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Developing Bacteriophage and the Predatory Bacterium *Bdellovibrio bacteriovorus* as an Alternative Therapy for Canine Otitis Externa

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Abstract

Canine otitis externa (OE) is a complex multifactorial disease and is one of the most common diagnoses for dogs seen in veterinary practices worldwide. *Pseudomonas aeruginosa* is not a typical constituent of the canine ear microbiota but when present in the canine ear, commonly results in severe chronic infections. Antimicrobial resistance and biofilm formation are common in chronic disease and highlights the need for alternative treatments. In the present study the antimicrobial susceptibility and biofilm forming ability of 253 isolates of *P. aeruginosa* from canine OE was assessed. This identified that resistance to fluroquinolones was high: namely enrofloxacin (25%), levofloxacin (15%) and ciprofloxacin (13%). Further analysis using genome sequencing implicated mutations in DNA gyrase and topoisomerase genes as the cause of this resistance. Similarly, the majority (82%) of canine *P. aeruginosa* clinical isolates were found to produce strong levels of biofilm. The use of bacteriophage and the predatory bacterium *Bdellovibrio bacteriovorus* have both been suggested for their use as novel therapies against a variety of pathogens. Subsequently, bacteriophage were isolated and characterised using the clinical *P. aeruginosa* isolates. Two lytic bacteriophage, with a wide host range (40%, 34%) in regard to the clinical *P. aeruginosa* isolates, *Pseudomonas* phage K9-6 and K9-7, were isolated from wastewater samples. Genotypic analysis suggested these bacteriophage belonged to the genus *Pbunavirus*. Finally, *B. bacteriovorus* was shown to prey upon a number of (16%) clinical isolates of *P. aeruginosa*. These results highlight the potential of bacteriophage and *B. bacteriovorus* to treat *P. aeruginosa* infection in cases of canine otitis externa.

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List of Abbreviations

%	Percentage
° C	Degrees Celsius
µL	Microlitre
µm	Micrometre
AMR	Antimicrobial Resistance
ATCC	American Type Culture Collection
b	Base(s)
B. bacteriovorus	*Bdellovibrio bacteriovorus*
$BaCl_2$	Barium Chloride
bp	Base Pair(s)
$CaCl_2$	Calcium Chloride
cAD	Canine Atopic Dermatitis
CFU	Colony-Forming Unit
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
exoS/ExoS	bi-functional type-III cytotoxin
FIC	Fractional Inhibitory Concentration
g	Gram(s)
g	Gravitational force equivalent
h	hour(s)
H_2O	Water
H_2SO_4	Sulfuric acid
ICTV	International Committee of Taxonomy of Viruses
K. pneumoniae	*Klebsiella pneumoniae*
L	Litre(s)
LB	Lysogeny Broth
LPS	Lipopolysaccharides
LTP	Long tail fibre
M	molar
M. pachydermatis	*Malassezia pachydermatis*
$MgSO_4$	Magnesium Sulfate
MIC	Minimum Inhibitory Concentration
min	Minutes
mL	Millilitre
mol	Mole
NAC	N-acetylcysteine
NaOH	Sodium Hydroxide
nm	Nanometre
OD_x	Optical Density at a given Wavelength (x)
OE	Otitis Externa
OM	Otitis Media
P. aeruginosa	*Pseudomonas aeruginosa*
PFU	Plaque-Forming Unit
plcH/PlcH	Haemolytic phospholipase C
QS	Quorum Sensing
R-M	Restriction-Modification
RO	Reverse Osmosis
rpm	Revolutions per minute

S. pseudintermedius	*Staphylococcus pseudintermedius*
SM buffer	Sodium Magnesium buffer
STP	Short tail fibres
TM	Tympanic Membrane
toxA/ToxA	Exotoxin A
V	Volt(s)
v/v	Volume per Volume Concentration
w/v	Weight per Volume Concentration
YT	Yeast extract tryptone

Chapter 1 Introduction

A portion of this introduction has been previously published (Secker, Shaw & Atterbury, 2023).

1.1 Canine otitis externa

Otitis externa (OE) is defined as inflammation of the external ear canal that may affect the entire length, or any part, of the canal from the tympanic membrane (TM) to the outer meatus and is often associated with concurrent pinnal changes. The disease can be painful and/or pruritic, acute, or chronic, and may affect one or both ears. Chronic OE is defined variably but is usually considered to be disease of over three months duration or which returns within three months (Bajwa, 2019; Nuttall, 2023).

1.1.1 Normal ear structure and function

Epidermal cells of the drum and ear wall are constantly being renewed. Corneocytes, shed from the surface, mix with sebaceous and ceruminous secretions to form cerumen (ear wax) that acts as a vehicle to carry material, including microorganisms, out of the ear. The underlying epidermis moves centrifugally towards the outer meatus to facilitate this (Tabacca et al., 2011).

A higher density of ceruminous glands has been associated with the development of OE in dogs predisposed to otitis (Huang, Little & McNeil, 2009; Stout-Graham et al., 1990). These secretions largely determine the acidity of the canine ear—at around pH 6—providing a buffered environment that regulates pH changes (Grono, 1970b). Acute ear infections can be accompanied by a reduction in the pH of the canal wall, whereas chronic disease may be more associated with an alkaline shift.

Innate and specific immune responses mediated by host defence peptides, such as β-defensin (cBD3)-like and cathelicidin (cCath), and the secretion of IgA, IgM, and (particularly) IgG in cerumen contribute to the maintenance of the normal microbiota (Santoro, 2023; Diamond et al., 2009). Bacterial and epidermal antimicrobial peptides may act to limit bacterial growth and alter the immunosurveillance of commensal organisms (Diamond et al., 2009). The disruption of the microbiota may lead to sustained dysbiosis and subsequent secondary infections. A further understanding of this may assist in the development of more effective treatments for OE.

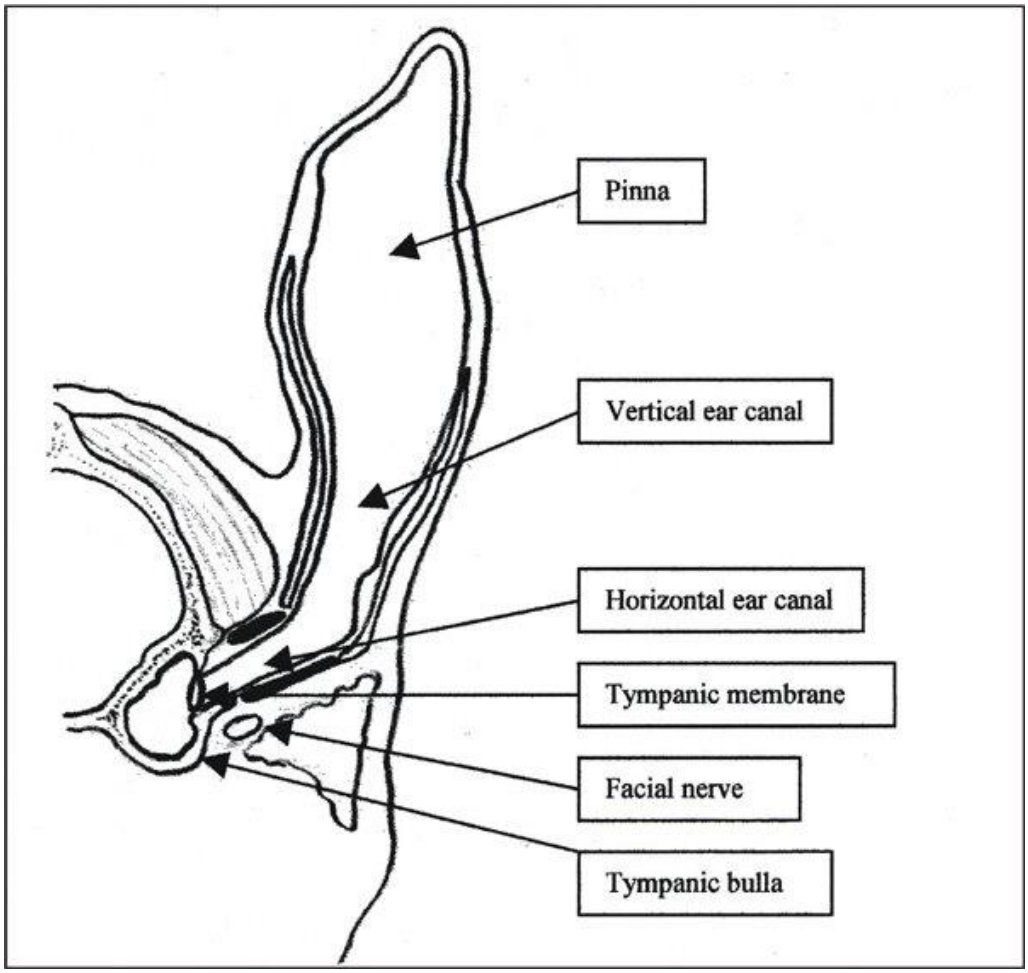

Figure 1.1 **Diagram of the external and middle canine ear** (Doyle, Skelly & Bellenger, 2004).

1.1.2 Normal ear microbiota

Microorganisms in the ear are characterised using three main techniques: cytological examination, bacterial culture, and deep sequencing (Dickson & Love, 1983; Tater et al., 2003; Leonard et al., 2022) (Figure 1.2). The organisms present in the normal canine ear have been investigated in only a few studies (Dickson & Love, 1983; Grono & Frost, 1969; Yamashita et al., 2005; Aoki-Komori et al., 2007). Traditional culturing has revealed the predominance of *Staphylococcus pseudintermedius* (previously termed *S. intermedius*), *Bacillus* spp., coagulase-negative *Staphylococcus* spp., *Micrococcus* spp., as well as *Malassezia* spp., with smaller numbers of *Corynebacterium* spp. and Gram-negative rods (Shaw, 2016; Fraser, 1961; Lyskova, Vydrzalova & Mazurova, 2007).

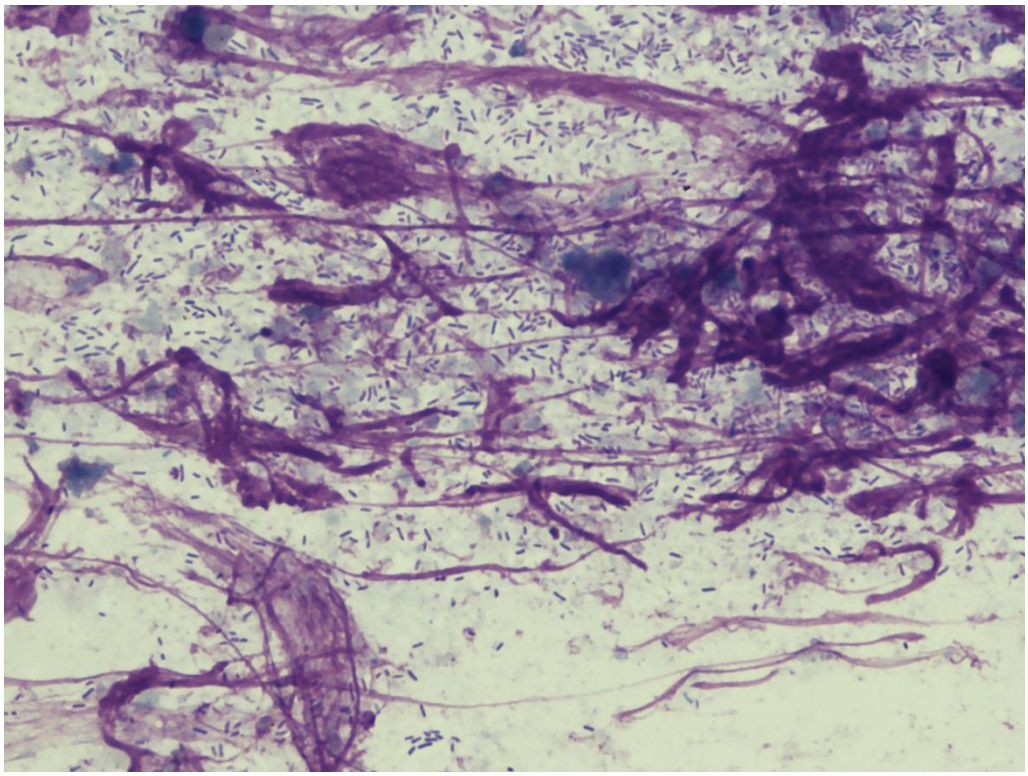

Figure 1.2 **Cytology showing rods and nuclear streaming indicative of an active infection with *Pseudomonas aeruginosa***—modified Romanovsky stain (×1000) (Secker, Shaw & Atterbury, 2023)

In OE, these organisms are over-represented, but the results of culture often reveal less commonly isolated organisms, such as *Staphylococcus schleiferi*, that are also likely part of the normal microbiota (Igimi, Takahashi & Mitsuoka, 1990).

More recently, deep sequencing and metagenomics have revealed a richer population of bacteria and yeasts that characterise the microbiome of normal ears. The normal microbiota of the ear is outside of the scope of this work but has been discussed elsewhere in detail (Tang et al., 2020; Hoffmann et al., 2014).

Dysbiosis of the ear may be characterised by an overgrowth of *Malassezia* spp. or bacterial populations, but there is not a consistent pattern of change from normal to infected ears, and this may also vary according to breed (Mauldin et al., 1997), dysbiosis often makes dogs more vulnerable to ear infections with *Pseudomonas*.

1.1.3 Clinical framework

August (1988) devised a useful clinical framework that separated the factors involved in the development of canine OE into primary disease, predisposing factors, and perpetuating factors. This allowed a structured approach to diagnosis and treatment. More recently, the perpetuating factors have been separated into physical changes and secondary infections resulting in the PSPP (primary, secondary, perpetuating, and predisposing factors) system (Figure 1.3).

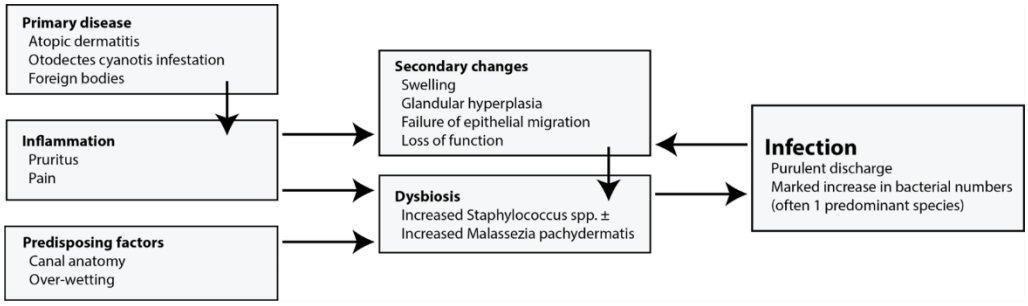

Figure 1.3 **Diagram showing the influences and progress of ear disease from primary disease to infection**. (Secker, Shaw & Atterbury, 2023).

1.1.3.1 Primary Factors

These describe the causes of initial inflammation in the ear. The most common are allergy, presence of foreign bodies, and infestations with *Otodectes cynotis*. Between 70 and 80% of dogs suffering from allergic skin disease (food and environmental allergy) have OE (Paterson & Matyskiewicz, 2018; Rosser, 1993). In particular, OE has been increasingly linked with canine atopic dermatitis (cAD) over the last few decades (Scott, 1981; Willemse, 1986; Saridomichelakis et al., 2007; Favrot et al., 2010). It is important to note that primary factors are not identified in some cases (Saridomichelakis et al., 2007).

The association between atopic dermatitis and OE complicates the PSPP system, as allergy causes inflammation and increases the probability of secondary infection. Studies comparing the microbiota of dogs suffering from atopic dermatitis with control animals identified significantly different numbers of some microbial species in the ears of dogs suffering from cAD compared with unaffected control animals (Ngo et al., 2018; Apostolopoulos et al., 2021).

The type of primary disease may influence the subsequent bacterial and yeast infections. Zur, Lifshitz & Bdolah-Abram (2011) reported *Malassezia* and

bacterial rods more commonly in allergy and endocrine disease, respectively. In contrast, Paterson & Matyskiewicz (2018) reported that allergy was a common cause of *Pseudomonas* infections in dogs (70%), likely a result of the chronic nature of the underlying disease.

1.1.3.2 Secondary Infections

Secondary infection usually causes a marked increase in the clinical signs experienced in canine OE, unfortunately many patients are not presented until this is noted. The normal canine ear is not sterile, and depending on the primary disease, some dysbiosis may be seen in the absence of clinical infection, blurring the line between traditionally binary infected versus non-infected ears. Inflamed ears are often dysbiotic, with an overgrowth of *S. pseudintermedius* and *M. pachydermatis* (Ngo et al., 2018). In the absence of perpetuating factors (below), reducing inflammation may be sufficient to resolve the overgrowth (Bergvall et al., 2017).

Table 1.1 summarises the microbial species found in OE using culture-based and metagenomic methods. *Staphylococcus* spp. (2.97–58.5%) and *P. aeruginosa* (5.83–35.5%) are both frequently isolated using culture, along with the fungus *M. pachydermatis* (8.75–30.9%). Shotgun metagenomics has further identified the obligate anaerobes *Peptostreptococcus canis* and *Porphyromonas cangingivalis* in 5.52% and 4.38% of OE cases respectively (Tang et al., 2020). The anaerobic nature of these organisms means that they have not yet been implicated in cases of OE; however, they have both been identified in the canine mouth (Lawson et al., 2012; Collins et al., 1994).

Genus	Species	Prevalence Using Traditional Culture	Prevalence Using Metagenomics
Gram-positive bacteria			
Staphylococcus	*S. intermedius*, coagulase-positive Staphylococci, *S. pseudintermedius*, *S. schleiferi*, *S. schleiferi* spp. Coagulans, coagulase-negative Staphylococci	58.5% + 6.2% [1] 36.83% + 2.97% [2] 24.30% [3]	22% [4] 11.25% + 8.54% [5]
Streptococcus	*S. canis*, β-haemolytic Streptococci, non-haemolytic Streptococci, *S. halichoeri*, *S. agalactiae*	29.9% + 4.1% [1] 6.2% [3] 2.97% [2]	5.42% + 3.80% + 0.83% [5] 2.2% [4]
Corynebacterium	*C. auriscanis*, *C. freneyi*, *C.* spp.	0.79% [2]	7.08% + 4.38% [5] 5.4% [4]
Finegoldia	*F. magna*		5.83% [5]
Peptostreptococcus	*P. canis*		5.52% [5]
Lactobacillus			5.5% [4]
Enterococcus	*E. faecium*, *E. faecalis*	5.2% [1]	2.30% [4] 1.04% [5]
Arcanobacterium	*A. canis*		4% [5]
Peptoniphilus	*P. harei*		2.29% [5]
Bacillus	*B.* spp.	0.99% [2]	
Gram-negative bacteria			
Pseudomonas	*P. aeruginosa*	35.5% [3] 16.24% [2] 7.2% [1]	18.6% [4] 5.83% [5]
Proteus	*P.* spp., *P. mirabilis*	14.4% [1] 6.8% [3] 3.56% [2]	5.60% [4] 2.29% [5]
Escherichia	*E. coli*	10.30% [1] 4.2% [3] 3.17% [2]	
Enterobacteriaceae (Unknown genus)			4.9% [4]
Porphyrimonas	*P. cangingivalis*		4.5% [4] 4.38% [5]
Fungi			
Malassezia	*M. pachydermatis*	30.9% [1] 30.01% [2]	8.75% [5]
Candida	*C.* spp.	2.38% [2]	
Others			
14 Others			2.08–0.63% [5]

Table 1.1 **Bacteria and fungi commonly isolated from cases of canine otitis externa using both culture-based and metagenomic methods.** (Secker, Shaw & Atterbury, 2023).

1 - Lyskova, Vydrzalova & Mazurova, 2007 2 - Petrov et al., 2019 3 - Bugden, 2013 4 - Borriello et al., 2020 5 - Tang et al., 2020

Corynebacterium spp. was isolated from 0.79% of canine OE patients in Bulgaria (Petrov et al., 2019). However, metagenomic sequencing suggests that this could be an underestimate, as its prevalence could be up to 5.4–7.08% (Tang et al., 2020; Borriello et al., 2020). This is in line with other studies showing the greater sensitivity of 16S rRNA sequencing compared with traditional culture methods (Leonard et al., 2022). However, the results of DNA sequence analysis should be interpreted with caution, as they do not distinguish between viable and dead cells.

Given the microbial diversity of healthy ears and the range of environmental and physical exposures that can initiate infection, it is unsurprising that the relative abundance of pathogens in canine OE also varies considerably between studies. However, despite this diversity, *Pseudomonas* spp. are the most common bacteria in chronic otitis, with up to 35% of cases affected (Figure 1.4) (Nuttall & Cole, 2007).

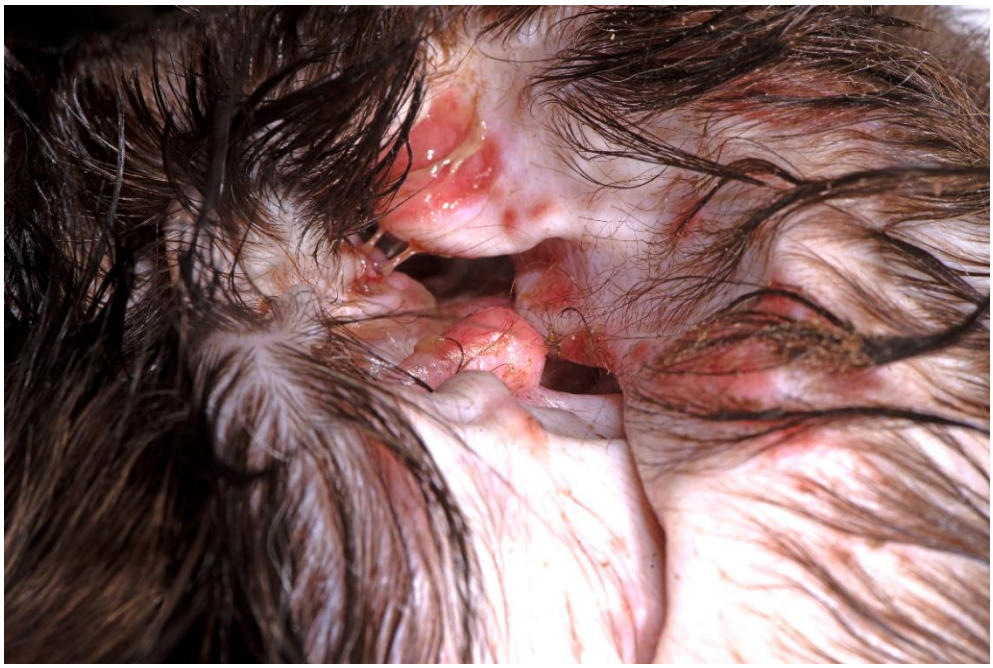

Figure 1.4 **An example of the clinical appearance of *Pseudomonas* otitis in a Cocker spaniel.** (Secker, Shaw & Atterbury, 2023).

1.1.3.3 Perpetuating Factors

Perpetuating factors represent the tissue changes in the ear canal that develop as a result of disease and that prevent the resolution of OE and make repeated infection more likely (August, 1988). Swelling and hyperplasia of the canal wall are followed by the enlargement of the ceruminous glands (modified sweat glands) and, finally, hidradenitis. Once the disease has progressed to this stage, it is often irreversible, and with increased surface area and ineffective physical and mechanical defences, bacteria such as *Pseudomonas* thrive. Bacterial toxins and physical accumulation of discharge can cause the rupture of the tympanic membrane, allowing infection to spread to the middle ear, resulting in otitis media (OM), which can then act as a perpetuating reservoir of infection (Paterson, 2016). Ear canal stenosis (38%) and OM (25%) were identified as the most prevalent perpetuating factors in Greece among 100 dogs of varying breeds (Saridomichelakis et al., 2007).

1.1.3.4 Predisposing Factors

Predisposing factors are not causative of inflammation but increase the probability of developing otitis. Predisposing factors include the physical traits of the dogs such as long, pendulous, hairy, or V-shaped drop pinna, as well as narrow external canals. These traits and external factors, such as swimming may disrupt normal functioning by increasing moisture within the ear canal, disrupting the normal microbiota and predisposing to OE (Saridomichelakis et al., 2007; O'Neill et al., 2021b).

1.1.4 Prevalence of canine otitis externa

Estimates of canine OE prevalence range between 5–20% (August, 1988). In the UK, canine OE was a frequent diagnosis (7.3%) in a random sample of 22,000 dogs attending primary care practices over a one-year period in 2016, which was second only to periodontal disease (12.52%) (O'Neill et al., 2021a). An earlier study in South-Eastern England found that OE was the most prevalent diagnosis for dogs (10.2%), followed by periodontal disease (9.3%) (O′Neill et al., 2014). A similar prevalence has been reported in South Korea (6.3%) (Kim et al., 2018), the US (13%) (Lund et al., 1999), and New Zealand (7.5%) (Baxter & Lawler, 1972).

1.1.5 Treatment

In cases where there are minimal perpetuating changes and mild dysbiosis, removing the primary disease may be sufficient to restore ear health. Steroids are often included in the treatment of canine OE; these aid in the management of inflammation of the ear canal and are administered orally, parenterally, or topically. Steroids do not have a direct effect on the ear microbiota (Léonard et al., 2021) and their use in infections, including *Pseudomonas*, is controversial as they may reduce useful inflammation and immune responses; however, severe swelling is often a marked impediment to successful treatment, and they are widely used. Negative effects are offset as steroids are usually part of combined steroid, antibiotic, antifungal products (cSAA products)—see Table 1.2 below.

Steroid	Antibiotic	Antifungal
Dexamethasone (as acetate) 0.9 mg/mL	Marbofloxacin 3 mg/mL	Clotrimazole 10 mg/mL
Prednisolone 2.5 mg/mL	Diethanolamine fusidate 5.0 mg/mL Framycetin sulphate 5.0 mg/mL	Nystatin 100,000 iu/mL
Hydrocortisone aceponate 1.11 mg/mL	Gentamicin sulfate 1505 iu/mL	Miconazole nitrate 15.1 mg/mL
Betamethasone valerate 0.88 mg/mL	Gentamicin sulfate 2640 iu/mL	Clotrimazole 8.80 mg/mL
Mometasone furoate 0.9 mg/mL	Orbifloxacin 8.5 mg/mL	Posaconazole 0.9 mg/mL
Prednisolone acetate 5 mg/mL	Polymyxin B sulfate 0.5293 mg/mL	Miconazole nitrate 23 mg/mL
Mometasone furoate 2.2 mg/dose	Florfenicol 16.7 mg/dose	Terbinafine 14.9 mg/dose
Betamethasone acetate 1 mg/dose	Florfenicol 10 mg/dose	Terbinifine 10 mg/dose
Note: no products have marketing authorisation for use in the middle ear.		

Table 1.2 **Otic treatments containing antibiotics in the UK**. (Secker, Shaw & Atterbury, 2023).

Topical antimicrobial therapy is generally preferred by clinicians for treating canine OE. This is because otic preparations provide a much higher local drug concentration than systemic treatments (Nuttall, 2016). The concentration used is such that culture and susceptibility data must be considered carefully as the antibiotic is being used at many times the minimum inhibitory concentration (MIC). For this reason, and following cytological examination, antibiotics are often used empirically (Nuttall & Cole, 2007). Bacterial resistance is a common feature of recurrent OE and constitutive resistance is a feature of *Pseudomonas* infections. For cases of *P. aeruginosa* OE, three classes of antibiotics are often used. These are fluoroquinolones, such as marbofloxacin, and aminoglycosides, particularly gentamicin and polymyxin antibiotics, such as polymyxin B. In addition, some clinicians prepare *ad hoc* solutions using injectable solutions of antibiotics when resistance is seen or suspected (Morris, 2004) e.g., ticarcillin–

clavulanic acid (Bateman et al., 2012), amikacin (Klinczar et al., 2022), ceftazidime (Hoff et al., 2021), and enrofloxacin (Metry et al., 2012).

Dogs with canine OE may be prescribed ear cleaners, usually as an adjunctive to antibiotics. This aids in the removal of debris from the ear canal, which is important for the proper function of some antimicrobials, namely aminoglycosides and polymyxin B, which have reduced efficiency in the presence of pus (Nuttall, 2016; Pye, 2018). The specific composition varies between products; however, they generally include cerumenolytics, surfactants, astringents, antimicrobials, and anti-inflammatories (Swinney et al., 2008), as well as containing ingredients that disrupt biofilms (Pye, Singh & Weese, 2014). When products with antimicrobial action are used, this can have a marked effect on the success of therapy. Such disinfectant cleaners can have a faster rate of action than antibiotics, and by providing an alternative mechanism to kill bacteria, make the development of resistance by mutant selection less likely. For fluoroquinolones, the mutant prevention concentration for *Pseudomonas* is many times the minimum inhibitory concentration and, in infections where high numbers of organisms are present, additional products are essential (Blondeau, 2009; Pasquali & Manfreda, 2007).

The efficacy of ear cleaners in inhibiting clinical *P. aeruginosa* from canine OE varies based on the composition. Multiple studies have failed to identify key components or properties of ear cleaners, e.g., pH. However, the combination of Tris-EDTA plus 0.15% chlorhexidine has shown some effect against *P. aeruginosa* (Swinney et al., 2008; Steen & Paterson, 2012), and products containing this formulation have been widely adopted for this reason.

In some cases of chronic OE, the disease may progress to a point where medical treatment will be unsuccessful in controlling infection. In such cases, marked canal hyperplasia, secondary OM, and biofilm production in the presence of a multi-drug-resistant *Pseudomonas* infection are often evident. At this stage, surgery is necessary, including total ear canal ablation with or without bulla osteotomy. This procedure involves the removal of the infected ear canal and bulla contents, resulting in the resolution of disease in most cases (Doyle, Skelly & Bellenger, 2004; Smeak, 2016).

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative bacterium usually found in soil and aqueous environments; it is not only an important opportunistic pathogen in canine otitis, but also in humans. Most infections in humans are seen from those with underlying health conditions, with 28.7% of infections in intensive care units across Europe as a result of *P. aeruginosa* (Vincent et al., 1995). Similarly, in England, 13% of hospital onset bacteraemia was due to *P. aeruginosa* (Sloot et al., 2022). While in the US it is estimated that 7.3% of all nosocomial infections can be attributed to this pathogen (Weiner et al., 2016). Perhaps most important are cystic fibrosis patients, where 70% of adults will suffer from untreatable *P. aeruginosa* infections for their entire life (Moradali, Ghods & Rehm, 2017). This highlights the importance of this pathogen from a one health perspective.

1.2.1 Prevalence

P. aeruginosa is often described as ubiquitous in soil and aqueous environments. However, it is more frequently isolated from soil and water sources linked to human activity, such as those contaminated with oil hydrocarbons or pesticides

(Crone et al., 2020). Urban rivers have been linked to an increased prevalence of *P. aeruginosa* (Crone et al., 2020). In an outbreak of human OE in the Netherlands involving *P. aeruginosa*, 83% of cases were linked to swimming in freshwater lakes, even though the water met quality standards (Asperen et al., 1995).

Contamination of the veterinary clinical environment has been reported in the findings of surveillance studies (Verdial et al., 2021; Stull & Weese, 2015; Fraser & Girling, 2009). In ear disease, the contamination of otic speculums is a particular risk, chiefly in practices with poor speculum availability and high case volumes where disinfection between patients is inadequate (Newton et al., 2006; Kirby et al., 2010), this is also a problem in human ear clinics (Korkmaz, Çetinkol & Korkmaz, 2013).

P. aeruginosa colonisation of environments in a hospital is also an important issue. As discussed above, *P. aeruginosa* nosocomial infections are common and reservoirs of this pathogen have been found in taps and sinks of intensive care units (Blanc et al., 2004; Cholley et al., 2008). Additionally, *P. aeruginosa* has been shown to persist on dry inanimate surfaces for up to 16 months (Kramer, Schwebke & Kampf, 2006).

P. aeruginosa has been isolated from multiple sites in the home, such as surfaces, water supplies, and dishwasher rubber seals. Several studies have identified household drains as important reservoirs of *P. aeruginosa* (Remold et al., 2011; Zupančič et al., 2019; Purdy-Gibson et al., 2015). Clonal isolates to those causing OE in dogs have been found in the oral cavity of affected dogs and other animals in the home, as well as water bowls and taps (Morris et al., 2017),

although the directionality of transmission could not be established. Clonal *P. aeruginosa* isolates have been found in household taps used to fill water bowls (Morris et al., 2017). The same study used statistical modelling to link swimming in pools and visiting dog parks to a 64% higher prevalence of *Pseudomonas* otitis (Morris et al., 2017). This makes sense given the occurrence of *P. aeruginosa* in environmental water samples and even in commercial swimming pools (Rice et al., 2012). *P. aeruginosa* can be isolated from the ears of apparently healthy dogs occasionally, suggesting an unknown history of OE (Park et al., 2020) or contamination from the environment. This could then result in the contamination of the home environment and subsequent reinfection or infection of other animals/humans in the household. Finally, grooming products in the home and professional salons have been found to harbour *P. aeruginosa* (Perry et al., 2022).

