Prophages present in *Acinetobacter pittii* influence bacterial virulence, antibiotic resistance, and genomic rearrangements

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Abstract

Antibiotic resistance and virulence are common among bacterial populations, posing a global clinical challenge. The bacterial species *Acinetobacter pittii*, an infectious agent in clinical environments, has shown increasing rates of antibiotic resistance. Viruses that integrate as prophages into *A. pittii* could be a potential cause of this pathogenicity, as they often contain antibiotic resistance or virulence factor gene sequences. In this study, we analyzed 25 *A. pittii* strains for potential prophages. Using virulence factor databases, we identified many common and virulent prophages in *A. pittii*. The analysis also included a specific catalogue of the virulence factors and antibiotic resistance genes contributed by *A. pittii* prophages. Finally, our results illustrate multiple similarities between *A. pittii* and its bacterial relatives with regards to prophage integration sites and prevalence. These findings provide broader insight into prophage behavior that can be applied to future studies on similar species in the *Acinetobacter calcoaceticus*-*baumannii* complex.

# Introduction

*Acinetobacter pittii* is a clinically significant, Gram-negative bacterial species of the *Acinetobacter* genus.1 It can cause nosocomial infections,2,3 particularly in intensive care units,4 and has been associated with increasing rates of antibiotic resistance and virulence, especially with regards to carbapenem antibiotics.5,6 Recently, it has also been associated with infections outside of a clinical setting.7 Although not as virulent and prevalent as the more well-known *Acinetobacter* species *A. baumannii*,8 *A. pittii*’s increasing antibiotic resistance and virulence warrants further study.

One mechanism of *A. pittii* pathogenicity could be through prophages. Prophages are formed by bacteriophages when they integrate their entire genome into bacterial genetic sequences during a lysogenic life cycle, and these prophages propagate by replicating with bacterial chromosomes or plasmids during cell division.9 Through lysogeny, prophages can spread throughout a bacterial population without killing its host. This allows prophage propagation to not only benefit the phage, but also confer advantages on the host bacterium, such as increased genetic diversity9 and the prevention of additional viral infection.10 Moreover, prophages often contain virulence factors (proteins that enhance the pathogenicity of their host) and thus increase the survival fitness of both phage and bacteria.9

Prophages have also been associated with virulence and antibiotic resistance in the *Acinetobacter* genus.11,12 In *A. baumannii*, prophages have been found in bacterial chromosomes and plasmids that encode a variety of virulence factors ranging from efflux pumps that remove toxins to antibiotic-inactivating enzymes.11 However, while related species like *A. baumannii* have been surveyed for integrated viral sequences, such prophage analysis has yet to be applied to *A. pittii*. Moreover, given the clinical infections resulting from *A. pittii*, it is imperative that the mechanisms and origins of its pathogenicity be better understood. As a result, by identifying the specific prophages and virulence factors that are most common in *A. pittii*, this study could provide new directions for *A. pittii* research and pinpoint existing or potential future causes of its virulence.

In our study, we characterized the distribution, lengths, and phylogenetic relationships of prophages found in *A. pittii*. We found that there is a clear but uneven distribution of prophages in *A. pittii* strains, and that the prophages exhibit differences with respect to viral families. In order to further understand their influence on bacterial hosts, we searched for and analyzed virulence factors and antibiotic resistance genes within these prophages. Our results indicate that virulence factors in general, compared to antibiotic resistance genes, are present in greater quantities and varieties. In addition, we present evidence of virulence factors potentially influencing genomic diversity and rearrangements.

# Materials and Methods

## Genome Collection

Our study used only complete *Acinetobacter pittii* genomes (24) and chromosomes (1), which were obtained from NCBI GenBank (last accessed January 2021).13 Each genome or chromosome belonged to a different strain of the bacteria. Genomes consisted of the fully sequenced chromosome for that specific strain as well as all associated plasmids. As a result, there were in total 25 chromosomes and 64 plasmids across the 25 *A. pittii* strains.

## Prophage Identification

Using the GenBank accession number for each chromosome or plasmid, as well as the PHAge Search Tool - Enhanced Release (PHASTER) web server,14 we identified potential prophages within each genetic sequence. In order to ensure high-confidence in the identity of sequences as potential prophages, those that were less than ten kilobases in length or did not contain structural genes and/or integrase were discarded, as per Costa, Rita *et al.*11

The selected prophages were then automatically classified as Intact, Questionable, or Incomplete by the PHASTER software based on the amount of phage genes in the prophage sequence.14 This automatic classification depends on factors like amount of phage coding regions and sequence length.14 In total, 94 prophages were identified for the *A. pittii* strains, of which 34 were labelled Intact by PHASTER. Due to their completeness, only these 34 intact prophage sequences were used for the analysis steps described below.

## Statistical and Graphical Analysis

Length data was retrieved from PHASTER for each of the 34 intact prophages. Statistical analysis of average prophage length within each viral family was then performed in Microsoft Excel (2019) using a one-tailed t-Test assuming unequal variances. The significance level was taken to be p < 0.05.

Graphical analysis on the spatial distribution of intact prophages within bacterial chromosomes was performed using data from PHASTER and the R package ggplot2.15 Specifically, the geom\_density() function of ggplot2 was employed to create a density plot of the intact prophages along bacterial chromosome sequences.

## Virulence Factor Identification

Chromosomes and plasmids with at least one intact prophage were selected for genome annotation using the myRAST annotation software and standard parameters.16 All protein encoding genes within the 34 intact prophages were thus identified and run in a BLAST search against the Virulence Factor DataBase (VFDB) with default parameters and the protein sequences from VFDB’s full dataset.17 An Expect value of less than 1 × 10-20 was used as a cutoff, and virulence factors were thus identified among the intact prophages.

