Prophages are Infrequently Associated With Antibiotic Resistance in *Pseudomonas aeruginosa* Clinical Isolates

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Abstract

Antimicrobial resistance (AMR) is a significant obstacle to the treatment of bacterial infections, including in the context of *Pseudomonas aeruginosa* infections in patients with cystic fibrosis (CF). Lysogenic bacteriophages can integrate their genome into the bacterial chromosome and are known to promote genetic transfer between bacterial strains. However, the contribution of lysogenic phages to the incidence of AMR is poorly understood. Here, in a set of 187 clinical isolates of *Pseudomonas aeruginosa* collected from 82 patients with CF, we evaluate the links between prophages and both genomic and phenotypic resistance to five anti-pseudomonal antibiotics: tobramycin, colistin, ciprofloxacin, meropenem, aztreonam, and tazobactam. We find that *P. aeruginosa* isolates contain on average 3.06 +/- 1.84 (SD) predicted prophages. We find no significant association between the number of prophages per isolate and the mean inhibitory concentration (MIC) for any of these antibiotics. We then investigate the relationship between particular prophages and AMR. We identify a single lysogenic phage that is associated with phenotypic resistance to the antibiotic tobramycin. Consistent with this association, we identify AMR genes associated with resistance to tobramycin in these strains and find that they are not encoded directly on prophage sequences. These findings suggest that prophages are infrequently associated with the AMR genes in clinical isolates of *P. aeruginosa*.

Introduction

Antimicrobial resistance (AMR) has emerged as a critical global health challenge, diminishing the efficacy of antibiotics and jeopardizing the treatment of bacterial infections. AMR is associated with increased morbidity, mortality, and healthcare costs, posing a significant burden on healthcare systems worldwide (Cassini et al., 2019; O'Neill, 2016). The rise of AMR is driven by the overuse and misuse of antibiotics in human medicine, agriculture, and veterinary practices, creating a selection pressure that favors resistant strains, and the subsequent transmission of AMR genes to other strains (Ventola, 2015; Levy & Marshall, 2004). Consequently, pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, commonly referred to as (ESKAPE) pathogens have developed resistance to multiple antibiotic classes, rendering standard treatments ineffective (WHO, 2019; CDC, 2019: The Lancet 2019). Strategies to combat AMR include the development of new antibiotics, alternative therapies, improved diagnostic tools, and enhanced infection control measures (Laxminarayan et al., 2013; Ventola, 2015). However, the current pipeline for antibiotic development cannot keep pace with the emergence of resistance, and new approaches are needed to address this issue (Kümmerer, 2009; Prestinaci 2015). Understanding the factors contributing to the spread of AMR is essential for devising effective interventions and preserving the efficacy of existing antibiotics (Bush et al., 2011; Martínez, 2009).

Mobile genetic elements (MGEs) such as plasmids, transposable elements, integrons and bacteriophages play an important role in the dissemination of antimicrobial resistance (AMR) genes across bacterial populations via horizontal gene transfer (HGT). Plasmids in particular often carry multiple resistance genes that confer multidrug resistance to their bacterial hosts (Carattoli, 2013; Partridge et al., 2018). Transposable elements, including insertion sequences (IS) and transposons, likewise facilitate the movement of resistance genes within and between DNA molecules, contributing to genomic plasticity and the spread of AMR (Partridge, 2011; Siguier et al., 2014). Bacteriophages, or phages, are viruses that infect bacteria and can mediate the transfer of AMR genes through transduction, a process where bacterial DNA is packaged into phage particles and delivered to new host cells (Colavecchio et al., 2017; Koonin & Makarova, 2013). The interplay between these MGEs and their bacterial hosts may contribute to the rapid and widespread dissemination of resistance determinants, complicating efforts to control AMR (Stokes & Gillings, 2011; Heuer et al., 2011; Frost et al., 2005; von Wintersdorff et al., 2016).