1.2.2 Isolates

The population structure of *P. aeruginosa* causing clinical infections in animals and people is largely non-clonal, i.e., the strains involved exhibit a high degree of diversity, with little or no association between groups and diseases (Haenni et al., 2015). Very few studies have examined the genomic profiles of *P. aeruginosa* strains causing canine otitis specifically. Whole-genome MLST has revealed a high diversity of *P. aeruginosa*, with 45 different sequence types (STs) identified from 80 isolates in one study (Hyun, Chung & Hwang, 2018) and 27 STs from 29 isolates in another (Elfadadny et al., 2023). This mirrors studies of *Pseudomonas* from human infections, which also found a largely non-clonal population structure punctuated by highly successful epidemic clones/clonal complexes (Maatallah et al., 2011). These highly successful clones

include STs such as ST235, ST175, ST111 and ST146, which are also frequently associated with extensively drug-resistant infections (Oliver et al., 2015). *P. aeruginosa* clone C is one example of a highly successful clone and is one of the most frequently isolated from chronic and acute human infections (Lee et al., 2020).

1.2.3 Virulence factors involved in canine otitis externa

P. aeruginosa is capable of growing at a wide range of temperatures from 4-42º C however its optimal growth temperature is 37º C, very similar to that of the canine ear (LaBauve & Wargo, 2012; Grono, 1970a). Growth at this temperature results in significant changes in gene expression such that 182 genes are upregulated when grown at 37º C compared to ambient temperatures (22º C); these changes include an upregulation in genes for metabolism, replication and various genes known to encode virulence factors (Barbier et al., 2014).

Few studies have investigated virulence factors specific to the development of canine OE. While it is widely accepted that biofilm formation and antibiotic resistance contribute to *P. aeruginosa* infection in canine OE (Nuttall, 2016), other virulence factors are relatively unexplored. In insect and plant infection models, clinical (hospital) and environmental (soil and water) isolates showed no statistically significant difference in pathogenesis (Vives-Flórez & Garnica, 2006), supporting the notion of infections originating from exposure to environmental reservoirs.

Hattab et al., (2021) investigated the presence of five virulence genes in *P. aeruginosa* isolates from dogs, including from the ear canal. Three genes, *lasB* (elastase A), *aprA* (alkaline protease), and *plcH* (haemolytic phospholipase C),

were present in all the tested isolates, while *exoS* (bi-functional type-III cytotoxin) and *toxA* (Exotoxin A) were present in 87.5% and 91.7%, respectively.

Bacterial proteases are a major contributor to disease. *P. aeruginosa* secretes several proteases, including alkaline protease (AprA), Elastase A and B (LasA and LasB), protease IV (PrpL), small protease (PASP), large exoprotease (LepA), and others (Galdino et al., 2017). Protease activity, measured by the degradation of azocasein, from *P. aeruginosa* infection has been identified in humans with OM (Avidano et al., 1998). The total protease activity of *P. aeruginosa* isolates from canine OE is more variable than that from other animal sources ($p < 0.0001$) but is the same on average ($p = 0.7538$) when assessed by the hide powder azure absorbance assay (Petermann, Doetkott & Rust, 2001). The treatment of a chinchilla model of *P. aeruginosa* OM with bacterial protease inhibitor (GM 6001) resulted in higher survival (66%) compared with the control, gentamicin and gentamicin + GM 6001 groups, although this was not significant ($p = 0.2674$) (Cotter et al., 1996).

P. aeruginosa isolates from chronic cases of canine OE have been found to have a reduced mean elastase activity compared with other animal isolates ($p < 0.0001$); moreover, some strains produce a stable elastase-negative phenotype due to deficiencies in the *rhl* quorum sensing system. Interestingly, the *rhl* phenotype was observed in an isolate with wild-type elastase activity, implicating an unknown constituent of quorum sensing being important in chronic otitis infections (Petermann, Doetkott & Rust, 2001; Tron et al., 2004). *P. aeruginosa* isolates from chronic lung infections such as those found in cystic fibrosis patients show a similar reduced virulence phenotype. This includes the

loss of flagella and mutations that inactivate *lasR* (Hogardt & Heesemann, 2010). One possible explanation for these findings is the presence of social cheats. These cells do not produce quorum sensing molecules, but still benefit from the products of quorum sensing and therefore have a selective advantage when growing in the presence of wild type cells (Chen et al., 2019; Diggle et al., 2007).

Haemolytic phospholipase C (*plcH*) is an exoenzyme that is able to lyse red blood cells (Shoriridge, Lazdunski & Vasil, 1992) and components of eukaryotic cell membranes (Berka & Vasil, 1982). No specific studies have been performed investigating the role of *plcH* in cases of OE. The intradermal inoculation of the protein in an *in vivo* mouse model resulted in a concentration-dependent response showing either no change, erythema, or dermonecrosis (Berk et al., 1987).

Bacterial secretion systems are important for the virulence of Gram-negative bacteria as they allow for the transport of proteins across the two membranes and, in some cases, directly into a target cell. Exotoxin A (ToxA) is exported via the SEC-dependent type II secretion system. The toxin is part of the AB toxin family, where the A subunit has enzymatic activity, and the B subunit facilitates attachment to CD91 (also known as alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein α2MR/LRP). Once inside the host cytoplasm, the protease furin cleaves the protein and it is transported to the trans Golgi network, where it inhibits the eukaryotic elongation factor-2 by ADP-ribosylation, inducing apoptosis. This allows the toxin to bind to and kill cells from many different tissues (Michalska & Wolf, 2015; Kounnas et al., 1992). Increased transcription of ToxA in human OE patients has been associated with patients suffering from more severe symptoms compared with those with ‘mild

to moderate' symptoms (Matar et al., 2002). Additionally, when applied to the middle ear, the toxin causes the apoptosis of epithelial cells and can penetrate the inner ear, causing damage to the cochlea in multiple animal studies (Lundman et al., 1992; Stenqvist, Anniko & Rask-Andersen, 1999; Stenqvist, Anniko & Pettersson, 1997). Whether the toxin is able to cross the tympanic membrane during OE and have this effect has not been investigated.

ExoS is part of the type III secretion system of *P. aeruginosa*. It possesses an N-terminal GTPase-activating protein region that triggers actin cytoskeleton disruption, and a C-terminal adenosine diphosphate ribosyl transferase, which is a major cause of host apoptosis (Horna & Ruiz, 2021). The presence of ExoS is commonly mutually exclusive with ExoU, although the reason for this and its role in infection are unclear (Feltman et al., 2001; Rutherford et al., 2018). *P. aeruginosa* isolates from human chronic OM cases were significantly more likely to be *exoU*+ than *exoS*+ compared with a control group of isolates from blood and respiratory infections (Park et al., 2017). In human cases of OE, *P. aeruginosa* isolates have been shown to produce significantly less pyocyanin and alginate than isolates from other sites of infection, in addition to increased deoxyribonuclease production (Sundström et al., 1997). Whether differences in these factors are seen in canine OE and their clinical significance remains to be elucidated.

1.2.4 Biofilm formation

Quorum sensing (QS) is present in many bacterial genera and allows populations to regulate gene expression based on the density of small molecules known as autoinducers. The *P. aeruginosa* QS system consists of four separate but interlinked systems - *las*, *rhl*, *pqs*, and *iqs*. These are responsible for the

production of some virulence factors such as proteases, toxins and hydrogen cyanide. Additionally, QS also controls biofilm formation (Moradali, Ghods & Rehm, 2017).

Biofilm formation is likely the most important virulence factor involved in chronic *P. aeruginosa* canine OE infections and is associated with poor treatment outcomes (Pye, Yu & Weese, 2013; Robinson et al., 2019; Chan et al., 2019a). Biofilms provide protection from antimicrobials and the immune system and facilitate dispersion to other environments. Bacteria form biofilms by attaching to a surface and producing extracellular polymeric substances comprising exopolysaccharides, extracellular DNA, and proteins.

Initial attachment is reversable and is facilitated by the flagella and type IV pili (O'Toole & Kolter, 1998). Adhered cells become irreversibly attached and produce extracellular polysaccharides. *P. aeruginosa* is known to produce three exopolysaccharides; Psl, Pel and alginate. One bacterial strain can have the genetic potential to synthesise all three polysaccharides, but it will typically only produce one. Alginate is the best characterised as it associated with chronic pulmonary infection while most environmental isolates will secrete Pel or Psl (Franklin et al., 2011). The biofilm then develops first into microcolonies and then into more complex mushroom-like structures (Allesen-Holm et al., 2006). In addition to the exopolysaccharides, extracellular DNA is highly structured within the biofilm at different stages of development and is essential for the formation of *P. aeruginosa* biofilm (Allesen-Holm et al., 2006; Whitchurch et al., 2002). Extracellular DNA has also been shown to chelate cations and induce antimicrobial resistance (Mulcahy, Charron-Mazenod & Lewenza, 2008).

Biofilms are produced by 40–95% of *P. aeruginosa* isolates from canine OE (Pye, Yu & Weese, 2013; Robinson et al., 2019; Chan et al., 2019a) and 92% of human patients with chronic OE, but only 20% from those with acute otitis (Fusconi et al., 2011). *Pseudomonas* isolates from human OE showed increased adherence to guinea pig epithelial cell lines compared with isolates from other sites of infection (Sundström et al., 1997). The removal of the biofilm using novel therapies, such as chemical ear peeling, significantly reduced the recurrence of symptoms compared with ciprofloxacin/hydrocortisone antibiotic treatment in humans (Fusconi et al., 2011).

1.2.5 Motility

P. aeruginosa employs a single polar flagellum which enables the cell to swim in aqueous environments and perform chemotaxis (Bouteiller et al., 2021). The presence of a flagellum is essential for early infection in both pulmonary and burn wound models (Feldman et al., 1998; Montie et al., 1982). Additionally, swarming motility is mediated by flagella and is important for growth on semisolid surfaces (Köhler et al., 2000). This likely means that this type of motility is important in mucous layers that overlay epithelial surfaces (Yeung et al., 2009). Swarmer cells are hyperflagellated, elongated and require the production of surfactants (Kearns, 2010). Moreover, swarmer cells overexpress virulence factors including type III secretion systems (Overhage et al., 2008). *P. aeruginosa* has a final method of motility that is twitching motility, unlike the method discussed above, twitching motility uses type IV pili to propel the cell across surfaces (Burrows, 2012).

1.2.6 Antimicrobial resistance (AMR) in *Pseudomonas aeruginosa*

AMR, both intrinsic and acquired, has allowed *P. aeruginosa* to become such a prevalent opportunistic pathogen (Hancock & Speert, 2000; Cox & Wright, 2013). The outer membrane of *P. aeruginosa* has limited permeability, 12-100-fold lower than *E. coli*, which confers some intrinsic resistance to antimicrobials and allows specific antibiotic resistance mechanisms to act more effectively. Resistance can also arise from efflux pumps and the expression of a chromosomal β-lactamase (Cox & Wright, 2013; Hancock, 1998). Acquired AMR can be due to mutation of a target gene or through horizontal gene transfer (Pang et al., 2019).

Resistance genes can be acquired via multiple mechanisms, namely, transformation, transduction, and conjugation (Pang et al., 2019). These genes usually encode enzymes that can modify the structure of the antibiotic, however, the main driver of resistance in *P. aeruginosa* is the development of mutations. *P. aeruginosa* rapidly develops mutations that confer resistance to a given antibiotic, in fact mutation of the *P. aeruginosa* genome can result in resistance to almost all antibiotics used to treat this organism. Mutations that are known to increase the AMR of *P. aeruginosa* have been reviewed in detail elsewhere (López-Causapé et al., 2018).

AMR, especially for Gram-negative rods, has been recognised as a problem in canine otitis for many years (Guedeja-Marrón et al., 1998), with numerous studies documenting resistance profiles for *P. aeruginosa* isolates. Fluoroquinolone resistance has frequently been reported, particularly with respect to enrofloxacin (27–68%), orbifloxacin (55–82%), and marbofloxacin

(33–35%). Gentamicin resistance seems to vary greatly depending on the study, with resistance reported in 3–43% of isolates (Petrov et al., 2019; Nuttall & Cole, 2007; Elfadadny et al., 2023; KuKanich, Bagladi-Swanson & KuKanich, 2022; Mekić, Matanović & Šeol, 2011; Bourély et al., 2019). Multidrug resistance, which is defined as resistance to at least one antibiotic from three or more classes (Sweeney et al., 2018), was recorded in 13–35% of isolates (Elfadadny et al., 2023; KuKanich, Bagladi-Swanson & KuKanich, 2022). Non-susceptibility to carbapenem antibiotics has also been reported in 15–23% of *P. aeruginosa* isolates from canine otitis (Hyun, Chung & Hwang, 2018; KuKanich, Bagladi-Swanson & KuKanich, 2022). This is important as the World Health Organisation identified a critical need for new antibiotics against carbapenem-resistant *P. aeruginosa* (WHO, 2017). Carbapenem resistance can arise in *P. aeruginosa* by either the production of carbapenemase, efflux pump over-expression, or reduced outer-membrane permeability (Nordmann & Poirel, 2019). Clinical canine otitis *P. aeruginosa* isolates producing metallo-β-lactamase VIM-2 have been identified in Korea (Hyun, Chung & Hwang, 2018).

Petrov et al., (2019) monitored AMR patterns in dogs suffering from both Gram-positive and Gram-negative infections in OE between 2007–2011, and again in 2013–2017. They found that, for most antibiotics tested against *P. aeruginosa* between these time points, resistance had increased. Notably, gentamicin resistance increased from 2% to 15%, as did resistance to tobramycin (20% to 26%), amikacin (0% to 18%), lincomycin/spectinomycin (40% to 93%), and polymyxin B (0% to 50%). In contrast, enrofloxacin resistance decreased (38% to 27%). In France, the investigation of *P. aeruginosa* resistance profiles from canine OE surveillance databases found that resistance to gentamicin remained

unchanged over a 5-year period, while resistance to enrofloxacin first increased and then slowly decreased over the following years. Worryingly, resistance to both enrofloxacin and gentamicin was reported in 19.4% of isolates. The decrease in resistance to enrofloxacin was likely due to the reduced exposure of companion animals to antibiotics over the study period (Bourély et al., 2019).

Park and colleagues (Park et al., 2020) investigated the antibiotic susceptibility of *P. aeruginosa* isolates from various body sites of healthy and diseased dogs. While antibiotic resistance was more prevalent in diseased samples, resistance was still present in isolates from otherwise healthy dogs. Specifically, resistance to ciprofloxacin (10.5%) was the most common, while other antibiotics all showed the same level of resistance (2.6%).

1.2.7 Previously investigated potential alternative treatments for use in canine otitis externa

Increasing AMR, particularly among the ESKAPE pathogens, a group of pathogens recognised for increasing levels of multidrug resistance, which includes *Pseudomonas*, has driven research into enhancing the efficacy of current therapeutic options and the development of novel alternatives. Prior to 2000, there was very little research on alternative treatments for *Pseudomonas* OE. One study investigated the use of new antibiotic, ticarcillin in treatment-resistant cases, resulting in the initial resolution of 11 out of 12 cases, although two of these later relapsed (Nuttall, 1998). More recently, synergy and partial synergy were reported when a combination of polymyxin B/miconazole and marbofloxacin/gentamicin was tested on *P. aeruginosa* canine otitis isolates (Pietschmann et al., 2013; Jerzsele & Pásztiné-Gere, 2015).

Ethylenediaminetetraacetic acid (EDTA), specifically Tris-EDTA, has been studied as an adjuvant in combination with antimicrobials in relation to OE for many years (Wooley & Jones, 1983; Sparks et al., 1994; Wooley et al., 1983), and is now a common component of many commercially available ear cleaners (Steen & Paterson, 2012). When used alone, Tris-EDTA typically produces a bacteriostatic effect on *P. aeruginosa*, unless applied in excess (Chan et al., 2019a). However, it has been used to eradicate biofilms produced by clinical *P. aeruginosa* isolates (Chan et al., 2019a; Buckley, McEwan & Nuttall, 2013) or reduce the MIC of certain antibiotics for *P. aeruginosa* present in biofilms (Pye, Singh & Weese, 2014).

Tris-EDTA has been found to act synergistically with amikacin (fractional inhibitory concentration, FIC = 0.1994) and neomycin (FIC = 0.1646) against *P. aeruginosa* isolates from canine OE (Sparks et al., 1994). It has also been found to complement the action of enrofloxacin (Gbadamosis & Gotthelf, 2003), marbofloxacin, and gentamicin (Buckley, McEwan & Nuttall, 2013) when used against AMR *P. aeruginosa* from cases of OE.

N-acetylcysteine (NAC) is another candidate being explored as an adjuvant for use in canine OE. NAC acts by inhibiting cysteine utilisation in addition to disrupting disulfide bonds in extracellular polymeric substances (Kregiel et al., 2019). Similarly to Tris-EDTA, it has been shown to inhibit the growth of *P. aeruginosa*, including in canine otitis clinical isolates (Chan et al., 2019a; May, Ratliff & Bemis, 2019; Chan et al., 2019b). Synergistic interactions between NAC and enrofloxacin or gentamicin have been observed for one *P. aeruginosa* isolate but were indifferent or antagonistic at the concentrations tested *in vitro* for most isolates (May, Ratliff & Bemis, 2019). NAC is also able to remove

biofilms formed by clinical *P. aeruginosa* (20,000–80,000 µg/mL minimum biofilm eradication concentration, MBEC) isolates; however, it is important to note that this was at potentially ototoxic concentrations (<20,000 µg/mL) (Chan et al., 2019a).

Narasin and monensin are ionophores which act by transporting mono/divalent cations across bacterial cell membranes (Chan et al., 2018). These compounds were unable to inhibit clinical *P. aeruginosa* and other Gram-negative otitis pathogens at the tested concentrations when used alone, but an additive effect for narasin (but not monensin) was seen in combination with Tris-EDTA (Chan et al., 2020, 2018). However, monensin was found to significantly reduce, but not eradicate, *P. aeruginosa* biofilm development (Chan et al., 2019a).

A combination of enrofloxacin plus silver sulfadiazine demonstrated increased antimicrobial susceptibility, with the mean MIC decreasing from 12.97 µg/mL to 1.52/3.05 µg/mL (Trott et al., 2007). Similarly, clinical canine OE *P. aeruginosa* isolates challenged with silver sulfadiazine alone were all found to be susceptible, with 80% of isolates having a mean MIC of <10 µg/mL. It was noted that a commercially available product should be effective *in vivo*, as it has a concentration much higher than the MICs seen in the study (von Silva-Tarouca, Wolf & Mueller, 2019).

Phytochemicals have been investigated for *P. aeruginosa* inhibition. An antimicrobial effect has been reported for cinnamon oil, cinnamaldehyde (Sim et al., 2019b), oregano oil, carvacrol, thyme oil, thymol (Sim et al., 2019a), basil oil, rosemary oil, clary sage oil (Ebani et al., 2017), and *Harungana madagascariensis* extract (Moulari et al., 2007) *in vitro*. In all cases, *P.*

aeruginosa required a higher MIC than other common otitis pathogens, specifically Gram-positive organisms, such as *S. pseudintermedius*. One group tested an EDTA combination, and a synergistic interaction was observed for cinnamon oil (FIC = 0.27) and cinnamaldehyde (FIC = 0.26) (Sim et al., 2019b). Song and colleagues (Song et al., 2020) showed that, when applied alone, *P. aeruginosa* was resistant to manuka oil (MIC > 8% v/v). However, when combined with Tris-EDTA, the MIC was reduced to 0.5% or less. This was also true for multidrug-resistant isolates. When investigating the use of a commercially available essential oil blend *in vivo* for cases of acute OE, 33.3% of dogs were "cured" and a further 20.9% showed strong improvements. Importantly, rods were not present in the cytology; therefore, more work is needed to assess its use in chronic cases of OE involving *P. aeruginosa*. Despite this, the blend did inhibit a clinical strain of *P. aeruginosa* when tested *in vitro* (Vercelli et al., 2021).

Another promising alternative treatment is the use of antimicrobial photodynamic therapy. The use of tetra-cationic porphyrins to inactivate multidrug-resistant *P. aeruginosa* isolates from canine OE and other infection sites has been demonstrated in vitro (Seeger et al., 2020; Machado et al., 2022). Antimicrobial photodynamic therapy has been successfully used *in vivo* for a case of OE caused by a VIM-2 Metallo-β-lactamase-producing *P. aeruginosa* that had been unresponsive to treatment with enrofloxacin (Sellera et al., 2019).

The effect of cold atmospheric microwave plasma was investigated using primarily Gram-positive canine otitis isolates, but the study did include an ATCC *P. aeruginosa*. Gram-negative bacteria, including the ATCC *P. aeruginosa*, were more susceptible to cold atmospheric microwave plasma than

other isolates. For example, after 10 s of exposure at a plasma intensity of 30 W, *P. aeruginosa* survival was only 22.9%, while that of *Staphylococcus aureus* was 50.3%. However, this has yet to be tested on canine OE isolates (Jin et al., 2021).

1.3 Bacteriophage

Bacteriophage, literally “bacteria eater”, are viruses that infect and replicate within bacterial cells. Bacteriophage were discovered independently by William Twort and Felix d’Herelle in 1915 and 1917 respectively (Twort, 1915; d’Herelle, 1917). Their antimicrobial nature was quickly exploited and in 1919, d’Herelle treated children suffering from dysentery using bacteriophage (Abedon et al., 2011). Subsequently, by 1922, bacteriophage had been applied for the treatment of dysentery in children in the US (Davison, 1922). Despite these early successes, research investigating bacteriophage therapy was abandoned in the west after the discovery of antibiotics (Clokie et al., 2011) while extensive research continued in the former Soviet Union (Sulakvelidze, Alavidze & Morris, 2001). The rapid development of antimicrobial resistance as discussed above has once again focused attention on bacteriophage as an alternative to antibiotics.

1.3.1 Bacteriophage morphology and classification

Initially, The International Committee of Taxonomy of Viruses (ICTV), classified bacteriophage according to multiple characteristics including the virus structure and genome sequence comparisons (Lefkowitz et al., 2018). In 2022, the taxonomy of bacteriophage was drastically changed using genome-level relationships, as a result, the families *Myoviridea*, *Podoviridae* and *Siphoviridae*

were abolished (Turner et al., 2023). With an estimated 10^{31} particles in the biosphere, bacteriophage are the most abundant biological entities on the planet (Comeau et al., 2008). Bacteriophage display a large amount of genomic diversity and can package their genome as double or single-stranded (ds or ss) DNA and RNA (Dion, Oechslin & Moineau, 2020).

The ICTV recognises 40 classes of bacteriophage, with the class *Caudoviricetes*, formally known as the order *Caudovirales*, making up the majority of bacteriophage comprising 1360 genera and 4079 species at the time of writing (Turner et al., 2023). This class encompasses all bacterial and archaeal tailed viruses with an icosahedral capsid and double-stranded DNA genome (Turner et al., 2023).

1.3.2 Lifestyle

Primary, bacteriophage can undergo two different life cycles, lytic and lysogenic. Upon infecting a host, a virulent bacteriophage will replicate and eventually kill its host. A temperate bacteriophage can either enter its lysogenic lifestyle or act as a virulent bacteriophage (Figure 1.5).

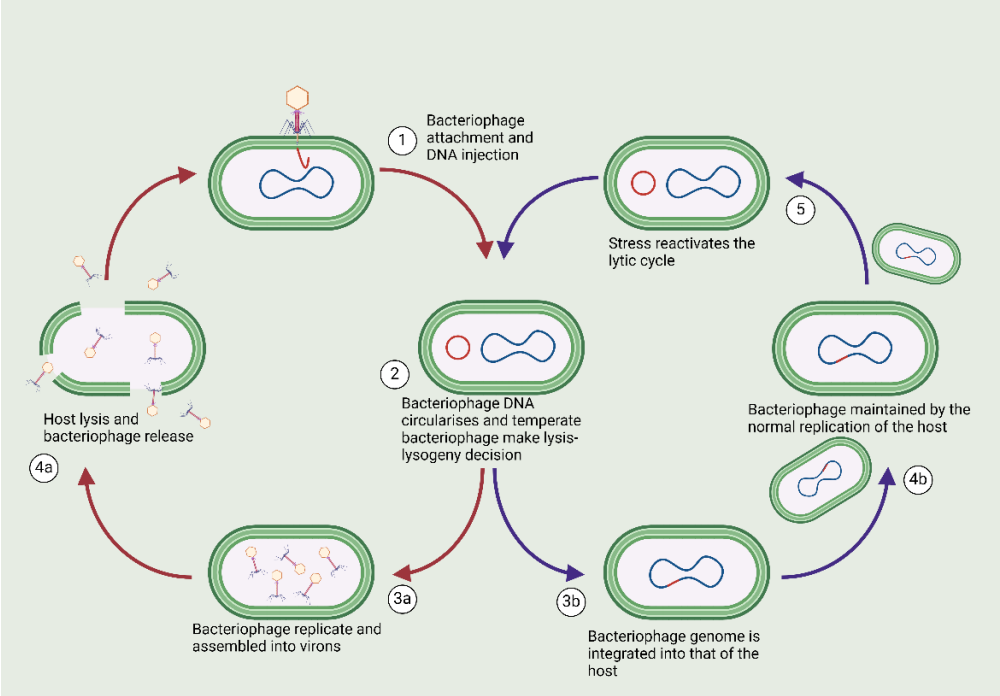

Figure 1.5 **A typical bacteriophage replication cycle highlighting both the lytic and lysogenic cycles.**

1.3.2.1 Host encounter, attachment and genome injection

Before a bacteriophage can infect a host, it must first encounter a susceptible host. When considering the movement of bacteriophage particles, diffusion is the primary mechanism by which this happens. This means that factors such as host and virus size, and the number of bacteriophage particles are important when considering the rate of adsorption (Abedon, 2023). Other forms for movement can also bring bacteriophage into contact with a host, namely turbulent movement and bacterial motility (Abedon, 2023).

Once a host cell has been encountered, there is an initial reversible attachment followed by an irreversible attachment, or separation of the bacteriophage and host when under unfavourable conditions (Storms & Sauvageau, 2015). The diversity of bacteriophage means that the mechanism by which this occurs is

distinct for a given virus. Attachment can also be influenced by other factors, namely: environmental factors such as temperature or acidity (Jończyk et al., 2011); or growth phase of the host, expression of surface structure is dependent on what stage of growth the host is in, common bacteriophage receptors are expressed during the exponential phase and therefore these cells will be infected more readily (Chai, 1983). Additionally, stochastic receptor expression can allow otherwise sensitive bacteria to resist bacteriophage infection (Chapman-McQuiston & Wu, 2008).

During attachment of the well-characterised tailed bacteriophage T4, a long tail fibre (LTP) will reversibly bind to its receptor, *E. coli* LPS or OmpC, a second LTP can then bind, and this process can allow the bacteriophage to 'walk' along the surface of the bacteria until an optimal site is reached. At this point, the short tail fibres (STP) irreversibly bind to the host, following this the bacteriophage genomic material is injected into the host cytoplasm from the action of the contractile tail (Washizaki, Yonesaki & Otsuka, 2016; Hu et al., 2015).

In contrast, bacteriophage MS2, which also infects *E. coli*, is a spherical (icosahedral) single-stranded RNA virus. The receptor binding protein of MS2 recognises and binds to the pili of its host. Upon retraction of the pili the receptor binding protein, which is also bound to the bacteriophage genome, is pulled into the host cytoplasm (Gorzelnik & Zhang, 2021).

Some bacteriophage are capable of infecting Gram-positive bacteria with peptidoglycan and teichoic acid being important receptors however Gram-positive bacteria are outside the scope of this work and so will not be discussed further (Dowah & Clokie, 2018; Leprince & Mahillon, 2023).

Bacteriophage that infect *P. aeruginosa* have been shown to recognise the O and Vi surface antigens in addition to PilA (Chaturongakul & Ounjai, 2014).

1.3.2.2 Replication, assembly and host lysis

The lytic-lysogenic decision has been documented in detail using bacteriophage lambda. This decision is determined by multiple factors, this includes genetic compatibility, host conditions such as starvation and the density of bacteriophage (Howard-Varona et al., 2017). Temperate bacteriophage are able to integrate their genome into that of the host at specific or random sites or produce extra-chromosomal elements which allows them to survive within the host without forming an active infection (Howard-Varona et al., 2017).

Once a lysogenic infection has been established the prophage is replicated as part of the bacterial chromosome however under conditions of stress such as changes in nutrients or exposure to antibiotics prophage can convert back to a lytic cycle (Howard-Varona et al., 2017).

A bacteriophage in the lytic cycle, either from a strict virulent bacteriophage or from a prophage entering the lytic cycle, replicate within the host producing new bacteriophage particles which are released upon the destruction of the host cell. The exact mechanism by which this happens varies by bacteriophage and the composition of the genome (DNA or RNA), as such, a variety of DNA replication mechanisms have been identified for bacteriophage (Weigel & Seitz, 2006).

Using the bacteriophage T7 as an example, host RNA polymerase transcribes the early genes that code for the T7 RNA polymerase as the bacteriophage dsDNA enters the host in addition to proteins that inhibit the host RNA

polymerase. From this point the T7 RNA polymerase transcribes the rest of the bacteriophage genes. This includes proteins for DNA replication and bacteriophage structural components (Madigan et al., 2010).

At the end of the infection cycle, virions are assembled, for dsDNA tailed phage there are several steps, the prohead is assembled and the genome is packed. Following this the proheads mature, and the tail proteins are attached (Aksyuk & Rossmann, 2011). Bacteriophage with dsDNA employ multiple methods to package their DNA. The packaging mechanism can vary based on the method that the bacteriophage used to replicate its genome. Some common packaging mechanisms include direct terminal repeats, cohesive ends and headful packaging (Merrill et al., 2016).

Once the virions are assembled, four classes of proteins have been identified with a role in host lysis. The first is a holin that forms pores in the host cell membrane before the second protein, an endolysin, degrades the cell wall peptidoglycan (Young, 2014). Bacteriophage endolysins have two distinct functional domains, the first binds the protein to its substrate although this is not always present in bacteriophage that target Gram-negative organisms. The second domain is responsible for catalysing the peptidoglycan of which there are five groups depending on the specific bond of the that is targeted (Schmelcher, Donovan & Loessner, 2012). The third protein is an antiholin that acts to regulate lysis under unfavourable conditions. The final class of protein are spanins, these are essential in Gram-negative hosts as they are responsible for outer membrane disruption (Young, 2014). At this point the host cell lyses and the viral progeny are released to infect new host cells.

1.3.3 Current use of bacteriophage for the treatment of infection

The use of bacteriophage *in vivo* is not a new idea (Abedon et al., 2011; Davison, 1922). Consequently, usage of bacteriophage *in vivo* for animals and humans has been investigated (Liu et al., 2021b). A focus of early bacteriophage work was veterinary medicine and the resurgence of bacteriophage therapy in the west was in part due to the work performed by William Simth in the 1980s on mice, calves, piglets and lambs (Gigante & Atterbury, 2019; Smith & Huggins, 1982, 1983; Smith, Huggins & Shaw, 1987). As a result, a number of commercial bacteriophage products are now marketed in countries such as the USA and the UK (Huang et al., 2022).

Bacteriophage therapies have already been assessed *in vivo* against antibiotic-resistant *P. aeruginosa* causing chronic otitis in humans and dogs. In humans, twenty-four patients were selected, of which twelve received bacteriophage. Those who were treated with the phage reported clinical improvement and no adverse effects, with three cases seeming to be cured after a single treatment (Wright et al., 2009). In the canine study, ten dogs suffering from antibiotic-resistant *P. aeruginosa* otitis were treated once with a cocktail of six bacteriophages and monitored. After 48 h there was a 67% reduction in the number of *P. aeruginosa*. After 18 months, three of the animals had recovered from the disease and another three had no detectable *P. aeruginosa*; again, no side effects were reported (Hawkins et al., 2010). Although these results are promising, bacteriophages are often strain-specific; therefore, a cocktail of bacteriophage targeting different receptors is required. Additionally, bacteriophage resistance may be seen, although this was not investigated in the

above studies. It is also potentially important to note that the chronic nature of *P. aeruginosa* OE might require multiple rounds of phage treatment, further increasing the risk of developing resistance and therefore potentially requiring multiple phage cocktails per treatment.

When examining the use of bacteriophage *in vivo* there are some factors that must be considered. One of these is the release of endotoxins such as LPS from bacterial lysis, LPS can induce an inflammatory response that can be lethal (Galanos & Freudenberg, 1993). This has been demonstrated *in vivo* in a *Galleria mellonella* wax month model where the application of bacteriophage to a *P. aeruginosa* infection resulted in a worsened condition (Olszak et al., 2015).

1.3.4 Limitations of bacteriophage

Despite these successes there are some limitations of bacteriophage therapy. Just like for antibiotics, resistance to bacteriophage can develop, however this is thought to happen at a lower rate than for antibiotics (Łusiak-Szelachowska et al., 2022). Resistance to bacteriophage can occur using three main mechanisms, masking of receptors, intracellular host systems and prophage associated defence (Labrie, Samson & Moineau, 2010). Mutations in the chromosome of *P. aeruginosa* have been identified that confer resistance to bacteriophage. This included deletions that result in the loss of the receptor (Le et al., 2014) but were also caused by single-nucleotide polymorphisms within a gene (Mi et al., 2023). These mutations are random and can therefore have a negative fitness cost associated with them in the form of decreased motility, biofilm formation (Mi et al., 2023) or an increased susceptibility to antibiotics (Chan et al., 2016). *P. aeruginosa* has also been shown to mask receptors through glycosylation which is less likely to cause these negative effects (Harvey et al., 2018). Additionally,

exopolysaccharide production, such as within a biofilm, is able to prevent bacteriophage attachment and therefore infection (Vidakovic et al., 2018).