## Synteny Comparison

Two of the most common virulence factors, as identified by VFDB, were further analyzed. These were anthranilate phosphoribosyltransferase and zinc binding alcohol dehydrogenase, each present in three intact prophages. Synteny maps for each set of prophages were generated using Mauve18 in order to compare the virulence factors shared within the set.

## Resistance Gene Identification

The sequence for each of the intact prophages were inputted into the Resistance Gene Identifier of the Comprehensive Antibiotic Resistance Database (CARD).19 By comparison with CARD, potential antibiotic resistance genes were identified within the prophages. Each resistance gene was labelled by CARD as Perfect, Strict, or Loose depending on the degree of confidence for the presence of that gene. “Perfect” sequences exactly matched an existing antibiotic resistance gene sequence in CARD, while Strict and Loose results were less exact matches.19

## Phylogenetic comparison

We constructed a phylogenetic tree comparing the Acinetobacter phage YMC/09/02/B1251\_ABA\_BP in *A. pittii* and *A. baumannii* strains. We retrieved the GenBank sequences for intact *A. pittii* prophages identified in our study, as well as the GenBank sequences for intact *A. baumannii* prophages. The 20 intact prophages were aligned using MAFFT version 7 and default parameters.47 The phylogenetic tree was then generated using MEGA, 48 with bootstrap replications set to 100.

# Results

## Prophage Presence

Table 1 shows the distribution of the 34 intact prophages across the 17 *A. pittii* strains that contained these high-confidence sequences. Intact prophages were distributed unevenly across the strains, with the strains ST220, HUMV-6483, and WCHAP005069 containing the most intact prophages (four each). The prevalence of these prophages in the different *A.pitti* strains is shown in Supplementary Figure 3. In terms of genetic element type, 2 out of 64 bacterial plasmids contained intact prophages, while 17 out of 25 bacterial chromosomes contained intact prophages. For these 19 sequences (2 plasmids and 17 chromosomes) that contained intact prophages, there averaged 1.00 prophages per plasmid and 1.88 prophages per chromosome.

## Prophage Distribution and Characteristics

All 94 prophages (intact, questionable, and incomplete) were predicted, by PHASTER and GenBank comparison, to belong to the viral order Caudovirales and the viral families *Myoviridae*, *Siphoviridae*, or *Podoviridae*. Of the three viral families, *Siphoviridae* accounted for most of the total prophages (65%, 61 out of 94), but *Myoviridae* accounted for the majority of intact prophages (56%, 19 out of 34). *Podoviridae* was present in the smallest amount, accounting for only five out of 94 total prophages and none of the intact prophages.

To analyze the effects of *A. pittii* prophage genome breakdown following integration, we compared the average lengths of intact *Myoviridae* and *Siphoviridae* prophages. *Siphoviridae* sequences averaged 49.3 kilobases (kb) in length, while *Myoviridae* sequences averaged 37.1 kb. Using a one-tailed t-Test assuming unequal variances, the lengths of the *Siphoviridae* were found to be significantly greater than those of the *Myoviridae*.

Using the geom\_density function of R package ggplot2,15 a density plot for the intact prophages present in *A. pittii* chromosomes was generated (Figure 1). Prophages present in plasmids were omitted due to the genetic differences and shortened lengths of these mobile genetic elements. The density plot demonstrated that there was significant prophage density around 1.4 megabase pairs (Mbp) and 3.2Mbp, with the 3.2Mbp peak having the higher prophage density. It can be seen in Figure 1 that there were many prophage sequences starting and ending at these two peaks. Thus, the preferential insertion of prophages within these two regions could help identification of novel prophages in other bacterial strains.

Hereafter, analysis is performed only with the 34 intact prophages, which are referred to as simply “prophages.”

## Virulence Factors within the Prophages

Using VFDB, 47 protein-encoding genes were identified in the prophages as encoding putative virulence factors (Table 2). The most commonly occurring virulence factors were invasion plasmid antigens (7 out of 47 virulence factors) and IS6 family transposases (5 out of 47). However, some virulence factors—such as the IS6 family transposases—were present numerous times in only one or a few of the 34 prophages. As a result, the virulence factors present in the most prophages were invasion plasmid antigens (4 out of 34 prophages), anthranilate phosphoribosyltransferases (3 out of 34), and zinc binding alcohol dehydrogenases (3 out of 34).

We categorized the virulence factors by their broader functions, based on a literature review (Table 4, Supplementary Figure 4). Based on functional categories, proteins involved in cellular metabolism and biosynthesis made up the largest fraction (12 out of 47 virulence factors, 25.5%). These proteins thus regulate key nutritional and energetic requirements in the cell that would help bacteria respond to stressful environments. The individual prophage (not species) that contained the most virulence factors was the Burkholderia phage phiE12-2 in the C54 strain’s plasmid (Table 2). It contained nine out of 47 total virulence factors (19.1%) within its genetic sequence, which included all of the IS6 family transposases (Table 2, gene name *mll6359*) and multiple invasion plasmid antigens (Table 2, gene name *ipaH2.5*).

## Virulence Factor Synteny Comparison

Since zinc binding alcohol dehydrogenases and anthranilate phosphoribosyltransferases were among the virulence factors present in the most intact prophages, we generated synteny maps for each virulence factor comparing the orientation of both in their respective prophage genomes. The synteny map of zinc binding alcohol dehydrogenase is shown in Figure 2, while that of anthranilate phosphoribosyltransferase is presented in Supplementary Figure 2.

In Figure 2, the virulence factor—zinc binding alcohol dehydrogenase—is present in the red synteny block for each of the three prophages. Other regions of these prophages are also highly conserved, with each homologous region delineated by a distinctly-colored block. However, it can be seen that genomic rearrangement of the prophage DNA occurred in the vicinity of the virulence factors. In the Acinetobacter phage YMC/09/02/B1251 (Figure 2, top), the aquamarine block seems to have undergone significant deletion in comparison with the aquamarine blocks of the two Acinetobacter phage YMC11/11/R3177 (Figure 2, center and bottom). In addition, the bottom Acinetobacter phage YMC11/11/R3177 possesses a large inversion of the light green and aquamarine blocks relative to the other two prophages. This change thus suggests that prophages and their virulence factors may have influences on genomic rearrangements in the bacterial genome.