Prophages are the genomes of lysogenic phages that are integrated into the bacterial chromosome, until induced to enter the lytic cycle, during which new phage particles are produced and the cell is lysed (Frost et al., 2005; Touchon et al., 2017). They can have an outsized influence on their host, affecting bacterial evolution and ecology (Casjens, 2003). The integration of prophages into bacterial genomes can also result in genetic rearrangements, influencing gene expression and phenotypic traits (Bobay et al., 2013; Brüssow et al., 2004). When integrated, some prophages can impose fitness costs under certain conditions (Howard-Varona et al., 2017; Touchon et al., 2016), while others contribute to bacterial fitness by conferring advantages such as immunity to superinfection by related phages and can carry genes that enhance survival under stress conditions (Wang et al., 2010; Paul, 2008).

Few phages directly encode AMR genes (Enault et al. 2017, Pfeiffer et al. 2022, Calero-Caceres et al 2019, Colavecchio et al., 2017), however, phages can contribute to the spread of AMR through either generalized or specialized transduction (Davies & Davies, 2010; Muniesa et al., 2013;Haaber et al., 2016; Enault et al., 2016). Generalized transduction is the mispackaging of

bacterial DNA into the phage capsid and is considered relatively uncommon (Volkova et al. 2014). Specialized transduction, on the other hand, occurs when the prophage is excised with some of the adjoining bacterial genetic material (Griffith et al. 2002, Chiang et al. 2019). Furthermore, the induction of prophages under stress conditions, such as antibiotic exposure, can lead to the release of phage particles carrying resistance genes, amplifying the spread of AMR and thus protecting vast amounts of bacteria at the same time (Keen et al., 2017; Mazel & Davies, 1999). While phage-mediated transduction has been observed in the lab, the relevance of phage-mediated transduction to the spread of AMR in clinical settings is still unclear.

This study investigates the relationship between prophages and both genotypic and phenotypic AMR in the context of bacterial lung infections of patients with cystic fibrosis (CF). We use whole-genome sequences and MIC measurements for 187 clinical isolates of *Pseudomonas aeruginosa* (*Pa*) from 82 patients from Stanford Hospital and the Cystic Fibrosis Clinic to address the following questions: 1) Is prophage abundance correlated with AMR in *Pa*? 2) Are specific prophages associated with phenotypic resistance to antibiotics and with AMR genes?

Results

Abundant prophage population is composed of a few high-frequency prophages and many low-frequency prophages.

We first identified all prophages integrated in the genomes of the *Pa* clinical isolates. Prophages were then assigned the same identification number if they were a similar length (within 90-110% of each other) and showed 90% pairwise similarity. We found that the distribution of prophage abundance varied, with most isolates containing between one and four prophages (mean = 3.06+/-1.84) (**Figure 1A**). Isolates harboring two prophages were most common, followed closely by those with one prophage. The occurrence of isolates with five or more prophages decreased significantly, with only a few instances of isolates containing up to ten prophages, which was the maximum number we observed.

The majority of prophages appeared only once or a few times, indicating a high diversity of prophage types among the isolates, with only a few prophages present in more than 10 isolates (**Figure 1B**). The most common prophage was found in 53 isolates out of 187 clinical isolates. This suggests that while a few prophages may be common, the prophage population is predominantly composed of diverse, low-frequency variants.

The number of prophages is not associated with phenotypic resistance to six commonly used antibiotics.

We hypothesized that a high number of prophages in an isolate may be used as a proxy for higher levels of transduction and be associated with high phenotypic AMR. We tested this association for six antibiotics commonly-used to treat *Pseudomonas* infections in patients with cystic fibrosis: tobramycin, colistin, ciprofloxacin, meropenem, aztreonam, and tazobactam. There was no significant association between the number of prophages per isolate and MIC for any of these antibiotics (**Figure 2**, t-test, $p > 0.05$). This suggests that prophage abundance alone is not a major factor influencing resistance profiles in these bacterial isolates.