Bacteria have also evolved their own mechanisms to defend against bacteriophage, the most well-known of these systems is the CRISPR-Cas system. CRISPR-Cas systems have been identified in up to 36% of clinical *P. aeruginosa* isolates from humans (Cady et al., 2011). CRISPR regions in the genome comprise multiple repeated sequences separated by spacers, the sequence of the spacer region will correspond to that of a bacteriophage or other foreign DNA. These are then transcribed and the mature small CRISPR RNA complexes with the Cas protein. Where a complementary sequence is encountered, it is cleaved, which prevents bacteriophage replication. Most interestingly CRISPR-Cas systems can incorporate DNA sequences from newly encountered bacteriophage into system subsequently becoming resistant to them (Cady et al., 2012). Another well characterised defence mechanism that targets the genomic material of the bacteriophage is the presence of restriction-modification (R-M) systems. R-M systems are classified into four groups and function by degrading DNA with self-DNA protected by modification, usually in the form of methylation. Interestingly, type IV systems target modified bacteriophage DNA (Bernheim & Sorek, 2020).

The final mechanism by which bacteria can be resistant to infection by bacteriophage is utilising bacteriophage derived mechanisms. These mechanisms are typically associated with prophages, in the case of *P. aeruginosa*, temperate bacteriophage have been shown to prevent superinfection by interacting with components of the type IV pili (Wang et al., 2022).

In addition to resistance, environmental factors can affect the efficiency of bacteriophage therapies. Factors such as temperature, acidity and salinity can have negative impacts on the viability of bacteriophage particles (Jończyk et al., 2011). Viscosity and the presence of inert obstacles can also significantly impact the rate at which bacteriophage can interact with host cells (Abedon, 2023). These factors will likely be important when considering otitis externa.

1.4 Bdellovibrio bacteriovorus

Bdellovibrio bacteriovorus is a small Gram-negative Deltaproteobacterium that can prey upon other Gram-negative bacteria via an unusual predatory lifestyle (Figure 1.6). Prey are encountered during its motile attack phase where attachment occurs before *B. bacteriovorus* enters the prey periplasm via a small pore which is resealed once inside. After a few minutes the prey is killed and the predator begins to reproduce inside of the periplasm, releasing enzymes to degrade macromolecules from the cytoplasm of the host (Negus et al., 2017; Lambert et al., 2011). *B. bacteriovorus* is ubiquitous in the environment having been isolated from soil, fresh water, and marine environments in addition to sewage and animal faeces (Chu & Zhu, 2010; Taylor et al., 1974; Klein & Casida, 1967). It was first reported as an accidental discovery by Stolp and Petzold in 1962 while trying to isolate bacteriophage due to the similar looking plaque morphology (Stolp & Petzold, 1962). In recent years *B. bacteriovorus* has become of greater interest for its potential use as an antimicrobial agent (Dashiff et al., 2011). *B. bacteriovorus* is not unique in its predatory lifestyle; other organisms such as *Micavibrio aeruginosavorus* and *Bdellovibrio exovorus* are both able to prey on Gram-negative organisms. *B. exovorus* was first identified in 1991 and later designated in 2013 and unlike its related counterpart

does not invade the periplasm, but rather attaches to the outside of prey, consuming them from outside (Koval et al., 2013). Similarly, *M. aeruginosavorus* is able to kill Gram-negative organisms but again doesn't invade the periplasm and interestingly has a smaller prey range which includes *P. aeruginosa* (Dashiff et al., 2011; Kadouri et al., 2013).

1.4.1 Lifecycle

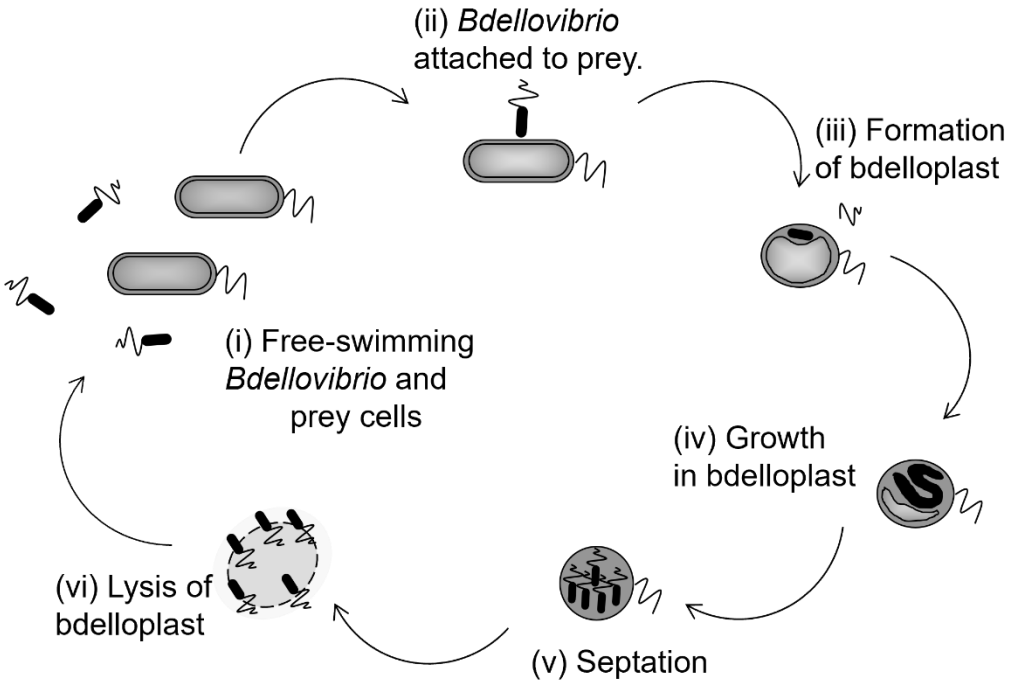

Figure 1.6 **Predatory life cycle of *Bdellovibrio bacteriovorus*.**

1.4.1.1 "Attack phase"

During the free-swimming "attack phase" of the life cycle of *B. bacteriovorus*, the cell is motile via a single polar sheathed flagellum and has been recorded swimming at up to 160 μm/s. The flagellum continues to grow as the cell ages. The flagellum also has an unusual, damped waveform (Thomashow & Rittenberg, 1985; Lambert et al., 2006; Iida et al., 2009). This is due to the

flagellar filament being a combination of six FliC proteins (Iida et al., 2009). Having so many copies is important as the flagellum is essential in liquid culture for both prey collisions and exit after the completion of replication. During knockout experiments only *fliC3* gave a complete loss in motility, however the sheath and basal filaments were still present. This suggests that FliC3 is the first filament protein in the flagellum and the others build upon it (Lambert et al., 2006; Iida et al., 2009). Additionally, *B. bacteriovorus* has three sets of *motAB* genes, encoding the flagellum motor proteins and further deletion experiments identified that just like the flagella, knockout of a single pair did not result in a loss of motility (Morehouse et al., 2011). This level of protection shows that even if natural acquisition of mutations were to occur, predation could continue. Moreover, flagella mediated motility is not the only method by which *B. bacteriovorus* is motile; in the absence of a high moisture environment, such as that encountered in biofilms, or when placed on a solid surface, *Bdellovibrio* is able to invade cells using gliding motility (Lambert et al., 2011).

Both types of motility are key for predation, however it is still unclear what role other factors play. Chemotaxis is the process by which bacteria move in relation to concentration gradients of a substrate. Early work on the role of chemotaxis in *Bdellovibrio* was contradictory. Some strains of *Bdellovibrio* can sense certain amino acids and other molecules by chemotaxis but also high concentrations of *E. coli* do not elicit a response (LaMarre, Straley & Conti, 1977; Straley & Conti, 1977; Straley et al., 1979). Since then, genome sequencing identified that *B. bacteriovorus* HD100 contains 21 genes with homology to methyl-accepting chemotaxis proteins (MCP) (Lambert et al., 2009). A study investigating a homolog of one of these genes in *B. bacteriovorus* 109J found that the wild-type

strain preyed more efficiently than the mutant, suggesting chemotaxis does have a role in predation (Lambert, Smith & Sockett, 2003). Additionally, the use of cyro-electron tomography identified chemosensory arrays during the attack phase (Kaplan et al., 2023).

1.4.1.2 Attachment and entry into prey

When *B. bacteriovorus* encounters a potential prey cell, there is a reversible attachment, before the cell becomes irreversibly attached, or in the case of non-prey, the initial attachment is reversed (Hobley, King & Sockett, 2006; Mahmoud & Koval, 2010). Recent work has identified a mosaic adhesive trimer (MAT) superfamily of proteins, localised on the predatory surface that are able to bind prey surface glycans potentially revealing the prey recognition mechanism (Caulton et al., 2024). Additionally, on the opposite pole to the flagellum, the presence of type IV pili has been shown using electron microscopy. Inactivation of these pili, either by mutation or through the use of antibodies, results in the loss of predation, as the *Bdellovibrio* cells are unable to enter into the prey (Evans, Lambert & Sockett, 2007; Mahmoud & Koval, 2010). *B. bacteriovorus* enters though a small pore formed shortly after attachment, and although the way in which this pore is formed is still unknown, pili could be responsible for movement though the pore via retraction (in other bacteria this retraction is due to the activity of PilT) (Evans, Lambert & Sockett, 2007). Although *B. bacteriovorus* possesses two homologs of the PilT protein, knockout experiments identified that PilT is not essential for invasion of *E. coli*. However, the study also noted that PilT2 is required for predation on biofilms (Chanyi & Koval, 2014). A further 10 genes have been identified as essential for prey attachment with only three having known function including *pilT*.

1.4.1.3 Intraperiplasmic growth and replication

During the switch from attack phase to intraperiplasmic growth, transcriptional analysis showed that 479 genes were up regulated, including several that are important for peptidoglycan modification (Lambert et al., 2010). As *B. bacteriovorus* enters the prey cell, peptidoglycan modification is needed in order to accommodate *B. bacteriovorus* in the periplasm, which results in a rounded bdelloplast (an osmotically stable structure of the dead prey cell in which *B. bacteriovorus* replicates) and ensures reinfection cannot occur (Lambert et al., 2016). Several genes have been identified to be important in peptidoglycan modification during infection, including bd3459 and bd0816, both of which encode DD-endopeptidases that cause de-crosslinking of the prey peptidoglycan. Deletion of both of these genes did not result in the loss of predation, however an atypical, less rounded bdelloplast shape was seen. Additionally, absence of these genes resulted in an increase in the number of double invasion events, where two *B. bacteriovorus* cells enter the same prey. A further two genes, bd3279 and bd0468, that encode for N-acetylglucosamine deacetylases, are important for peptidoglycan degradation (Lerner et al., 2012; Lambert et al., 2016). Inside the bdelloplast the prey macromolecules and genomic material are rapidly degraded by extracellular enzymes secreted by the *Bdellovibrio* into the cytoplasm of the prey, and incorporated into the predator, which is possible due to the large number of hydrolytic enzymes that have been identified in the genome (Engelking & Seidler, 1974; Rendulic et al., 2004).

Like most aspects of its life cycle, cell division in *B. bacteriovorus* is unusual. Bacteria will most commonly divide by binary fission, however *B. bacteriovorus* can be seen forming a long filament which will then divide into multiple cells.

The length of the filament and number of progeny depends on the host cell; both odd and even numbers of progeny can be seen which allows for the maximum number of new *Bdellovibrio* to be produced from the limited resources of one prey cell (Eksztejn & Varon, 1977; Fenton et al., 2010). The exact method by which *B. bacteriovorus* regulates this division is still being investigated however DivIVA has been identified at the poles of the filament with a suggested role in septal position selection, as well as four proteins that potentially interact with it (Milner et al., 2020). Once the prey cell content has been depleted and division has produced progeny *Bdellovibrio*, the new cells mature producing flagella. Additionally, in most cases, exit from the bdelloplast is facilitated by one or two small pores in the outer membrane of the remaining bdelloplast rather than a complete breakdown of the structure (Fenton et al., 2010).

1.4.2 Prey range and decoys

B. bacteriovorus preys on a wide range of Gram-negative bacteria, with one study identifying 68 different strains from 18 separate genera successfully preyed upon, including pathogens such as *E. coli* and *K. pneumoniae* (Dashiff et al., 2011). The primary focus of this work is predation of *P. aeruginosa*. Interestingly, reports of predation of *P. aeruginosa* by *Bdellovibrio* are contradictory. Two studies have shown that *B. bacteriovorus* was unable to produce plaques on agar or reduce *P. aeruginosa* numbers in liquid coculture (Dashiff et al., 2011; Markelova, 2010), while other groups have reported predation in liquid coculture but no plaque formation on agar (Waso, Khan & Khan, 2019). Finally, when testing 10 different strains for *P. aeruginosa*, Shanks and co-workers saw that *B. bacteriovorus* strain HD100 was able to prey on all 10 *P. aeruginosa* strains while *B. bacteriovorus strain* 109J was only able to

prey on 7 of the 10 tested. This was again analysed in liquid coculture and suggests that some strains of *P. aeruginosa* possess protection from predation (Shanks et al., 2013). Other groups have also reported *B. bacteriovorus* HD100 predation on *P. aeruginosa* but have only investigated a single isolate (Iebba et al., 2014; Pantanella et al., 2018; Liu et al., 2023b).

When considering the use of *B. bacteriovorus* as a novel therapy we must also consider the effects of the presence of Gram-positive pathogens as well as the natural microbiota. *Bacillus* spp. are a common Gram-positive commensal found in healthy dog ears (Shaw, 2016). The presence of this non-susceptible 'decoy' organism has been shown, *in vitro*, to introduce a lag period when compared to predation in its absence due to the *Bdellovibrio* reversibly attaching, then detaching after it recognises it is attached to a non-susceptible cell. However, its presence does not reduce the overall yield of *Bdellovibrio* (Hobley, King & Sockett, 2006). *S. pseudintermedius* is a common Gram-positive pathogen isolated from cases of canine otitis, and there are no published investigations of the interaction between *B. bacteriovorus* and this organism. However, in the presence of *S. aureus*, it has been shown that *B. bacteriovorus* is able to attach to *S. aureus* as well as the *Bdellovibrio* population increasing in the presence of the *S. aureus*, suggesting epibiotic predation as seen in *B. exovorus* (Pantanella et al., 2018; Iebba et al., 2014). There is also a chance of two Gram-negatives co-existing in a case of otitis with the second species being such bacteria as *Proteus* or *E. coli* (Morris et al., 2017). In a culture containing multiple prey organisms, not all are preyed upon equally, with evidence of prey preference shown (Rogosky, Moak & Emmert, 2006). Together, the presence of more than one species within an infected site will increase the number of interactions

between different species during *Bdellovibrio* treatment, and further investigation of these interactions *in vitro* is required to inform the best approaches before applying *Bdellovibrio* therapeutically.

1.4.3 Resistance and inhibition of predation

Bacterial resistance (for example to antibiotics or bacteriophage) is typically due to genetic change, be that acquiring an enzyme or changing a target site (Pang et al., 2019). One of the major benefits to using *Bdellovibrio* as 'a living antibiotic' is that prey bacteria do not become resistant via genetic mutation. Instead 'plastic' resistance is seen, a temporary resistance that is not genetically encoded. Thus, when a suspension of prey is left overnight in the presence of *Bdellovibrio* the prey population will initially decrease in number, however a small proportion of the population will survive and be able to increase in number (if sufficient nutrients are available for growth). However, if the resistant cells are recovered and grown on nutrient agar before being re-introduced to *Bdellovibrio*, they are cleared as efficiently as the original culture, suggesting this is a phenotypic change (Shemesh & Jurkevitch, 2004). The precise cause and mechanism of this resistance is unknown, but recent work suggests that it may arise due to the prey sensing predation of other prey cells within the culture (Hobley et al., 2020). *Bdellovibrio* is a very successful predator and can overcome LPS modification, capsules and S-layers (Negus et al., 2017).

1.4.4 Animal trials

Recent research has been performed in order to assess the safety of *B. bacteriovorus*, including both *in vitro* and *in vivo* studies. *In vitro* human corneal-limbal epithelial cells were inoculated with *B. bacteriovorus* and no

damage was reported for the cells (Shanks et al., 2013). Whilst a recent study has shown that when incubated alongside human phagocytic cells, the *Bdellovibrio* were engulfed and ultimately degraded by the phagocytes similarly to other bacteria (Raghunathan et al., 2019). As the aim of this project is to evaluate the possibility for a treatment for canine otitis it is important to consider the risks of using predatory bacteria *in vivo*. Atterbury and colleagues were the first to publish *in vivo* of a complete live-animal with *B. bacteriovorus*; layer healthy chicks were inoculated orally and monitored for 28 days, after which there was no difference in health or weight observed between the study and control groups. Additionally, when used therapeutically in birds colonised by *Salmonella*, the presence of the predator significantly reduced *Salmonella* carriage, which in turn resulted in a reduction of caecal abnormalities. A change in microbiota was seen, specifically a statistically significant reduction in the population of *Lactobacillus* and increase of streptococcal populations (Atterbury et al., 2011). Which has since been shown to also occur in rats, and so this is something that will need to be considered when considering the types and locations of infections that could be treated with *Bdellovibrio* (Shatzkes et al., 2017b). Another study inoculated high concentrations (1.1×10^9 PFU) of *B. bacteriovorus* into rat lungs; after 24 h there was a non-sustained increase in inflammatory cytokines and after 10 days no *B. bacteriovorus* could be recovered. Additionally, no animals showed any signs of illness, and lung tissue was shown to be normal. In the same study rats were challenged with *Klebsiella pneumoniae* in the presence and absence of the predator, which was shown to reduce the burden of *K. pneumoniae* (Shatzkes et al., 2016). At the time of writing there have been numerous *in vivo* experiments that show similar results

regarding the safety of *B. bacteriovorus*, although none specifically in canine ears (Shatzkes et al., 2017a; Willis et al., 2016; Tajabadi et al., 2023; Romanowski et al., 2023, 2016). These results show considerable evidence that when an animal is challenged by an inoculation of *B. bacteriovorus* there are no serious adverse effects.

1.5 Summary

Canine OE is a common disease in veterinary practices, with the most recent study from the UK reporting a prevalence of 7.3%. Cases are often only seen after an increase in clinical signs from secondary infection. When addressing this disease, it is important to consider the primary, secondary, perpetuating, and predisposing factors. *P. aeruginosa* is commonly isolated from cases of canine OE and, due to widely reported antimicrobial resistance and biofilm formation, poses a major issue for clinicians. Research investigating alternative treatments has seen promising results *in vitro*, with only a few being tested *in vivo*. Further work and *in vivo* experiments will be required in order to provide a better prognosis for the future. *B. bacteriovorus* and bacteriophage have both been investigated previously for their use as a novel therapeutic and could be an alternative therapy for this disease.

1.6 Aims

The primary aim of this study was to investigate the use of bacteriophage and *Bdellovibrio bacteriovorus* as novel therapies for treating canine otitis externa caused by *Pseudomonas aeruginosa*. To that end the project was split into three main aims that form the basis for the three results chapters of this thesis.

I. **Characterisation of *Pseudomonas aeruginosa* from cases of canine otitis externa.** Use a combination of phenotypic and bioinformatics methods to analyse the antimicrobial susceptibility, biofilm formation capacity and swarming ability of a range of clinical and non-clinical *P. aeruginosa* isolates. Determine the genomic diversity of these isolates and evaluate the complement of key virulence factors in these strains.

II. **Isolation and characterisation of bacteriophage that infect isolates of *P. aeruginosa* from clinical cases of canine otitis externa.** Isolate bacteriophage from a range of environmental sources including wastewater. Characterise the bacteriophage for attributes important in clinical applications including host range and efficiency of plating. Employ whole genome sequencing to predict their lifestyles and suitability for use as a therapeutic agent.

III. **Investigate the ability of *Bdellovibrio bacteriovorus* to prey upon clinical *P. aeruginosa* isolates.** Determine the ability of *B. bacteriovorus* to prey upon a collection of clinical and non-clinical *P. aeruginosa* isolates. Assess if factors within the ear will affect the predatory efficiency of *B. bacteriovorus*. And finally, investigate is a combination of *B. bacteriovorus* and bacteriophage result in a greater reduction of *P. aeruginosa in vitro*.

Chapter 2 Materials and Methods

2.1 Preparation of buffers, culture and storage media

Unless otherwise stated all media was prepared using reverse osmosis (RO) water according to the manufacturer's instructions and sterilised by autoclaving at 121° C for 15 minutes. Most media and chemicals were purchased from Fisher Scientific and Sigma Aldrich unless otherwise stated, product codes were included for each item.

Solutions, buffers, broth, and storage media were stored at room temperature until required. Agar plates were stored at 4° C for up to two weeks.

2.1.1 Solutions and buffers

2.1.1.1 Calcium chloride stock solution

Calcium chloride stock (25 g/L $CaCl_2 \cdot 2H_2O$, C/1500/53) was prepared using analytical grade water (10626852).

2.1.1.2 Sodium hydroxide solution

Sodium hydroxide (NaOH aq, S/4880/60) solution was prepared to a final concentration of 2 M and was not sterilised.

2.1.1.3 Congo red stock

Congo red stocks (1 g/L Congo red dye, C6767-100G) were prepared, and filter sterilised using a 0.22 µm filter (Sartorius, Germany FIL6570) before use. Stock solutions were protected from light using aluminium foil and stored at 4° C.

2.1.1.4 Coomassie blue stock

Coomassie blue stocks (20 g/L Coomassie brilliant blue dye, 27815-25G-F) were prepared, and filter sterilised before use. Stock solutions were protected using aluminium foil and stored at 4° C.

2.1.1.5 Ca/HEPES buffer

Ca/HEPES buffer (5.94 g/L HEPES, 44034-1KG, 0.284 g/L $CaCl_2 \cdot 2H_2O$) was prepared using analytical grade water and corrected to pH 7.6 using 2 M NaOH.

2.1.1.6 Sodium magnesium (SM) buffer

SM buffer (5.8 g/L NaCl, S/3160/60; 2 g/L $MgSO_4 \cdot 7H_2O$, M/1050/53; 5% v/v 1 M Tris-Cl, adjusted to pH 7.5) was prepared and aliquoted into 50 mL working stocks before use.

2.1.1.7 McFarland standard

A 0.5 McFarland Standard (0.5% v/v, 0.048 mol/L $BaCl_2$ stock, 217565-500G; 99.5% v/v, 0.18 mol/L H_2SO_4 stock, 339741) was prepared as previously described (CLSI, 2012). The absorbance was checked to be between 0.08 to 0.13 at 625 nm before being aliquoted into 15 mL falcon tubes (Sarstedt, UK 62.554.502), the same type used for the bacterial inoculum in §2.5.

2.1.2 Culture media

2.1.2.1 Lysogeny broth (LB) / agar

LB Miller (25 g/L, L3152-1KG), supplemented with 15 g/L agar (15129303) for plates.

2.1.2.2 LB double layer agar plates

LB was prepared as described in §2.1.2.1 but was instead supplemented with 15 g/L agar select (A5054-1KG) for base agar or 7 g/L agar select for top agar.

2.1.2.3 Yeast extract tryptone (YT) broth / agar

YT broth (5.0 g/L yeast extract Gibco 212750, 8.0 g/L tryptone Gibco 211705, 5.0 g/L NaCl) was prepared using analytical grade water and adjusted to pH 7.5 using 2 M NaOH. For plates 10 g/L agar select was included.

2.1.2.4 YPSC broth / double layer agar plates

YPSC broth (1.0 g/L yeast extract, 1.0 g/L meat peptone 70174-500G, 0.5 g/L anhydrous sodium acetate S/2080/60, 0.25 g/L $MgSO_4 \cdot 7H_2O$) was prepared using analytical grade water and adjusted to pH 7.6 using 2 M NaOH. For double layer agar plates 10 g/L agar select was added for base agar and 6 g/L agar select for top agar. After sterilisation, $CaCl_2$ was added to a final concentration of 0.25 g/L.

2.1.2.5 Mueller-Hinton broth

Mueller-Hinton (MH) broth (21 g/L 70195-500G).

2.1.2.6 Mueller-Hinton agar 2

MH agar 2 (38 g/L 97580-500G).

2.1.2.7 Tryptone broth / agar

Tryptone broth (10 g/L tryptone, 5 g/L NaCl) supplemented with 10 g/L agar for plates.

2.1.2.8 Swarming agar

Swarming agar (8 g/L nutrient broth Oxoid, UK SM001, 5 g/L agar select) was prepared and after sterilisation, glucose was added to a final concentration of 0.5% v/v using a filter sterilised stock solution (20% w/v glucose G/0500/61).

2.1.3 Storage media preparation

2.1.3.1 Glycerol solution

A 50% v/v glycerol stock solution was prepared by combing an equal volume of 100% glycerol (Honeywell, UK 49770-25L) and analytical grade water.

2.2 Bacterial strain isolation

A summary of the bacterial strains used in this work are described below (Table 2.1), a complete list can be found in the appendix Table 8.1:

Species/Strain	**Origin**	**Original Isolation**
Escherichia coli		
E. coli S17-1	University of Nottingham, Prof. L Sockett	Simon *et al.*, 1983
E. coli ATCC25922	University of Nottingham, Dr J Hobman	
Pseudomonas aeruginosa		
P. aeruginosa PAO1-QUB	Queens University Belfast, Dr R Ingram	Holloway, WB. 1955
P. aeruginosa PAO1-N	University of Nottingham, Prof. Paul Williams	
P. aeruginosa PAO1-UW	University of Nottingham, Prof. Paul Williams	
P. aeruginosa PAO1-L	University of Nottingham, Prof. Paul Williams	Dieter Haas laboratory, University of Lausanne
P. aeruginosa ATCC 27853	University of Nottingham, Prof. Paul Williams	
P. aeruginosa isolates from cases of canine otitis externa (n = 46)	Royal Veterinary Collage	
P. aeruginosa isolates from cases of canine otitis externa (n = 93)	NationWide Laboratories	Veterinary practices across the UK
P. aeruginosa isolates from cases of canine otitis externa (n = 104)	University of Copenhagen, School of Veterinary Medicine	EU collection
P. aeruginosa isolates from cases of canine otitis externa (n = 10)	Unknown	

Table 2.1. **A summary of the bacterial strains used in this work**.

2.3 Growth and laboratory maintenance

Bacterial strains were maintained at -80° C in 25% v/v glycerol stocks.

2.3.1 *P. aeruginosa*

Prior to each experiment LB agar plates were inoculated with the required *P. aeruginosa* strain and streaked to obtain single colonies. Plates were inverted and incubated statically at 37° C for 16 h. Following incubation, 10 mL of LB was inoculated with a single colony using a sterile inoculating loop (302774) and incubated at 37° C with shaking at 200 rpm for 16 h.

P. aeruginosa PAO1-QUB was used as a control where appropriate.

2.3.2 *E. coli*

E. coli S17-1 was first cultured on YT agar to obtain single colonies. Plates were incubated statically at 37° C for 16 h. Following incubation, a single colony was used to inoculate YT broth and grown at 37° C with shaking at 200 rpm for 16 h. *E. coli* S17-1 cultured in YT was used for the maintenance of *B. bacteriovorus* as described in §2.3.3.

For use as a control in predation experiments (§2.14, §2.15, §2.16, and §2.18) *E. coli* S17-1 instead grown in LB.

E. coli ATCC 25922 was grown using LB media as for *P. aeruginosa*.

2.3.3 *B. bacteriovorus*

Bdellovibrio bacteriovorus was maintained as described in (Lambert & Sockett, 2008).

2.3.3.1 Bacterial lawn preparation

The required bacterial cultures were prepared as described above (§2.3.1, §2.3.2). Lawns were prepared by combining 150 µL of a prey overnight culture (~10^9 CFU/mL) with 5 mL of YPSC top agar in a 7 mL bijou (STARLAB, UK E1412-0711). The inoculated agar was immediately poured onto YPSC base agar plates and rotated gently to produce an even lawn. Plates were left to set at room temperature before being used.

2.3.3.2 Recovery of *B. bacteriovorus* from frozen stocks

First a bacterial lawn containing *E. coli* S17-1 prey cells was produced as described in §2.3.3.1. *B. bacteriovorus* HD100 glycerol stocks were then partially defrosted, and the lawn was inoculated with 100 µL of stock. This was incubated at 29° C for 4-5 days. The plates were observed for an area of clearing, indicative of predation by *B. bacteriovorus*, a P1000 pipette set to 250 µL was used to pick this into 2 mL of Ca/HEPES buffer containing 150 µL of *E. coli* S17-1 and incubated at 29° C with shaking at 200 rpm for 48 h. The presence of a complete predatory culture, i.e., the presence of motile *B. bacteriovorus* in the absence of *E. coli* or bdelloplast was confirmed by light microscopy, the predatory culture could be sub-cultured for up to two weeks keeping the ratio of 5:3:1, Ca/HEPES buffer: prey: predator. Alternatively, the predatory culture can be filtered through a 0.45 µm filter (Sartorius, Germany 10109180) and used in an experiment.

2.3.3.3 Enumeration of *B. bacteriovorus*

Enumeration of *B. bacteriovorus* was performed by producing a lawn as described in §2.3.3.1 with slight modifications. First, 150 µL of *E. coli* S17-1 prey cells were combined with 100 µL aliquots of *B. bacteriovorus* decimal

dilutions within an appropriate range to allow for counting (typically 10^{-4}, 10^{-5}, 10^{-6}) before 5 mL of YPSC top agar was added. Once dried the plates were incubated at 29° C for 4-5 days.

Due to the small size of attack phase cells, *B. bacteriovorus* cannot be quantified by optical density readings. As a result, the concentration (PFU/mL) of *B. bacteriovorus* used in a given experiment could only be calculated retrospectively. Therefore, the input *B. bacteriovorus* predatory culture was always enumerated to ensure a concentration ~ 2×10^{8} PFU/mL

2.3.4 Bacteriophage

The majority of standard methods used here have been previously described by Sambrook & Russell, (2001) and Clokie & Kropinski, (2009) with some modifications.

2.3.4.1 Preparation of a bacterial lawn

The preparation of bacterial lawns for use with bacteriophage is very similar to that for *B. bacteriovorus* as described in §2.3.3.1 with some differences. In a 7 mL bijou tube, 100 µL of host overnight culture was combined with 5 mL LB top agar and then promptly poured onto LB base agar plates, gently rotating to create a uniform lawn.

2.3.4.2 Enumeration of bacteriophage

Enumeration of bacteriophage was performed using two methods, double layer agar overlay and Miles and Misra plating. For the first method, serial dilutions of bacteriophage were prepared using SM buffer within the required range. Then a bacterial lawn was prepared as in §2.3.4.1 however, before adding the molten

top agar, 100 µL of the dilution was added. Once dry, the plates were incubated at 37° C for 16 h.

Secondly, the Miles and Misra method was performed by producing a bacterial lawn was produced as described in §2.3.4.1, once the top agar had dried 3× 10 µL spot of each bacteriophage dilution was spotted onto the lawn and left to dry. Once the spots had dried, they could be incubated as above. This method could be used to quickly enumerate multiple bacteriophage using a Micro-Dilution Tube System (STARLAB, UK E1750-0410) with a multichannel pipette.

2.3.4.3 Plaque picks

Plaque picks were achieved by resuspending a plaque in 500 µL of SM buffer using a P1000 set to 250 µL followed by a 2 h incubation period at room temperature.

2.3.4.4 Preparing bacteriophage stocks

In a 50 mL falcon tube (Sarstedt, UK 62.547.254) 100 µL of host overnight culture was combined with 50 µL of phage adjusted to 1×10^6 PFU/mL and incubated at 37° C for 20 min. Following incubation 30 mL of pre-warmed LB as added and the mixture was incubated for a further 16 h at 37° C with shaking (150 rpm). Next, the culture was centrifuged for 30 min at 1935 × *g* and the supernatant collected. DNase and RNase were then added to the supernatant to a final concentration of 1 µg/µL and incubated at room temperature for 30 min. Subsequently, NaCl was added to a final concentration of 1M and mixed until dissolved followed by PEG-8000 (10407773) to a final concentration of 10% (w/v). The lysate was then stored at 4° C overnight.

The precipitated bacteriophage was recovered by centrifugation for 30 min at 1935 × *g* and the supernatant removed, leaving the tube inverted to allow the pellet to dry. Once the supernatant had been removed, the pellet was resuspended in 800 µL of SM buffer and transferred to a 2 mL microcentrifuge tube and combined with an equal volume of chloroform. The mixture was gently mixed for 30 seconds and then centrifuged for 1 min at 13000 × *g*, the aqueous layer was recovered and washed again until all the PEG-8000 has been removed. Finally, the purified phage was filtered using a 0.22 µm filter.

2.4 Agarose gel electrophoresis

A 1% (w/v) agarose gel was prepared by combining 1 g of agarose (A9539-500G) to 100 mL of 1x TAE buffer and dissolving the agarose by microwave heating. The solution was allowed to cool until no longer steaming before ethidium bromide was added to a final concentration of 0.2 µg/mL and the mixture poured into a casting tray. Once set, 5 µL of sample was combined with 1 µL of loading dye (New England Biolabs, USA B7025S) and loaded into a well in the agarose gel. An aliquot of the DNA ladder was loaded into each gel as appropriate. Finally, the gel was electrophoresed at 100 V for 45-60 min and imaged using a UV transilluminator (Bio-Rad).