## Prophages contain Antibiotic Resistance Genes

Using the Resistance Gene Identifier of the Comprehensive Antibiotic Resistance Database (CARD),19 37 antibiotic resistance genes were identified within 19 of the 34 intact prophages (Table 3). Resistance genes were classified as Loose, Strict, or Perfect within CARD in increasing degrees of similarity between the prophage gene and the CARD sequence.19 The majority (73.0%), were classified as Loose, although the remaining ten resistance genes did fulfill the Strict or Perfect criteria. Interestingly, nine out of these ten genes were found in the Burkholderia phage phiE12-2 present in the C54 strain’s plasmid, which was also the prophage which contained the most virulence factors.

Of the 37 resistance genes (Table 3), efflux pumps constituted the largest category of resistance genes (48.6%). These proteins remove toxic substances, including antibiotics, from the cell interior.38 Notably, *adeL* genes were present in the highest quantity (13.5% of total resistance genes). *adeL* is significant because it regulates the expression of the AdeFGH Resistance Nodulation Division (RND) efflux pump system, which can confer multidrug resistance that includes resistance to tetracycline, trimethoprim, and chloramphenicol.39,40

# **Discussion**

*A. pittii* is a bacterial pathogen that has the ability to cause nosocomial infections.2 It belongs to the *A. calcoaceticus*- *baumannii* complex, which consists of four similar bacterial species: *A. pitti*, *A. baumannii*, *A. calcoaceticus*, and *A. nosocomialis*.41 Other species in this complex have been found to have virulence-enhancing prophages,11,12 but *A. pittii* has yet to be extensively analyzed for prophage sequences. Thus, this study probes and analyzes the prophages in *A. pittii*, as well as their effects on the bacteria’s virulence.

*A. baumannii* and *A. nosocomialis*, the other two species in the *A. calcoaceticus*-*baumannii* complex that can cause clinical infection,23 have both been shown to possess virulence-strengthening prophages.11,12,42 As a result, the observation of a similar trend in *A. pittii* suggests a similar mechanism for virulence in the complex.

The *A. pittii* C54 strain contained a plasmid (Genbank accession: NZ\_CP042365.1) that harbored a Burkholderia phage phiE12-2. This phage contained almost a fifth of all virulence factors within *A. pittii* prophages, as well as almost all of the antibiotic resistance genes present with high-confidence within prophages. Given that *A. pittii* has been shown to harbor R (resistance) plasmids,43,44 it is possible that genetic recombination and rearrangement within the C54 plasmid led to insertion of a larger amount of bacterial virulence genes into the Burkholderia phage phiE12-2 compared to other prophages in the study. If this is the case, then the C54 plasmid should be studied as a potentially novel R plasmid.

We identified a general effect of prophages in bacterial genomes that could also be linked to their virulence genes. Genomic rearrangement of prophage sequences was observed in proximity to the virulence factor zinc binding alcohol dehydrogenase. While the influence of the alcohol dehydrogenase virulence factors on these genomic changes is unclear, the presence of such changes itself could provide evidence for the role of prophages in mediating inversions and other rearrangements in bacterial genomes.45 The increased genetic diversity could aid bacteria in adapting to stressful environments during infection.

Two prophages were identified in the study as being both prevalent within *A. pittii* and containing a large quantity of virulence factors: Acinetobacter phages YMC11/11/R3177 (Genbank accession NC\_041866.1) and YMC/09/02/B1251\_ABA\_BP (Genbank accession NC\_019541.1). While the former has not been extensively researched, studies on the latter have demonstrated that YMC/09/02/B1251\_ABA\_BP is a ubiquitous and mobile prophage shared among many *A. baumannii* strains.46 The similarity between the *A.pitti*and *A.baumannii* prophages is shown in Supplementary Figure 2. As a result, these two prophages could be major mediators of virulence gene transfer within and between *Acinetobacter* species, and it is necessary that they be studied further.

This study provides conclusive evidence for the existence of virulent prophages within *A. pittii*. However, it was limited by a few factors. The sample of 25 *A. pittii* strains used in the study was relatively small, as only these strains were available at the time of the study. The small sample size could also have affected our search for the most prophage-dense regions in *A. pittii* chromosomes. Given *A. pittii*’s increasing clinical relevance, it is expected that more strains will be catalogued in the future. This will allow subsequent *A. pittii* analysis to be more comprehensive and revealing. Additionally, this study focused on a bioinformatics approach to analyze bacterial genomes and the evidence provided is not supported by experimental data. Finally, our study was stringent in applying high cutoff standards: we analyzed only intact prophages and virulence genes which were present with an E-value of less than 1 × 10-20 when compared to VFDB. This allowed a high-degree confidence in our results.

In general, our study revealed the existence of numerous prophages within *A. pittii*. Furthermore, we catalogued these sequences’ effects on bacterial virulence, antibiotic resistance, and genome structure. Our results further understanding of this nosocomial pathogen, its pathogenicity mechanisms, and its bacterial relatives.

# Conclusion

*Acinetobacter pittii*, a nosocomial pathogen, contains prophages that could impact its virulence, antibiotic resistance, and genomic rearrangements. The prophage sequences contain many virulence factors and antibiotic resistance genes, some of which—such as beta lactamases—mirror existing resistance phenotypes in *A. pittii*. This provides evidence for prophages serving as current and future influences on the bacteria’s pathogenicity mechanisms. Moreover, the results demonstrate that *A. pittii* and other members of the *Acinetobacter calcoaceticus*-*baumannii* complex exhibit similar patterns with regard to spatial distribution of prophages, prophage phylogeny, and prevalence of common prophages (i.e. YMC/09/02/B1251\_ABA\_BP). Further analysis of common virulence and prophage trends in *A. pittii* and other members of the complex is warranted. Such research will help illuminate the extent to which prophages have and continue to influence the pathogenic phenotypes of bacteria, which could have clinical ramifications.