Specific prophage is associated with an increase in phenotypic resistance to tobramycin.

Most prophages were not present in enough isolates to evaluate their relationship with resistance. We selected the five most prevalent phages, which we named vB_Tem_CfSt1-5 and evaluated their association with the MIC for the four antibiotics tobramycin, colistin, ciprofloxacin and meropenem (**Figure 3A**, Wilcoxon test). We found a significant increase in tobramycin resistance when prophage vB_Tem_CfSt1 was present (**Figure 3B**, Wilcoxon test).

Prophage associated with increases in phenotypic AMR is also associated with AMR genes.
We next asked if the same prophages were associated with the presence of AMR genes. Among all AMR genes described in the CARD-RGI dataset found in our isolates, only two were significantly associated with resistance to tobramycin (Figure 4A & 4B, Wilcoxon test, $p < 0.001$). Both of these genes (aadA and AAC(6')-lb9) are aminoglycosides-modifying enzymes (Hollingshead & Vapnek 1985, Mugnier et al, 1998). Finally, we found that the presence of prophage vB_Tem_CfSt1 was significantly associated with the presence of these genes (Figure 4C & 4D, Fisher test, $p < 0.01$). **We did not find any AMR genes directly within the prophage sequence. We could not determine the relative location of prophages and antibiotic resistance genes due to the small size of many scaffolds containing prophages and AMR genes.**

Discussion
Phage-mediated transduction of AMR genes has been shown to occur in the lab but its relevance to the spread of AMR in patients remains unclear. In this work, we investigated the relationship between the presence of prophages in clinical isolates of *Pa* and both phenotypic and genotypic resistance to clinically-relevant antibiotics. Our results show that prophage abundance cannot predict phenotypic AMR but that specific prophages are infrequently associated with phenotypic AMR and AMR genes.

We first hypothesized that prophage abundance could be used as a proxy for transduction frequency, susceptibility to horizontal transfer of AMR genes and phenotypic AMR. Despite a relatively high variance in the number of prophages found in the bacterial genomes, we did not find a relationship between prophage abundance and MIC for any of the antibiotics considered here. These results suggest that phage-mediated transduction is rare and that most phages do not introduce AMR genes during infection.

We then asked if specific prophages were associated with MIC and identified two prophages (vB_Tem_CfSt1 and vB_Tem_CfSt3) showing significant and near-significant association with resistance to tobramycin and ciprofloxacin respectively. For tobramycin, two genes were found to be associated with phenotypic resistance and to be more likely to be present in isolates infected with the phage vB_Tem_CfSt1. On the other hand, we were not able to identify AMR genes that were associated with ciprofloxacin resistance. The genome reconstructions from the short-read sequencing performed for this work did not allow us to establish the relative locations of prophages and AMR genes. In addition, these findings are limited by low sample size for many phages and AMR genes, the majority of which were only found in one isolate. Very few phages were found in more than twenty isolates and AMR genes associated with tobramycin resistance were only found in 14 isolates. The low prevalence of most AMR genes also prevented us from assessing additive effects from the presence of multiple genes. While whole-genome sequencing is becoming easier to perform at a larger scale, the assessment of phenotypic resistance in the lab remains slow and labor intensive. The prediction of resistance phenotype from bacterial genomes represents a

potential path forward but prediction accuracy varies between bacterial species and antibiotics and is also limited by the sample size of the training data (Wang et al, 2022; Vanstokstraeten et al, 2023).

