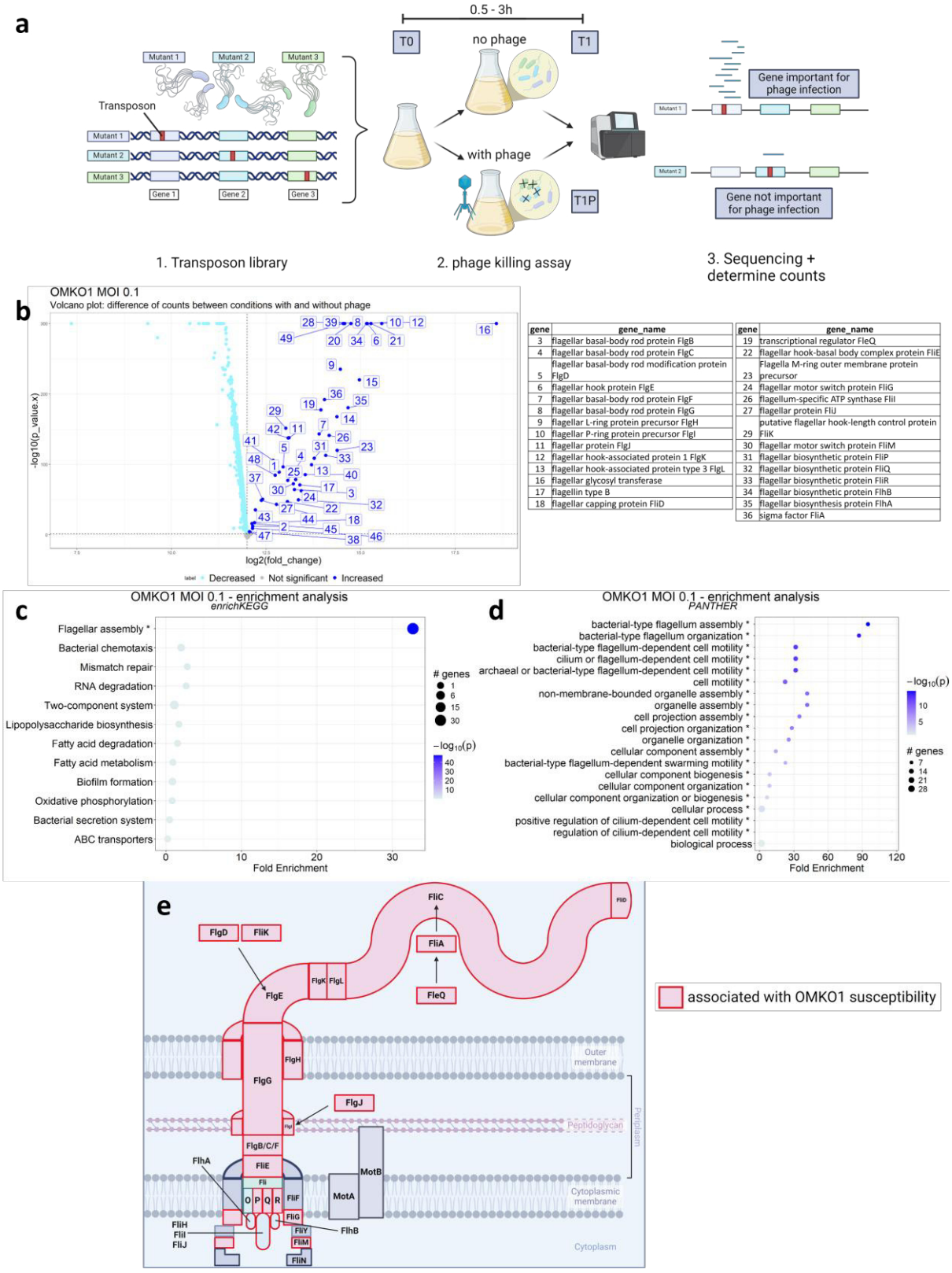
Contributions
Dea M Müller, Julie D Pourtois, Minyoung Kevin Kim, Bobby Targ, Robert McBride and Paul L. Bollyky

P.L.B., J.P. and D.M.M. conceptualized the main idea of this research project. D.M.M. performed the majority of the experiments and analysis. P.L.B., L.A.G and J.P. provided feedback on the results. D.M.M. wrote the paper. P.L.B., L.A.G, J.P., M.K.K and D.M.M. contributed to the revision of the paper. M.K.K supported with the Experimental validation and Maryam Hajfathalian[1] provided the TEM pictures. D.M.M. and Bobby Targ created the LPS schematic.

Rob McBride (Co-Founder & CEO of Felix Biotechnology) and the members of Felix Biotechnology, Inc (San Francisco, CA) provided the data from the Transposon mutagenesis experiment and lytic plaque assay.

Competing interests
The authors declare no competing interests.