# Tables

**Table 1.** Intact prophage distribution across *A. pittii* strains

|  |  |
| --- | --- |
| *A. pittii* strain | Prophage |
| Name | Genetic element type | GenBank accession | Name | Frequency in strain | GenBank accession |
| ST220 | Chromosome | NZ\_CP029610.1 | Mannheimia phage vB\_MhM\_3927AP2 | 1 | NC\_028766.1 |
|  | Acinetobacter phage YMC11/11/R3177 | 1 | NC\_041866.1 |
|  | Acinetobacter phage vB\_AbaS\_TRS1 | 1 | NC\_031098.1 |
|  | Pseudomonas virus phiCTX | 1 | NC\_003278.1 |
| XJ88 | Chromosome | NZ\_CP018909.1 | Pseudomonas virus phiCTX | 1 | NC\_003278.1 |
| WP2-W18-ESBL-11 | Chromosome | NZ\_AP021936.1 | Acinetobacter phage YMC/09/02/B1251 | 1 | NC\_019541.1 |
|  | Pseudomonas virus phiCTX | 1 | NC\_003278.1 |
| HUMV-6483  | Chromosome | NZ\_CP021428.1 | Acinetobacter phage YMC/09/02/B1251 | 2 | NC\_019541.1 |
|  | Mannheimia phage vB\_MhM\_3927AP2 | 2 | NC\_028766.1 |
| WCHAP005046 | Chromosome | NZ\_CP028574.2 | Acinetobacter phage vB\_AbaS\_TRS1 | 2 | NC\_031098.1 |
| WCHAP005069 | Chromosome | NZ\_CP026089.2 | Salmonella phage SEN34 | 1 | NC\_028699.1 |
|  | Acinetobacter phage YMC11/11/R3177 | 1 | NC\_041866.1 |
|  | Burkholderia cenocepacia phage BcepMu | 1 | NC\_005882.1 |
|  | Acinetobacter phage YMC/09/02/B1251 | 1 | NC\_019541.1 |
| YMC2010/8/T346 | Chromosome | NZ\_CP017938.1 | Pseudomonas virus phiCTX | 1 | NC\_003278.1 |
| NQ-003 | Chromosome | NZ\_CP035109.1 | Burkholderia cenocepacia phage BcepMu | 1 | NC\_005882.1 |
|  | Mannheimia phage vB\_MhM\_3927AP2 | 1 | NC\_028766.1 |
| WCHAP100004 | Chromosome | NZ\_CP027250.2 | Mannheimia phage vB\_MhM\_3927AP2 | 1 | NC\_028766.1 |
|  | Pseudomonas virus phiCTX | 1 | NC\_003278.1 |
| AP43  | Chromosome | NZ\_CP043052.1 | Burkholderia cenocepacia phage BcepMu | 1 | NC\_005882.1 |
| IEC338SC  | Chromosome | NZ\_CP015145.1 | Salmonella phage SEN34 | 1 | NC\_028699.1 |
| C54  | Chromosome | NZ\_CP042364.1 | Acinetobacter phage YMC11/11/R3177 | 1 | NC\_041866.1 |
|  | Mannheimia phage vB\_MhM\_3927AP2 | 1 | NC\_028766.1 |
| C54  | Plasmid | NZ\_CP042365.1 | Burkholderia phage phiE12-2 | 1 | NC\_009236.1 |
| AB17H194  | Chromosome | NZ\_CP040911.1 | Acinetobacter phage YMC/09/02/B1251 | 1 | NC\_019541.1 |
|  | Pseudomonas phage Dobby | 1 | NC\_048109.1 |
| AB17H194  | Plasmid | NZ\_CP040912.1 | Enterobacteria phage BP-4795 | 1 | NC\_004813.1 |
| WCHAP100020  | Chromosome | NZ\_CP027254.3 | Acinetobacter phage YMC11/11/R3177 | 1 | NC\_041866.1 |
| 2010C01-170  | Chromosome | CP029489.1 | Acinetobacter phage YMC/09/02/B1251 | 1 | NC\_019541.1 |
| 2014S07-126  | Chromosome | CP033530.1 | Salmonella phage SEN34 | 1 | NC\_028699.1 |
| 2014N21-145 | Chromosome | CP033568.1 | Acinetobacter phage YMC11/11/R3177 | 1 | NC\_041866.1 |

**Table 2.** Virulence factor distribution in 34 *A. pittii* prophages, as classified by the Virulence Factor DataBase (VFDB). Identified virulence factors were all part of VFDB.