The two genes linked to tobramycin resistance and associated with the presence of phage vB_Tem_CfSt1 were identified as aadA and AAC(6')-lb9, which both encode aminoglycosides-modifying enzymes. These enzymes are some of the most widespread mechanisms for tobramycin resistance and are known to be highly mobile (Garneau-Tsodiko & Labby 2016). Other common mechanisms for tobramycin resistance include other mobile genes such as efflux pumps and ribosome methyltransferases. Mutations in the ribosome genes targeted by aminoglycosides are rarer on the other hand. This is in contrast with ciprofloxacin resistance, which is often acquired through mutations of the A subunit DNA gyrase targeted by quinolones like ciprofloxacin, rather than mobile genetic elements (Hooper et al, 1989). Other works also show a lower association between known genotypic markers of resistance and phenotypic resistance for ciprofloxacin compared to other antibiotics like aminoglycosides (Vanstokstraeten et al 2023), which is consistent with our findings. Phage-mediated transduction of AMR genes may thus be more relevant to antibiotics for which mobile genes, rather than point mutations, confer resistance and will be easier to detect for antibiotics with strong genotype to phenotype associations for antibiotic resistance.

In summary, we identified one prophage associated with higher MIC for tobramycin and found that known AMR genes conferring resistance to tobramycin were more likely to be found in isolates infected with that phage. Prophage abundance, on the other hand, was not associated with antibiotic resistance. These findings suggest that the spread of AMR through prophages is rare overall. Future work should focus on new phenotype prediction tools to increase sample sizes, as well as long read sequencing to explore causal relationships between phage and AMR through the relative locations of mobile elements in bacterial genomes.

Materials and Methods

Sample Collection

From June 2020 to June 2023, 187 *Pseudomonas aeruginosa (Pa)* isolates from respiratory cultures of cystic fibrosis patients were collected at Stanford Hospital under IRB approvals #11197. Samples were biobanked with patient consent and de-identified using unique codes.

DNA Extraction and Sequencing

DNA was extracted from Pa isolates using the DNeasy Blood and Tissue Kit (Qiagen, 69504) and sequenced at the Stanford Illumina sequencing core on an Illumina NovaSeq (100bp paired-end) and an Illumina NextSeq (150bp paired-end). The extraction involved bacterial lysis, DNA purification, and elution, ensuring high-quality DNA suitable for sequencing. Sequencing reads were quality-checked using FASTQualityControl and trimmed with Trimmomatic 0.39 or 'trim galore' to remove Nextera adapters from raw reads sequenced on Illumina NextSeq. Quality reports were assembled using MultiQC55. Trimmed reads were then assembled with SPAdes using –isolate and --cov-cutoff auto (Prjibelski *et al.*, 2020).

Prophage identification
We identified prophages using VIBRANT (Kieft, Zhou and Anantharaman, 2020). We then performed a BLAST search (word size = 28, e-value = 0.005) on all predicted prophages against their own sequences. Prophages were grouped under the same name if they were of similar length (less than 10% difference in length) and showed at least 90% pairwise identity.

Antibiotic resistance measurements
Clinical isolates were streaked on LB agar plates and incubated at 37°C overnight. The ~~Next~~ day, the colonies were picked and suspended in 2 ml of sterile 0.85% saline solution. The inoculum was vortexed and its turbidity was adjusted until it reached 0.5Mc. Farland standard (OD 0.08-0.1). The standardized bacterial suspension was evenly streaked onto 150mm Mueller Hinton agar plates (Thermo Fisher Scientific) using a sterile cotton swab. The plates were allowed to dry for 15 min and the E test strips (Fisher Scientific) were placed onto the plates and incubated at 37°C. The MICs were read after 16-20 hrs.

Identification of antibiotic resistance genes
Antibiotic resistance genes were identified with a BLAST search (-word size 28 -culling_limit 1 evalue 0.005) of bacterial genomes against the homolog model of the CARD-RGI database. This model includes sequences that are determinants of resistance without mutation.

Statistical Analysis
Statistical analyses were performed using R. Tests used include t-tests for linear regression, Wilcoxon tests and fisher tests. Sample sizes were not predetermined. Investigators were blinded during DNA extraction, library preparation, and phage genome annotation, but not during the final analysis.

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Competing interests
The authors declare no competing interests.