Main Figures

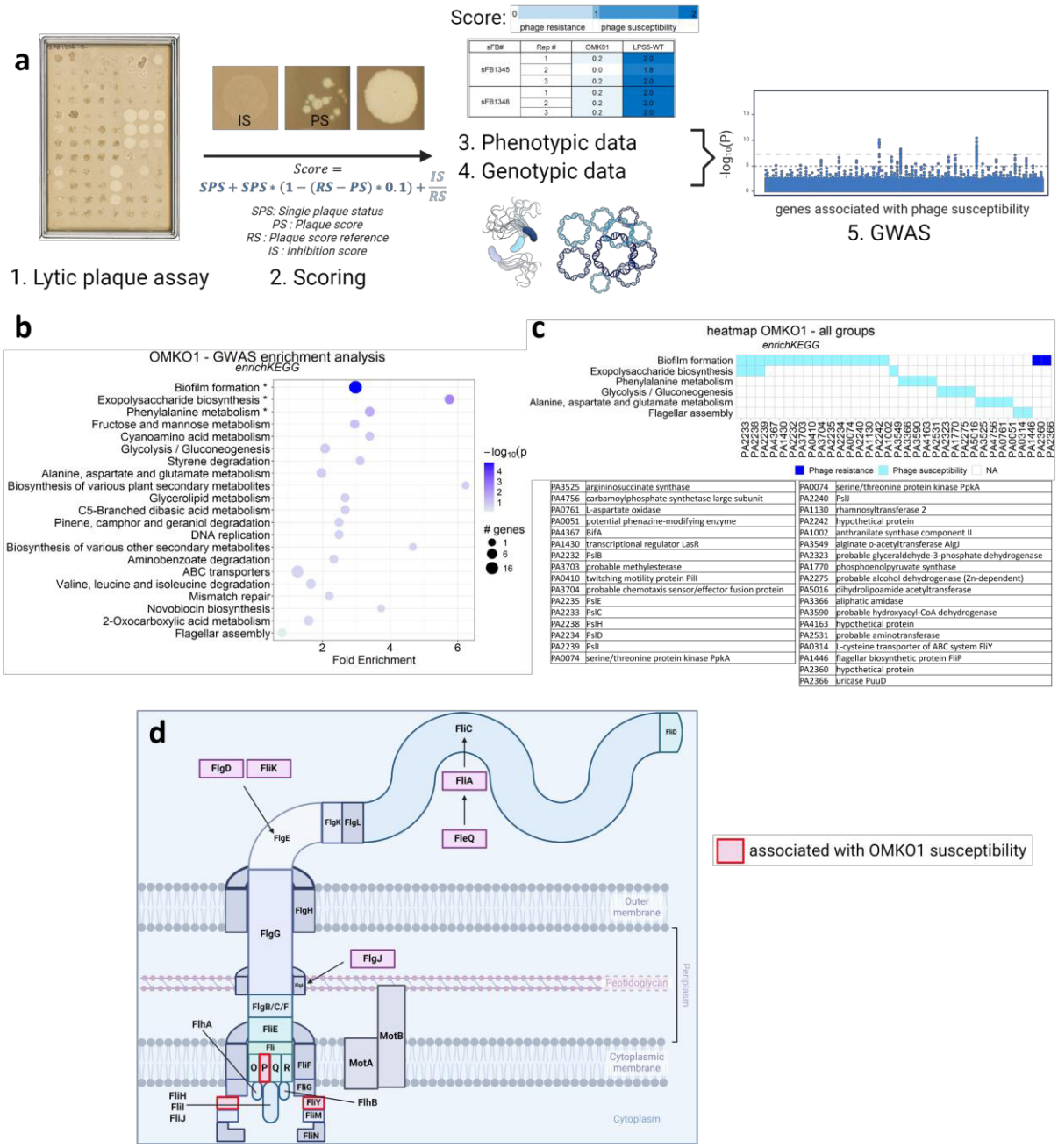


gene	gene_name
3	flagellar basal-body rod protein FlgB
4	flagellar basal-body rod protein FlgC
5	flagellar basal-body rod modification protein FlgD
6	flagellar hook protein FlgE
7	flagellar basal-body rod protein FlgF
8	flagellar basal-body rod protein FlgG
9	flagellar L-ring protein precursor FlgH
10	flagellar P-ring protein precursor FlgI
11	flagellar protein FlgJ
12	flagellar hook-associated protein 1 FlgK
13	flagellar hook-associated protein type 3 FlgL
16	flagellar glycosyl transferase
17	flagellin type B
18	flagellar capping protein FliD

gene	gene_name
19	transcriptional regulator FleQ
22	flagellar hook-basal body complex protein FliE
23	Flagella M-ring outer membrane protein precursor
24	flagellar motor switch protein FliG
26	flagellum-specific ATP synthase FliI
27	flagellar protein FliJ
29	putative flagellar hook-length control protein FliK
30	flagellar motor switch protein FliM
31	flagellar biosynthetic protein FliP
32	flagellar biosynthetic protein FliQ
33	flagellar biosynthetic protein FliR
34	flagellar biosynthetic protein FlhB
35	flagellar biosynthesis protein FlhA
36	sigma factor FliA

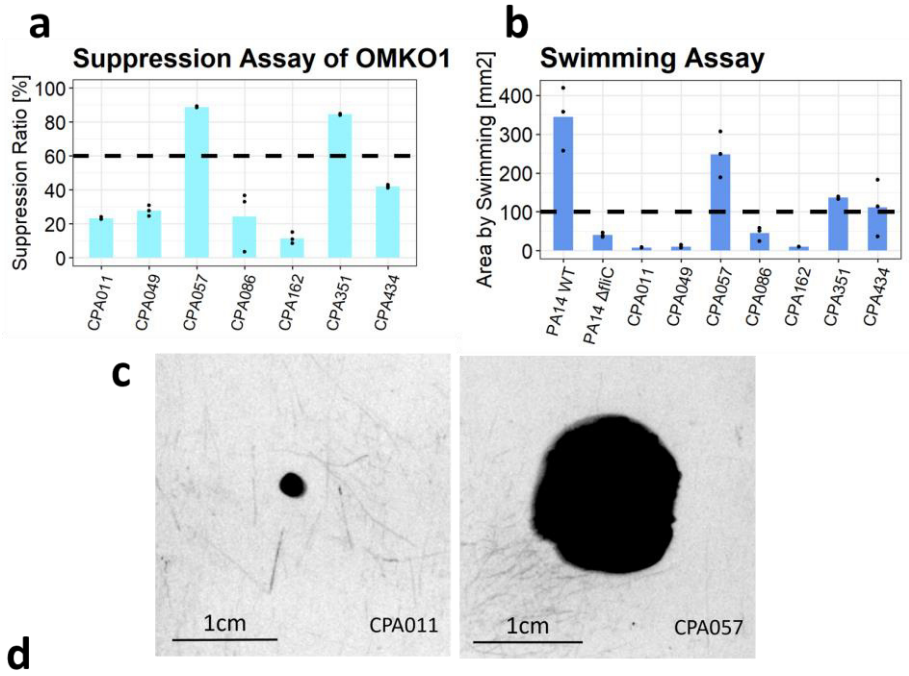
Figure 1: **Transposon Mutagenesis Experiments show that Flagella-Associated Genes are required for OMKO1 Phage Infection. a,** In the transposon mutagenesis library, a distinct gene in each mutant strain was disrupted via a transposon insertion. Following co-cultivation with phages, transposons were sequenced and quantified. Abundant transposon counts indicated bacterial survival, suggesting that the disrupted gene played a protective role against phage infection. This allowed the identification of genes crucial for phage infection. Conversely, minimal or absent transposon counts signified bacterial death

despite the non-functioning gene. This observation indicated that the disrupted gene was not essential for phage infection. **b,** This volcano plot highlights genes with a significant difference in counts (fold change) between conditions with and without OMKO1 phage at MOI 20. Each point represents a gene, with the x-axis showing the log2 fold change and the y-axis representing statistical significance. Significant increase in gene count is showed in dark blue, significant decrease in bright blue and non significant genes in gray. Notably, a majority of genes showing a significant increase in counts in the presence of phage, indicative of their importance in phage infection, are associated with flagella for the phage OMKO1. (The complete list of significant genes can be found in Supplementary Figure 1) **c,** This plot visually summarizes functional enrichment analysis results conducted with enrichKEGG, where each bubble represents a functional category. The size of each bubble corresponds to the number of associated genes, and the colour intensity indicates the significance level. Significant pathways are labelled with a star. Specifically for the phage OMKO1 at MOI 20, significant functional categories are linked to flagella assembly. **d,** Functional enrichment analysis using PANTHER enables a more detailed examination. Significant functional categories are associated with flagellum-dependent cell motility and flagellum assembly, particularly for the phage OMKO1. **e,** Schematic representation of flagella and essential structural proteins. Proteins identified through Tn mutagenesis experiments as associated with OMKO1 phage susceptibility are highlighted in red.