|  |  |  |
| --- | --- | --- |
| *A. pittii* strain | Prophage | Virulence Factor |
| Name (genetic element type) | Name (frequency in strain) | Name | Occurrence (out of 34 prophages) |
| ST220 (chromosome) | Acinetobacter phage YMC11/11/R3177 | (*boaB*) hemagluttinin motif-containing protein [BoaB] | 1 |
| (*csrA*) global regulator CsrA [Carbon storage regulator A] | 2 |
| Acinetobacter phage vB\_AbaS\_TRS1 | (*ptxR*) transcriptional regulator PtxR [pyoverdine] | 2 |
| (*kdtB*) lipopolysaccharide core biosynthesis protein [LPS] | 1 |
| (*KPN\_02501*) acyltransferase [Capsule] | 1 |
| (*ABZJ\_00085*) IS4 family transposase ORF 1 [Capsule] | 2 |
| (*ABZJ\_00086*) IS4 family transposase ORF 2 [Capsule] | 1 |
| WP2-W18-ESBL-11 (chromosome) | Acinetobacter phage YMC/09/02/B1251 | (*lptA*) hypothetical protein [Phosphoethanolamine modification] | 1 |
| HUMV-6483 (chromosome) | Acinetobacter phage YMC/09/02/B1251 (2) | (*csrA*) global regulator CsrA [Carbon storage regulator A] | 2 |
| (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | 4 |
| (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | 4 |
| (*adhD*) zinc binding alcohol dehydrogenase [MymA operon] | 3 |
| (*MG\_301*) glyceraldehyde-3-phosphate dehydrogenase [GAPDH] | 2 |
| (*vfr*) cAMP-regulatory protein [type IV pili] | 2 |
| Mannheimia phage vB\_MhM\_3927AP2 (2) | (*oppF*) oligopeptide ABC transporter, permease component [Capsule] | 1 |
| WCHAP005046 (chromosome) | Acinetobacter phage vB\_AbaS\_TRS1 (2) | (*ddrA*) drrA [PDIM (phthiocerol dimycocerosate) and PGL (phenolic glycolipid) biosynthesis and transport] | 1 |
| (*CBU\_1566*) Coxiella Dot/Icm type IVB secretion system translocated effector [T4SS effectors] | 1 |
| (*fbpC*) iron(III) ABC transporter ATP-binding protein [ABC transporter] | 1 |
| WCHAP005069 (chromosome) | Salmonella phage SEN34 | hypothetical protein [Biofilm-associated protein] | 1 |
| (*htpB*) molecular chaperone GroEL [Hsp60] | 2 |
| Acinetobacter phage YMC11/11/R3177 | (*trpD*) anthranilate phosphoribosyltransferase [Tryptophan synthesis] | 3 |
| C54 (chromosome) | Acinetobacter phage YMC11/11/R3177 | (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | 4 |
| (*adhD*) zinc binding alcohol dehydrogenase [MymA operon] | 3 |
| (*MG\_301*) glyceraldehyde-3-phosphate dehydrogenase [GAPDH] | 2 |
| (*vfr*) cAMP-regulatory protein [type IV pili] | 2 |
| Mannheimia phage vB\_MhM\_3927AP2 | (*ptxR*) transcriptional regulator PtxR [pyoverdine] | 2 |
| C54 (plasmid) | Burkholderia phage phiE12-2 | (*mll6359*) transposase [T3SS] | 1 |
| (*ABZJ\_00085*) IS4 family transposase ORF 1 [Capsule] | 2 |
| (*mll6359*) transposase [T3SS] | 1 |
| (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | 4 |
| (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | 4 |
| (*mll6359*) transposase [T3SS] | 1 |
| (*mll6359*) transposase [T3SS] | 1 |
| (*mll6359*) transposase [T3SS] | 1 |
| (*cap5H*) capsular polysaccharide biosynthesis protein Cap5H [Capsule] | 1 |
| AB17H194 (chromosome) | Acinetobacter phage YMC/09/02/B1251 | (*trpD*) anthranilate phosphoribosyltransferase [Tryptophan synthesis] | 3 |
| AB17H194 (plasmid) | Enterobacteria phage BP-4795 | (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | 4 |
| (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | 4 |
| (*B565\_1123*) CobQ/CobB/MinD/ParA family protein [Polar flagella] | 1 |
| WCHAP100020 (chromosome) | Acinetobacter phage YMC11/11/R3177 | (*kpsF*) arabinose-5-phosphate isomerase [Capsule biosynthesis and transport] | 1 |
| 2010C01-170 (chromosome) | Acinetobacter phage YMC/09/02/B1251 | (*trpD*) anthranilate phosphoribosyltransferase [Tryptophan synthesis] | 3 |
| 2014S07-126 (chromosome) | Salmonella phage SEN34 | (*htpB*) molecular chaperone GroEL [Hsp60] | 2 |
| (*htpB*) Hsp60, 60K heat shock protein HtpB [Hsp60] | 1 |
| (*A225\_1326*) RND efflux system [AcrAB] | 1 |
| (*Kvar\_3938*) hydrophobe/amphiphile efflux-1 (HAE1) family transporter [AcrAB] | 1 |
| (*ETAE\_0884*) putative transglycosylase signal peptide protein [T3SS] | 1 |
| 2014N21-145 (chromosome) | Acinetobacter phage YMC11/11/R3177 | (*adhD*) zinc binding alcohol dehydrogenase [MymA operon] | 3 |

**Table 3.** Presence of antibiotic resistance genes within *A. pittii* prophages, as classified by the Comprehensive Antibiotic Resistance Database.