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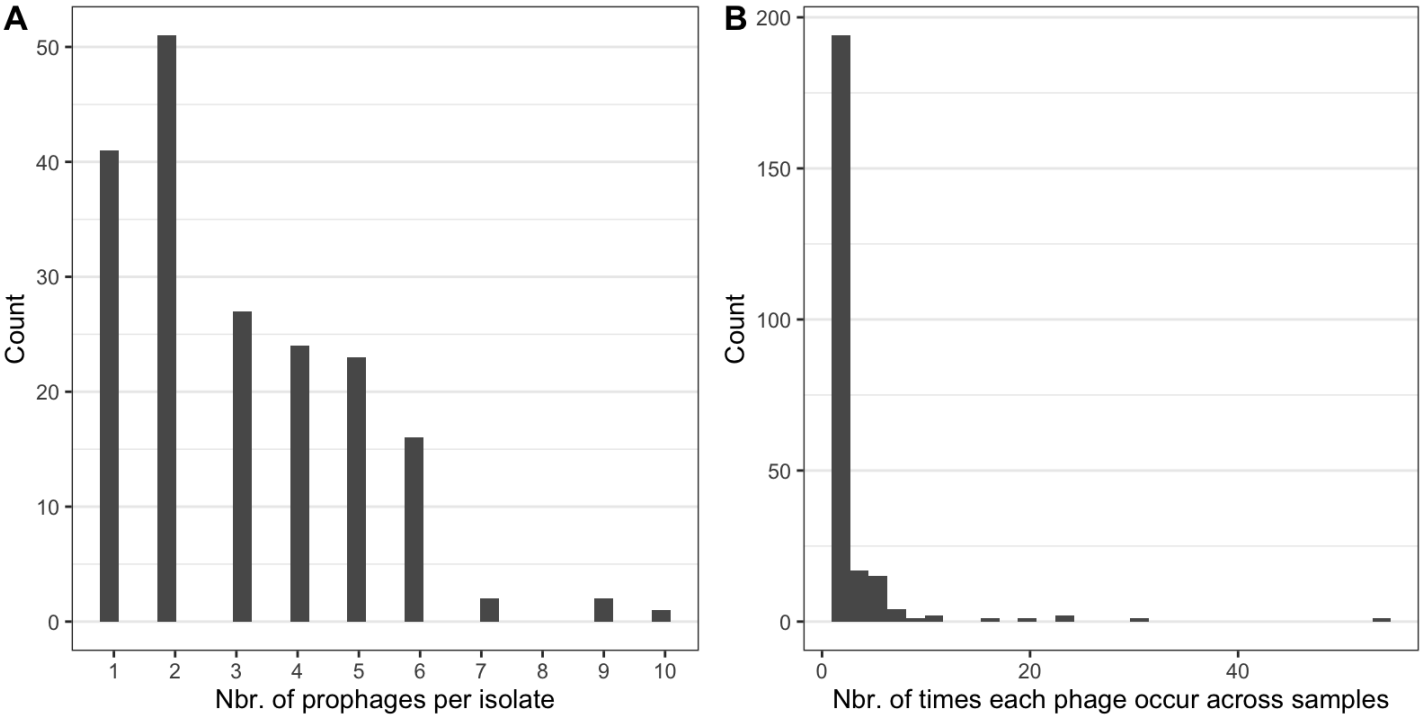

Figure 1: Prophages are common in clinical samples. A. Number of prophages per isolate. **B.** Distribution of phage occurrence across the samples. Most phages are present only once or twice with a few phages present many times.