PA3525	argininosuccinate synthase	PA0074	serine/threonine protein kinase PpkA
PA4756	carbamoylphosphate synthetase large subunit	PA2240	PslJ
PA0761	L-aspartate oxidase	PA1130	rhamnosyltransferase 2
PA0051	potential phenazine-modifying enzyme	PA2242	hypothetical protein
PA4367	BifA	PA1002	anthranilate synthase component II
PA1430	transcriptional regulator LasR	PA3549	alginate o-acetyltransferase AlgJ
PA2232	PslB	PA2323	probable glyceraldehyde-3-phosphate dehydrogenase
PA3703	probable methylesterase	PA1770	phosphoenolpyruvate synthase
PA0410	twitching motility protein PilI	PA2275	probable alcohol dehydrogenase (Zn-dependent)
PA3704	probable chemotaxis sensor/effector fusion protein	PA5016	dihydrolipoamide acetyltransferase
PA2235	PslE	PA3366	aliphatic amidase
PA2233	PslC	PA3590	probable hydroxyacyl-CoA dehydrogenase
PA2238	PslH	PA4163	hypothetical protein
PA2234	PslD	PA2531	probable aminotransferase
PA2239	PslI	PA0314	L-cysteine transporter of ABC system FliY
PA0074	serine/threonine protein kinase PpkA	PA1446	flagellar biosynthetic protein FliP
		PA2360	hypothetical protein
		PA2366	uricase PuuD

Figure 2: **GWAS with lytic plaque assay. a,** During the lytic plaque assay, plaques assessment included single plaque status, plaque score of the sample and reference, and inhibition score. These factors collectively contributed to the derived score, which was used alongside genomic data for the Genome-Wide Association Study (GWAS). **b,** Each bubble represents a specific functional category, with the size of the bubble indicating the number of associated genes, and the color intensity reflecting the statistical significance level of the enrichment. Significant pathways are labelled with a star. Notably, the most significant pathway identified is Biofilm formation. Additionally, the pathway for flagellar assembly is also detected in the analysis. **c,** This heatmap illustrates the categorization of genes on the x-axis into distinct functional groups on the y-axis. The color scheme distinguishes genes associated with phage susceptibility, depicted in bright blue, from those linked to phage resistance, represented in dark blue. Notably, two genes within the flagellar assembly pathway can be identified, revealing an association with phage susceptibility. **d,** Schematic representation of flagella and essential structural proteins. Proteins identified through the above described experiments as associated with OMKO1 phage susceptibility are highlighted in red.