2.5 Antimicrobial sensitivity testing

Antimicrobial sensitivity testing (AST) was performed as described by the Clinical & Laboratory Standards Institute (CLSI) using the disc diffusion method (CLSI, 2012). A suspension of bacterial cells was prepared by inoculating 8 mL of MH broth in a 15 mL falcon tube with bacterial colonies selected from an LB plate until a turbidity equivalent to a 0.5 McFarland

standard had been reached, as determined by visual comparison using a Wickerham card. Within 15 min of adjusting the turbidity, a sterile cotton swab (VWR, USA 115-8270) was saturated with the suspension and excess removed by rotating inside of the tube. The swab was then used to inoculate the surface of a Mueller-Hinton agar plate ensuring the entire plate is covered evenly ensuring to rotate the plate 90° between swabbing the first time with a final diagonal swab. Again, within 15 min antibiotic discs were dispensed onto the surface of the plate using an antibiotic disc dispenser. Finally, within 15 min of the discs being applied the plates were placed in a 37° C incubator for 16 h. The zones of inhibition were inspected for uniform circles before being measured using a digital calliper (11777105).

AST was performed using an antibiotic panel comprised of six antibiotic classes and is shown below (Table 2.2). All antibiotics were tested against all strains with the exception of Ticarcillin + Clavulanic acid which was discontinued part way through this work and were acquired from two suppliers, ProLabs, UK and Oxoid, UK. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were the control strains used in this assay.

Antibiotic sensitivity was determined using breakpoints outlined by the CLSI, where available canine veterinary breakpoints were used (CLSI, 2020) otherwise human breakpoints were used (CLSI, 2018).

Antibiotic	Concentration (µg)
Penicillin	
Piperacillin	100
Piperacillin + Tazibactam	100 + 10
Ticarcillin + Clavulanic acid	75 + 10
Cephalosporin	
Ceftazidime	30
Cefepime	30
Carbapenem	
Imipenem	10
Meropenem	10
Monobactam	
Aztreonam	30
Aminoglycosides	
Amikacin	30
Gentamicin	10
Tobramycin	10
Fluroquinolone	
Enrofloxacin	5
Levofloxacin	5
Ciprofloxacin	5

Table 2.2 **List of antibiotics used in the disc diffusion assay**

Due to being discontinued, most of the antibiotics were acquired from two suppliers. 1. ProLabs (Cirencester, UK) 2. Oxoid (Basingstoke, UK)

2.6 Submerged biofilm assay

The ability of clinical *P. aeruginosa* isolates to form biofilm was tested *in vitro* using U-bottom polystyrene 96-well microtiter plates (Greiner bio-one, Austria 650161) as described previously (Coffey & Anderson, 2014). Overnight cultures were diluted 1 in 100 into LB and 100 µL aliquots were separately transferred to wells of the 96-well microtiter plates in triplicate in the same microtiter plate, two microtiter plates were used for each technical repeat. A negative control, consisting of three wells containing sterile LB, was included on each plate. The cultures were then grown statically at 37° C for 24 h. Following incubation, planktonic cells were removed, and the wells were washed using 125 µL of Ca/HEPES buffer; the wells were then stained using 0.1% (v/v) crystal violet solution (HT901-8F02). Excess crystal violet was removed, and the wells were then de-stained using ethanol (100%), and absorbance measured at 595 nm using a Tecan GENios Pro (Tecan Group AG, Switzerland). The assay was repeated three times to achieve three biological repeats.

2.6.1 Biofilm analysis

Biofilm production was scored as either as either strong, moderate, weak, or non-biofilm producing as previously described (Stepanović et al., 2000). Briefly, the mean absorbance of the negative control wells was used to blank the samples. For each plate three times the standard deviation of the negative control wells was calculated (ODc) and subsequentially used to categorise biofilm production as shown:

$$Non - Biofilm\ Producing\ \leq ODc\ < Weak\ Biofilm\ Producing$$
$$\leq 2ODc\ < Moderate\ Biofilm\ Producing\ \leq 4ODc$$
$$< Strong\ Biofilm\ Producing$$

2.7 Congo red biofilm assay

Congo red agar plates were used to detect colony associated biofilm formation adapted from (McCready et al., 2019). *P. aeruginosa* cultures were first grown in tryptone broth before 10 µL aliquots were spotted onto tryptone agar supplemented with Congo red dye (0.04 mg/mL) and Coomassie brilliant blue dye (0.02 mg/mL) from filter sterilised stock solutions (§2.1.1.3, §2.1.1.4). The plates were then incubated at 25° C for 120 h. Images were taken at a set distance of 12 cm using a Google Pixel 5 camera at 2× zoom. Three biological repeats were performed.

2.8 Swarming motility assay

Swarming motility assays were modified from (Ugurlu et al., 2016). Swarming motility agar was prepared, poured, and used on the same day. To ensure plates were evenly dried, they were left within the sterile area around a lit Bunsen burner for 40 min with the lid ajar. Once dry, 1 µL of bacterial culture was spotted into the centre of the plate and incubated at 37° C for 16 h. The distance travelled from the point of inoculation was measured using a digital caliper, and swarming motility assessed by phenotype on the plates in combination with the distance travelled from the point of inoculation. Three biological repeats were performed.

2.9 Isolation of bacteriophage

An existing collection of 109 bacteriophage isolated using *P. aeruginosa*, as the target, was assessed using four *P. aeruginosa* host strains PAO1-QUB, 80664, 84269 and 484098. This was performed by preparing a lawn as described in §2.3.4.1, followed by spotting 10 µL of the bacteriophage stock onto the lawn and incubating at 37° C for 16 h. The appearance of clearing indicated viable bacteriophage which were then purified as described below.

Aqueous samples were collected from 19 sites. A summary of the samples is shown in Table 2.3

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Sample	Collection Date	Location	Collected by
School A effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
School B effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
School C effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
School D effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
School E effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
School F effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
Prison A effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
Prison B effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
Prison C effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
Prison D effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
Care home A effluent	Feb - July 2021	Unknown	UK Centre of Ecology & Hydrology
Sutton Bonington effluent	10/11/2022	52°49’54”N 1°15’30”W	Dr. Jon Hobman – University of Nottingham
Sutton Bonington effluent	24/11/2022	52°49’54”N 1°15’30”W	This work
Sutton Bonington dairy farm effluent	10/11/2022	52°50’20.805”N 1°15’1.988”W	Dr. Jon Hobman – University of Nottingham
Sutton Bonington muck heap runoff	10/11/2022	52°50’20.805”N 1°15’1.988”W	Dr. Jon Hobman – University of Nottingham
Beeston canal	24/11/2022	52°55’9.095”N 1°11’26.381”W	This work
Highfield Park lake	24/11/2022	52°56’1.846”N 1°11’59.197”W	This work
Wollaton Park lake	24/11/2022	52°56’28.799”N 1°12’49.482”W	This work
BABS moat	24/11/2022	52°59’52.586”N 1°15’2.35”W	This work

Table 2.3 **Summary of environmental samples collected and used for bacteriophage isolation.**

Aliquots (50 mL) of the samples were then centrifuged for 30 min at 1935 × *g* to remove large particles. Subsequently, the supernatant was filtered using a 0.22 µm filter and stored at 4° C until required. The filtered samples were then used to isolate environmental bacteriophage using five hosts, *P. aeruginosa* PAO1-QUB, 84269, 488958, 26820-3 and 484098. To achieve single plaques, decimal dilutions (10^0 to 10^{-4}) were used as described in §2.3.4.2. In cases where individual plaques were present, they underwent passage to ensure the presence of only a single bacteriophage. Plaques were picked as described above §2.3.4.3 and a dilution series produced to achieve single plaques. This was repeated at least three times or until only a single plaque morphology was present. Any bacteriophage identified were subsequently named as described by Adriaenssens and Brister (Adriaenssens & Brister, 2017).

2.9.1 Isolation using enriched samples

In addition to the filtered raw samples, enrichments were also performed. This was performed by combining 20 mL of sterile LB with 8 mL of the filtered raw sample and 150 µL of the target host in a 50 mL falcon tube. Following incubation for 16 h at 37° C, the enrichment was centrifuged for 30 min at 1935 × *g*. The resulting supernatant was filtered using a 0.22 µm filter. The enriched sample could then be stored at 4° C and used for bacteriophage isolation as described above §2.9.

2.10 Bacteriophage host range

Host range and efficiency of plating was performed as previously described (Kutter, 2009).

Host range was determined by first producing a bacterial lawn as described in §2.3.4.1 before applying 10 µL of each phage at a concentration of 1×10^8 PFU/mL. Following incubation at 37° C for 16 h, any clearing was classified either as complete clearing (4), clearing throughout but with faintly hazy background (3), substantial turbidity throughout the cleared zone (2), a few individual plaques (1) or no clearing (0). A SM only spot was included as a control.

2.10.1 Bacteriophage efficiency of plating

Efficiency of plating was performed in a similar manner as host range however, a serial dilution of the 1×10^8 PFU/mL bacteriophage stock was prepared 10^{-1} to 10^{-6}. Subsequently, the overlay was inoculated with 20 µL of each bacteriophage dilution. The PFU/mL was calculated for the host strain that each phage was isolated from, and this was used to calculate the efficacy of plating as a percentage. For each host two technical and three biological repeats were performed. An SM only spot was included as a control.

Mean efficiency of plating values were then ranked as previously described (Viazis et al., 2011). Where no lysis was recorded (0%) the bacteriophage were ranked as ‘Not effective’, ‘High’ efficiency was recorded for values ≥50%, ‘Medium’ ≥20%<50% and ‘Low’ >0%<20%.

2.11 Bacteriophage genomic DNA extraction

Bacteriophage DNA extraction was performed with a Promega Wizard DNA cleanup kit (Promega, USA A7280) using a method from the Center for Phage Technology (CPT, 2011). First a lysate was made as described in §2.3.4.4 with some modifications, when adding the DNase and RNase, a higher concentration of 10 µg/µL of each was used. Additionally, a precipitant stock solution consisting of 30% (w/v) PEG-8000 and 3M NaCl was prepared in advance by combining 110 mL ddH_2O, 35g NaCl and 60g PEG-8000 in a Duran bottle and incubating at 55° C stirring occasionally, once dissolved ddH_2O was added to a final total volume of 200 mL. This was combined with 10 mL of DNase and RNase treated lysate at a rate of 1:2 precipitant:lysate and incubated at 4° C overnight.

Following this, the mixture was centrifuged at 1935 × *g* for 30 min and the supernatant removed. The pellet was then resuspended in 500 µL of 5mM $MgSO_4$, transferred to a 1.5 mL microcentrifuge tube, centrifuged at 13,000 × *g* for 5-10 seconds and the supernatant transferred to a clean microcentrifuge tube. Next, 1 mL of purification resin was added to the tube and pushed through the minicolumn using a syringe. The resin was then washed using 2 mL of 80% isopropanol and subsequently dried by centrifugation at 13,000 × *g* for 2 min. Finally, the column was transferred to a clean microcentrifuge tube and 100 µL of MilliQ water pre-heated to 80° C was added, the column was then immediately centrifuged at 13,000 × *g* for 1 min. The eluted DNA was then quantified using a BioDrop uLite+ Spectrophotometer (BioDrop, UK) and ran on an agarose gel to determine integrity, DNA was stored at -20° C until required.

2.12 Restriction digestion

All reagents were purchased from New England Biolabs, USA. Restriction digestions were performed using the manufacturer's instructions by combining sample DNA diluted to 1 µg, 5 µL 10x CutSmart buffer, 1 µL enzyme and MilliQ water to 50 µL total volume. The mixture was then incubated at 37° C for 2 h. The digestions were subsequently separated by gel electrophoresis as described in §2.4 using λ-HindIII (N3013) and λ-BstEII (N3014) as a ladder. Enzymes were selected based on a literature search. Initially, three enzymes were used, EcoRI-HF (R3101), NdeI (R0111) and HindIII (R0104), these alone did not provide a good separation for all the phage genomes so two further enzymes, HincII (R0103) and XbaI (R0145) were included.

2.13 *P. aeruginosa* growth kinetics in the presence of bacteriophage

The indirect measurement of bacteriophage growth was assessed using an Omnilog plate reader (Biolog, USA) assay.

First, 10 mL of fresh LB was inoculated with 100 µL of overnight culture and incubating at 37° C with shaking (150rpm) until an OD_{600} of 0.5 was reached.

Meanwhile, 10 µL of 2,3,5-Triphenyltetrazolium chloride (TTC T8877-5G) was combined with 10 mL of LB per 96 well plate to a final concentration of 0.002% (w/v). Subsequently, 90 µL of the mixture was loaded into each well of the 96 well plate.

Once the broth had been added, 10 µL of bacteriophage suspension at 1.5×10^7 PFU/mL was added to wells A7+B7, subsequently, different bacteriophage were pipetted into wells C1+D1, C7+D7 until all of the wells had been inoculated.

Once the bacteriophage had been added to the plate, serial dilutions from wells 1 to 6 and 7 to 12 were performed.

Once the host refresh had reached 0.5 OD_{600}, the culture was diluted to reach a bacterial suspension of 1×10^4 CFU/mL, 10 µL of this was finally added to all of the wells excluding column 6 and 12 which acted as controls. The microtiter platers were then loaded into the Omnilog plate reader and incubated at 37° C for 24 h with readings every 5 min.

Technical repeats were performed within the microtiter plates and three biological repeats were performed.

2.13.1 Optimising host input

The microtiter plate was set up as described above however the host refresh at 0.5 OD_{600} was added in place of the bacteriophage and diluted. Following this, 10 µL of fresh LB was used in place of additional host.

2.14 Predation on YPSC double layer agar plates

B. bacteriovorus was prepared as in 2.3.3, serial dilutions (10^0 to 10^{-6}) of the filtered predatory culture were prepared and the bacterial lawn was inoculated with 3 x 5 µL spots of each dilution. Duplicates of each host were prepared, and plates were incubated for 5 days at 29° C and 37° C. The ability of *B. bacteriovorus* to prey upon each strain was assessed by the appearance of clearing of the bacterial lawn, indicative of prey killing. Ca/HEPES only spots were used as a control and three biological repeats were performed.

2.15 Detection of secreted inhibitory products

The detection of products secreted by *P. aeruginosa* isolates that may inhibit *B. bacteriovorus* was adapted from work previously described (Hoshiko et al., 2021). In a 7 mL bijou bottle 100 µL of *B. bacteriovorus* was combined with 100 µL of *E. coli* S17-1 and 3 mL molten YPSC top agar before being poured into a YPSC base agar plate and left to cool. Once dry the middle of the top agar was inoculated with 2 µL of *P. aeruginosa* overnight culture. Duplicate plates of each isolate were prepared, and plates were incubated statically at 29° C and 37° C for 5 days. The zone of inhibition, indicated by *E. coli* growth, was measured using a digital calliper. Of the 253 *P. aeruginosa* isolates that were screen, 69 of these were screened by undergraduate project students, Michael Rousell, Jade Brewster-Mahon and Annice Norman.

2.16 Predation in liquid culture

An overnight predatory culture (§2.3.3.2) was centrifuged at 4000 × *g* for 30 min and the pellet resuspended in YPSC broth, this was then repeated. Similarly, overnight cultures of *P. aeruginosa* were centrifuged at 4000 × *g* for 10 min and the pellet resuspended in YPSC broth, again this was then repeated. Subsequently, in a 50 mL falcon tube, 12 mL YPSC broth was combined with 1.5 mL of prey in YPSC broth and either 1.5 mL washed predatory culture or 1.5 mL YPSC broth. Immediately, the OD_{600} was collected, and the mixture was incubated at 37° C with shaking (200 rpm). The input of prey and *B. bacteriovorus* was then enumerated. The OD_{600} was then measured every 24 h for 72 h, where a large drop in OD_{600} was observed, confirmation of predation was performed using light microscopy.

2.17 Microscopy

Fluorescence microscopy using a phase contrast lens was performed by Dr Jess Tyson using the following method. A filtered overnight predatory culture of *B. bacteriovorus* HD100:Bd0064mCherry was concentrated and resuspended in YPSC broth. Subsequently, 500 µL of the predatory culture was combined with a prey culture at OD_{600} 1 and incubated at 37° C statically. Images were then taken within 1 h 15 min to 2 h post addition. Images were then processed using Fiji (Schindelin et al., 2012), a distribution of ImageJ, using the sharpen and smooth tools iteratively a maximum of two times in addition to adjustments to brightness and contrast.

2.18 Predation of pre-formed biofilm

Initially, biofilms were produced as described in §1.1; however, after 24 h, planktonic cells were removed, and the wells were washed using Ca/HEPES buffer. Then 125 µL of either a dilution of 1in10 *B. bacteriovorus* or Ca/HEPES control was added to the wells and incubated at 29°c or 37°c statically for a further 24 h. Following incubation, the media was removed, and the wells were washed using Ca/HEPES buffer. Staining and biofilm quantification was then performed as described before (§1.1).

2.19 Survival in ear wash

An overnight predatory culture was aliquoted into 2 mL samples and centrifuged at 4000 × *g* for 20 min. Subsequently, the pellet was resuspended in either 2 mL Ca/HEPES or ear wash (TrizAural) and incubated at 29°c with shaking at 200 rpm for 24 h. The PFU/mL of *B. bacteriovorus* was enumerated every 15 min

for the first two hours, after 24 h, 1 mL was enumerated over 10 agar plates to get an accurate measurement of PFU/mL.

2.20 Bioinformatics

Genome sequencing was performed by MicrobesNG (Birmingham, UK) using their standard protocols. Clinical *P. aeruginosa* were sent as strain samples for the enhanced sequencing platform consisting of short read (Illumina MiSeq) and long read (Oxford Nanopore) sequencing.

Bacteriophage were sent as DNA samples (§2.11) for short read (Illumina MiSeq) sequencing only.

2.21 Genome assembly and annotation

2.21.1 *P. aeruginosa* genome sequencing

The quality of the Illumina paired-end reads was initially assessed using FastQC v0.11.8 (Andrews, 2010) before trimming adapters and poor quality reads using FastP v0.12.4 (Chen et al., 2018) with default settings except phred quality which was set to 26. Assembly was performed using a 'long read first' approach where initially Flye v2.9.2-b1786 (Kolmogorov et al., 2019) was used to create a *de novo* assembly specifying high quality nanopore reads, followed by Circlator v1.5.5 to circularise the genome assembly (Hunt et al., 2015). The integrity of the assemblies, in addition to the presence of secondary circular contigs which indicated the presence of a plasmid, was then assessed using Bandage v0.8.1 (Wick et al., 2015). Finally polishing was performed with one round of long read polishing using Medaka v 1.11.1 (ONT, 2023) and two rounds of short read polishing using the trimmed Illumina reads, first using Polypolish v0.5.0 (Wick & Holt, 2022) and then POLCA (Zimin & Salzberg, 2020). Quast

v5.2.0 (Gurevich et al., 2013) was used to assess the quality of the assemblies. This was done by evaluating measurements such as total contigs, GC content, N50 values and the percentage of reads that mapped to the assembly. Similarly, Quast was used to compare the quality of the assembly and subsequent polishing steps. Once the assemblies were deemed complete, i.e. once the assemblies were in as few contigs as possible and were at an appropriate quality as determined by QUAST, the genomes were reoriented to begin with *dnaA* using Dnaapler v0.4.0 (Bouras et al., 2024) before being annotated using Bakta v1.8.2 (Schwengers et al., 2021). Multilocus sequence typing (MLST) was performed *in silico* using mlst v2.23.0 (Seemann, 2023) which scans contigs against the PubMLST database (Jolley, Bray & Maiden, 2018). At the same time, another *de novo* assembly was produced using Unicycler v0.5.0 (Wick et al., 2017). This was done as small plasmids can be underrepresented in long read sequencing and so this assembly was used as a comparison.

2.21.2 Bacteriophage genome sequencing

Bacteriophage genome sequences were processed as previously described with some modifications (Shen & Millard, 2021). Similar to above, prior to assembly the Illumina paired-end reads were assessed using FastQC followed by trimming adapters and poor-quality reads with a Phred value lower than 26 using FastP while retaining unpaired reads. Trimmed and unpaired reads were used to produce a *de novo* genome assembly with Unicycler. The integrity of the assemblies was assessed using Bandage. This assembly was then used to establish a preliminary genome size and the number of reads required to achieve 100x coverage was calculated. Subsequently, the trimmed reads were subsampled using Seqtk v1.4 (Li, 2013) specifying the seed value as 100 for

each of the paired end reads. *De novo* genome assembly was repeated using Unicycler and again assessed using Bandage. Additional quality control was performed by first aligning the reads used to the assembly with Minimap2 v2.26-r1175 (Li, 2018), the alignment file was then processed using Samtools v1.19 (Li et al., 2009) using the utilities fixmate, sort, markdup and index. The alignment was finally assessed with Qualimap2 v2.3 (Okonechnikov, Conesa & García-Alcalde, 2016). The assemblies then underwent short read polishing with Polypolish. Preliminary checks for completeness of the draft genomes were performed by comparing similarity and size to closely related bacteriophage identified using PhageClouds (Rangel-Pineros et al., 2021)

PhageTerm (Garneau et al., 2017) was used to predict the DNA termini, packaging mechanisms and reorder the genomes accordingly. Where the termini were not identified, manual genome reordering was performed using the closest relative as a reference where a complete genome was available. This was determined using Blastn percentage identity (≥95%) specifying Viruses (taxid:10239) in addition to PhageClouds (Rangel-Pineros et al., 2021). The first coding sequence of this relative was subsequently used as an arbitrary start of the genome. Once a reordered sequence had been achieved, a final round of polishing was performed using Polypolish. The finished genomes were then annotated using Pharokka (Bouras et al., 2023a).

2.22 Whole genome analysis of *P. aeruginosa* isolates

2.22.1 Selection of additional *P. aeruginosa* genomes

The genome sequences of 70 *P. aeruginosa* isolates were downloaded from the *Pseudomonas* genome database (PGDB) v.22.1 (Winsor et al., 2016). This included the genomes of three common reference strains, PAO1, PA14 and PA7. Genomes were selected from the PGDB as it contains assembled and annotated *Pseudomonas* genomes. The genome sequences of isolates from a variety of human and animal infections in addition to environmental strains were chosen to ensure diversity amongst the genomes Table 2.4

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Isolate	ST	Source		Assembly Accession
PAO1	549	Human	Wound	GCF_000006765.1*
UCBPP-PA14	253	Human	Burn wound	GCF_000014625.1*
PA7	1195	Human	Non-respiratory clinical isolate	GCF_000017205.1
VA-134	3090	Human	Burn wound	GCF_001447845.1*
PA_D22	1971	Human	Ventilator-associated pneumonia	GCF_001721825.1*
Pa124	309	Human	Ventilator-associated pneumonia	GCF_002192475.1*
CCBH4851	277	Human	Catheter	GCF_000763245.3*
RP73	198	Human	Cystic fibrosis	GCF_000414035.1*
Pa1207	155	Human	Blood	GCF_002208645.1*
BAMC 07-48	313	Human	Combat injury wound	GCF_001632245.1*
FRD1	111	Human	Cystic fibrosis	GCF_000829885.1*
PA34	1284	Human	Keratitis	GCF_003332705.2*
DH01	395	Human	Nose (asymptomatic carrier)	GCF_000496455.2*
X78812	257	Human	Cancer	GCF_001542795.2*
W16407	244	Human	Cancer	GCF_001516165.2*
VRFPA04	-	Human	Keratitis	GCF_000473745.2*
24Pae112	235	Human	Blood	GCF_003433235.1*
2856	111	Human	Tracheal aspirate	GCF_028403965.1*
NY5510	1976	Human	Phlegm	GCF_030121895.1*
YT12746	292	Human	Blood	GCF_013305645.2*
F22031	485	Human	Pubic bone (cancer)	GCF_000816985.1*
L1a	549	Human	Sputum	GCF_022213165.2*
PB350	235	Human	Sputum	GCF_002812905.2*
97	234	Human	Urine	GCF_002411865.3*

BWHPSA016	2317	Human	Ear	GCF_000481385.1
20/27	27	Human	Chronic otitis media	GCF_028895265.1
019	446	Human	Otitis externa	GCF_016792065.1
2021CK-01162	309	Human	Abscess	GCF_029961325.1*
PALA36	532	Human	Cystic Fibrosis	GCF_027571115.1*
MRSN1612	207	Human	Ear	GCF_003969725.1
BK2	2719	Human	Keratitis	GCF_002243265.1
AZPAE14828	1858	Human	Intra-abdominal tract infection	GCF_000795385.1
LIM1136	277	Human	Blood	GCF_003204365.1
PB353	-	Human	Urine	GCF_002812865.1*
LES431	146	Human	Healthy carrier	GCF_000508765.1*
DK2	386	Human	Cystic fibrosis	GCF_000271365.1*
PACS2	1394	Human	Cystic fibrosis	GCF_000168335.1*
F23197	1295	Human	Cancer	GCF_001516245.2*
PA7790	277	Human	Tracheal aspirate	GCF_001870265.1*
USDA-ARS-USMARC-41639	-	Cow	Respiratory Disease	GCF_001518975.1*
K19PSE24	313	Dog	Ear	GCF_013201015.1
AUS207	145	Koala	Ear	GCF_003839865.1
PSE305	244	Sheep	Mastitis	GCF_000750905.1
19660	-	Mouse	Ocular infection	GCF_000481765.1
KCJK8014	2853	Coyote	Missing	GCF_003053445.1
ICB10P	644	Magellanic penguin	Pododermatitis	GCF_002156655.1
VET-33	2450	Cow		GCF_003628935.1
VET-36	2450	Cat	Ear	GCF_003628985.1
CA19603	235	Cat	Ear	GCF_014596275.1
PA59	260	Mink	Hemorrhagic pneumonia	GCF_009497675.1

UFMG-H10	549	Zebu	Urine	GCF_012102115.1
OSIRIS	146	Cat	Upper Resp Tract	GCF_013619435.1
FMDP001	1249	Forest musk deer	Lung	GCF_007896855.1
BCW_7428	-	Dolphin	Rectum	GCF_009916345.1
VET-58	1744	Cow	Internal Organs	GCF_003633515.1
B-20-37098-1-1	2683	Cow	Unknown	GCF_025791395.1
CA12133	1600	Dog	Ear	GCF_015704805.1
AUS456	179	Goat	Milk	GCF_003976335.1
A237	1207	Leporidae	Unknown	GCF_003836985.1
3K-6	-	Env		GCF_025209925.1
WH-SGI-V-07166	1990	Env		GCF_001449485.1
S-8	381	Env		GCF_028970225.1
PDNC003	-	Env		GCF_016919425.1
F1-DN-2	-	Env		GCF_003833465.1
C41	164	Env		GCF_000480455.1
C20	17	Env		GCF_000480515.1
M8A.4	918	Env		GCF_000480555.1
M8A.1	257	Env		GCF_000480615.1
PB25	-	Env		GCF_025209735.1
PA1-1	27	Env		GCF_026624905.1

Table 2.4 **Additional *P. aeruginosa* genomes used for bioinformatics analysis.**

P. aeruginosa genomes (n=70) were selected from the *Pseudomonas* genome database from human, animal, and environmental (Env) sources. - denotes unknown sequence type * - denotes a complete genome assembly

2.22.2 Identification of resistance, virulence genes and mutations

Antibiotic resistance genes were screened for using ABRicate v1.0.1 (Seemann, 2015) with the CARD database (Alcock et al., 2023). Numerous databases are available as a result of the rise in high throughput sequencing used to test for genes associated with antibiotic resistance. CARD was chosen because it has a vast amount of gene sequences and can detect mutations more effectively than comparable methods (Papp & Solymosi, 2022).

The presence of virulence genes was assessed using ABRicate v1.0.1 with the VFDB database (Liu et al., 2021a). To avoid repeat analysis, genes recognised to be related to alginate biofilm formation were removed as they are investigated below (§2.22.3). Percentage coverage was then adjusted to analyse presence and absence of genes.

To investigate the presence of mutations that confer resistance, the amino acid sequences of genes of interest were extracted using a custom python script (Figure 8.1). Subsequently, *P. aeruginosa* PAO1 reference genes were downloaded from the PSDB. The amino acid sequences of each gene were then aligned using the ClustalW algorithm (Larkin et al., 2007) in MEGA v11.0.13 (Tamura, Stecher & Kumar, 2021) and amino acid substitutions and deletions were recorded.

2.22.3 Identification of plasmids

Plasmids were assembled from the whole genome sequencing reads using Plassembler v1.3.0 (Bouras et al., 2023b) with default settings. Where the plasmid was assembled as a single contig, the sequence was reoriented to begin with *repA* using Dnaapler. Subsequently, the plasmid sequence were investigated for antimicrobial resistance and virulence genes using ABRicate as described above (§2.22.2).

2.22.4 Identification of biofilm associated genes

A custom database using gene sequences from *P. aeruginosa* PAO1 (NC_002516.2), downloaded from NCBI, was created as described by (Seemann, 2015) using genes located within the *psl*, *pel* and alginate (*alg*) operons (Franklin et al., 2011) in addition to other associated biofilm genes (Kiel et al., 2022; Liu et al., 2021a). A complete list of the genes is shown in Table 2.5.

Gene	**Locus Tag**	**Description**
pelA	PA3064	hypothetical protein
pelB	PA3063	pellicle/biofilm biosynthesis protei
pelC	PA3062	pellicle/biofilm biosynthesis outer membrane protein
pelD	PA3061	pellicle/biofilm biosynthesis protein
pelE	PA3060	pellicle/biofilm biosynthesis protein
pelF	PA3059	pellicle/biofilm biosynthesis glycosyltransferase
pelG	PA3058	pellicle/biofilm biosynthesis Wzx-like polysaccharide transporter
pslA	PA2231	biofilm formation protein PslA
pslB	PA2232	biofilm formation protein PslB
pslC	PA2233	biofilm formation protein PslC
pslD	PA2234	biofilm formation protein PslD
pslE	PA2235	biofilm formation protein PslE
pslF	PA2236	biofilm formation protein PslF
pslG	PA2237	biofilm formation protein PslG

pslH	PA2238	biofilm formation protein PslH
pslI	PA2239	biofilm formation protein PslI
pslJ	PA2240	biofilm formation protein PslJ
pslK	PA2241	biofilm formation protein PlsK
pslL	PA2242	biofilm formation protein PslL
pslM	PA2243	bFAD-binding dehydrogenase
pslN	PA2244	hypothetical protein
algD	PA3540	GDP-mannose 6-dehydrogenase AlgD
alg8	PA3541	glycosyltransferase alg8
alg44	PA3542	alginate biosynthesis protein Alg44
algK	PA3543	alginate biosynthesis protein AlgK
algE	PA3544	alginate production protein AlgE
algG	PA3545	alginate-c5-mannuronan-epimerase AlgG
algX	PA3546	alginate biosynthesis protein AlgX
algL	PA3547	alginate lyase
algI	PA3548	alginate o-acetylase AlgI
algJ	PA3549	alginate o-acetylase AlgJ
algF	PA3550	alginate o-acetyltransferase AlgF
algA	PA3551	bifunctional mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase
mvfR	PA1003	transcriptional regulator MvfR
pqsA	PA0996	anthranilate--CoA ligase
pqsB	PA0997	hypothetical protein

pqsC	PA0998	hypothetical protein
pqsD	PA0999	3-oxoacyl-ACP synthase
pqsE	PA1000	thioesterase PqsE
algU	PA0762	RNA polymerase sigma factor AlgU
mucA	PA0763	sigma factor AlgU negative regulator MucA
mucB	PA0764	sigma factor AlgU regulator MucB
mucC	PA0765	positive regulator for alginate biosynthesis MucC
mucD	PA0765	serine protease MucD
mucP	PA3649	metalloprotease protease
mucE	PA4033	small envelope protein MucE
algW	PA4446	AlgW protein
algP	PA5253	alginate regulatory protein AlgP
algQ	PA5255	anti-RNA polymerase sigma 70 factor
algR	PA5261	alginate biosynthesis regulatory protein AlgR
algZ	PA5262	alginate biosynthesis protein AlgZ/FimS
algC	PA5322	phosphomannomutase
algB	PA5483	two-component response regulator AlgB

Table 2.5 **Genes that are associated with biofilm formation and were investigated in the present study.**

2.22.5 Phylogeny

A neighbour joining phylogenetic tree was constructed using Mashtree v1.4.6 (Katz et al., 2019) with bootstrapping with 100 replicates and mindepth 0. Trees were then visualised using MEGA.

2.22.6 Identification of CRISPR-Cas arrays

CRISPRCasFinder (Couvin et al., 2018) was performed to identify CRISPR arrays and Cas proteins using default settings.

2.22.7 Prediction of prophage sequences

The presence of prophage regions in the genome sequences was predicted using PHASTEST (Wishart et al., 2023) with default settings.

2.22.8 Identification of predation-inhibitory products

Bakta annotation produced GFF3-files which were used in combination with Roary v3.13.0 (Page et al., 2015) to create a gene presence and absence file for the 35 *P. aeruginosa* genomes which was used to identify genes known to encode for products that inhibit predation (Mun et al., 2017; Hoshiko et al., 2021).

Subsequently, the Roary analysis was paired with a trait table containing sensitivity to predation in the liquid predation assay (§2.16), and used to calculated genes that were overrepresented in the groups with Scoary (-p 5E-2 -c I --no_pairwise) v1.6.16 (Brynildsrud et al., 2016).