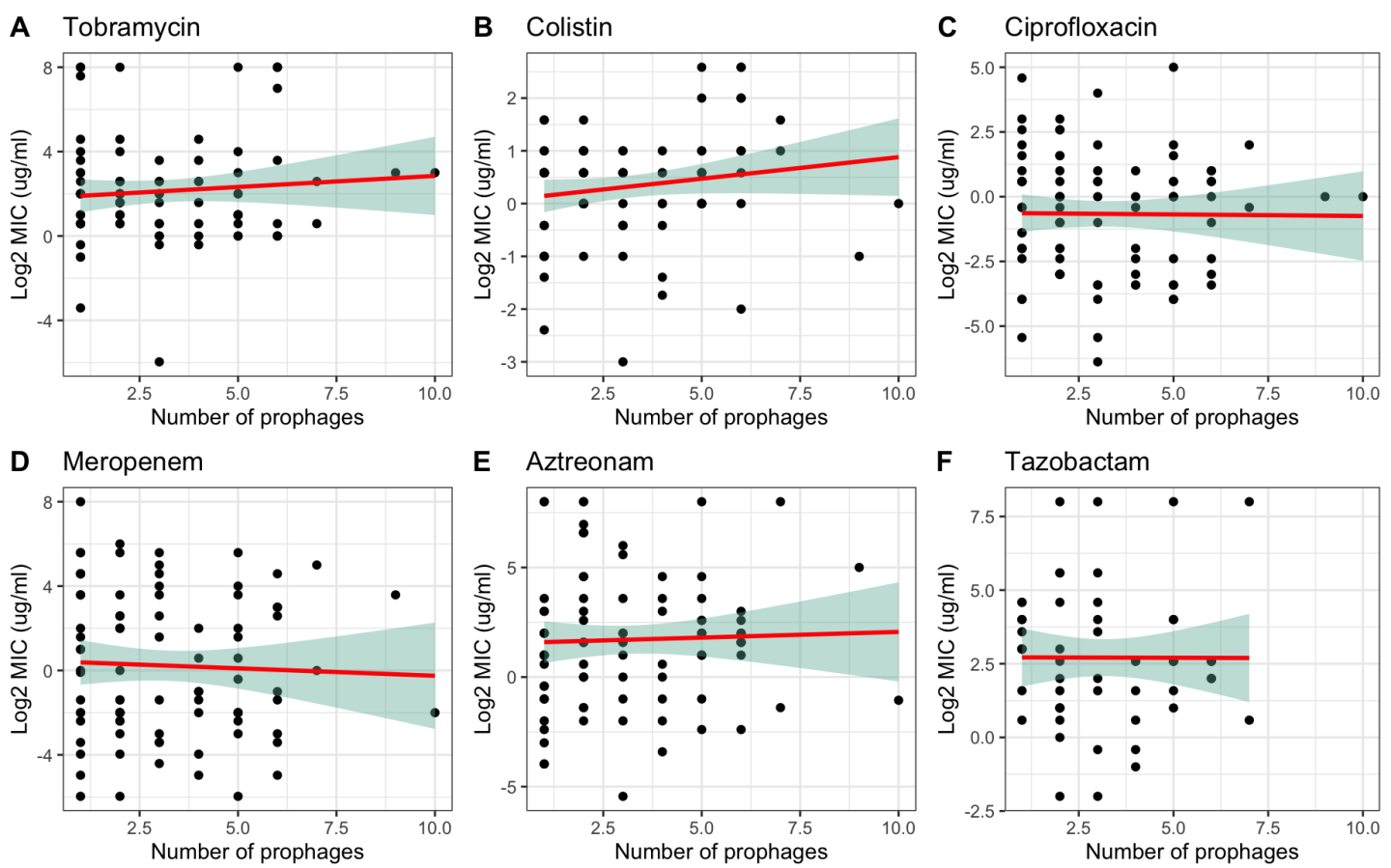

Figure 2: There is no relationship between the Number of Prophages and the Minimum Inhibitory Concentration (MIC) for various antibiotics. A. Tobramycin **B.** Colistin **C.** Ciprofloxacin **D.** Meropenem **E.** Aztreonam **F.** Tazobactam. Each plot includes the linear regression line (red) with a shaded 95% confidence interval (green).