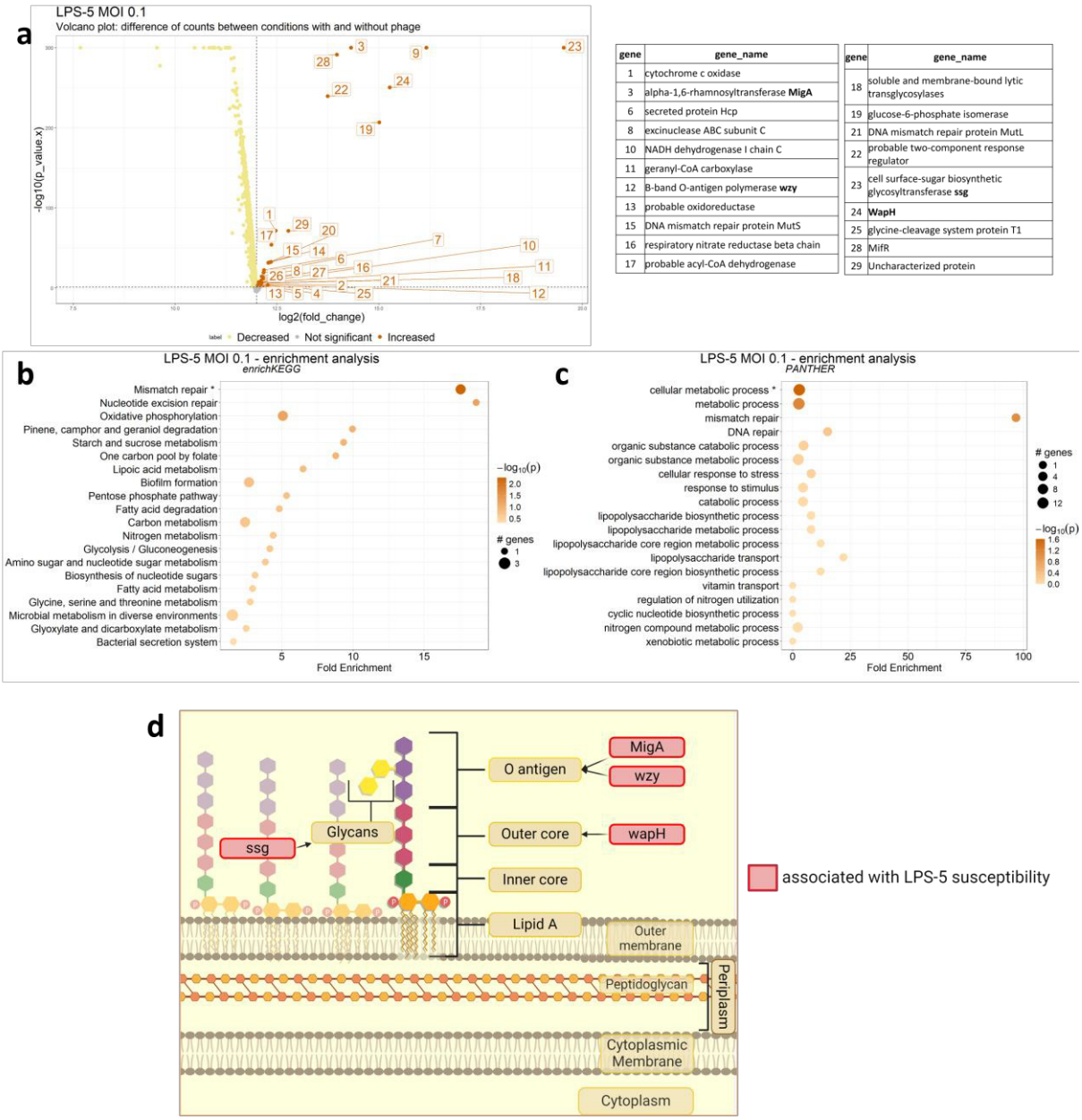


d

Strains	Susceptibility	Swimming area [mm2]	Gene	Position (amino acid sequence)	Mutation (reference → strain)
CPA011	Resistant	8.1087	flgE	397	S->A
			fliH	56	P->A
			flgH	21	N->S
			fliR	60	S->P
			fliE	34	V->A
			fliY	67	A->E
			fliY	125	E->D
			fliY	130	K->R
CPA049	Resistant	10.30024	fliH	259	L->F
			fliH	10	N->S
			fliR	165	S->N
			fliR	60	S->P
CPA086	Resistant	44.48828	fliE	34	V->A
			fliR	60	S->P
CPA162	Resistant	10.30024	flgB	63	T->I
			flhA	26	V->I
			fliE	34	V->A
			fliR	60	S->P
			fliE	28	V->A
			fliF	21	K->Q
CPA434	Resistant	110.892	fliE	34	V->A
			flgF	92	A->T
CPA057	Susceptible	248.0824	flhA	26	V->I
			flgE	397	S->A
			flhB	49	I->M
			fliR	60	S->P
CPA351	Susceptible	136.7521	fliE	34	V->A
			fliR	60	S->P
			fliO	7	A->V

Figure 3: **Experimental validation of Flagella as the receptor for OMKO1 a,** Phage suppression (Suppression ratio) was assessed for CPA. A value above the threshold indicates susceptibility to the OMKO1 phage, while values below indicate phage resistance. The suppression ratio was calculated as the area under the curve (AUC) for the non-phage-treated condition minus the phage-treated condition, divided by the AUC of the non-treated sample. **b,** The area covered by swimming was measured for various clinical Pseudomonas isolates (CPA). Growth areas similar to those of the ΔfliC strain indicate non-functional flagella, while swimming behavior comparable to that of the WT strain indicates functional flagella. **c,** Large colony growth area indicates the presence functional flagella allowing

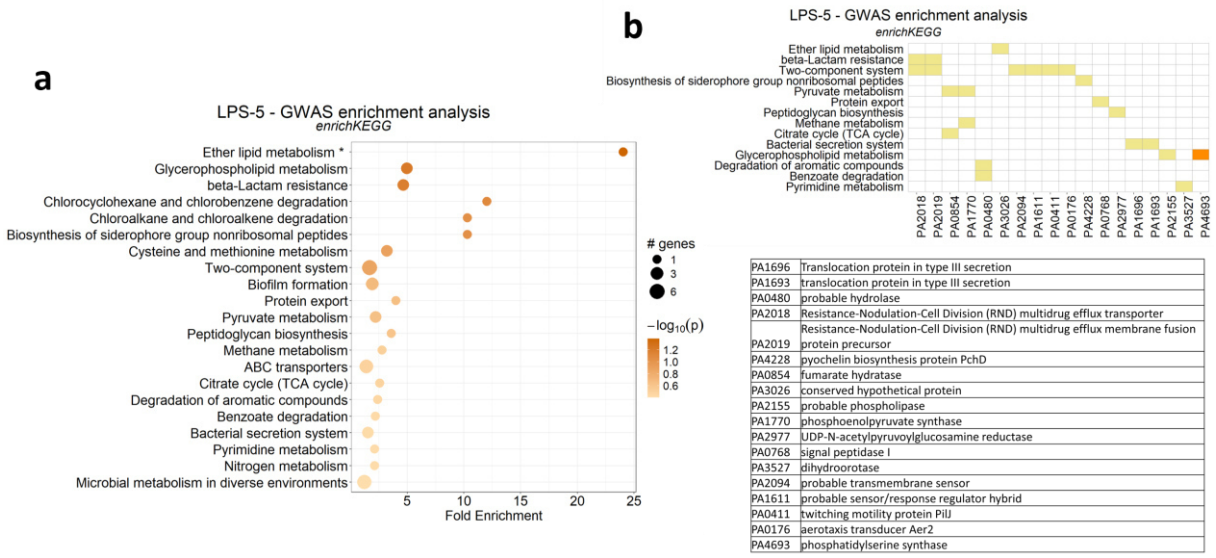
swimming (CPA057), whereas small colony growth area indicates non-functional flagella (CPA011). **d,** Various bacterial sequences and mutations were identified to be associated with OMKO1 phage infection. The most significant differences were observed in the genes FliC, FliD, FlgK, and FlgL. Sequence similarity for these genes within resistant and susceptible strains was clustered, and consensus sequences for both resistant and susceptible strains were generated. (Supplementary Figure 3). Single mutations were identified in other flagella genes.



gene	gene_name
1	cytochrome c oxidase
3	alpha-1,6-rhamnosyltransferase **MigA**
6	secreted protein Hcp
8	excinuclease ABC subunit C
10	NADH dehydrogenase I chain C
11	geranyl-CoA carboxylase
12	B-band O-antigen polymerase **wzy**
13	probable oxidoreductase
15	DNA mismatch repair protein MutS
16	respiratory nitrate reductase beta chain
17	probable acyl-CoA dehydrogenase

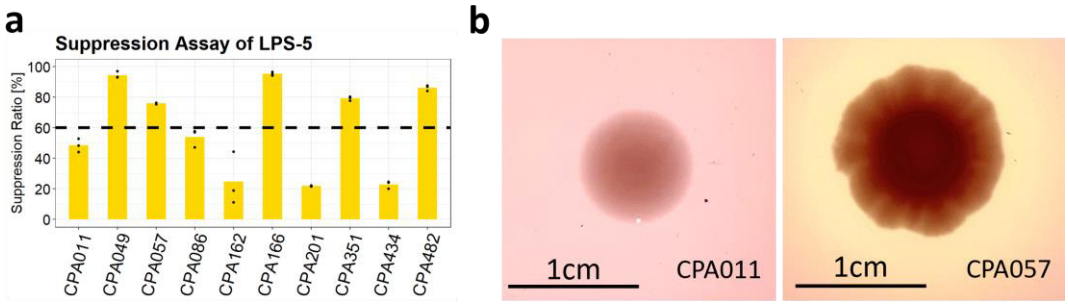
gene	gene_name
18	soluble and membrane-bound lytic transglycosylases
19	glucose-6-phosphate isomerase
21	DNA mismatch repair protein MutL
22	probable two-component response regulator
23	cell surface-sugar biosynthetic glycosyltransferase **ssg**
24	**WapH**
25	glycine-cleavage system protein T1
28	MifR
29	Uncharacterized protein

Figure 4: **Identification of LPS-Associated Genes Crucial for LPS-5 Phage Infection through Transposon Mutagenesis Experiments. a,** This volcano plot highlights genes with a significant difference in counts (fold change) between conditions with and without LPS-5 phage at MOI 20. Each point represents a gene, with the x-axis showing the log2 fold change and the y-axis representing statistical significance. Significant increase in gene count is showed in orange, significant decrease in yellow and non significant genes in gray. Notably, several genes showing a significant increase in counts in the presence of phage, indicative of their importance in phage infection, are associated with LPS (The complete list of significant genes can be found in Supplementary Figure 4) **b,** This plot visually summarizes functional enrichment analysis results conducted with enrichKEGG, where each bubble represents a functional category. The size of each bubble corresponds to the number of associated genes, and the colour intensity indicates the significance level. Significant pathways are labelled with a star. **c,** Functional enrichment analysis using PANTHER enables a more detailed examination. Several functional categories are associated with LPS and LPS biosynthesis, particularly for the phage LPS. **d,** Schematic representation of LPS and related pathways. Proteins identified through Tn mutagenesis experiments as associated with LPS-5 phage susceptibility are highlighted in red.