2.23 Whole genome analysis of bacteriophage

Taxonomic classification at the genus and species level was performed using taxmyPHAGE v0.2.7 (Millard, 2023). Bacteriophage lifestyle predictions were performed using three tools, PhaTYP (Shang, Tang & Sun, 2023), BACPHLIP v1.0

(Hockenberry & Wilke, 2021) and PhageScope (Wang et al., 2024). Intergenomic similarities were performed using VIRIDIC (Moraru, Varsani & Kropinski, 2020). Finally, a proteomic tree was generated using ViPTree (Nishimura et al., 2017).

2.24 Statistical analysis

Statistical analysis was performed using Python 3.8.5 and the SciPy library version 1.9.1. Results were first assessed for normality by first plotting a histogram of the data followed by a Shapiro-Wilk test. This was followed by a T-test or ANOVA (parametric data), or Mann-Whitney U or Kruskal-Wallis test (non-parametric data). The type of statistical test used is stated where appropriate.

2.24.1 Principal Component Analysis

Principal component analysis (PCA) was performed using Scikit-learn (Pedregosa et al., 2011) with AST data of isolates from the UK and Denmark. Ticarcillin/Clavulanic Acid discs were discontinued part way though this work and so some isolates were missing this data and so this antibiotic was removed from the analysis. Following this, the data was normalised using StandardScaler. PCA was then performed by linear dimensionality reduction using Singular Value Decomposition.

2.24.2 Cluster analysis

Hierarchical clustering analysis was performed using the results from six experiments, namely the crystal violet biofilm and swarming motility assays, the total number of resistances to the tested antibiotics, predation by *B. bacteriovorus* on YPSC agar and in YPSC broth and finally bacteriophage host range. Hierarchical clustering was performed using Euclidean distance with Ward linkage, both of these metrics were selected as are widely considered the most appropriate in most cases. A dendrogram that shows the clusters' merging or breaking patterns was used to calculate the optimal

number of clusters. Where the data was categorical, such as predation by *B. bacteriovorus* on YPSC double agar or in YPSC broth, ordinal encoding was used to encode categorical data to numbers.

Chapter 3 Characterisation of *Pseudomonas aeruginosa* Isolated from Canine Otitis Externa

3.1 Introduction

Canine otitis externa (OE) is a common condition seen in veterinary practices, a recent study in the UK identified that canine OE was a frequent diagnosis (7.3%), second only to periodontal disease (12.52%) (O'Neill DG, 2021). Canine OE is a multifactorial disease; however, cases are often only seen after an increase in clinical signs due to secondary infection. In some cases of chronic OE, the disease may progress to a point where medical treatment will be unsuccessful in controlling infection. In such cases, biofilm production in the presence of a multi-drug-resistant *Pseudomonas* infection are often evident. At this stage, surgery is necessary, including total ear canal ablation with or without bulla osteotomy. This procedure involves the removal of the infected ear canal and bulla contents, resulting in the resolution of disease in most cases (Doyle RS, 2004).

P. aeruginosa is not a typical constituent of the canine ear microbiota but is frequently isolated from cases of chronic OE in up to 35.5% of cases (Budgen DL, 2013). Despite this there are only a limited number of studies investigating isolates of *P. aeruginosa* from cases of canine OE. The nature of this pathogen often makes treatment difficult; biofilm formation is identified in 40–95% of *P. aeruginosa* from cases of OE (Pye CC, 2013; Robinson VH, 2019; Chan WY, 2019) and intrinsic and acquired antibiotic resistance, especially resistance to clinically important antibiotics, highlights the need for alternative treatments. Very few studies have examined the genomic profiles of *P. aeruginosa* strains causing canine otitis specifically, however the population structure of *P. aeruginosa* causing clinical infections in animals consists of genetically diverse isolates. This highlights the need to investigate this pathogen further, with a focus on bioinformatic analysis.

3.1.1 Aims and Hypothesis

The aim of this chapter was to identify factors that reduce the success of treatment in a clinical setting. Additionally, to use bioinformatics to get a better understanding of *P. aeruginosa* isolates from cases of canine otitis externa.

1. Hypothesis: **Clinical *Pseudomonas aeruginosa* isolates from canine OE will form biofilms under the tested conditions and have resistance to antibiotics commonly used in veterinary medicine.** Biofilm will be quantified using two methods and antibiotic resistance assessed using a panel of antibiotics important for *P. aeruginosa* infections in veterinary medicine. Subsequent genomic analysis will be utilised to further characterise these important virulence factors.
2. Hypothesis: **Clinical *P. aeruginosa* from canine OE will be genetically diverse.** Analysis of the genomes of clinical *P. aeruginosa* will be performed and compared to a selection of other isolates from human, animal and environment sources.

3.2 Results

3.2.1 Strain collection

A collection of 253 clinical isolates from cases of canine OE collected in veterinary practices from the UK (88), Denmark (103), Dubai (2), Malta (1) and unknown (59) were previously identified by Anette Loeffler (Royal Veterinary College), Arshnee Moodley (University of Copenhagen), and Stephen Steen (NationWide Laboratories) using growth on cetrimide agar, the oxidase test and 16s ribosomal RNA (rRNA) amplification (Table 8.1).

In addition to clinical isolates four sublines of *P. aeruginosa* PAO1 were included in this study. *P. aeruginosa* PAO1-QUB (PAO1-QUB) previously existed within the lab group and originates from Queens University Belfast. The other sublines used in the present study were selected as they have been previously characterised and sequenced. *P. aeruginosa* PAO1 subline Lausanne (PAO1-L) originally from the Dieter Haas laboratory, Washington (PAO1-UW), maintained at the University of Washington, and the University of Nottingham (PAO1-N).

3.2.2 Antimicrobial sensitivity testing

3.2.2.1 Selection of antimicrobials

Antibiotics were selected based on clinical relevance for the treatment of dogs with *P. aeruginosa* infections seen in the veterinary practice (Morris, 2004; Nuttall & Cole, 2007). This was established using CLSI Vet (CLSI, 2018), presence in the literature (Arais et al., 2016; Dégi et al., 2021; Mekić, Matanović & Šeol, 2011) and personal communication with Dr Stephen Shaw.

3.2.2.2 Clinical *P. aeruginosa* isolates show varied antibiotic resistance

P. aeruginosa canine otitis isolates were tested for resistance to 14 antibiotics representing 6 different classes (Table 2.2). One strain, 29130, failed to grow under the tested conditions and was excluded from this assay. The greatest level of resistance was recorded for Enrofloxacin (25.4%; 64/252) while Tobramycin showed the highest sensitivity (99.6%; 251/252). Intermediate resistance was mainly seen against Ticarcillin + Clavulanic acid (60.7%; 127/252 of strains tested) (Figure 3.1). Importantly, 8.3% (21/252) and 0.79% (2/252) of isolates were resistant to imipenem and meropenem respectively.

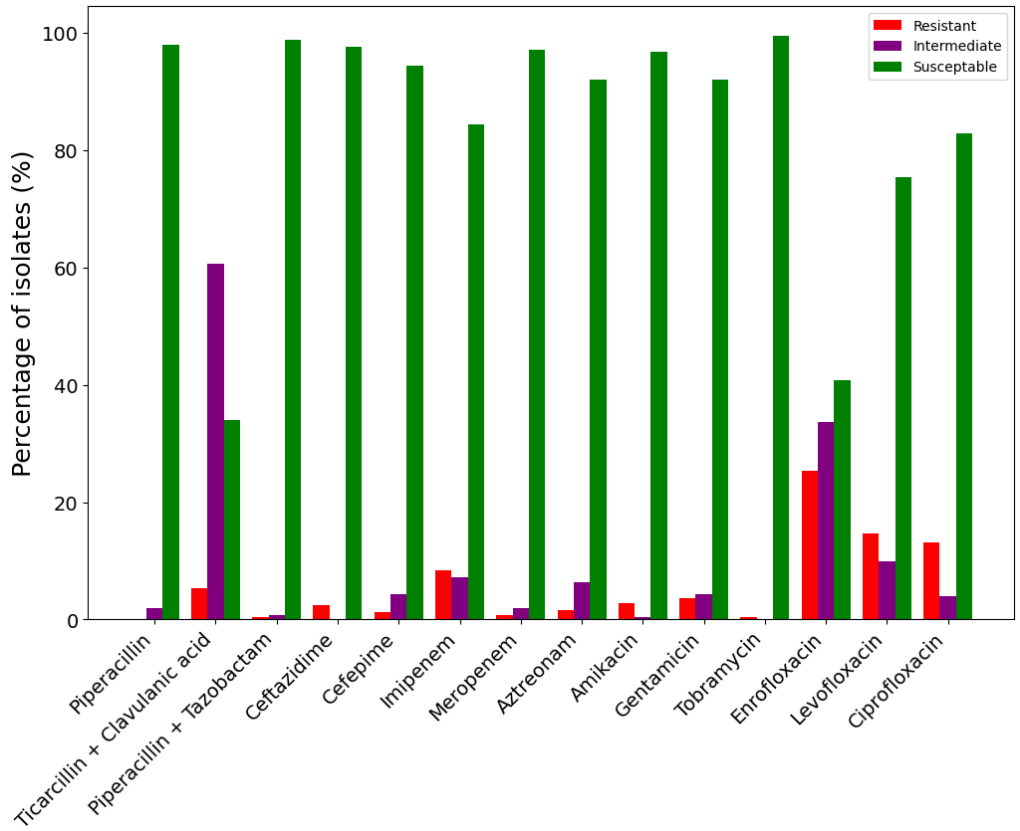


Figure 3.1 **Percentage of isolates susceptibility to 14 antibiotics.**

Antimicrobial susceptibility of 252 isolates of *P. aeruginosa* from clinical cases of canine otitis extra was determined using the disc diffusion method as described by the Clinical & Laboratory Standards Institute. Red bars indicate resistance, purple intermediate resistance and green sensitive.

Of the 252 isolates tested, 65% (164/252) of the isolates were susceptible to all of the tested antibiotics. The remaining 35% (88/252) were resistant to at least one antibiotic. Moreover, 1.6% (4/252) were classified as multidrug resistant (Figure 3.2), multidrug resistance was determined by resistance to three or more antibiotic classes as previously described (Sweeney et al., 2018).

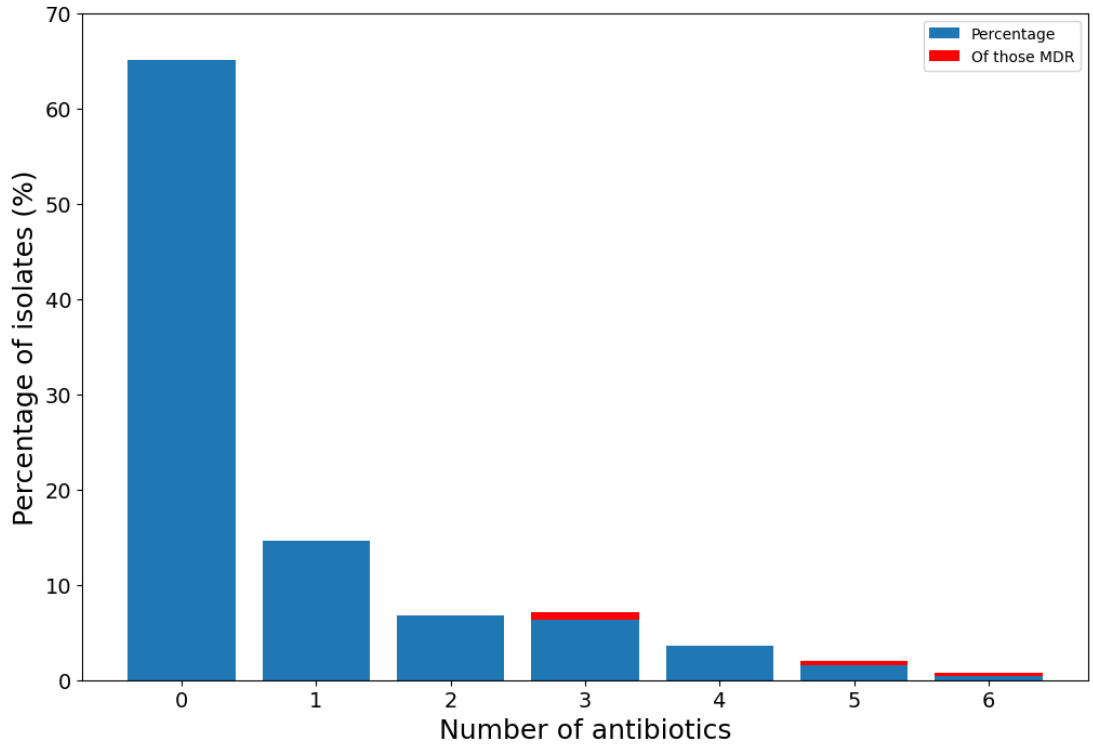


Figure 3.2 **Percentage of *P. aeruginosa* isolates from canine otitis extra resistant to multiple antibiotics.**

Bar chart identifying the percentage of isolates resistant to a different number of antibiotics as determined by the disc diffusion method. Multidrug resistance is reported for resistance to three or more classes, strains found to meet this criterion are noted in red.

The relationship between resistance and origin of isolation was investigated by comparing the number of antibiotics strains from the UK and EU were resistant to using a Mann-Whitney U test. Strains for which the source of isolation was unknown were excluded. Due to Ticarcillin + Clavulanic acid being discontinued part way though this work, several European isolates were not tested with this antibiotic and so it was also removed from the test. This resulted in 88 isolates from the UK and 103 isolates from the EU with a mean number of resistances per isolate being 0.52 and 0.95 respectively; the distributions of these two groups differed significantly ($p=0.0164$).

To further investigate this, Principal Component Analysis (PCA) was performed based on the size of the zone of clearing for each antibiotic. The results for Ticarcillin +

Clavulanic acid was excluded as PCA does not allow missing values. The results shown in Figure 3.3 show two components which account for 60.1% of the variation of the original data. Principal component 1 explaining 40.9% of the total variation separates isolates based on their sensitivity to all antibiotics - isolates with the largest zones of clearing and therefore the most sensitive migrate to the left of the graph. The UK isolates cluster more towards the left-hand side of the graph suggesting they are more susceptible to all antibiotics. The loadings for each antibiotic across both principal components range from 0.45 to -0.30 indicating that a lot of antibiotics played a role in separating the samples rather than just one or two. Similarly, there are no clusters from a particular location for any individual antibiotic.

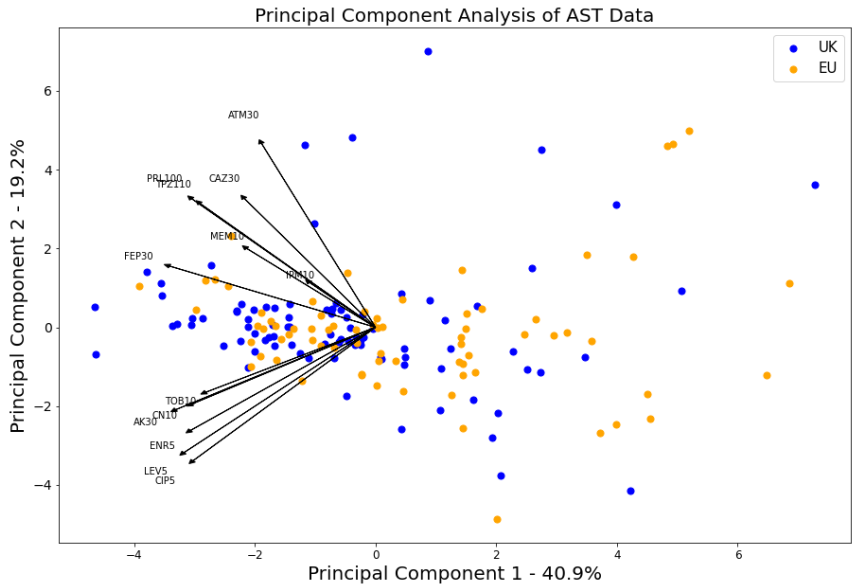


Figure 3.3 **Principal component analysis of antimicrobial sensitivity data.**

Principal component analysis was performed using AST data for UK (n=88) and EU (n=103) isolates. The first and second principal components explain 40.9% and 19.2% of the total variation respectively.

3.2.2.3 Presence of antimicrobial resistance genes

The genomes of clinical *P. aeruginosa* isolates from canine OE were screened for the presence of antimicrobial resistance genes using the CARD database (§0).

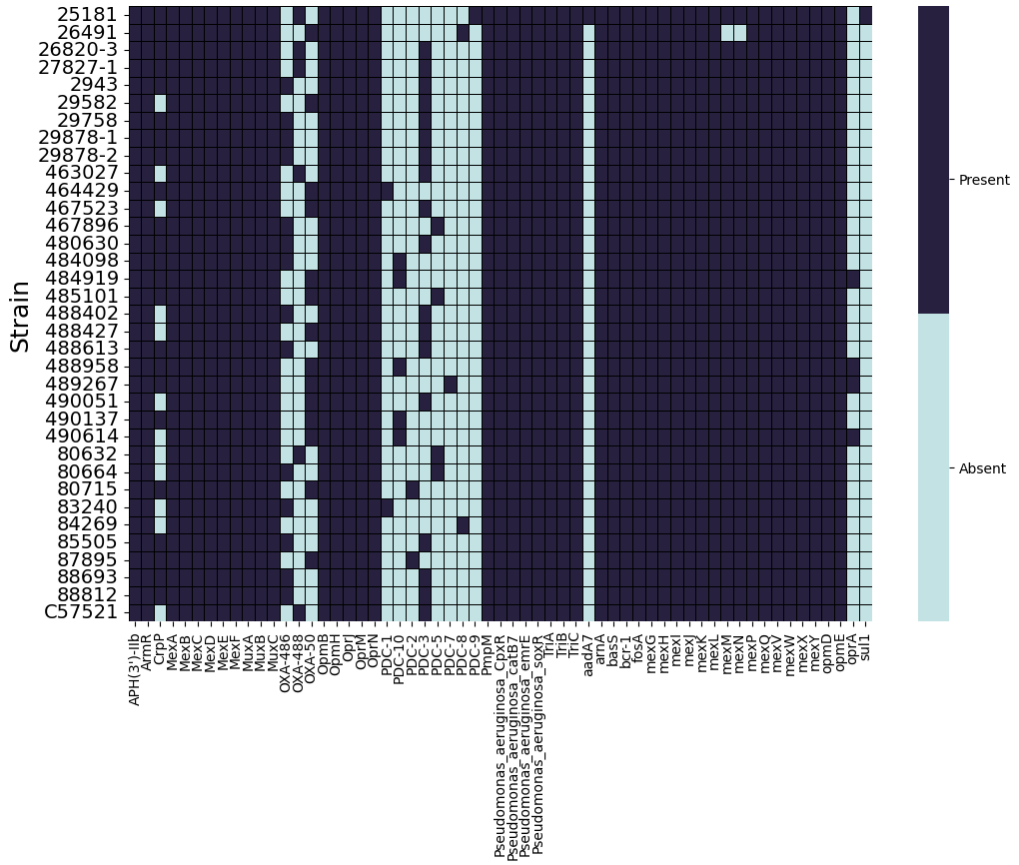


Figure 3.4 **Presence and absence of antimicrobial resistance genes**.

A selection of 35/253 isolates of *P. aeruginosa* from clinical cases of canine otitis externa were selected for whole genome sequencing. The genomes were subsequently screened for the presence of antimicrobial resistance genes using the CARD database.

The results shown in Figure 3.4 are interesting as they show that the presence of resistance genes is largely the same across the isolates despite having variable phenotypic susceptibility. The main exceptions being *mexM* and *mexN* being absent from *P. aeruginosa* 26491 and *aadA7* plus *sul1* being present in *P. aeruginosa* 25181. A summary of the genes identified can be found in Table 3.1.

Product	**Gene(s) and prevalence (%)**	**Resistance**
aminoglycoside 3'-phosphotransferase type IIb (APH(3')-IIb)	*aph* (100%)	Chromosomal aminoglycoside phosphotransferase that provides resistance to kanamycin A and B, neomycin B and C, butirosin and seldomycin F5 (Hächler, Santanam & Kayser, 1996).
MexAB-OprM	*mexA* (100%), *mexB* (100%), *oprM* (100%), *armR* (100%), *cpxR* (100%)	MexAB-OprM is a constitutively expressed efflux pump providing intrinsic resistance (Aeschlimann, 2003).
MexCD-OprJ	*mexC* (100%), *mexD* (100%), *oprJ* (100%)	MexCD-OprJ mutations that give rise to overexpression can result in resistance to several classes of antibiotics (Jeannot et al., 2008).
MexEF-OprN	*mexE* (100%), *mexF* (100%), *OprN* (100%)	Generally inactive under normal conditions, can cause resistance when overexpressed (Köhler et al., 1997).
MuxABC-OpmB	*muxA* (100%), *muxB* (100%), *muxC* (100%), *opmB* (100%)	MuxABC-OpmB contributes to the intrinsic resistance of *P. aeruginosa*, mutations resulting in overexpression produce an increased resistance phenotype (Mima et al., 2009).
TriABC-OpmH	*triA* (100%), *triB* (100%), *triC* (100%), *opmH* (100%)	TriABC-OpmH, another efflux pump, seems to only provide resistance to triclosan when overexpressed due to mutations (Mima et al., 2007).
MexGHI-OpmD	*mexG* (100%), *mexH* (100%), *mexI* (100%),	The efflux pump MexGHI-OmpD has been shown to confer resistance to some antibiotics but also appears to be important for quorum sensing (Aendekerk et al., 2005).

	opmD (100%), *soxR* (100%)	
MexJK	*mexJ* (100%). *mexK* (100%), *mexL* (100%)	Similarly, MexJK seems to be primarily involved in quorum sensing and not antimicrobial resistance (Amieva et al., 2022).
MexMN-OprM	*mexM* (97.14%), *mexN* (97.14%), *oprM* (100%)	Multidrug efflux pumped expressed silently or in low levels in wild type *P. aeruginosa* but provides a resistance phenotype when expressed (Mima et al., 2005).
MexPQ-OpmE	*mexP* (100%), *mexQ* (100%), *opmE* (100%)	Multidrug efflux pumped expressed silently or in low levels in wild type *P. aeruginosa* but provides a resistance phenotype when expressed (Mima et al., 2005).
MexVW-OprM	*mexV* (100%), *mexW* (100%), *oprM* (100%)	Similar to the other efflux pumps, expression of MexVW-OprM in wild type *P. aeruginosa* is silent or weak but overexpression results in resistance to multiple classes of antibiotics (Li et al., 2003).
MexXY-OprM	*mexX* (100%), *mexY* (100%), *oprM* (100%)	MexXY-OprM efflux pump important for intrinsic resistance to aminoglycosides. Similar to the other efflux pumps, overexpression leads to a greater resistance phenotype (Aires et al., 1999).
PmpM	*pmpM* (100%)	PmpM is a H^+ drug antiporter providing resistance to multiple classes of antibiotics (He et al., 2004).
CatB7	*catB7* (100%)	Chromosomal chloramphenicol acetyltransferase (White et al., 1999).
EmrE	*emrE* (100%)	Constitutively expressed efflux transporter that provides intrinsic resistance to aminoglycosides but also ethidium bromide and acriflavine (Li, Poole & Nikaido, 2003).
ArnA	*arnA* (100%)	Modifies lipid A with 4-amino-4-deoxy-L-arabinose (Ara4N) which provides resistance to cationic antimicrobial peptides and antibiotics such as polymyxin (Gatzeva-Topalova, May & Sousa, 2005).

BasS	*basS* (100%)	
Bcr-1	*bcr1* (100%)	Transmembrane protein that removes bicyclomycin from the cell (Fonseca et al., 2015).
FosA	*fosA* (100%)	Metalloglutathione transferase which inactivates Fosfomycin (Beharry & Palzkill, 2005).
CrpP	*crpP* (65.71%)	Phosphotransferase with unknown function, first thought to confer resistance to ciprofloxacin (Chávez-Jacobo et al., 2018) but later disproven (Zubyk & Wright, 2021).
OXA-486, OXA-488, OXA-50	40%, 17.14%, 40%	OXA-50 is a constitutively expressed oxacillinase gene that provides resistance to ampicillin, ticarcillin and other β-lactams (Girlich, Naas & Nordmann, 2004).
PDC-1, PDC-2, PDC-3, PDC-5, PDC-7, PDC-8, PDC-9, PDC-10	5.71%, 5.71%, 51.43%, 11.43, 2.86%, 5.71%, 2.86%, 14.29%	Chromosomal AmpC variations identified by (Rodríguez-Martínez, Poirel & Nordmann, 2009).
aadA7	2.68%	Aminoglycoside nucleotidyltransferase that confers resistance to gentamicin and sisomicin (Ahmed et al., 2004).
MexXY-OprA	*mexX* (100%), *mexY* (100%), *oprA* (11.43%)	Some *P. aeruginosa* contain *oprA* which can form a complex with MexXY providing more resistances (Singh et al., 2020).
Sul1	2.86%	Sulfonamide resistant dihydropteroate synthase (Domínguez et al., 2019).

Table 3.1 **Prevalence of antimicrobial resistance genes and a summary of their function.**

A selection *P. aeruginosa* from clinical cases of canine otitis externa were selected for whole genome sequencing. The genomes were subsequently screened for the presence of antimicrobial resistance genes using the CARD database. Above shows a summary of the findings including the prevalence (%) of each gene.

3.2.2.4 Identifying mutations in DNA Gyrase, DNA Topoisomerase and *fusA1* genes

Strain	*gyrA*	*gyrB*	*parC*	*parE*	*fusA1*	Enr	Lev	Cip	Ak	Cn	Tob
80664	T83I, S912_E913del, A916T				I186V	R	I	S	S	S	S
87895	T83I, S912_E913del		S87L, H262Q		I186V, Q678L	R	R	R	S	S	S
88812	T83I, D87H		S87L, E91K		A595V, M648V	R	R	R	R	R	S
484919	T83I, S912_E913del	T235S, E239Q, L268I, I529V, A627V	S331T, I388V, A528T	D533E		R	R	R	S	I	S
488958	T83I, S912_E913del	T235S, E239Q, L268I, I529V, A627V	S331T, I388V, A528T	D533E	T671A	R	R	R	R	R	R
489267	T83I, S912_E913del	T235S, E239Q, L268I, S466F, I529V, A627V	S87L, S331T, I388V, A528T	D533E		R	R	R	S	S	S
490614	D87N, S912_E913del	T235S, E239Q, L268I, I529V, A627V	I388V, A528T, P752A	D533E		R	I	S	S	S	S
29582	I380L		D754N	H50Y, I57V		R	I	S	S	S	S
2943	D652Y	S466F				R	R	R	S	S	S
29758	D652Y	S466F				R	R	R	S	S	S

29878-1	D652Y	S466F			M1_R137del, I138M, V139F, Y140P, V141I, N142L	R	I	S	S	S	S
29878-2	D652Y	S466F				R	R	I	S	S	S
490051	E783D		H262Q			S	S	S	S	S	S
488402	T847A, S912_E913del					I	S	S	S	S	S
25181	S912_E913del			D533E		S	S	S	S	S	S
26491	S912_E913del			D533E		R	I	S	S	S	S
88693	S912_E913del		V646L			S	S	S	S	S	S
467896	S912_E913del					S	S	S	S	S	S
485101	S912_E913del		G634S	D533E		I	S	S	S	S	S
488613	S912_E913del			A473V, D533E		I	S	S	S	S	S

490137	S912_E913del			A473V, D533E		I	S	S	S	S	S
484098			V238I	D533E		S	S	S	S	S	S
488427			H262Q			S	S	S	S	S	S
85505			V646L	D533E		I	S	S	S	S	S
26820-3			V646L			S	S	S	S	I	S
80715				D533E		I	S	S	S	S	S
27827-1				D533E		I	S	S	S	S	S
84269						R	I	S	S	S	S
83240						I	I	S	S	S	S
80632						I	S	S	S	S	S
463027						S	S	S	S	S	S
464429						S	S	S	S	S	S

480630						S	S	S	S	S	S
C5752-1						I	S	S	S	S	S

Table 3.2 **Amino acid substitutions and deletions of *gyrA*, *gyrB*, *parC*, *parE* and *fusA1*.**

The amino acid sequences of five genes were extracted from the genome sequences of 35 *P. aeruginosa* from cases of canine otitis externa using a custom python script (§8.1.2). The amino acid sequence of each gene was compared to PAO1 by alignment using MEGA and compared to the resistance profile from the disc diffusion assay. Amino acids are reported using standard single letter abbreviations. Enr – Enrofloxacin, Lev – Levofloxacin, Cip – Ciprofloxacin, Ak – Amikacin, Cn – Gentamicin, Tob – Tobramycin, R – Resistant, I – Intermediate, S – Susceptible.

Development of resistance through the acquisition of mutations is one of the main drivers of resistance in *P. aeruginosa* (López-Causapé et al., 2018). In order to get a better understanding of the resistance patterns observed in the clinical *P. aeruginosa* isolates, and given the high proportion of resistance to fluoroquinolones the amino acid sequence of *gyrA*, *gyrB*, *parC* and *parE* was investigated for quinolone resistance-determining region (QRDR) mutations and compared to *P. aeruginosa* PAO1. The same was then performed for *fusA1* to explain aminoglycoside resistance.

QRDR mutations were identified in almost all of the isolates that were resistant to at least one fluroquinolone antibiotic with the exception of 84269 that was resistant to enrofloxacin but did not contain a QRDR mutation. However, QRDR mutations were also identified in isolates susceptible to all of the investigated fluoroquinolones suggesting these mutations do not play a role in resistance. An example of this is V646L in *parC*, despite having a moderate prevalence 11.4% (4/35), all of the isolates in which this mutation was seen were sensitive to all of the tested fluroquinolones.

Looking at genes individually, *gyrA* was the most likely to have a mutation with 65.7% (23/35) of the sequenced *P. aeruginosa* having at least one mutation. Interestingly, in 40% (14/35) of the sequenced strains studied a deletion was identified (S912_E913del). The mutation T83I was the most common amongst strains resistant to at least one fluroquinolone. S466F, S87L/S331T and D533E were the most common mutations for *gyrB, parC, parE* respectively.

As for *fusA1*, fewer mutations were seen overall with only 11.4% (4/35) of the sequenced isolates containing a mutation in this gene, of these, two showed resistance to at least one of the tested aminoglycosides. Strain 488958, which was resistant to all of the tested aminoglycosides, contained T671A. Similarly, 88812 was resistant to amikacin and gentamicin and contained A595V and M648V.

3.2.2.5 PAO1 sublines show different resistance profiles

The antibiotic resistance patterns of four *P. aeruginosa* PAO1 sublines was investigated as described above. PAO1-QUB and *P. aeruginosa* PAO1-L (PAO1-L) were sensitive to all the tested antibiotics, while *P. aeruginosa* PAO1-N (PAO1-N) was resistant to imipenem and meropenem and *P. aeruginosa* PAO1-UW (PAO1-UW) was resistant to imipenem and enrofloxacin and showed intermediate resistance to levofloxacin (Figure 3.5).

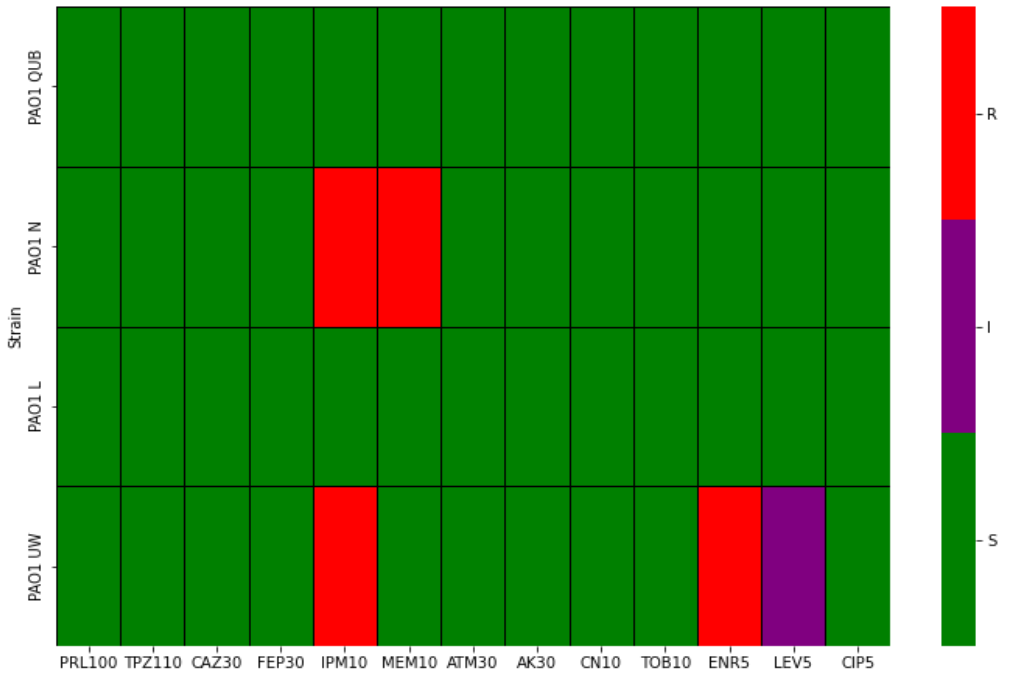


Figure 3.5 **Antimicrobial susceptibility of *P. aeruginosa* PAO1 sublines.**

Shown are the susceptibility patterns of three common lab sublines *P. aeruginosa* PAO1-L, PAO1-N and PAO1-UW, in addition to *P. aeruginosa* PAO1-QUB to 13 antibiotics using the disc diffusion method. Red indicates resistance, purple intermediate resistance and green sensitive as described by the CLSI.