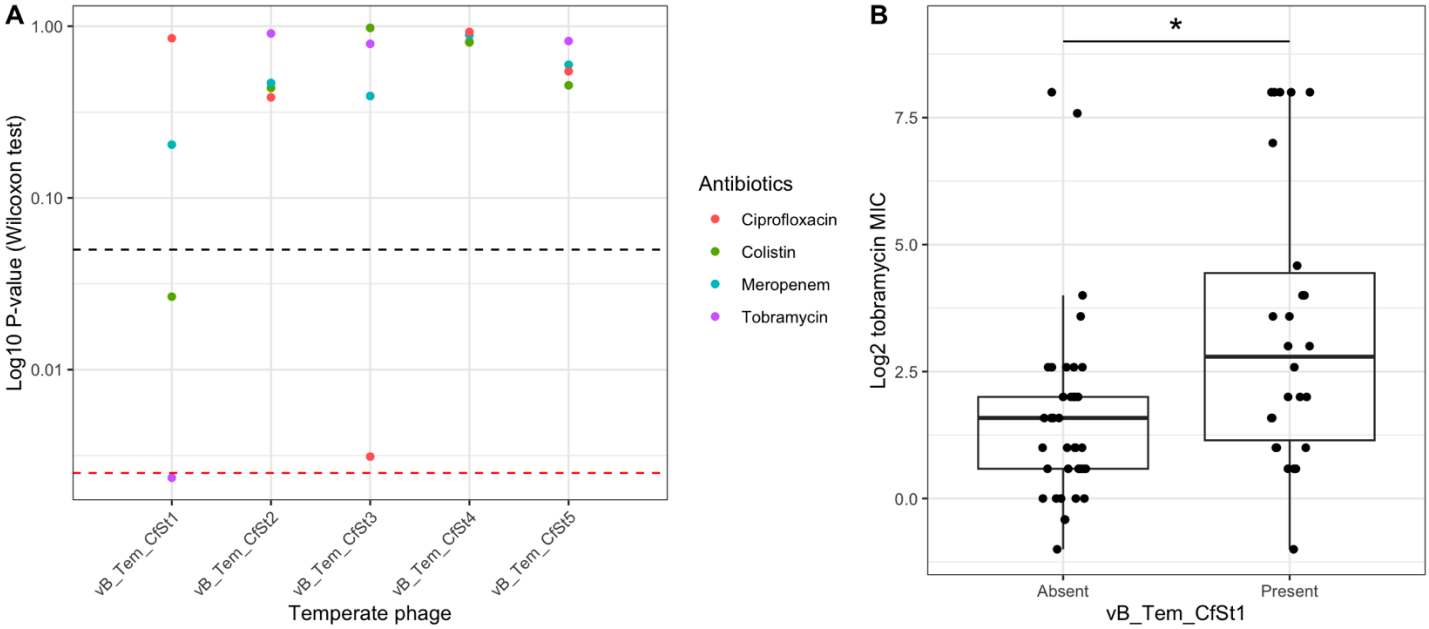

Figure 3: Certain phages are associated with antibiotic resistance. A. P-values from Wilcoxon tests assessing the relationship between the presence of individual phages and MIC values for various antibiotics. **B.** Tobramycin resistance (Log2 Tobramycin MIC (µg/mL)) for isolates with and without phage vB_Tem_CfSt1 showing a significant difference in MIC when the phage is present.