PA1696	Translocation protein in type III secretion
PA1693	translocation protein in type III secretion
PA0480	probable hydrolase
PA2018	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter
PA2019	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein precursor
PA4228	pyochelin biosynthesis protein PchD
PA0854	fumarate hydratase
PA3026	conserved hypothetical protein
PA2155	probable phospholipase
PA1770	phosphoenolpyruvate synthase
PA2977	UDP-N-acetylpyruvoylglucosamine reductase
PA0768	signal peptidase I
PA3527	dihydroorotase
PA2094	probable transmembrane sensor
PA1611	probable sensor/response regulator hybrid
PA0411	twitching motility protein PilJ
PA0176	aerotaxis transducer Aer2
PA4693	phosphatidylserine synthase

Figure 5: **GWAS with lytic plaque assay. a,** With the results of the GWAS, a comprehensive functional enrichment analysis was conducted and illustrated in the bubble graph. Each bubble represents a specific functional category, with the size of the bubble indicating the number of associated genes, and the color intensity reflecting the statistical significance level of the enrichment. Significant pathways are labelled with a star. Different metabolic pathways are significantly enriched and relevant to phage susceptibility. **b,** This heatmap illustrates the categorization of genes on the x-axis into distinct functional groups on the y-axis. The color scheme distinguishes genes associated with phage susceptibility, depicted in yellow, from those linked to phage resistance, represented in orange. This facilitates the identification of genes within pathways and demonstrates whether these pathways are linked either to phage resistance or susceptibility.



c

Strains	Susceptibility	Rugae	Gene	Position (amino acid sequence)	Mutation (reference → strain)
CPA011	Resistant	no rugae	migA	92	R->Q
			wapH	312	V->I
			wzy	No hits in blast	
CPA049	Resistant	no growth	migA	92	R->Q
CPA086	Resistant	no rugae	ssg	47	K->N
			migA	92	R->Q
CPA162	Resistant	no growth	migA	92	R->Q
			wzy	No hits in blast	
CPA434	Resistant	no rugae	migA	92	R->Q
			wzy	25	T->I
			wapH	322	T->A
			wzy	155	V->A
			wzy	24	A->V
			wzy	262	V->I
			wzy	85	L->I
			wzy	376	V->I
			wzy	182	V->A
			wzy	326	Q->H
CPA057	Susceptible	rugae	migA	92	R->Q
			wapH	160	A->G
			wzy	No hits in blast	
CPA166	Susceptible	no rugae	migA	92	R->Q
			wzy	No hits in blast	
CPA201	Susceptible	rugae	migA	92	R->Q
			wzy	No hits in blast	
CPA351	Susceptible	no rugae	migA	12	G->D
			wapH	322	T->A
			wzy	No hits in blast	
CPA482	Susceptible	no rugae	wapH	322	T->A
			migA	92	R->Q
			wzy	No hits in blast	

Figure 6: **Experimental validation of O-antigen/LPS as the receptor for LPS-5. a,** Phage suppression (Suppression ratio) was assessed for clinical CPA. A value above the treshold indicates susceptibility to the LPS-5 phage, while values below indicate phage resistance. The suppression ratio was calculated as the area under the curve for the non-phage-treated condition minus the phage-treated condition, divided by the AUC of the non-treated sample. **b,** Colony morphology displaying rugae indicates the presence of LPS containing O-antigen (O-AG), whereas smooth colonies lacking rugae suggest mutations in the O-AG within the LPS. **c,** Mutations in LPS biosynthetic genes in these strains were identified to be associated with LPS-5 phage infection. The most mutations were observed in the genes MigA and wzy, with some mutations also found in the genes ssg and wapH.