|  |  |  |  |
| --- | --- | --- | --- |
| *A. pittii* strain | Prophage |  | Resistance Gene |
| Name (genetic element type) | Name (frequency in strain) | Name | Function | Accession (ARO term) | Confidence |
| ST220 (chromosome) | Mannheimia phage vB\_MhM\_3927AP2 | chloramphenicol phosphotransferase | Antibiotic inactivation | cmlv | Loose |
|  | Acinetobacter phage YMC11/11/R3177 | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | rsmA | Loose |
|  | Acinetobacter phage vB\_AbaS\_TRS1 | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | adeL | Loose |
| WP2-W18-ESBL-11 (chromosome) | Acinetobacter phage YMC/09/02/B1251 | pmr phosphoethanolamine transferase | Antibiotic target regulation | eptA | Loose |
| HUMV-6483 (chromosome) | Acinetobacter phage YMC/09/02/B1251 (2) | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | rsmA | Loose |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | CRP | Loose |
|  | Mannheimia phage vB\_MhM\_3927AP2 (2) | ATP-binding cassette (ABC) antibiotic efflux pump | Efflux pump | RanA | Loose |
|  |  | chloramphenicol phosphotransferase | Antibiotic inactivation | cmlv | Loose |
| WCHAP005046 (chromosome) | Acinetobacter phage vB\_AbaS\_TRS1 (2) | major facilitator superfamily (MFS) antibiotic efflux pump | Efflux pump | tetA(58) | Loose |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | adeL | Loose |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | adeL | Loose |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | MexL | Loose |
|  |  | ATP-binding cassette (ABC) antibiotic efflux pump | Efflux pump | macB | Loose |
| WCHAP005069 (chromosome) | Salmonella phage SEN34 | SPG beta-lactamase | Antibiotic inactivation | SPG-1 | Loose |
|  | Burkholderia cenocepacia phage BcepMu | chloramphenicol phosphotransferase | Antibiotic inactivation | cmlv | Loose |
|  |  | NmcA beta-lactamase | Antibiotic inactivation | NmcR | Loose |
| NQ-003 (chromosome) | Burkholderia cenocepacia phage BcepMu | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | adeL | Loose |
| Mannheimia phage vB\_MhM\_3927AP2 | chloramphenicol phosphotransferase | Antibiotic inactivation | cmlv | Loose |
| AP43 (chromosome) | Burkholderia cenocepacia phage BcepMu | chloramphenicol phosphotransferase | Antibiotic inactivation | cmlv | Loose |
|  |  | NmcA beta-lactamase | Antibiotic inactivation | NmcR | Loose |
| C54 (chromosome) | Acinetobacter phage YMC11/11/R3177 | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | CRP | Loose |
| Mannheimia phage vB\_MhM\_3927AP2 | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | adeL | Loose |
| C54 (plasmid) | Burkholderia phage phiE12-2 | sulfonamide resistant sul | Antibiotic target regulation | sul2 | Perfect |
|  |  | ABC-F ATP-binding cassette ribosomal protection protein | Antibiotic target regulation | msrE | Perfect |
|  |  | macrolide phosphotransferase (MPH) | Antibiotic inactivation | mphE | Perfect |
|  |  | sulfonamide resistant sul | Antibiotic target regulation | sul1 | Perfect |
|  |  | AAC(6') | Antibiotic inactivation | AAC(6')-Ib4 | Perfect |
|  |  | IMP beta-lactamase | Antibiotic inactivation | IMP-26 | Perfect |
|  |  | major facilitator superfamily (MFS) antibiotic efflux pump | Efflux pump | floR | Strict |
|  |  | chloramphenicol acetyltransferase (CAT) | Antibiotic inactivation | catB3 | Strict |
|  |  | trimethoprim resistant dihydrofolate reductase dfr | Antibiotic target regulation | dfrA19 | Strict |
| AB17H194 (plasmid) | Enterobacteria phage BP-4795 | tetracycline inactivation enzyme | Antibiotic inactivation | Tet(X5) | Perfect |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | golS | Loose |
| 2014S07-126 (chromosome) | Salmonella phage SEN34 | subclass B3 LRA beta-lactamase | Antibiotic inactivation | LRA-2 | Loose |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | acrB | Loose |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | AcrF | Loose |
|  |  | resistance-nodulation-cell division (RND) antibiotic efflux pump | Efflux pump | MexB | Loose |

**Table 4.** Virulence factor distribution by function based on literature search.

|  |  |
| --- | --- |
| Functional category | Virulence factors in category |
| Category name | Fraction of total *A. pittii* virulence factors | Name | Function |
| Cellular metabolism and biosynthesis | 25.5% | (*trpD*) anthranilate phosphoribosyltransferase [Tryptophan synthesis] | Tryptophan synthesis; metabolism (Uniprot, 2021b) |
| (*kdtB*) lipopolysaccharide core biosynthesis protein [LPS] | Adenylyltransferase pantetheine function; coenzyme A biosynthesis (Geerlof *et al.*, 1999) |
| (*csrA*) global regulator CsrA [Carbon storage regulator A] | Metabolism; response to stress (Gangaiah *et al.*, 2013) |
| (*htpB*) molecular chaperone GroEL [Hsp60] | Protein refolding; prevention of protein misfolding (Uniprot, 2021a) |
| (*KPN\_02501*) acyltransferase [Capsule] | Acyltransferase function; fatty acid and lipid catabolism (Röttig & Steinbüchel, 2013) |
| (*MG\_301*) glyceraldehyde-3-phosphate dehydrogenase [GAPDH] | Glycolysis (Uniprot, 2020b) |
| Toxins, effector proteins, and associated transcriptional regulators | 21.3% | (*CBU\_1566*) Coxiella Dot/Icm type IVB secretion system translocated effector [T4SS effectors] | Secreted effector proteins (Weber *et al.*, 2013) |
|  |  | (*ipaH2.5*) invasion plasmid antigen, fragment [Mxi-Spa TTSS effectors controlled by MxiE] | Secreted effector proteins (Uniprot, 2020d) |
|  |  | (*ptxR*) transcriptional regulator PtxR [pyoverdine] | Transcriptional activator of exotoxin genes (Uniprot, 2020c) |
| Transposases | 17.0% | (*mll6359*) transposase [T3SS] | Transposase |
|  |  | (*ABZJ\_00085*) IS4 family transposase ORF 1 [Capsule] | Transposase |
|  |  | (*ABZJ\_00086*) IS4 family transposase ORF 2 [Capsule] | Transposase |
| Cell wall/capsule metabolism and maintenance | 12.8% | (*kpsF*) arabinose-5-phosphate isomerase [Capsule biosynthesis and transport] | Synthesis of bacterial capsules (Uniprot, 2020a) |
|  |  | (*ETAE\_0884*) putative transglycosylase signal peptide protein [T3SS] | Cell wall metabolism and maintenance (Scheurwater *et al.*, 2008) |
|  |  | (*cap5H*) capsular polysaccharide biosynthesis protein Cap5H [Capsule] | Synthesis of bacterial capsules |
|  |  | (*adhD*) zinc binding alcohol dehydrogenase [MymA operon] | Regulation of bacterial cell wall composition (Uniprot, 2021c) |
| Transport | 10.6% | (*fbpC*) iron(III) ABC transporter ATP-binding protein [ABC transporter] | Iron acquisition and transport |
| (*oppF*) oligopeptide ABC transporter, permease component [Capsule] | Protein transport (Uniprot, 2021d) |
| (*A225\_1326*) RND efflux system [AcrAB] | RND efflux system; toxin and antibiotic removal (Anes *et al.*, 2015) |
| (*Kvar\_3938*) hydrophobe/amphiphile efflux-1 (HAE1) family transporter [AcrAB] | Efflux transport |
| (*ddrA*) drrA [PDIM (phthiocerol dimycocerosate) and PGL (phenolic glycolipid) biosynthesis and transport] | Virulence factor transport and synthesis |
| Bacterial motility and adherence | 8.5% | hypothetical protein [Biofilm-associated protein] | Biofilm maintenance |
| (*vfr*) cAMP-regulatory protein [type IV pili] | Twitching motility (Beatson *et al.*, 2002) |
| (*boaB*) hemagluttinin motif-containing protein [BoaB] | Bacterial adhesion (Balder *et al.*, 2010) |
| Enzymes conferring antibiotic resistance | 2.1% | (*lptA*) hypothetical protein [Phosphoethanolamine modification] | Polymyxin resistance (Wanty *et al.*, 2013) |
| Cell division | 2.1% | (*B565\_1123*) CobQ/CobB/MinD/ParA family protein [Polar flagella] | Plasmid partitioning during cell division (Uniprot, 2021e) |

# Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# Author Contributions

R.Z. conceived of the project idea, drafted the methodology, and performed the data analysis. V.M. and R.Z. worked together to interpret the analysis results, after which R.Z. wrote the manuscript. V.M. provided general structural guidance during the manuscript-writing process. V.M. also supervised the project during its entire duration.

# Non-standard Abbreviations

PHASTER: PHAge Search Tool - Enhanced Release

MEGA: Molecular Evolutionary Genetics Analysis

VFDB: Virulence Factor DataBase

CARD: Comprehensive Antibiotic Resistance Database

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# Figures



**Figure 1. Spatial distribution of prophages in *A. pittii* genomes.** A density plot was generated in R for all intact prophages within *A. pittii* chromosomes (n=32). The x-axis indicated positions along the *A. pittii* genome in base pairs, while the y-axis indicated the probability of containing prophages per base pair unit. The area under the curve for an x-axis interval thus provided the prophage density for that particular genome region. The resulting plot showed increased prophage density at two peaks along the bacterial chromosome: 1.4 megabase pairs (Mbp) and 3.2 Mbp. The red line denotes the density in terms of prophage start locations, while the green line denotes the density in terms of prophage end locations. Both show similar peaks.



**Figure 2. Synteny map comparing conserved alcohol dehydrogenase within *A. pittii* prophages.** A MAUVE synteny map was generated in order to analyze the zinc binding alcohol dehydrogenase genes (*adhD*) present in *A. pittii* prophages. The map compares the three prophages that possess *adhD* by looking at the complete nucleotide sequence of each prophage. Each colored block represents a segment of genomic material conserved across sequences, and the height of the colored similarity profile within each block correlates with the degree of conservation between sequences.18 The virulence factor—zinc binding alcohol dehydrogenase—is present in an area of high similarity profile height (boxed in black), which suggests that the virulence factor is conserved. Genomic rearrangement of the prophage DNA occurred in the vicinity of the virulence factor, with the aquamarine block undergoing significant deletion and inversion relative to the light green block in some sequences.

***Supplementary Material***

# Supplementary Figures and Tables

## Supplementary Figures



**Supplementary Figure 1. Synteny map comparing conserved anthranilate phosphoribosyltransferase within *A. pittii* prophages.**A MAUVE synteny map was generated in order to analyze the anthranilate phosphoribosyltransferases (trpD) present in *A. pittii*prophages. The map compares the three prophages that possess trpD by looking at the complete nucleotide sequence of each prophage. The virulence factor—anthranilate phosphoribosyltransferase—is present at the leftmost edge of each red block (boxed in block), where the similarity profile is high but not at its maximum. While this suggests that the virulence factor is mostly conserved, little genomic rearrangement is observed in the vicinity of the virulence factor.



**Supplementary Figure 2. Phylogenetic comparison of Acinetobacter phages in *A. pittii* and *A. baumannii* strains.** The 20 intact prophages retrieved from GenBank were aligned using MAFFT version 7 and default parameters. The phylogenetic tree was then generated using MEGA, with bootstrap replications set to 100. *A. pittii* prophages are shaded in green and *A. baumannii* prophages are shaded in purple.

 