3.2.3 Biofilm-forming ability of *P. aeruginosa* isolates

Clinical isolates of *P. aeruginosa* were tested for their ability to form a biofilm *in vitro* (§1.1). In total, 82% (207/253) of strains produced strong levels of biofilm, 9% (23/253) produced moderate biofilm, 5% (13/253) produced weak biofilm and 4% (10/253) made no quantifiable biofilm under the tested conditions (Figure 3.6; Figure 8.2). Additionally, each strain as was compared to PAO1-QUB. Strain 29051 produced the most biofilm followed by 464009(1) which produced 181% and 173% more biofilm on average than PAO1 respectively.

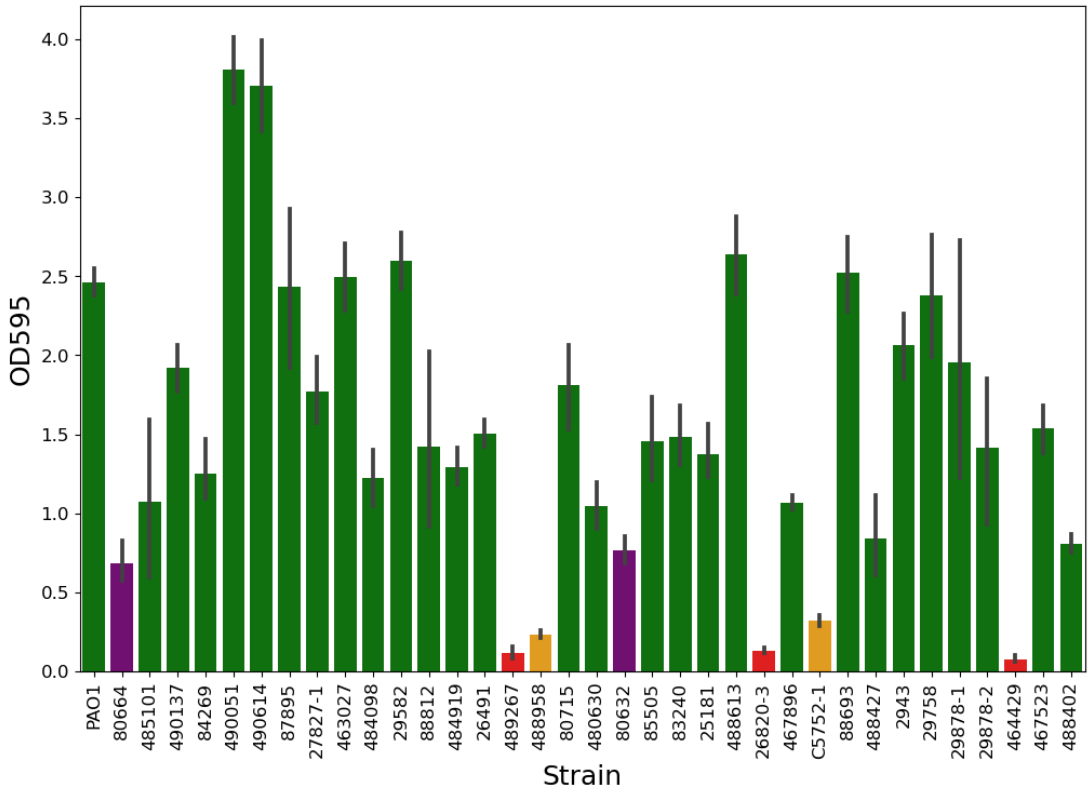


Figure 3.6 **Biofilm formation of *P. aeruginosa* from canine otitis externa.**

The biofilm forming ability of 253 *P. aeruginosa* strains was tested in a crystal violet, 96 well plate assay, a representative subset of 35, which were selected for whole genome sequencing, are shown here. Green – Strong biofilm producing - Purple – Moderate biofilm producing - Orange – Weak biofilm producing - Red – No quantifiable biofilm. The values here are from three biological and six technical repeats represented by the mean with 95% confidence interval.

3.2.3.1 Comparison of biofilm formation of PAO1 sublines

The *in vitro* biofilm forming ability of different *P. aeruginosa* PAO1 sublines was assessed as described above (Figure 3.7). All of the isolates were strong biofilm formers. Each strain produced a similar amount of biofilm under the tested conditions however a one-way Welch's ANOVA test revealed a statistically significant difference in biofilm formation ($p<0.0001$). Dunnett's T3 multiple comparisons test found that all the groups were statistically significantly different (Figure 8.3).

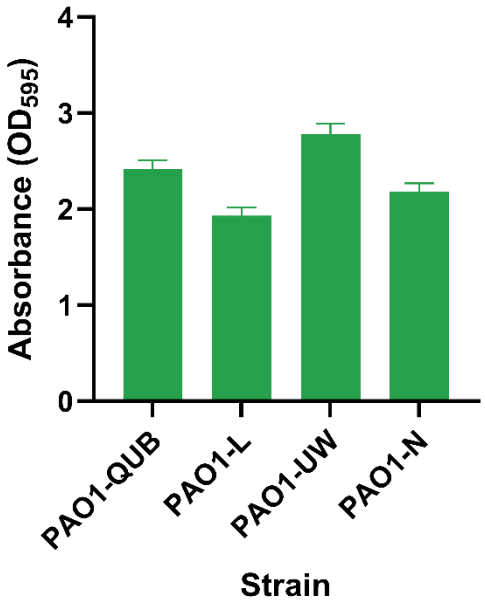


Figure 3.7 **Comparison of biofilm formation between *P. aeruginosa* sublines.**

The ability of three common *P. aeruginosa* PAO1 sublines, PAO1-L originally from the Dieter Haas laboratory, PAO1-UW, maintained at the University of Washington, and PAO1-N from the University of Nottingham to form biofilm in vitro was compared using a 96 well microtiter plate assay to PAO1-QUB from the present study. Green – Strong biofilm producing. Three biological repeats with 24 technical repeats are represented by the mean with 95% confidence interval.

3.2.3.2 Congo Red agar

Congo red in combination with Coomassie blue was used to complement the biofilm work done in section §3.2.3 with surface associated biofilm production. Tryptone agar containing Congo red and Coomassie blue dyes was inoculated with an overnight tryptone broth culture of *P. aeruginosa* and visualised after 120 h (Figure 3.8).

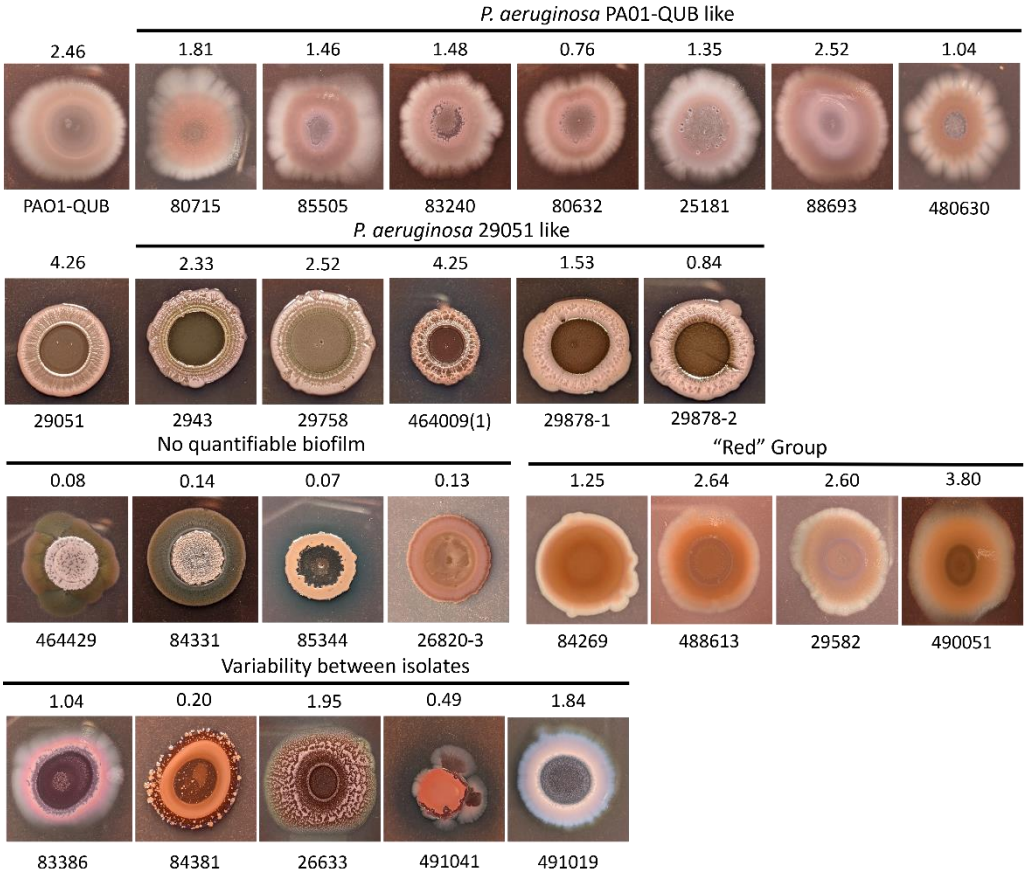


Figure 3.8 ***P. aeruginosa*** **colony morphology in the presence of Congo red and Coomassie blue dyes.**

Overnight cultures grown in tryptone broth were spotted onto agar plates containing Congo red and Coomassie blue and imaged after incubation at 25° C for 120 h. A subset of strains were separated based on morphology. Isolates that resembled *P. aeruginosa* PAO1-QUB are shown in the top row, while the second row shows isolates that appeared similar to isolate 29051 which produced the most biofilm in the crystal violet assay. The third row shows a selection of isolates that produced no quantifiable biofilm in the crystal violet assay in addition to a selection of isolates grouped based on their "red" appearance. Finally, the bottom row shows a selection of isolates that produced varying morphologies in comparison to the other groups. Above each image is the OD_{595} of that isolate from the crystal violet assay. Images shown are representative of three biological repeats.

A subset of isolates were grouped based morphology in the Congo red assay. Despite these groups having a similar appearance on Congo red agar, when the amount of biofilm they produced in the crystal violet assay was assessed it highlighted there is still variation. There were also some isolates that were not able to be grouped as there is such a large amount of variation in the assay. Interestingly, the non-biofilm forming

group also had varied morphologies suggesting that something other than Pel and Psl is impacting their ability to form biofilm.

3.2.3.3 Comparison of PAO1 sublines colony morphology

To further compare biofilm formation between different PAO1 lab strains, colony morphology on agar containing Congo red and Coomassie blue was assessed as done above. There was no difference in the appearance of colony morphology between the strains.

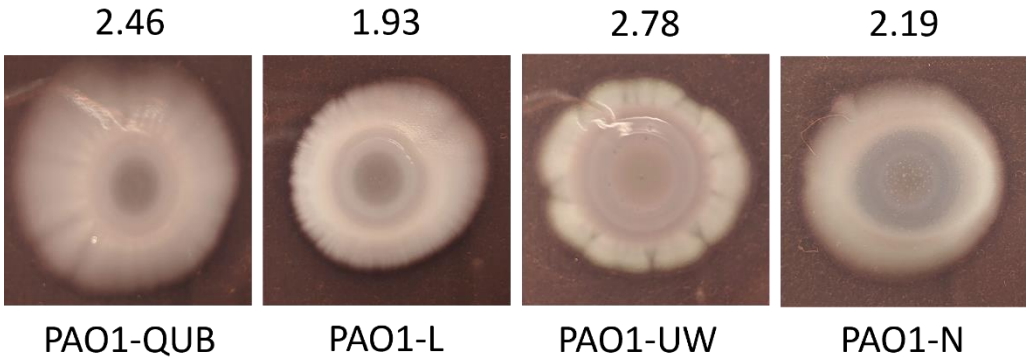


Figure 3.9 **Comparison of *P. aeruginosa* PAO1 sublines on agar containing Congo red and Coomassie blue dyes.**

Overnight cultures of three *P. aeruginosa* PAO1 sublines, PAO1-N, PAO1-L and PAO1-UW in addition to PAO1-QUB were grown in tryptone broth and spotted onto agar plates containing Congo red and Coomassie blue and imaged after 120 h at 25° C. Above each image is the OD_{595} of that isolate from the crystal violet assay. Images shown are representative of three biological repeats.

3.2.3.4 Detection of biofilm associated genes

To further understand the differences between levels of biofilm formation, the genomes of 35 *P. aeruginosa* isolates were screened for the presence of 53 genes known to be associated with biofilm formation (§2.22.4). This identified that 97% (34/35) of the isolates contained all the screened genes with the exception of 464429 which did not contain *pslABCD* (Figure 3.10). Additionally, with the exception of *algP*, all of the genes were highly conserved when compared to PAO1.

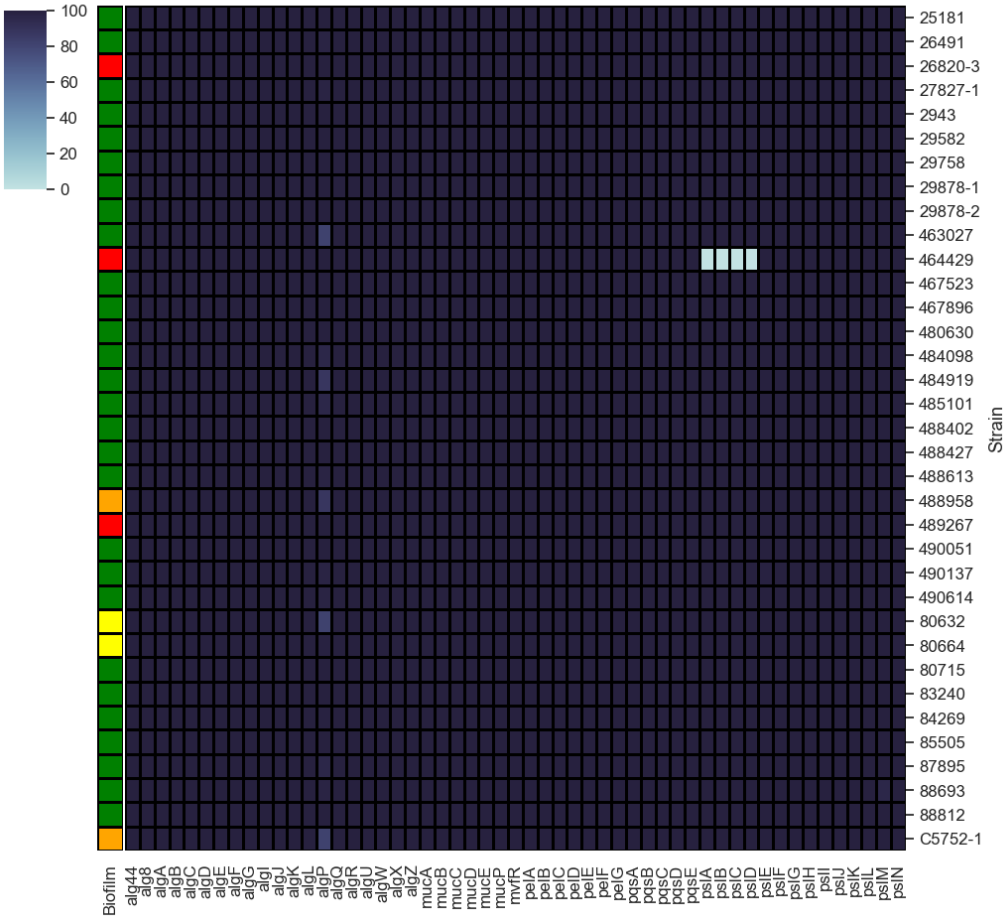


Figure 3.10 **Percentage coverage of the nucleotide sequence of 53 genes associated with biofilm formation.**

Heatmap displaying percentage coverage of the genomes of 35 *P. aeruginosa* canine OE isolates to 53 genes from PAO1 that are associated with biofilm formation. Additionally, the level of biofilm produced in a crystal violet microtiter plate assay is shown Green – Strong biofilm producing - Purple – Moderate biofilm producing - Orange – Weak biofilm producing - Red – No quantifiable biofilm.

3.2.4 Swarming motility

To determine the prevalence of swarming motility among *Pseudomonas aeruginosa* from canine otitis externa, the isolates were tested for their ability to swarm (§2.8). Agar plates containing 0.5% agar select were inoculated with overnight cultures of *P. aeruginosa* (§2.1.2.8) and the distance travelled in addition to comparison to PAO1-QUB was measured (Figure 3.11), this identified that 71% (179/253) were positive for swarming motility. PAO1 consistently travelled to the edge of the agar plate, 16% (41/253) of the isolates showed a similar swarming ability. Of the 205 isolates that produced strong levels of biofilm, 72% (147/205) were also tested positive for swarming. Additionally, Strain 29051, the highest biofilm former, was swarming negative.

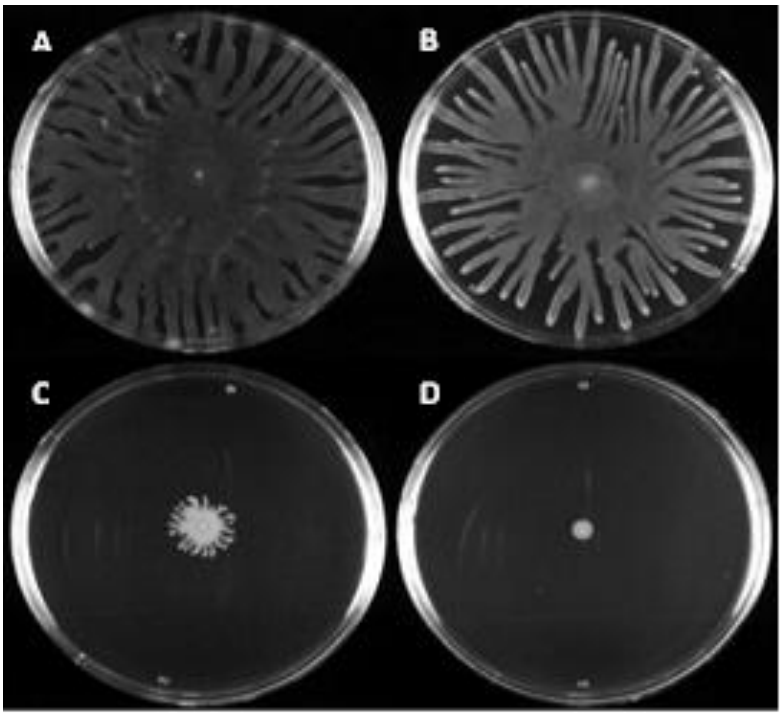


Figure 3.11 **Examples of *P. aeruginosa* swarming motility.**

Overnight cultures grown in LB were spotted onto swarming motility agar and incubated at 37° C for 16 h before imaging. Above shows selection of *P. aeruginosa* clinical canine otitis externa isolates with varying levels of swarming motility. A-C shows strains PAO1, 83240 and C3524. D shows strain 29775 which is swarming negative under the tested conditions. Images shown are representative of three biological repeats.

3.2.4.1 Relationship Between Swarming Motility and Biofilm Formation

The relationship between biofilm and swarming motility was investigated (Figure 3.12) using a Mann-Whitney U test and found there was no significant difference (p=0.52) between the populations when testing the biofilm formation of non-swarmers (n=74) with swarmers (n=179).

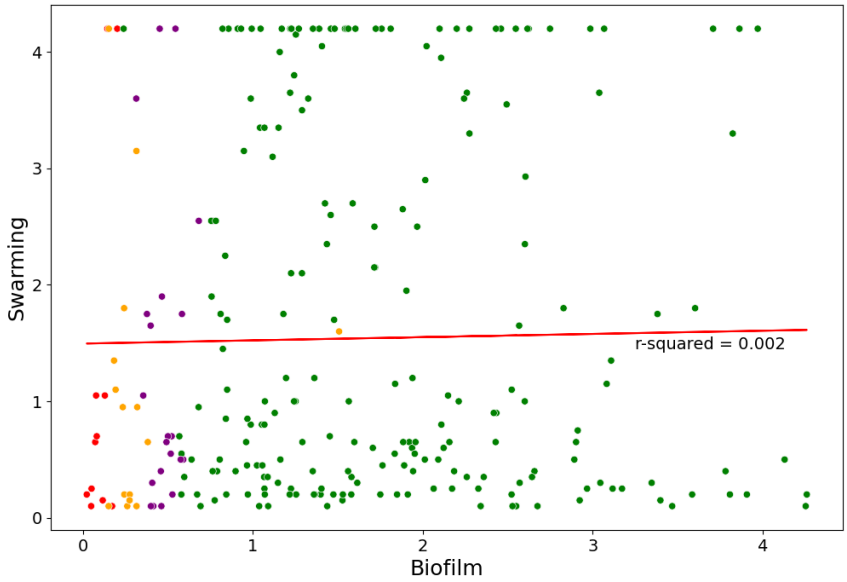


Figure 3.12 **Relationship between biofilm formation and swarming motility.**

A scatter plot showing the relationship between absorbance in a crystal violet biofilm assay and distance travelled in a swarming motility assay for 253 clinical *P. aeruginosa* isolates from canine otitis externa. Results from the biofilm assay are indicated on the graph - Green – Strong biofilm producing - Purple – Moderate biofilm producing - Orange – Weak biofilm producing - Red – No quantifiable biofilm. Swarming motility was measured in cm.

3.2.4.2 Comparing Swarming Motility Between PAO1 Sublines

The swarming motility assay was performed as described above using PAO1 sublines (Figure 3.13). Interestingly, differences in the morphologies of the different sublines were identified. *P. aeruginosa* PAO1-QUB and PAO1-L both consistently swarmed to the edge of the plate while PAO1-UW and PAO1-N displayed different swarming patterns in comparison and a reduction in distance travelled.

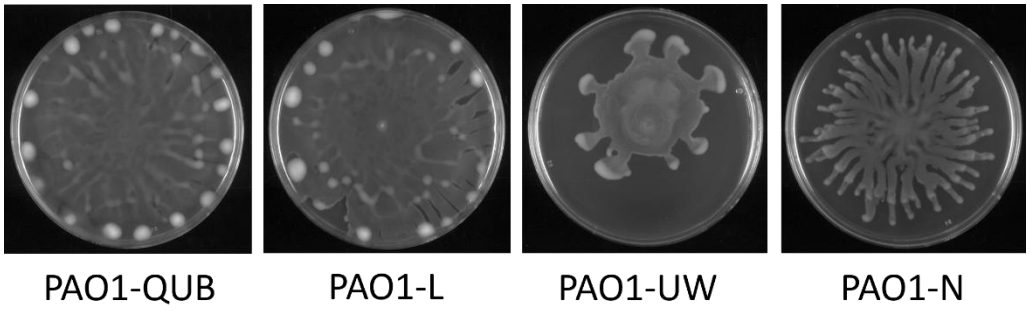


Figure 3.13 **Swarming motility patterns of four *P. aeruginosa* PAO1 sublines.**

Overnight cultures grown in LB were spotted onto swarming motility agar and incubated for 16 h at 37° C before images were taken. Above shows the results of three different PAO1 sublines and PAO1-QUB. Images shown are representative of three biological repeats.

3.2.5 Cluster analysis

In order to aid in the selection of isolates for whole genome sequencing, hierarchical cluster analysis was performed using results from crystal violet biofilm and swarming motility assays, the total number of resistances to the tested antibiotics, predation by *B. bacteriovorus* on YPSC agar and in YPSC broth and finally bacteriophage host range (§2.24.2). From the dendrogram, four distinct clusters were identified coloured yellow (Cluster A), green (Cluster B), red (Cluster C) and purple (Cluster D). This indicates that these isolates have separated based on results from the different assays (Figure 3.14).

Further analysis (Figure 8.4) identified that Cluster A contained isolates that were susceptible to *Bdellovibrio* in liquid predation, whereas isolates susceptible to *Bdellovibrio* on double layer agar were found in all clusters but primarily in cluster A and B. Cluster C contained only strains that swarmed a long distance while Cluster D contained non and lower distance swarmers. Finally, Cluster B contained strains that were susceptible to the broadest range bacteriophage (§4.2.2.2). Notably, this cluster also contained the strains resistant to the most antibiotics. Interestingly, biofilm formation did not seem to impact clustering with the mean biofilm formation remaining the same across the clusters.

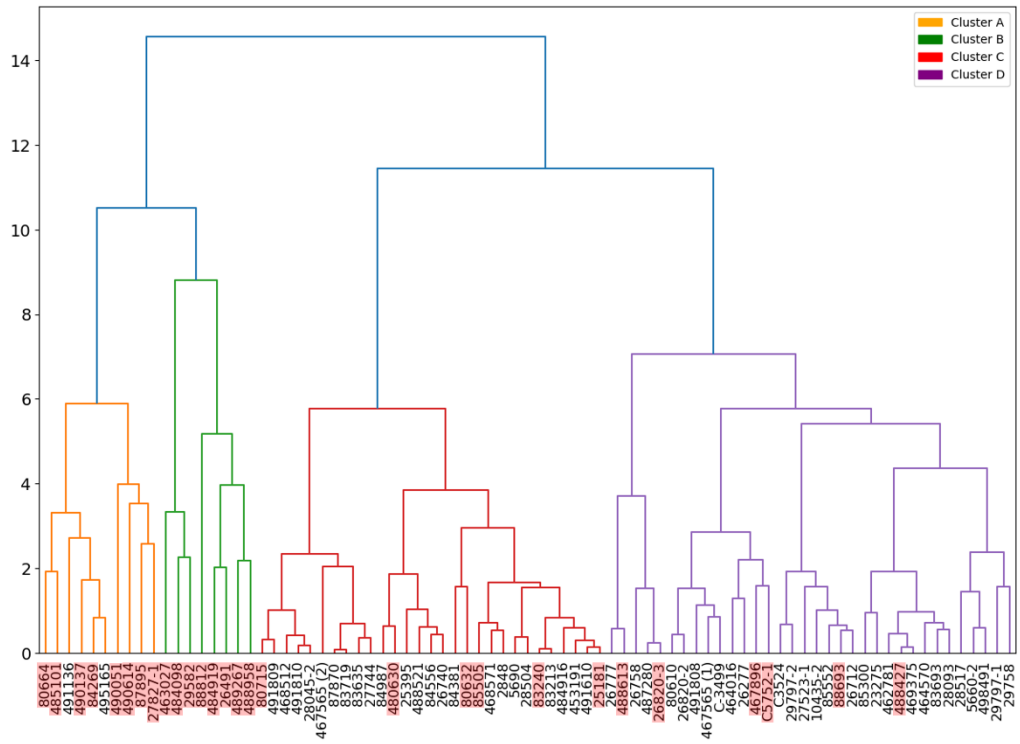


Figure 3.14 **Hierarchical clustering of clinical *P. aeruginosa* isolates.**

Dendrogram from hierarchical clustering using clinical *P. aeruginosa* isolates from canine otitis externa with Euclidean distance with Ward linkage. Isolates selected for sequencing are highlighted in red.

3.2.6 Bioinformatic analysis

A total of 35 clinical isolates were selected for whole genome sequencing based on cluster analysis described above (§3.2.5) or as part of a separate project. The seven additional genomes were 464429, 467523, 488402, 2943, 29758, 29878-1, 29878-2.

3.2.6.1 Genome assembly

Prior to assembly the quality of the reads were assessed using FastQC, and subsequently adaptor sequences and low-quality score bases were trimmed using FastP (§2.21.1). Genome assembly was initially performed using Flye and Circlator, genomes were assessed using Bandage. Subsequently polishing with Medaka, Polypolish and POLCA was performed. Following assembly, the genomes were reoriented to *dnaA* and MLST was performed *in silico* (§2.21.1). A summary of the findings is shown in Table 3.3. Interestingly, strain 484919 and 488958 originally assembled into one contig using Flye, however, upon investigation of the Unicycler assembly, each was found to be missing a small plasmid which was subsequently added to the genome.

Strain	Sequence type (ST)	Number of contigs	GC (%)	Genome Length (bp)
2943	Unknown	1	66.42	6,503,316
25181	253	2	65.42	7,421,260
26491	Unknown	2	66.24	6,771,923
26820-3	1437	2	66.46	6,325,486
27827-1	1284	2	66.21	6,612,504
29582	1527	1	66.53	6,309,761
29758	Unknown	1	66.43	6,499,987
29878-1	Unknown	1	66.42	6,503,340
29878-2	Unknown	1	66.42	6,503,319
80632	471	1	66.43	6,380,312
80664	Unknown	3	66.39	6,453,488
80715	1248	1	66.27	6,624,527
83240	Unknown	2	65.78	6,918,464
84269	Unknown	1	66.04	6,906,640
85505	1228	1	66.34	6,426,992
87895	3514	1	65.80	7,118,680
88693	Unknown	1	66.11	6,785,458
88812	Unknown	2	66.32	6,597,535
463027	267	1	66.23	6,558,666
464429	111	7	66.03	6,629,067
467523	3014	1	66.50	6,376,100
467896	2451	1	66.36	6,458,191
480630	569	1	66.43	6,389,087
484098	815	1	66.11	6,846,060
484919	705	2	66.37	6,374,283
485101	2961	1	65.84	6,980,998
488402	557	1	66.42	6,337,671
488427	381	1	65.87	7,012,748
488613	Unknown	4	65.95	6,997,611
488958	705	2	66.36	6,330,233
489267	Unknown	2	66.22	6,541,895
490051	381	5	65.99	6,915,769
490137	1720	1	66.27	6,509,478
490614	3690	2	66.35	6,342,491
C5752-1	267	1	66.37	6,408,360

Table 3.3 **Summary of the genome features of 35 isolates of *P. aeruginosa* from clinical cases of canine otitis externa.**

Whole genome sequencing of 35 isolates of *P. aeruginosa* from cases of canine otitis externa. Above is a table showing genome length and GC content for each isolate in addition to the number of contigs comprising each assembly and the sequence type of each strain. Assembled using Flye followed by polishing with Medaka, Polypolish and POLCA.

MLST analysis identified 28 unique sequence types (STs) among the 35 isolates, with 8 of these belonging to unknown STs. *P. aeruginosa* 2943, 29758, 29878-1 and 29878-2 belonged to the same unknown ST while 463027 and C5752-1 belonged to ST 267, 484919 and 488958 belonged to ST 705 and 488427 and 490051 belonged to ST 381. The remaining isolates all belonged to unique STs, indicating a large degree of diversity among the isolates.

3.2.6.2 Identification and analysis of plasmids

Analysis of Bandage results highlighted the presence of secondary circular contigs in some of the assemblies, this indicated a high likelihood of plasmids (Figure 3.15).

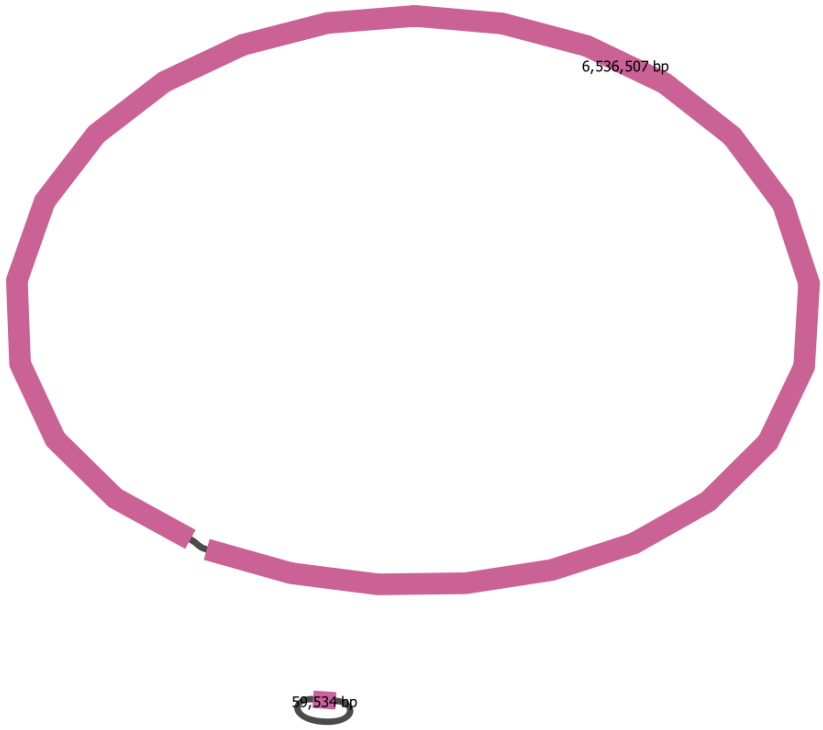


Figure 3.15 **Bandage output of an assembly graph from Flye.**

Bandage output of the assembly graph produced using Flye for *P. aeruginosa* 88812. The small secondary circular contig is typical of a plasmid.

Plasmid sequences were assembled using Plassembler and - similarly to genome sequences where a circular contig was produced - were aligned to the *repA* gene using Dnaapler (§2.22.3).

A summary of the results can be seen in Table 3.4

***Pseudomonas* strain isolates from**	**Plasmid name**	**Circular (Y/N)**	**Plasmid Length (bp)**
25181	p25181	Y	353,950
27827-1	p278271	Y	15,017
80664	p80664	Y	46,902
83240	p83240	Y	128,992
88812	p88812	Y	59,209
484098	p484098	N	43,517
484919	p484919	Y	47,781
488427	p488427	N	25,546
488958	p488958	Y	47,781
489267	p489267	Y	46,272
490051	p490051	N	515,872
490137	p490137	Y	11,837
490614	p490614	Y	47,052

Table 3.4 **Summary of plasmids identified from genome sequencing.**

Plasmid sequences were assembled from whole genome sequencing reads of 35 isolates of *P. aeruginosa* from clinical cases of canine otitis externa using Plassembler.