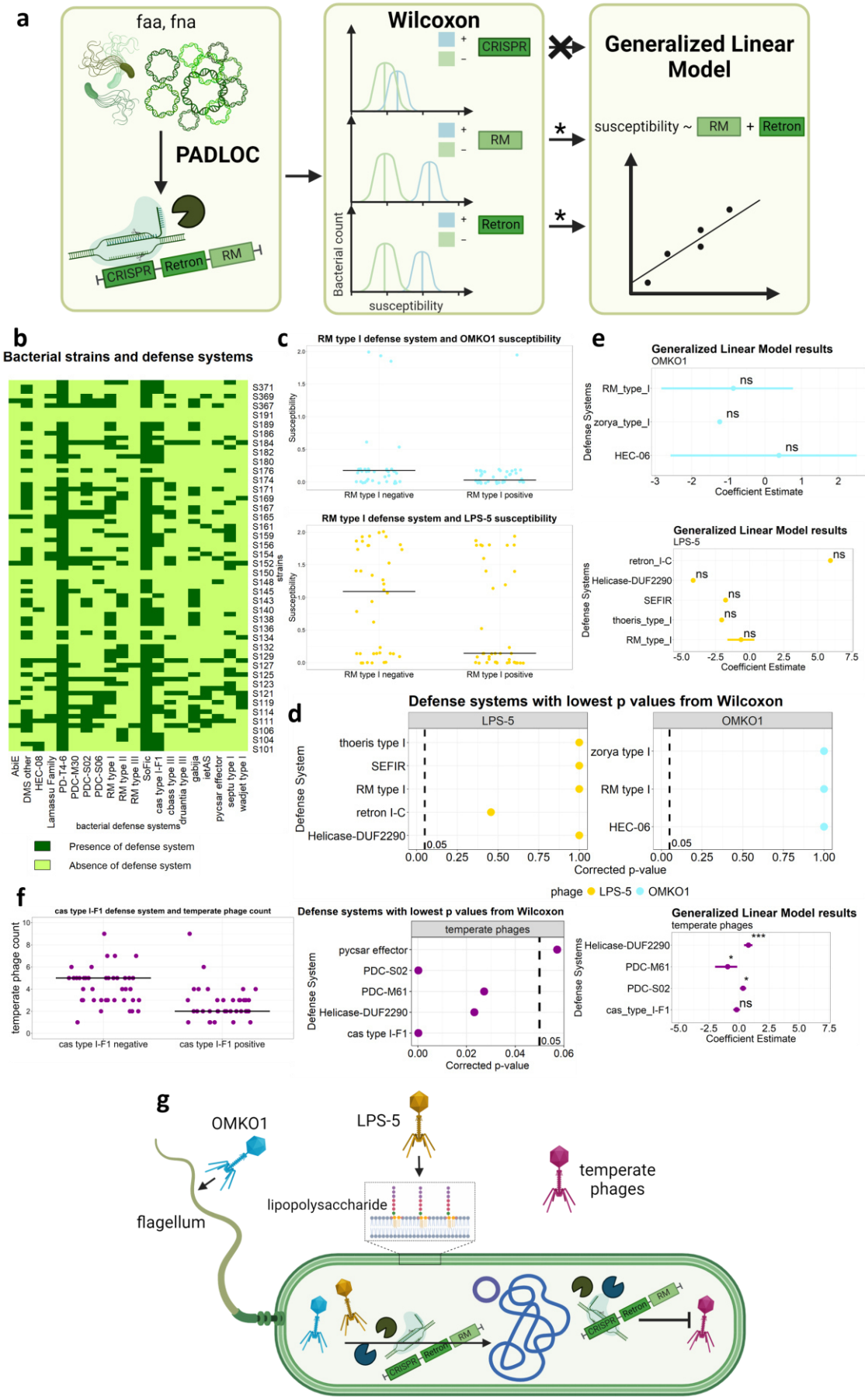

a
faa, fna
PADLOC
CRISPR
Retron
RM
Wilcoxon
CRISPR
RM
Retron
Bacterial count
susceptibility
Generalized Linear Model
susceptibility ~ RM + Retron
b
Bacterial strains and defense systems
bacterial defense systems
Presence of defense system
Absence of defense system
c
RM type I defense system and OMKO1 susceptibility
RM type I negative
RM type I positive
RM type I defense system and LPS-5 susceptibility
e
Generalized Linear Model results
OMKO1
RM_type_I
zorya_type_I
HEC-06
Coefficient Estimate
Generalized Linear Model results
LPS-5
retron_I-C
Helicase-DUF2290
SEFIR
thoeris_type_I
RM_type_I
d
Defense systems with lowest p values from Wilcoxon
LPS-5
OMKO1
thoeris type I
SEFIR
RM type I
retron I-C
Helicase-DUF2290
zorya type I
RM type I
HEC-06
Defense System
Corrected p-value
phage LPS-5 OMKO1
f
cas type I-F1 defense system and temperate phage count
temperate phage count
cas type I-F1 negative
cas type I-F1 positive
Defense systems with lowest p values from Wilcoxon
temperate phages
pycsar effector
PDC-S02
PDC-M61
Helicase-DUF2290
cas type I-F1
Generalized Linear Model results
temperate phages
Helicase-DUF2290
PDC-M61
PDC-S02
cas_type_I-F1
Coefficient Estimate
g
OMKO1
LPS-5
temperate phages
lipopolysaccharide
flagellum
CRISPR
Retron
RM

Figure 7: **Presence of bacterial defense systems is not associated with lytic phage susceptibility. a,** Defense System Analysis Workflow. Clinical isolates were screened for defense systems using the Prokaryotic Antiviral Defence LOCator (Padloc). Pre-selection of defense systems was conducted via a Wilcoxon signed-rank test, followed by their inclusion in a generalized linear model to assess potential associations between the presence or absence of these defense mechanisms and phage susceptibility. **b,** Presence or absence of bacterial defense systems across bacterial strains. On the x-axis are the identified defense systems, while the y-axis represents individual bacterial strains. Presence or absence of a defense system is represented in colours. **c,** Phage susceptibility for isolates with and without RM Type I. Each point represents one bacterial strain grouped into RM type I negative and positive categories. The median is represented by the horizontal line. The first plot illustrates the data for OMKO1, while the second plot includes all data points for LPS-5. **d,** Wilcoxon Results. The plot illustrates the defense systems with the lowest p-values against their corrected p-values, indicating that none of the p-values fall below the significance threshold of 0.05 for any phage **e,** Whisker Plot of the GLM. The defense systems identified in the Wilcoxon analysis were incorporated into a Generalized Linear Model (GLM), demonstrating that none of the systems are significantly associated with OMKO1 susceptibility or LPS-5 susceptibility. Defense systems are plotted against their coefficient estimates, indicating their non-significance (ns). **f,** Bacterial defense systems are associated with the number of temperate phages in the bacterial genome. Similar to the previous analysis, we first investigated the association between the count of temperate phages and the presence of defense systems using a Wilcoxon test, followed by a GLM analysis. **g,** Concluding Schematic. This study suggests that receptors alone determine the host range of lytic phages. We identified the flagellum as a receptor of OMKO1 and lipopolysaccharide as the receptor of LPS-5. Although no significant relationship was found between bacterial defense systems and lytic phage infection, defense mechanisms may play a role in protecting against temperate phages.

References

1. Murray CJL, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*. 2022;399(10325):629-655. doi:10.1016/S0140-6736(21)02724-0

2. Pachori P, Gothalwal R, Gandhi P. Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review. *Genes Dis*. 2019;6(2):109-119. doi:10.1016/j.gendis.2019.04.001

3. Jorth P, Staudinger BJ, Wu X, et al. Regional Isolation Drives Bacterial Diversification within Cystic Fibrosis Lungs. *Cell Host Microbe*. 2015;18(3):307-319. doi:10.1016/j.chom.2015.07.006

4. Wood SJ, Kuzel TM, Shafikhani SH. Pseudomonas aeruginosa: Infections, Animal Modeling, and Therapeutics. *Cells*. 2023;12(1):199. doi:10.3390/cells12010199

5. Du Toit A. The language of phages. *Nat Rev Microbiol*. 2017;15(3):135-135. doi:10.1038/nrmicro.2017.8

6. Hulo C, de Castro E, Masson P, et al. ViralZone: a knowledge resource to understand virus diversity. *Nucleic Acids Res*. 2011;39(Database issue):D576-582. doi:10.1093/nar/gkq901

7. Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. *Nat Rev Microbiol*. 2010;8(5):317-327. doi:10.1038/nrmicro2315

8. Hampton HG, Watson BNJ, Fineran PC. The arms race between bacteria and their phage foes. *Nature*. 2020;577(7790):327-336. doi:10.1038/s41586-019-1894-8

9. McCallin, Sacher, Zheng, Chan. Current State of Compassionate Phage Therapy. *Viruses*. 2019;11(4):343. doi:10.3390/v11040343