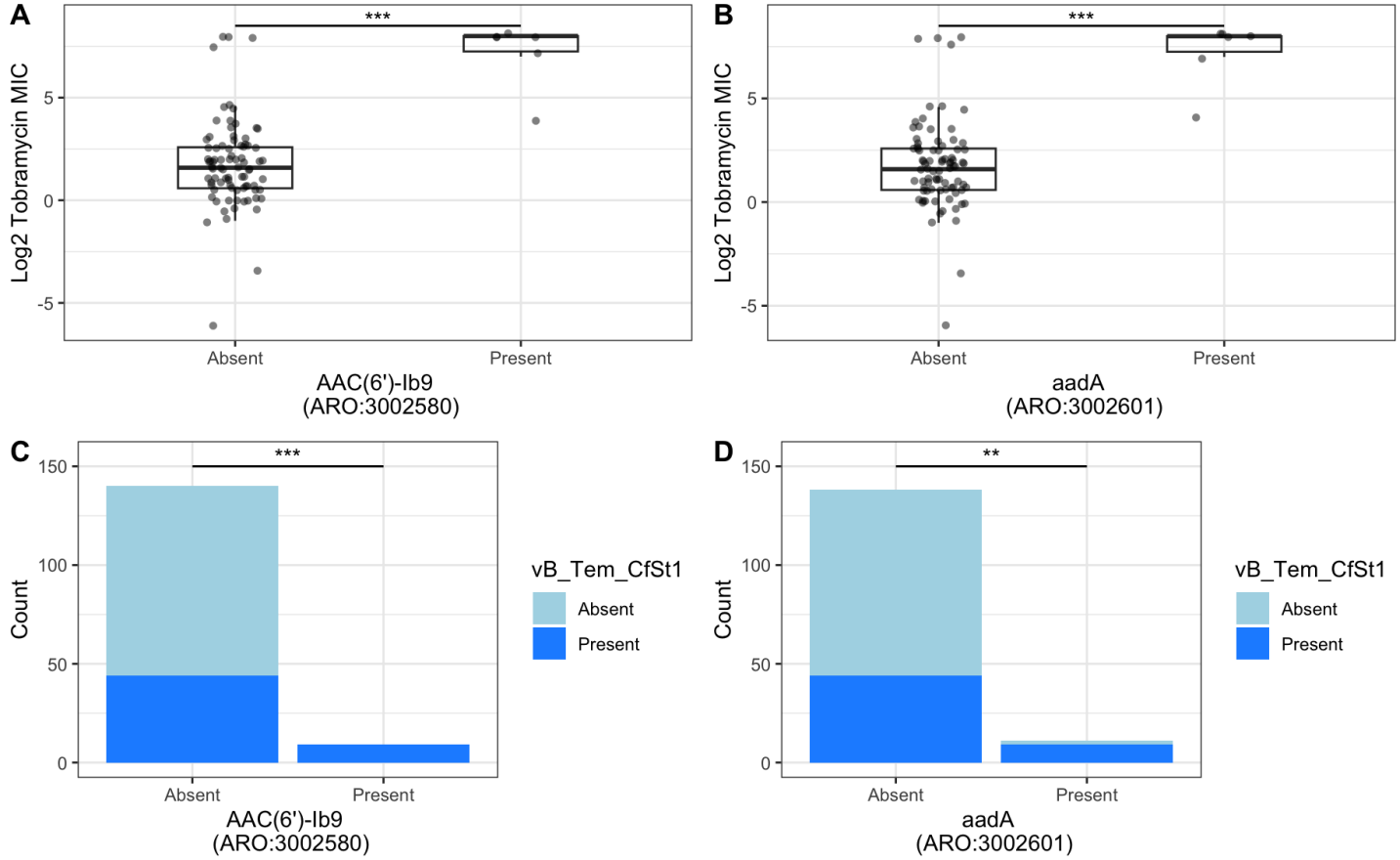

Figure 4: Presence of antibiotic resistance genes is associated with increased tobramycin resistance and prophage presence. **A.** Tobramycin resistance (Log2 Tobramycin MIC) for isolates with and without resistance genes AAC(6')-Ib9 (ARO:3002580) **B.** Tobramycin resistance (Log2 Tobramycin MIC) for isolates with and without resistance genes aadA (ARO:3002601)**C.** Presence of temperate phage vB_Tem_CfSt1 for isolates with and without antibiotic resistance genes AAC(6')-Ib9 **D.** Presence of temperate phage vB_Tem_CfSt1 for isolates with and without antibiotic resistance genes aadA.