Analysis of the plasmid sequences for antimicrobial resistance and virulence genes only identified genes in p490051 (Table 3.5).

Plasmid	**CARD**	**VFDB**
p490051	*Pseudomonas_aeruginosa_catB7, mexV, mexW*	*algU, algW, mucA, mucB, mucC, mucD*

Table 3.5 **Summary of antimicrobial resistance and virulence genes identified in plasmid sequences.**

Plasmid sequences from the genomes of 35 isolates of *P. aeruginosa* from cases of canine otitis externa were investigated for the presence of antimicrobial resistance and virulence genes using CARD and VFDB.

3.2.6.3 Presence of virulence genes in clinical isolates

Presence and absence of virulence genes was investigated using ABRicate with VFDB (Figure 3.16). Comparison of the canine OE isolates from this work to other *P. aeruginosa* isolates from human, animal and environmental sources using hierarchical clustering highlighted that there is no specific virulence profile that predisposed an isolate for causing a canine OE infection.

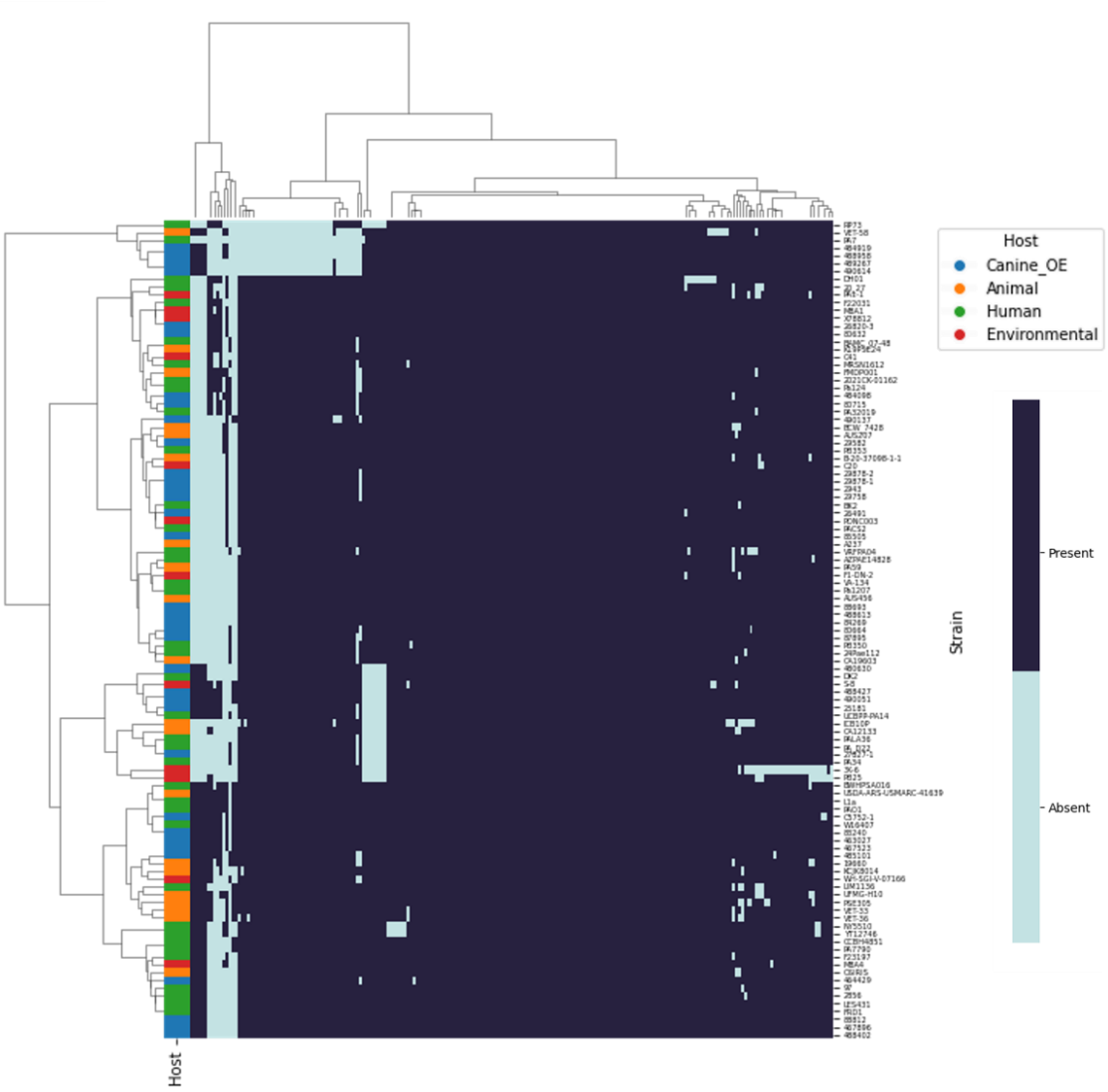


Figure 3.16 **Presence and absence of virulence genes in the genomes of *P. aeruginosa* isolates.**

Heatmap with hierarchical clustering using Euclidean distance with Ward linkage of presence and absence of virulence genes from 35 canine otitis externa isolates from the present study and an additional 70 genomes from human, animal, and environmental sources.

3.2.6.4 Phylogenetic Tree

A total of 107 *P. aeruginosa* genomes including PAO1, PA14, PA7 in addition to 70 isolates listed in PGBD and 35 from this study were compared using Mashtree. The distances calculated were then used to construct phylogenetic trees (Figure 3.17). This found that the isolates were largely part of two groups with some in a smaller third group. Additionally, the canine isolates from the present study were distributed across the tree amongst isolates from human, animal and environmental sources indicating that the canine isolates were not closely related, even when considering geography of isolation, isolates from either Denmark or the UK can be found across the tree.

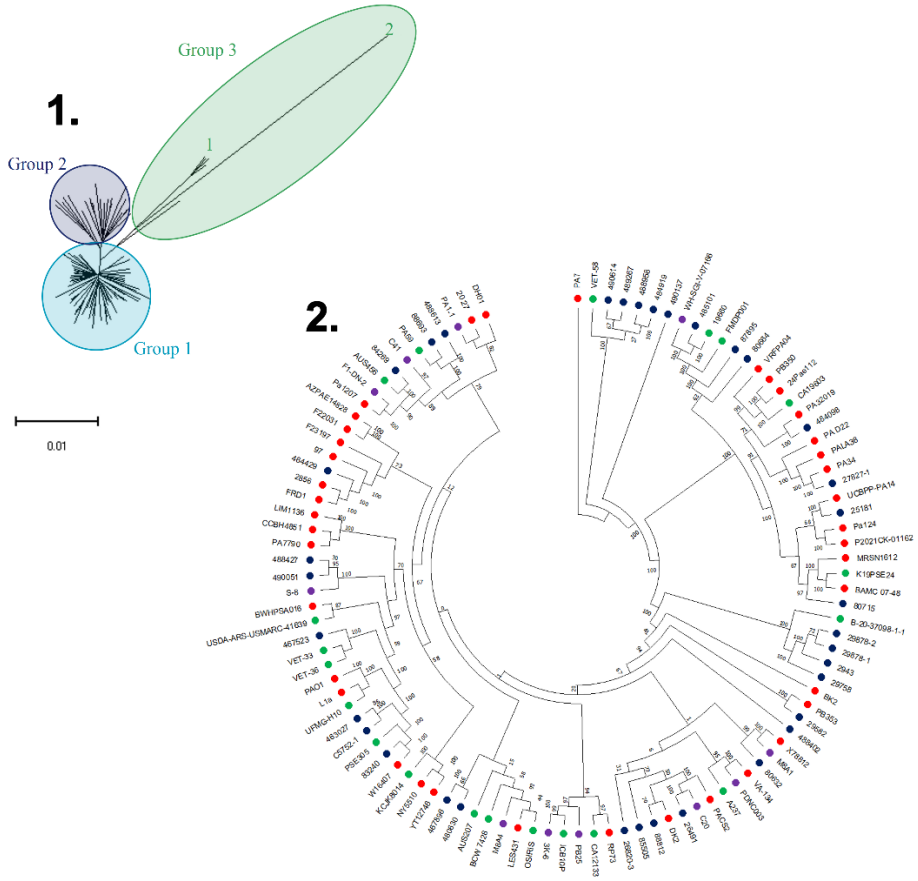


Figure 3.17 **Phylogenetic trees of *P. aeruginosa* strains.**

Mashtree with bootstrapping (100 replicates) was used to calculate the phylogenetic distances of the *P. aeruginosa* isolates from this work in addition to 70 isolates from other sources to create a neighbour joining tree which was visualised using MEGA. 1. The scale represents 1 base difference per 100 bases. 2. Strains from the present study (blue), human infections (red), animal infections (green) and environmental sources (purple) were included as a comparison.

3.3 Discussion

3.3.1 Characterisation of clinical *P. aeruginosa* isolates from cases of canine otitis externa

Canine otitis externa is a common condition seen in veterinary practice worldwide with *P. aeruginosa* being frequently isolated from chronic cases. The nature of this pathogen makes treatment difficult, as part of the present study, isolates of *P. aeruginosa* from clinical cases of canine OE were characterised using culture based and bioinformatic methods.

Antimicrobial resistance has been recognised in cases of canine otitis externa for many years (Guedeja-Marrón et al., 1998). In the UK, otic treatments for dogs are topical and often combine steroid, antibiotic, and antifungal products, (Table 1.2) and, in comparison to systemic treatments, offer a higher drug concentration. These products primarily contain four classes of antibiotics: aminoglycosides, fluroquinolones, amphenicols and polymyxin B. The disc diffusion assay identified that resistance to fluroquinolones was high, enrofloxacin (25%; 64/252), levofloxacin (15%; 37/252) and ciprofloxacin (13%; 33/252) but resistance to gentamicin was lower (3.6%). Antimicrobial resistance has been extensively studied for cases of *Pseudomonas* canine OE. Similar to what was shown in this study, fluroquinolone resistance is frequently reported, notably for enrofloxacin (27-72.2%) and marbofloxacin (33-35%). Highlighting the prevalence of fluroquinolone resistance in *P. aeruginosa* from cases of canine OE. According to different studies, gentamicin resistance varies significantly, with reports of resistance ranging from 3 to 43% (Petrov et al., 2019; Nuttall & Cole, 2007; Elfadadny et al., 2023; KuKanich, Bagladi-Swanson & KuKanich, 2022; Mekić, Matanović & Šeol, 2011; Bourély et al., 2019; Arais et al., 2016).

The finding of levofloxacin resistance is worrying as the use of levofloxacin for the treatment of animals is banned in the EU (Sitovs, Sartini & Giorgi, 2021) and is licensed for use in multiple human infections including the treatment of *P. aeruginosa* infections in cystic fibrosis patients (Podder & Sadiq, 2024; Waters & Smyth, 2015). Similarly, ciprofloxacin is not routinely used in dogs, although some veterinary dermatologists have reported the successful use of this antibiotic (Nuttall, 2016; Morris, 2004). All strains resistant to ciprofloxacin were also resistant to levofloxacin and enrofloxacin suggesting that it is the most effective fluroquinolone against *Pseudomonas in vitro*. This is similar to what has been seen in other studies (Mekić, Matanović & Šeol, 2011) and is most likely due to its lower affinity to efflux pumps compared to other fluroquinolones (Teresa Tejedor et al., 2003). This means ciprofloxacin is likely a better measure of fluroquinolone resistance and relying on enrofloxacin alone could result in an overestimation of resistance, conversely, in a clinical setting relying on ciprofloxacin alone could conceal resistance to antibiotics relevant to a veterinary setting.

While enrofloxacin is not a component of any cSAA listed above, some clinicians use injectable antibiotics as *ad hoc* solutions when resistance is seen or suspected (Morris, 2004), including the use of enrofloxacin (Metry et al., 2012). This could explain the high percentage of resistance that was identified here. Alternatively, although marbofloxacin shows better antimicrobial activity than enrofloxacin against *P. aeruginosa* isolated from companion animals (Farca et al., 2007), continuous exposure studies using *E. coli* from canine infections shows that quinolone resistance-determining region (QRDR) mutations occur after treatment with marbofloxacin that can provide protection against other fluroquinolone antibiotics (Gebru et al., 2011). This could explain the high amount of fluroquinolone resistance observed.

Despite having different resistances in the disc diffusion assay, isolates were found to have very similar antimicrobial resistance gene profiles according to CARD. One exception is the identification of CrpP, an enzyme initially thought to confer resistance to ciprofloxacin (Chávez-Jacobo et al., 2018) but later found to not be the case (Zubyk & Wright, 2021). This is consistent with what was found in this study as the gene was present in 65.7% (23/35) of the sequenced *P. aeruginosa* isolates but 69.6% (16/23) of those were susceptible to ciprofloxacin suggesting that this is not the only factor involved in ciprofloxacin resistance.

P. aeruginosa rapidly develops mutations that can cause resistance to most antibiotics used to treat the organism, resulting in resistance to almost all available treatments (López-Causapé et al., 2018). Point mutations are difficult to detect, databases such as PointFinder (Zankari et al., 2017) are available but have not been developed for *P. aeruginosa*. Mutations in the QRDR are a primary way in which *P. aeruginosa* can become resistant to fluoroquinolones (Piddock, 1999). In *E. coli* these mutations occur between amino acid residues 67-107 for GyrA, 63-102 for ParC (Egorov, Ulyashova & Rubtsova, 2018). *P. aeruginosa* researchers have reported the QRDR regions in GyrB as 429-585 and ParE as 357-503 (Bruchmann et al., 2013). From this, multiple common substitutions were identified, namely T83I, S466F, S87L/S331T and D533E. Three of these, G*yrA*, T83I; G*yrB*, S466F and P*arC*, S87L were all found in strains with resistance to at least one of the tested fluroquinolones, are within the QRDR region reported above, and have been previously associated with resistance (Bruchmann et al., 2013; Akasaka et al., 2001). This implicates these mutations as the source of fluroquinolone resistance in these isolates.

The presence of QRDR mutations has been studied in *P. aeruginosa* isolates from dog infections in Korea, where *gyrA* T83I was identified as a prevalent mutation but also

reported high numbers of a novel mutation, *parC* P116R which was not identified as part of the present study (Park et al., 2020). Another study in Brazil also frequently identified T83I in *P. aeruginosa* resistant to fluroquinolone antibiotics from canine otitis externa and pyoderma (Arais et al., 2016). This highlights that the high levels of fluroquinolone resistance seen in this work is likely due to QRDR mutations although further sequencing work would be needed to confirm this for all the isolates.

Two of the sequenced strains were resistant to gentamycin; strain 88812 was resistant to amikacin and gentamicin while 488958 was resistant to all the tested aminoglycosides. Resistance to aminoglycosides can be due to inactivation by enzymes but also though reduced permeability or increased efflux (Poole, 2005). More recently, mutations to *fusA1* in *P. aeruginosa* have been shown to confer resistance to aminoglycosides (Bolard, Plésiat & Jeannot, 2018). CARD was able to identify one chromosomal aminoglycoside 3'-phosphotransferase (APH(3')-IIb) that provides resistance to kanamycin A and B, neomycin B and C, butirosin and seldomycin F5 in all the sequenced isolates but this does not explain the resistances observed in the ASTs. As this did not explain any of the resistances observed, the amino acid sequence of FusA1 was investigated. This identified a few mutations - two strains contained an I186V substitution, but this has been shown to not impact aminoglycoside resistance (Bolard, Plésiat & Jeannot, 2018) which is consistent with what was shown here as both of the isolates were sensitive to all of the tested aminoglycosides. Strain 488958 was resistant to all the tested aminoglycosides and contained a T671A substitution which has been shown to significantly decrease the susceptibility of *P. aeruginosa* to the tested aminoglycosides including gentamicin, amikacin and tobramycin (Bolard, Plésiat & Jeannot, 2018). Interestingly, strain 88812 was resistant to gentamicin and amikacin and contained two amino acid substitutions A595V and M648V, at the time

of writing A595V has not been reported in the literature and M648V has been but is unlikely to affect protein function (Thacharodi & Lamont, 2023). This could mean that A595V is a novel substitution that confers resistance to some aminoglycosides or that other factors such as decreased permeability or overexpression of efflux pumps might be responsible for the resistance seen.

Mutations alone do not account for all of the resistance seen in this study, namely, *P. aeruginosa* 84269 was resistant to enrofloxacin under the tested conditions but did not contain any mutations in the genes investigated. However, other well characterised fluoroquinolone resistance mechanisms such as efflux pump overexpression and decreased outer membrane permeability were not investigated in the present study but could be responsible for the resistance seen (Hooper & Jacoby, 2016). CARD (Table 3.1) identified 14 efflux mechanisms. Mutations in regulatory genes can result in overproduction of these pumps resulting in resistances to many antibiotics including fluroquinolones and aminoglycosides (Pang et al., 2019). Additionally, *P. aeruginosa* encodes a chromosomal encoded AmpC protein for which 10 mutations have been identified with increased resistance to ceftazidime and imipenem (Rodríguez-Martínez, Poirel & Nordmann 2009) although no clear pattern in the presence of one of the AmpC mutations and resistance was found in the present study.

Worryingly, resistance to two carbapenem antibiotics, meropenem and imipenem, was identified, with the latter showing resistance in 8.33% of isolates. This is significant as the World Health Organisation (WHO) recognises a critical need for new antimicrobials for the treatment of carbapenem resistance *P. aeruginosa* (WHO, 2017). Of the isolates sent for whole genome sequencing, only one showed resistance to imipenem although no carbapenemase genes were identified. Carbapenemases have

been identified in Korea in a *P. aeruginosa* isolate from a case of canine otitis externa (Hyun, Chung & Hwang, 2018).

In addition to the use of injectable enrofloxacin as a topical solution, other antibiotics have also been used including, ticarcillin–clavulanic acid (Bateman et al., 2012), amikacin (Klinczar et al., 2022), tobramycin (Morris, 2004), and ceftazidime (Hoff et al., 2021). Resistance to these antibiotics was also relatively low in the present study 5.2%, 2.4%, 0.4% and 2.8% respectively, with the exception of ticarcillin-clavulanic which had the highest intermediate resistance of the antibiotics tested of 60.8%. This shows that resistance in cases of canine OE is most prevalent against the antibiotic classes commonly found in prescribable ear cleanings, which is expected.

Antibiotic resistance, in combination with biofilm formation, are some of the major challenges that need to be overcome when thinking about the treatment of *P. aeruginosa* infections. The formation of biofilms allows *P. aeruginosa* to better survive in its environment and significantly reduces the effectiveness of treatment in the case of otitis externa. This can result in prolonged chronic infections which in extreme cases can only be solved with surgery (Pye, 2018).

The ability of *P. aeruginosa* to form biofilm *in vitro* was investigated using a microtiter plate assay. Among the strains tested, 82% produced strong levels of biofilm, 9% produced moderate, 5% produced weak and 4% produced no detectable biofilm. Other groups have investigated biofilm formation in *P. aeruginosa* isolates from cases of canine OE and found biofilm formation in between 40-100% of clinical *P. aeruginosa* from canine OE (Robinson et al., 2019; Pye, Yu & Weese, 2013; Chan et al., 2019; Pye, Singh & Weese, 2014). Interestingly, like the present study, some non-biofilm formers were identified (Chan et al., 2019; Robinson et al., 2019). To further

investigate the identification of non-biofilm strains, the presence of 53 biofilm associated genes was assessed. Only one of the strains that produced no biofilm, 464429, was missing any of the biofilm genes that were screened. Specifically, *pslABCD* were absent in this strain. Deletion of *pslD* has been shown to eliminate the ability of *P. aeruginosa* PAO1 to attach to a microtiter dish (Colvin et al., 2012). Interestingly, some *P. aeruginosa* isolates have been shown to be deficient in *psl* production the most notable being PA14 which is also missing *pslABCD* (Friedman & Kolter, 2004). As a result, PA14 uses *pel* as its primary biofilm matrix polysaccharide. Despite this *P. aeruginosa* PA14 is still able to adhere to a microtiter dish and form a biofilm after 24 h (Colvin et al., 2011). This could mean that the strains that produce no quantifiable biofilm have an attachment deficiency. The presence of non-biofilm producing clinical strains is not yet fully understood - while it is accepted that the presence of biofilm-related genes correlates with biofilm forming capacities of *P. aeruginosa* strains, it is unknown why some strains show no biofilm production, as the presence of biofilm associated genes can also be seen in non-biofilm producers. Quorum sensing deficiencies due to mutations in the LasR protein could be the reason why these non-biofilm formers exist (Kamali et al., 2020; Lima et al., 2018). To get a better understanding of biofilm production, a second widely used technique was added, namely the use of Congo red and Coomassie brilliant blue dye in agar. Congo red binds to amyloid fibres in addition to several bacterial polysaccharides and other species-specific polysaccharides (Jones & Wozniak, 2017). Congo red has previously been used to quantify Psl in a liquid assay (Ma et al., 2006) and Pel production on agar (Friedman & Kolter, 2004). The combination of Congo red and Coomassie brilliant blue dyes in agar is used as Psl appears to form a wrinkled colony morphology and binds brilliant blue dye, while Pel will bind Congo red (Jones & Wozniak, 2017). This

should allow for the identification of the primary biofilm polysaccharide and addition to matrix overproducers (Jones & Wozniak, 2017).

Unfortunately, due to the large variety of morphologies that were identified, nothing was uncovered from this method. While this method may be appropriate to investigate individual mutations in polysaccharide genes, the results from this experiment suggest that it may not be sensitive enough to make meaningful comparisons between clinical isolates. The wide variation in colony morphology using Congo red agar has been reported by other researchers (Colvin et al., 2012).

Due to its ability to help bacterial cells colonise their host, motility is a crucial component of virulence. Additionally, the flagella can act as an adhesin during the initial stages of colonisation (O'Toole & Kolter, 1998). In burn would and pulmonary models, it is crucial for full clinical outcomes from *P. aeruginosa* infection (Arora et al., 2005; Feldman et al., 1998). Swarming motility specifically has been implicated in the dispersion of *P. aeruginosa* around the body (Coleman, Pletzer & Hancock, 2021) in addition to the upregulation of virulence genes (Overhage et al., 2008).

The relationship between swarming motility and biofilm formation in *P. aeruginosa* is complex, various knock out experiments using PA14 seem to indicate an inverse relationship between swarming and biofilm formation (Yeung et al., 2009; Caiazza et al., 2007). Similar results have been found using clinical non-cystic fibrosis *P. aeruginosa* from humans (Murray et al., 2010). Contradictorily, others have demonstrated that mutants with altered swarming motility were also incapable of forming biofilms, implicating swarming motility with early biofilm formation (Overhage et al., 2008). As such the relationship between swarming motility and biofilm formation was investigated. Which highlighted that there is no correlation

between swarming and biofilm formation. This was confirmed by a Mann-Whitney U test where the populations were shown to be equal (p=0.52).

Very few studies have examined the genomic profiles of *P. aeruginosa* strains causing canine otitis specifically. The use of MLST has revealed a high diversity of *P. aeruginosa*, with 45 different sequence types (STs) identified from 80 isolates in one study (Hyun, Chung & Hwang, 2018) and 27 STs from 29 isolates in another (Elfadadny et al., 2023). MLST analysis of the OE isolates from the present study identified that 80% (28/35) of the isolates belonged to unique ST. The STs found in the present study vary from those found in the other studies. This is consistent with research on *Pseudomonas* infections in humans, which also revealed a predominantly non-clonal population structure interspersed with highly successful epidemic clones and clonal complexes (Maatallah et al., 2011). These 'high-risk' clones include STs such as ST235, ST175, ST111 and ST146 (Oliver et al., 2015). Interestingly, one isolate was identified as ST111 in this study which is known to be associated with resistant nosocomial infections (Oliver et al., 2015). Transmission of *Pseudomonas* between dogs and people has been reported in both directions (Santaniello et al., 2020; Fernandes et al., 2018). The identification of ST111 in the present study might indicate human to animal spread. Transfer of this pathogen between dogs and people is particularly concerning considering the isolation of resistance to antibiotics important in human medicine.

Interestingly, 8 unknown STs were identified amongst the canine OE isolates, the identification of unknown STs has also been reported in isolates from Australia (Subedi et al., 2018), Pakistan (Irum et al., 2021) and from dog isolates in Japan (Elfadadny et al., 2023) and Korea (Hyun, Chung & Hwang, 2018), therefore the identification of unknown sequence types in this collection was not atypical. Future

work would involve the submission of these isolates to the pubMLST database to be assigned a new ST.

Analysis of phylogeny using Mashtree highlights similar trends. Isolates from the present study were compared using 70 isolates from human, animal and environmental sources and found to be distributed across the tree indicating that the canine isolates were not closely related. Further analysis identified that the majority of the isolates belonged to two groups as previously described (Ozer et al., 2019; Stewart et al., 2014) where one group contains PAO1 and the other contains PA14. A third group was also identified, containing the known taxonomic outlier PA7, which in some cases can exist as two sub-groups as identified in this work (Ozer et al., 2019; Roy et al., 2010).

Not much is known about virulence factors specific to the development of canine OE. Investigation of five virulence genes in *P. aeruginosa* from canine sources, including the ear canal, found that three genes, *lasB*, *aprA*, and *plcH*, were present in all of the tested isolates, while *exoS* and *toxA* were present in 87.5% and 91.7%, respectively (Hattab et al., 2021). Similarly, in the present study *lasB*, *aprA*, and *plcH* were identified in all of the isolates and *toxA* in 91%.

The population of *P. aeruginosa* isolates either contain *exoU* or *exoS* although the reason for this is currently unclear. This is important because *exoU* positive isolates tend to cause more severe infections and seem to be involved in chronic OM infections (Ozer et al., 2019; Park et al., 2017). In the present study, *exoS* positive strains constituted a larger proportion of the population with 69% (24/35) of isolates containing this gene while only 14% (5/35) contained *exoU*. Interesting, one isolate 3% (1/35) contained both genes and 14% (5/35) had neither gene. All of the *exoU* positive isolates in addition to the isolate containing both genes were located on the

same branch of the phylogenetic tree as PA14 as previously described (Ozer et al., 2019).

Comparison of the virulence factor genes from isolates in the present study to other human, animal and environmental strains did not implicate any virulence genes as having a role in this disease. Environmental *P. aeruginosa* isolates were found in clusters with clinical isolates reiterating what is already known (Vives-Flórez & Garnica, 2006).

In summary, the findings from the present study have identified that antimicrobial resistance, in particular resistance to fluroquinolones is common amongst isolates of *P. aeruginosa* from clinical cases of canine OE. Moreover, this resistance is largely due to mutations in QRDR genes. Additionally, a majority of isolates were found to be strong biofilm formers. Finally, whole genome sequencing of 35 isolates confirmed the non-clonal nature of these infections and highlighted that no virulence factor was commonly found in this disease. This supports the notion that *P. aeruginosa* from these infections originates from environmental sources.

3.3.2 Comparison of different *P. aeruginosa* PAO1 sublines

Despite being a common reference strain, comparison of PAO1 isolates maintained in laboratories highlighted genotypic and phenotypic differences (Klockgether et al., 2010; Chandler et al., 2019) which might be important for the conclusions of past and future research using PAO1. Differences between four different PAO1 sublines were investigated, PAO1-QUB originated from Queens University Belfast. PAO1-UW maintained at the University of Washington, in addition to PAO1-L from the Dieter Haas laboratory and PAO1-N from Nottingham were also investigated as part of the present study.

Results from the disc diffusion assay found that PAO1-QUB and PAO1-L were sensitive to all of the tested antibiotics while PAO1-N showed resistance to imipenem and meropenem and PAO1-UW was resistant to imipenem and enrofloxacin and showed intermediate resistance to levofloxacin. Differences in the antimicrobial resistance has been reported previously (Chandler et al., 2019) however, this is the first report to use the disc diffusion assay to establish a resistance phenotype.

When investigating differences in the swarming motility of the sublines, different swarming phenotypes were found with PAO1-N and PAO1-UW having a reduced swarming phenotype in comparison to PAO1-QUB and PAO1-L. This reduction swarming phenotype between PAO1-L and PAO1-N has been previously shown by other groups (Dubern et al., 2022). Differences in swarming phenotype has also been reported in different sublines of PAO1 from across North America (Chandler et al., 2019). At the time of writing, this is the first report of a reduced swarming phenotype of PAO1-UW.

In the crystal violet microtiter plate biofilm assay, all of the sublines were strong biofilm formers but there was a statistically significant difference ($p<0.0001$) in the levels of biofilm between the four sublines. PAO1-UW produced the most biofilm under the tested conditions and PAO1-L produced the least.

Similar to what found with the clinical isolates, the Congo red agar assay was unable to uncover any differences between the sublines. The morphologies were similar for all of the sublines. Other studies have observed that for wild type PAO1, there are large, smooth colonies that do not bind either dye (Jones & Wozniak, 2017).

Dubern and colleagues (Dubern et al., 2022) identified a large deletion in PAO1-N that included *toxR* in addition to a Y442D mutation in *bifA* that were responsible for the

reduced swarming phenotype that has been seen for this subline. Additionally, they found that biofilm formation was increased in the absence of *toxR* expression due to an increase in the expression of *psl* and *pel*.

Chapter 4 Isolation and Characterisation of Bacteriophage

4.1 Introduction

P. aeruginosa infections from cases of canine otitis externa are complicated by antimicrobial resistance and biofilm formation (Pye, 2018). As part of the present study, a collection of isolates from clinical cases of canine OE were characterised (Chapter 3). Antimicrobial susceptibility testing identified that resistance to fluroquinolones was common. Whole genome sequencing of the *P. aeruginosa* isolates identified that this resistance was likely the result of amino acid substitutions in DNA gyrase and topoisomerase genes. Moreover, a majority of isolates were found to produce strong levels of biofilm under the tested conditions. Prolonged infection can result in the progression of disease such that treatment will be unsuccessful in controlling infection, at this stage, surgery is necessary (Doyle, Skelly & Bellenger, 2004; Smeak, 2016).

Bacteriophage, literally “bacteria eater”, are viruses that infect and subsequently lyse bacterial cells. Bacteriophage are the most abundant biological entities on the planet and have been isolated from a variety of environments (Comeau et al., 2008). Bacteriophage can undergo two different life cycles, lytic and lysogenic. Upon infecting a host, a virulent bacteriophage will replicate and eventually kill its host. A temperate bacteriophage can either enter its lysogenic lifestyle or act as a virulent bacteriophage (§1.3.2). Due to their ability to kill important pathogens, bacteriophage have been investigated as alternative therapies in both humans and animals (Liu et al., 2021b).

4.1.1 Aims and Hypothesis

The aim of this work was to isolate bacteriophage from environmental samples that showed potential for the use of as a novel therapeutic for *P. aeruginosa* infections in cases of canine otitis externa.

1 Hypothesis: **Bacteriophage that infect isolates of *Pseudomonas aeruginosa* from clinical cases of canine otitis externa will be recoverable from environmental samples.** Bacteriophage isolated previously from environmental sources will first be screened for recoverable bacteriophage. Following this, environmental samples will be collected and used to isolate new bacteriophage.

2 Hypothesis: **Bacteriophage are able to infect and control the growth of clinical *P. aeruginosa*.** *In vitro* experiments will show that *P. aeruginosa* isolated from clinical instances of canine otitis externa can be infected and killed by bacteriophage from the environment.

4.2 Results

4.2.1 Screening existing phage collection

A collection of 109 bacteriophage isolated using *P. aeruginosa* from environmental and wastewater samples had been previously collected by Dr Joan Colom and was initially assessed for use in the present study. Overlays were produced using four *P. aeruginosa* strains, PAO1-QUB, 80664, 84269 and 484098 (§2.3.4.1) the top agar was then inoculated with the bacteriophage stocks (§2.9). Only two bacteriophage, 491171-1-30 and 464570-1-10 were successfully recovered using PAO1-QUB. Only one of those, 491171-1-30 was carried forward for further investigation.

4.2.1.1 Isolation of novel bacteriophage

As the existing bacteriophage collection was largely unrecoverable, environmental samples from 19 sources (Table 2.3) were used to isolate bacteriophage as described in §2.9 and §2.9.1. A majority of the collected samples (68%, 13/19), were directly from wastewater treatment plants (WWTPs), a further 5% (1/19) were from bodies of water associated with WWTPs. Of the remaining samples 11% (2/19) were from rumen-associated samples and the final 16% (3/19) from environmental bodies of water.

Using these samples 18 bacteriophage were isolated primarily from WWTPs or samples associated with WWTPs (78%, 14/18). The remaining (22%, 4/18) were isolated from rumen-associated samples (Table 4.1). A majority (89%, 16/18) were isolated using *P. aeruginosa* PAO1-QUB and the final 11% (2/18) were isolated using *P. aeruginosa* 484098.