10. Aslam S, Lampley E, Wooten D, et al. Lessons Learned From the First 10 Consecutive Cases of Intravenous Bacteriophage Therapy to Treat Multidrug-Resistant Bacterial Infections at a Single Center in the United States. *Open Forum Infect Dis*. 2020;7(9):ofaa389. doi:10.1093/ofid/ofaa389

11. Wright A, Hawkins CH, Änggård EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa* ; a preliminary report of efficacy. *Clin Otolaryngol*. 2009;34(4):349-357. doi:10.1111/j.1749-4486.2009.01973.x

12. Terwilliger AL, Gu Liu C, Green SI, et al. Tailored Antibacterials and Innovative Laboratories for Phage (Φ) Research: Personalized Infectious Disease Medicine for the Most Vulnerable At-Risk Patients. *PHAGE*. 2020;1(2):66-74. doi:10.1089/phage.2020.0007

13. Khatami A, Foley DA, Warner MS, et al. Standardised treatment and monitoring protocol to assess safety and tolerability of bacteriophage therapy for adult and paediatric patients (STAMP study): protocol for an open-label, single-arm trial. *BMJ Open*. 2022;12(12):e065401. doi:10.1136/bmjopen-2022-065401

14. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*. *Evol Med Public Health*. 2018;2018(1):60-66. doi:10.1093/emph/eoy005

15. Ackermann HW. Who went into phage research? *Bacteriophage*. 2012;2(1):55-59. doi:10.4161/bact.18680

16. Sarker SA, Sultana S, Reuteler G, et al. Oral Phage Therapy of Acute Bacterial Diarrhea With Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh. *EBioMedicine*. 2016;4:124-137. doi:10.1016/j.ebiom.2015.12.023

17. Jault P, Leclerc T, Jennes S, et al. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by Pseudomonas aeruginosa (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect Dis*. 2019;19(1):35-45. doi:10.1016/S1473-3099(18)30482-1

18. Jia HJ, Jia PP, Yin S, Bu LK, Yang G, Pei DS. Engineering bacteriophages for enhanced host range and efficacy: insights from bacteriophage-bacteria interactions. *Front Microbiol*. 2023;14:1172635. doi:10.3389/fmicb.2023.1172635

19. Alqahtani A. Bacteriophage treatment as an alternative therapy for multidrug-resistant bacteria. *Saudi Med J*. 2023;44(12):1222-1231. doi:10.15537/smj.2023.44.12.20230366

20. Du J, Meile S, Baggenstos J, et al. *Enhancing Bacteriophage Therapeutics through* in Situ *Production and Release of Heterologous Antimicrobial Effectors*. Bioengineering; 2022. doi:10.1101/2022.03.09.483629

21. Pourcel C, Midoux C, Vergnaud G, Latino L. The Basis for Natural Multiresistance to Phage in Pseudomonas aeruginosa. *Antibiotics*. 2020;9(6):339. doi:10.3390/antibiotics9060339

22. Bickle TA. Restricting restriction. *Mol Microbiol*. 2004;51(1):3-5. doi:10.1046/j.1365-2958.2003.03846.x

23. Vasu K, Nagaraja V. Diverse Functions of Restriction-Modification Systems in Addition to Cellular Defense. *Microbiol Mol Biol Rev*. 2013;77(1):53-72. doi:10.1128/MMBR.00044-12

24. Barrangou R, Fremaux C, Deveau H, et al. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science*. 2007;315(5819):1709-1712. doi:10.1126/science.1138140

25. Mohanraju P, Makarova KS, Zetsche B, Zhang F, Koonin EV, Van Der Oost J. Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science*. 2016;353(6299):aad5147. doi:10.1126/science.aad5147

26. Dupuis MÈ, Villion M, Magadán AH, Moineau S. CRISPR-Cas and restriction–modification systems are compatible and increase phage resistance. *Nat Commun*. 2013;4(1):2087. doi:10.1038/ncomms3087

27. Ando H, Lemire S, Pires DP, Lu TK. Engineering Modular Viral Scaffolds for Targeted Bacterial Population Editing. *Cell Syst*. 2015;1(3):187-196. doi:10.1016/j.cels.2015.08.013

28. Nobrega FL, Vlot M, De Jonge PA, et al. Targeting mechanisms of tailed bacteriophages. *Nat Rev Microbiol*. 2018;16(12):760-773. doi:10.1038/s41579-018-0070-8

29. Guerrero-Ferreira RC, Viollier PH, Ely B, et al. Alternative mechanism for bacteriophage adsorption to the motile bacterium *Caulobacter crescentus*. *Proc Natl Acad Sci*. 2011;108(24):9963-9968. doi:10.1073/pnas.1012388108

30. Choi Y, Shin H, Lee JH, Ryu S. Identification and Characterization of a Novel Flagellum-Dependent Salmonella-Infecting Bacteriophage, iEPS5. *Appl Environ Microbiol*. 2013;79(16):4829-4837. doi:10.1128/AEM.00706-13

31. Raimondo LM, Lundh NP, Martinez RJ. Primary Adsorption Site of Phage PBS1: the Flagellum of *Bacillus subtilis*. *J Virol*. 1968;2(3):256-264. doi:10.1128/jvi.2.3.256-264.1968

32. Lovett PS. PBP1: A flagella specific bacteriophage mediating transduction in Bacillus pumilus. *Virology*. 1972;47(3):743-752. doi:10.1016/0042-6822(72)90564-8

33. Esteves NC, Scharf BE. Flagellotropic Bacteriophages: Opportunities and Challenges for Antimicrobial Applications. *Int J Mol Sci*. 2022;23(13):7084. doi:10.3390/ijms23137084

34. Harvey H, Bondy-Denomy J, Marquis H, Sztanko KM, Davidson AR, Burrows LL. Pseudomonas aeruginosa defends against phages through type IV pilus glycosylation. *Nat Microbiol*. 2017;3(1):47-52. doi:10.1038/s41564-017-0061-y

35. Huszczynski SM, Lam JS, Khursigara CM. The Role of Pseudomonas aeruginosa Lipopolysaccharide in Bacterial Pathogenesis and Physiology. *Pathog Basel Switz*. 2019;9(1):6. doi:10.3390/pathogens9010006

36. Kortright KE, Chan BK, Evans BR, Turner PE. Arms race and fluctuating selection dynamics in *Pseudomonas aeruginosa* bacteria coevolving with phage OMKO1. *J Evol Biol*. 2022;35(11):1475-1487. doi:10.1111/jeb.14095

37. Mukherjee S, Moustafa DA, Stergioula V, Smith CD, Goldberg JB, Bassler BL. The PqsE and RhlR proteins are an autoinducer synthase–receptor pair that control virulence and biofilm development in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci*. 2018;115(40). doi:10.1073/pnas.1814023115