**Supplementary Figure 3. The prevalence of the 34 intact prophages in the different *A.pitti* strains**



**Supplementary Figure 4. The classification of Virulence factors based on their function from a literature review**

## Supplementary Tables

**Supplementary Table 1.***A. pittii*strains in GenBank and associated chromosomes or plasmids.

|  |
| --- |
| *A. pittii* strain  |
| Name | Genetic element type | GenBank accession |
| ST220 | Chromosome | NZ\_CP029610.1 |
|   | Plasmid | NZ\_CP029611.1 |
| XJ88 | Chromosome | NZ\_CP018909.1 |
|   | Plasmid | NZ\_CP018910.1 |
| WP2-W18-ESBL-11 | Chromosome | NZ\_AP021936.1 |
|   | Plasmid | NZ\_AP021937.1 |
|   | Plasmid | NZ\_AP021938.1 |
|   | Plasmid | NZ\_AP021939.1 |
| HUMV-6483 | Chromosome | NZ\_CP021428.1 |
| Plasmid | NZ\_CP021429.1 |
| A1254 | Chromosome | NZ\_CP049806.1 |
|   | Plasmid | NZ\_CP049807.1 |
|   | Plasmid | NZ\_CP049808.1 |
|   | Plasmid | NZ\_CP049809.1 |
|   | Plasmid | NZ\_CP0498010.1 |
| WCHAP005046 | Chromosome | NZ\_CP028574.2 |
|   | Plasmid | NZ\_CP028569.1 |
|   | Plasmid | CP028570.1 |
|   | Plasmid | CP028571.1 |
|   | Plasmid | CP028572.2 |
|   | Plasmid | NZ\_CP028573.2 |
| WCHAP005069 | Chromosome | NZ\_CP026089.2 |
| Plasmid | NZ\_CP026087.2 |
| Plasmid | NZ\_CP026088.1 |
| Plasmid | NZ\_CP026086.2 |
| AP\_882 | Chromosome | NZ\_CP014477.1 |
|   | Plasmid | NZ\_CP014478.1 |
|   | Plasmid | NZ\_CP014479.1 |
| YMC2010/8/T346 | Chromosome | NZ\_CP017938.1 |
|   | Plasmid | NZ\_CP017939.1 |
| NQ-003 | Chromosome | NZ\_CP035109.1 |
| WCHAP100004 | Chromosome | NZ\_CP027250.2 |
| Plasmid | NZ\_CP027247.2 |
|   | Plasmid | NZ\_CP027248.1 |
|   | Plasmid | NZ\_CP027249.2 |
| AP43 | Chromosome | NZ\_CP043052.1 |
|   | Plasmid | NZ\_CP043054.1 |
|   | Plasmid | NZ\_CP043055.1 |
|   | Plasmid | NZ\_CP043053.1 |
| IEC338SC | Chromosome | NZ\_CP015145.1 |
|   | Plasmid | NZ\_CP015147.1 |
|   | Plasmid | NZ\_CP015146.1 |
|   | Plasmid | NZ\_CP015148.1 |
| C54 | Chromosome | NZ\_CP042364.1 |
| Plasmid | NZ\_CP042365.1 |
|   | Plasmid | NZ\_CP042366.1 |
|   | Plasmid | NZ\_CP042367.1 |
|   | Plasmid | NZ\_CP042368.1 |
|   | Plasmid | NZ\_CP042369.1 |
|   | Plasmid | NZ\_CP042370.1 |
| AB17H194 | Chromosome | NZ\_CP040911.1 |
| Plasmid | NZ\_CP040912.1 |
|   | Plasmid | NZ\_CP040913.1 |
| WCHAP100020 | Chromosome | NZ\_CP027254.3 |
|   | Plasmid | NZ\_CP027251.3 |
|   | Plasmid | NZ\_CP027252.3 |
|   | Plasmid | NZ\_CP027253.1 |
| AP007 | Chromosome | CP040903.1 |
| 2010C01-170 | Chromosome | CP029489.1 |
| 2014S06-099 | Chromosome | CP033540.1 |
|   | Plasmid | CP033541.1 |
|   | Plasmid | CP033542.1 |
|   | Plasmid | CP033543.1 |
|   | Plasmid | CP033544.1 |
| 2012N21-164 | Chromosome | CP033535.1 |
|   | Plasmid | CP033536.1 |
|   | Plasmid | CP033537.1 |
|   | Plasmid | CP033538.1 |
|   | Plasmid | CP033539.1 |
| 2014S07-126 | Chromosome | CP033530.1 |
|   | Plasmid | CP033531.1 |
|   | Plasmid | CP033532.1 |
|   | Plasmid | CP033533.1 |
|   | Plasmid | CP033534.1 |
| 2012N08-034 | Chromosome | CP033520.1 |
|   | Plasmid | CP033521.1 |
|   | Plasmid | CP033522.1 |
|   | Plasmid | CP033523.1 |
|   | Plasmid | CP033524.1 |
| 2014N21-145 | Chromosome | CP033568.1 |
|   | Plasmid | CP033569.1 |
|   | Plasmid | CP033570.1 |
|   | Plasmid | CP033571.1 |
| 2014N05-125 | Chromosome | CP033525.1 |
|   | Plasmid | CP033526.1 |
|   | Plasmid | CP033527.1 |
|   | Plasmid | CP033528.1 |
|   | Plasmid | CP0335259.1 |
| PHEA-2 | Chromosome | NC\_016603.1 |

**Supplementary Table 2.**The prevalence of Actinobacter prophages in the different *A.pitti* strains

|  |  |  |
| --- | --- | --- |
| Prophage Name | *A. pittii* Strains | Total Strains with Prophage |
| Mannheimia phage vB\_MhM\_3927AP2 | ST220HUMV-6483NQ-003WCHAP100004C54 | 5 |
| Acinetobacter phage YMC11/11/R3177 | ST220WCHAP005069C54WCHAP1000202014N21-145 | 5 |
| Acinetobacter phage vB\_AbaS\_TRS1 | ST220WCHAP005046 | 2  |
| Pseudomonas virus phiCTX | ST220XJ88WP2-W18-ESBL-11YMC2010/8/T346WCHAP100004 | 5 |
| Acinetobacter phage YMC/09/02/B1251 | WP2-W18-ESBL-11HUMV-6483WCHAP005069AB17H1942010C01-170 | 5 |
| Salmonella phage SEN34 | WCHAP005069IEC338SC2014S07-126 | 3 |
| Burkholderia cenocepacia phage BcepMu | WCHAP005069NQ-003AP43 | 3 |
| Burkholderia phage phiE12-2 | C54 | 1 |
| Pseudomonas phage Dobby | AB17H194 | 1 |
| Enterobacteria phage BP-4795 | AB17H194 | 1 |