38. Markwitz P, Lood C, Olszak T, Van Noort V, Lavigne R, Drulis-Kawa Z. Genome-driven elucidation of phage-host interplay and impact of phage resistance evolution on bacterial fitness. *ISME J*. 2022;16(2):533-542. doi:10.1038/s41396-021-01096-5

39. De Smet J, Zimmermann M, Kogadeeva M, et al. High coverage metabolomics analysis reveals phage-specific alterations to *Pseudomonas aeruginosa* physiology during infection. *ISME J*. 2016;10(8):1823-1835. doi:10.1038/ismej.2016.3

40. Olszak T, Augustyniak D, García-Romero I, et al. Phage treatment of Pseudomonas aeruginosa yields a phage-resistant population with different susceptibility to innate immune responses and mild effects on metabolic profiles. *Microbiol Res*. 2024;282:127609. doi:10.1016/j.micres.2024.127609

41. Pitcher RS, Tonkin LM, Daley JM, et al. Mycobacteriophage Exploit NHEJ to Facilitate Genome Circularization. *Mol Cell*. 2006;23(5):743-748. doi:10.1016/j.molcel.2006.07.009

42. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Sci Rep*. 2016;6(1):26717. doi:10.1038/srep26717

43. Seed KD. Battling Phages: How Bacteria Defend against Viral Attack. Miller VL, ed. *PLOS Pathog*. 2015;11(6):e1004847. doi:10.1371/journal.ppat.1004847

44. Deveau H, Garneau JE, Moineau S. CRISPR/Cas System and Its Role in Phage-Bacteria Interactions. *Annu Rev Microbiol*. 2010;64(1):475-493. doi:10.1146/annurev.micro.112408.134123

45. Costa AR, Van Den Berg DF, Esser JQ, et al. Accumulation of defense systems in phage-resistant strains of *Pseudomonas aeruginosa*. *Sci Adv*. 2024;10(8):eadj0341. doi:10.1126/sciadv.adj0341

46. Essoh C, Blouin Y, Loukou G, et al. The Susceptibility of Pseudomonas aeruginosa Strains from Cystic Fibrosis Patients to Bacteriophages. Kaufmann GF, ed. *PLoS ONE*. 2013;8(4):e60575. doi:10.1371/journal.pone.0060575

47. Mutalik VK, Adler BA, Rishi HS, et al. High-throughput mapping of the phage resistance landscape in E. coli. De Visser JAGM, ed. *PLOS Biol*. 2020;18(10):e3000877. doi:10.1371/journal.pbio.3000877

48. Patel PH, Maxwell KL. Prophages provide a rich source of antiphage defense systems. *Curr Opin Microbiol*. 2023;73:102321. doi:10.1016/j.mib.2023.102321

49. Rollie C, Chevallereau A, Watson BNJ, et al. Targeting of temperate phages drives loss of type I CRISPR–Cas systems. *Nature*. 2020;578(7793):149-153. doi:10.1038/s41586-020-1936-2

50. Mutalik VK, Novichkov PS, Price MN, et al. Dual-barcoded shotgun expression library sequencing for high-throughput characterization of functional traits in bacteria. *Nat Commun*. 2019;10(1):308. doi:10.1038/s41467-018-08177-8

51. Payne LJ, Todeschini TC, Wu Y, et al. Identification and classification of antiviral defence systems in bacteria and archaea with PADLOC reveals new system types. *Nucleic Acids Res*. 2021;49(19):10868-10878. doi:10.1093/nar/gkab883

52. Bernheim A, Sorek R. The pan-immune system of bacteria: antiviral defence as a community resource. *Nat Rev Microbiol*. 2020;18(2):113-119. doi:10.1038/s41579-019-0278-2

53. Chan B, Stanley G, Kortright K, et al. *Personalized Inhaled Bacteriophage Therapy Decreases Multidrug-Resistant* Pseudomonas Aeruginosa. Respiratory Medicine; 2023. doi:10.1101/2023.01.23.22283996

54. Jacobs MA, Alwood A, Thaipisuttikul I, et al. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci*. 2003;100(24):14339-14344. doi:10.1073/pnas.2036282100

55. Fisher RA. The Logic of Inductive Inference. *J R Stat Soc*. 1935;98(1):39. doi:10.2307/2342435

56. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *OMICS J Integr Biol*. 2012;16(5):284-287. doi:10.1089/omi.2011.0118

57. Mi H, Ebert D, Muruganujan A, et al. PANTHER version 16: a revised family classification, tree-based classification tool, enhancer regions and extensive API. *Nucleic Acids Res*. 2021;49(D1):D394-D403. doi:10.1093/nar/gkaa1106

58. Jaillard M, Lima L, Tournoud M, et al. A fast and agnostic method for bacterial genome-wide association studies: Bridging the gap between k-mers and genetic events. Didelot X, ed. *PLOS Genet*. 2018;14(11):e1007758. doi:10.1371/journal.pgen.1007758

59. ncbi. BLAST® Command Line Applications User Manual. Published online June 23, 2008. https://www.ncbi.nlm.nih.gov/books/NBK279670/

60. Katoh K. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*. 2002;30(14):3059-3066. doi:10.1093/nar/gkf436

61. Rice P, Longden I, Bleasby A. EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet*. 2000;16(6):276-277. doi:10.1016/S0168-9525(00)02024-2

62. Hornik K. Wilcoxon test. https://web.mit.edu/~r/current/arch/i386_linux26/lib/R/library/stats/html/Wilcoxon.html

63. Davies S, Ihaka R. glm. https://stat.ethz.ch/R-manual/R-devel/library/stats/html/glm.html

64. Perkel JM. The software that powers scientific illustration. *Nature*. 2020;582(7810):137-138. doi:10.1038/d41586-020-01404-7

65. Siryaporn A, Kim MK, Shen Y, Stone HA, Gitai Z. Colonization, Competition, and Dispersal of Pathogens in Fluid Flow Networks. *Curr Biol*. 2015;25(9):1201-1207. doi:10.1016/j.cub.2015.02.074

66. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FSL. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res*. 2016;44(D1):D646-653. doi:10.1093/nar/gkv1227

67. Conover WJ. On Methods of Handling Ties in the Wilcoxon Signed-Rank Test. *J Am Stat Assoc*. 1973;68(344):985-988. doi:10.1080/01621459.1973.10481460

68. Portet S. A primer on model selection using the Akaike Information Criterion. *Infect Dis Model*. 2020;5:111-128. doi:10.1016/j.idm.2019.12.010

69. Hartig F. DHARMa. Published online September 8, 2022. https://cran.r-project.org/web/packages/DHARMa/vignettes/DHARMa.html