Name	**Isolated using**	**Source**	**Enriched (Y/N)**
Pseudomonas phage 491171-1-30	PAO1-QUB	Original Collection	NA
Pseudomonas phage K9-1	PAO1-QUB	School D	Y
Pseudomonas phage K9-2	PAO1-QUB	School F	Y
Pseudomonas phage K9-3	PAO1-QUB	School F	Y
Pseudomonas phage K9-4	PAO1-QUB	Prison B	Y
Pseudomonas phage K9-5	PAO1-QUB	Prison B	Y
Pseudomonas phage K9-6	PAO1-QUB	Prison D	Y
Pseudomonas phage K9-7	PAO1-QUB	School E	Y
Pseudomonas phage K9-8	PAO1-QUB	Carehome A	Y
Pseudomonas phage K9-9	PAO1-QUB	SB Effluent	N
Pseudomonas phage K9-10	PAO1-QUB	SB Effluent	N
Pseudomonas phage K9-11	PAO1-QUB	SB Dairy	N
Pseudomonas phage K9-12	PAO1-QUB	SB Muckheap	N
Pseudomonas phage K9-13	PAO1-QUB	SB Effluent	N
Pseudomonas phage K9-14	PAO1-QUB	SB Effluent	N
Pseudomonas phage K9-15	PAO1-QUB	SB Effluent	N
Pseudomonas phage K9-16	PAO1-QUB	Beeston Canal	N
Pseudomonas phage K9-17	484098	SB Dairy	N
Pseudomonas phage K9-18	484098	SB Muckheap	N

Table 4.1 **Collection of bacteriophage characterised as part of the present study**.

The source of isolation and propagation strain are shown. Samples were enriched as described in §2.9.1. Y-Yes N-No

4.2.2 Characterisation of bacteriophage

4.2.2.1 Selection of *P. aeruginosa* isolates

Due to evidence of lysogeny, of the 253 *P. aeruginosa* isolates, only 81 were carried forward for further investigation in the bacteriophage work to mitigate any confounding results.

4.2.2.2 Host range and Efficiency of plating

The host range of the isolated bacteriophage was determined using a selection of 81 isolates of *P. aeruginosa* from clincal cases of caine otitis externa in addition to PAO1-QUB (§2.10). Overlays were inoculated with each of the bacteriophage, the results were subsequently characterised between 0-4 with 0 being no lysis and 4 complete clearing (Figure 4.1).

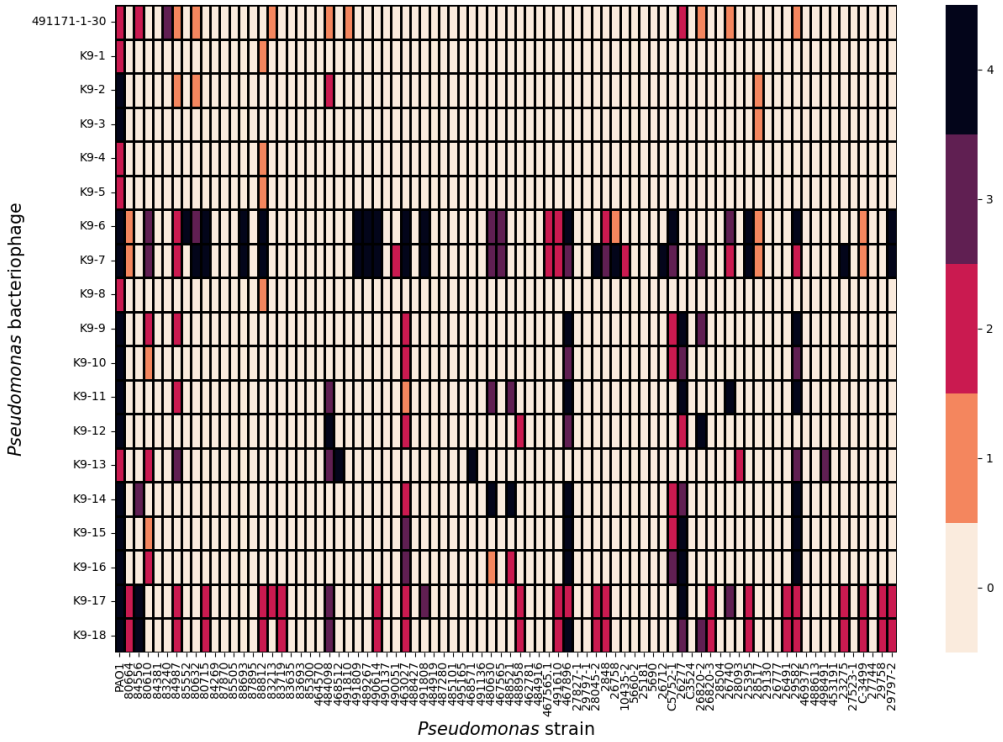


Figure 4.1 **Host range of a collection of 19 bacteriophage.**

Overlays of 80 *P. aeruginosa* isolates from canine otitis externa, PAO1-QUB and 484098 were inoculated with 10 µL of each bacteriophage in the collection at a concentration of 1×10^8 PFU/mL. After incubation for 16 h at 37° C the area of clearing was characterised. 0 – no clearing. 1 – individual plaques. 2 – turbidity throughout the cleared zone. 3 – clearing with a hazy background. 4 – complete clearing.

A majority of the isolates (61%, 50/82) were susceptible to infection by at least one of the bacteriophage in the collection. The bacteriophage with the largest host range was *Pseudomonas* phage K9-7 followed by *Pseudomonas* phage K9-6 which were able to produce at least individual plaques in 40% (33/82) and 34% (28/82) of the *P. aeruginosa* isolates respectively. Similarly, *Pseudomonas* phage K9-7 and *Pseudomonas* phage K9-6 were able to produce complete clearing on 20% (16/82) and 18% (15/82) of *P. aeruginosa* isolates respectively.

P. aeruginosa isolates that were insensitive to infection for all the bacteriophage (32/82) were excluded and efficiency of plating was performed with the remaining 50 isolates (Figure 4.2).

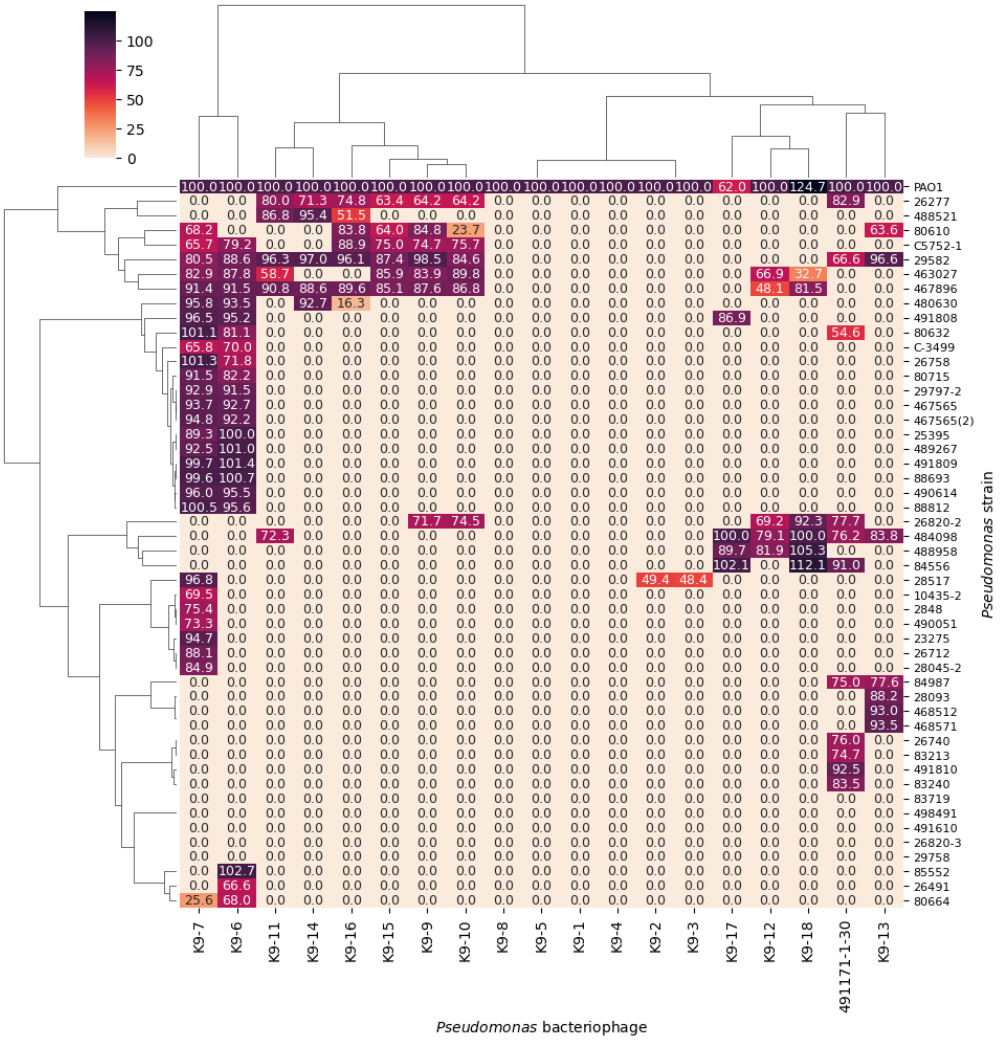


Figure 4.2 **Efficiency of plating of 19 bacteriophage.**

Overlays of 50 *P. aeruginosa* isolates from canine otitis externa were inoculated with decimal dilutions (10^{-1} to 10^{-6}) of each bacteriophage. The result shown is the mean efficiency of plating (%) where the PFU/mL of the propagation strain is 100% with hierarchical clustering using Euclidean distance and Ward linkage. Values are representative of two technical and three biological repeats.

Despite removing the non-susceptible strains based on the host range assay, 10% (5/50) of isolates were identified as resistant to all the tested bacteriophage based on the efficiency of plating results.

Efficiency of plating was calculated as a percentage of infection in comparison to the isolation host. The proportion of bacteriophage where efficiency of plating was greater than 100% was low with only 22% (11/50) of host isolates displaying

increased sensitivity to a given bacteriophage. Clustering analysis identified that the bacteriophage fell into three main clusters when considering efficiency of plating.

Where a bacteriophage was able to infect a given host strain, it did so with 'High' efficiency (Figure 4.3) as described by (Viazis et al., 2011).

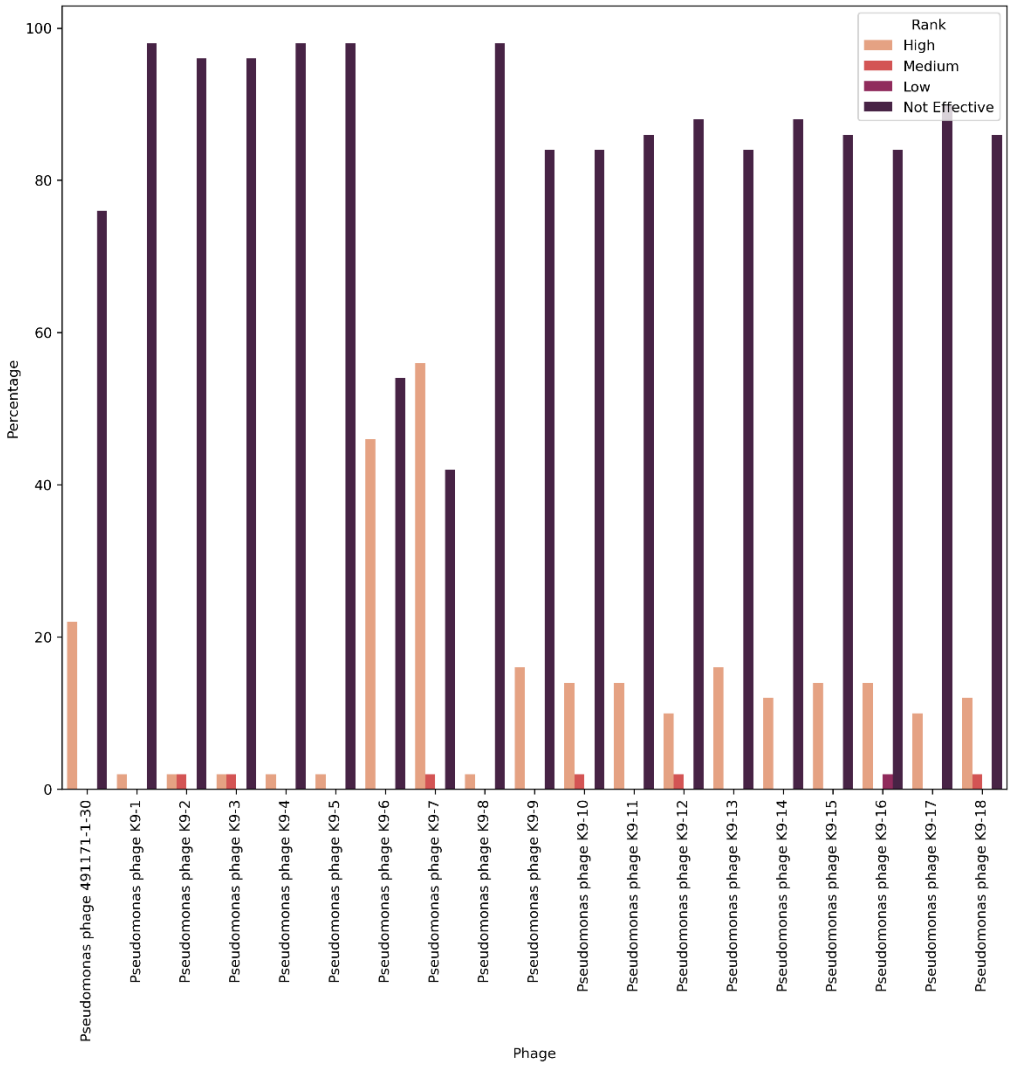


Figure 4.3 **Efficiency of bacteriophage infection**.

Efficiency of plating values were ranked as previously described (Viazis et al., 2011). High efficiency was defined as a mean efficient of plating value ≥50%, medium ≥20%<50% and 'Low' >0%<20%.

4.2.2.3 *in vitro* replication kinetics

Growth curves of *P. aeruginosa* PAO1-QUB were optimised to ensure the effect of bacteriophage infection could be monitored (§2.13.1). Initially, the input CFU/mL of the host was assessed. An aliquot of overnight culture was combined with sterile media and incubated until an OD_{600} of 0.5 was reached and enumerated, which was equivalent to 5×10^8 CFU/mL. Decimal dilutions of this culture (10^{-1} to 10^{-4}) were used to inoculate a 96 well microtiter plate and incubated at 37° C statically for 24 h with readings every 5 min (Figure 4.4).

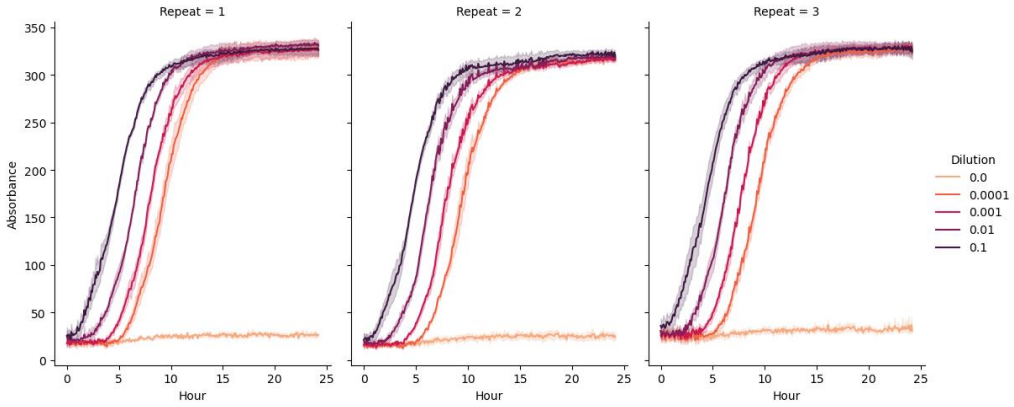


Figure 4.4 **Growth curves of *P. aeruginosa* PAO1-QUB.**

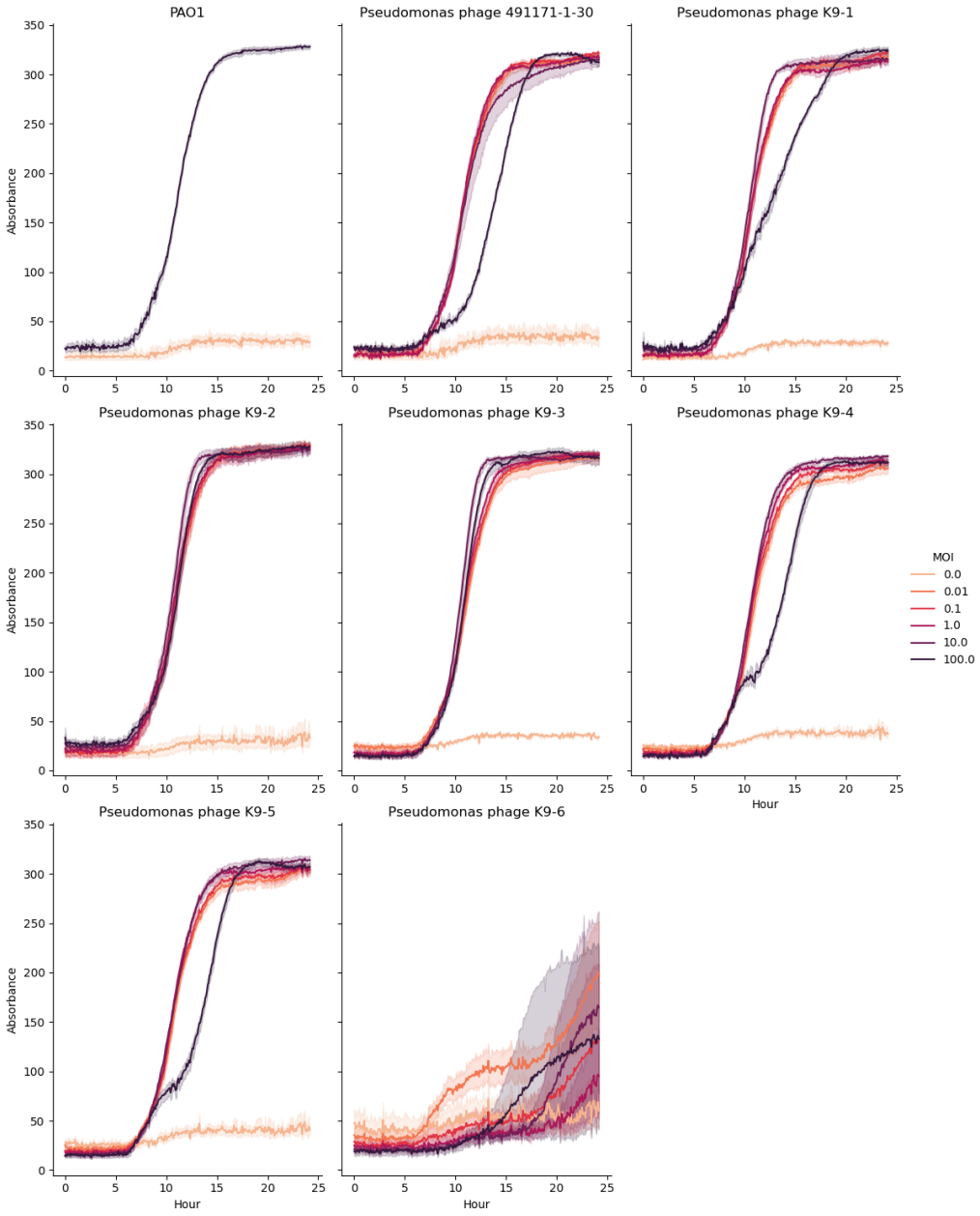
A culture of *P. aeruginosa* PAO1-QUB at an OD_{600} equivalent to 5×10^8 CFU/mL was prepared. Decimal dilutions of this culture (10^{-1} to 10^{-4}) were used to inoculate a 96 well microtiter plate and grown in LB containing 2,3,5-Triphenyltetrazolium chloride at 37° C for 24 h with readings every 5 min using an OmniLog plate reader. The values shown are the mean of two technical repeats with 95% confidence intervals for each biological repeat.

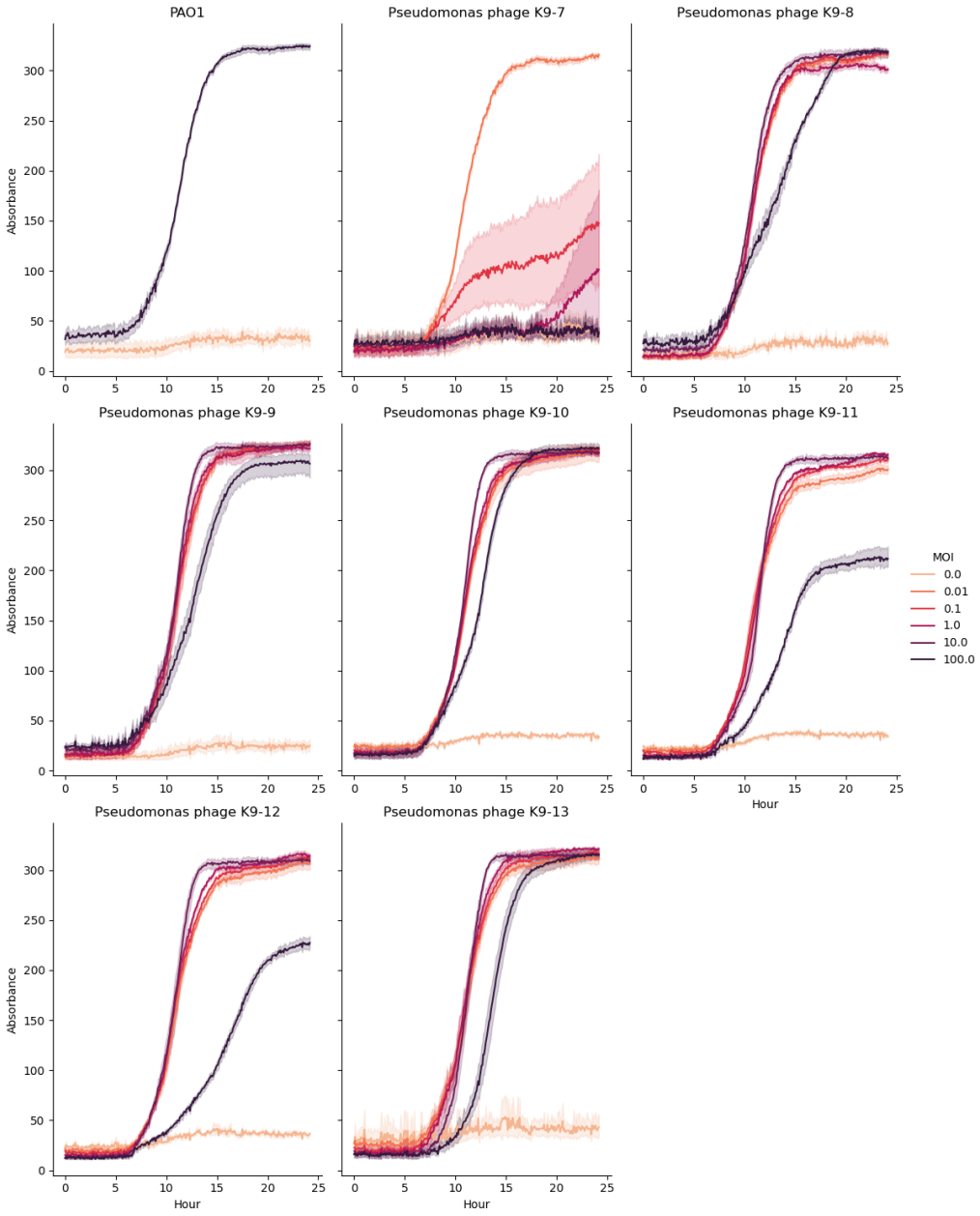
Analysis of the growth curves identified that the 10^{-4} dilution equivalent to an input of 5×10^4 CFU/mL was the optimum condition as a clear lag phase was seen in addition to still reaching the maximum absorbance within 24 h.

Bacteriophage infection was measured indirectly by monitoring the kinetics of infection using an OmniLog plate reader (§2.13). As a result of the findings presented above, to achieve a multiplicity of infection of 100, 10, 1, 0.1 and 0.01

a starting CFU/mL of 1×10^4 was used to measure bacteriophage lysis (Figure 4.5).

Examination of the results identified that *P. aeruginosa* PAO1 alone followed a typical sigmoid logarithmic curve. With the exception of *Pseudomonas* phage K9-6 and K9-7, an MOI lower than 100 did not have a noticeable effect on the growth of *P. aeruginosa* PAO1-QUB. Looking specifically at MOI 100, five patterns were observed; no effect (2/19), interruption of exponential growth but with no effect to the maximum growth (3/19) as seen for *Pseudomonas* phage K9-4, slower exponential growth but with no effect to the maximum growth (8/19), slower exponential growth and reduced total growth (4/19) and finally complete disruption of normal growth (2/19).


PAO1
Pseudomonas phage 491171-1-30
Pseudomonas phage K9-1
Pseudomonas phage K9-2
Pseudomonas phage K9-3
Pseudomonas phage K9-4
Pseudomonas phage K9-5
Pseudomonas phage K9-6
Absorbance
Hour
MOI
0.0
0.01
0.1
1.0
10.0
100.0


PAO1
Pseudomonas phage K9-7
Pseudomonas phage K9-8
Pseudomonas phage K9-9
Pseudomonas phage K9-10
Pseudomonas phage K9-11
Pseudomonas phage K9-12
Pseudomonas phage K9-13
Absorbance
Hour
MOI
0.0
0.01
0.1
1.0
10.0
100.0

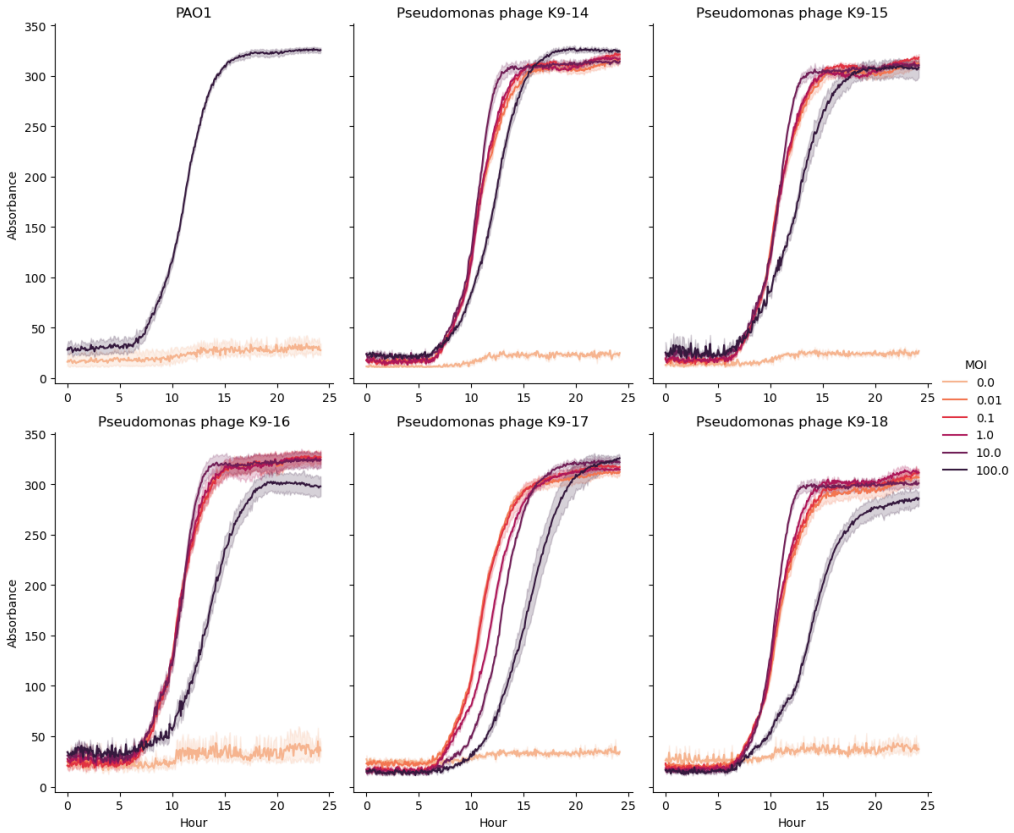


Figure 4.5 **Growth curves of *P. aeruginosa* PAO1-QUB in the presence of bacteriophage.**

LB containing 2,3,5-Triphenyltetrazolium chloride was inoculated with a culture of *P. aeruginosa* PAO1-QUB equivalent to 1×10^3 CFU/mL in addition to bacteriophage ranging from 1×10^6 to 1×10^2 PFU/mL. For each bacteriophage, a phage only control was included shown as the orange line. Similarly, a host only control was included in each microtiter plate shown in the top left of each figure. The values shown are representative of three biological and two technical repeats.

The error bars for *Pseudomonas* phage K9-6 were much larger than those from the other samples suggesting a large variation in the growth of the host. Further investigation of the growth curves (Figure 4.6) highlighted variable regrowth within individual technical repeats after a long lag period of 10-15 h.

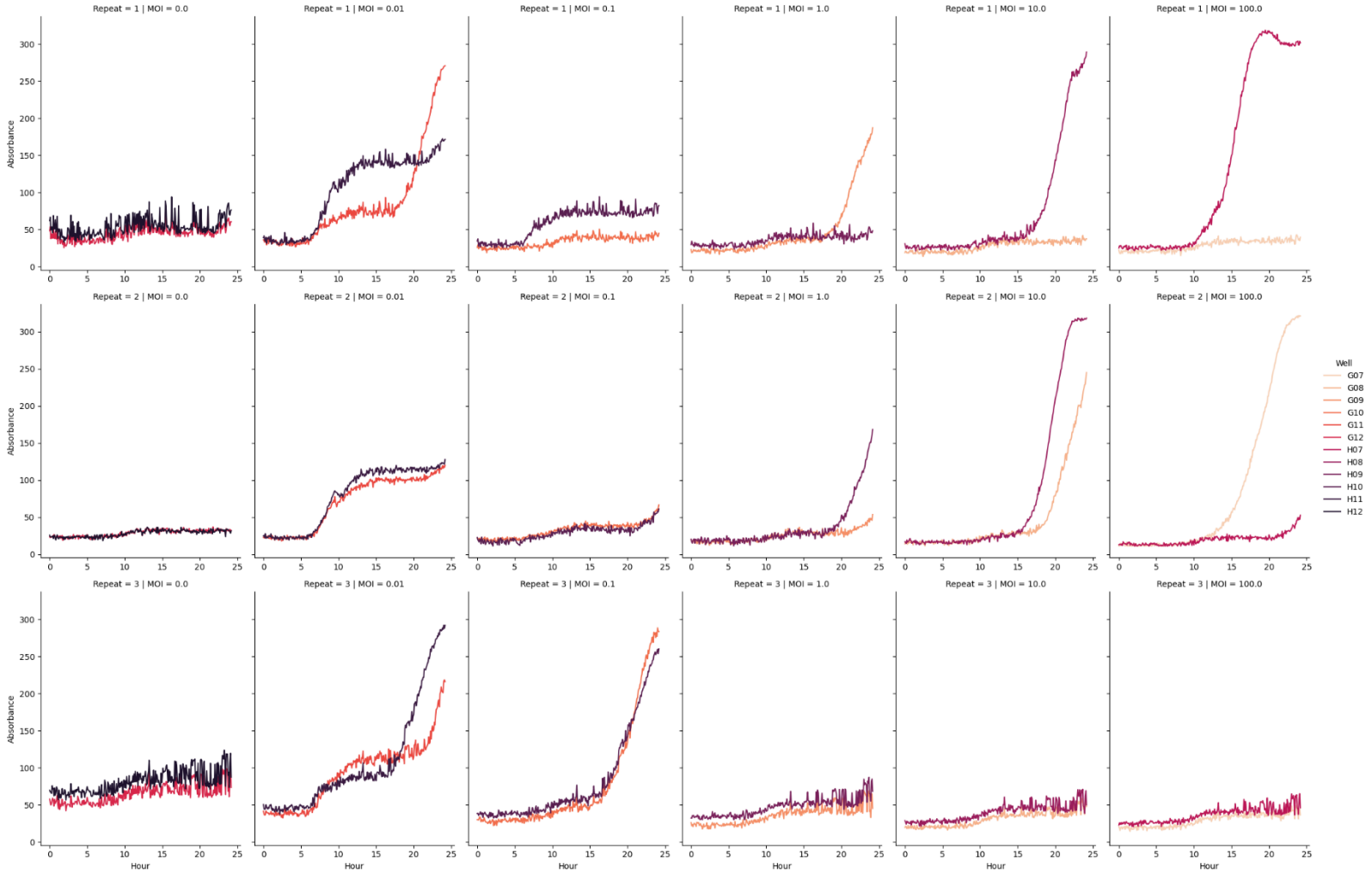

Repeat = 1 | MOI = 0.0
Repeat = 1 | MOI = 0.01
Repeat = 1 | MOI = 0.1
Repeat = 1 | MOI = 1.0
Repeat = 1 | MOI = 10.0
Repeat = 1 | MOI = 100.0
Repeat = 2 | MOI = 0.0
Repeat = 2 | MOI = 0.01
Repeat = 2 | MOI = 0.1
Repeat = 2 | MOI = 1.0
Repeat = 2 | MOI = 10.0
Repeat = 2 | MOI = 100.0
Repeat = 3 | MOI = 0.0
Repeat = 3 | MOI = 0.01
Repeat = 3 | MOI = 0.1
Repeat = 3 | MOI = 1.0
Repeat = 3 | MOI = 10.0
Repeat = 3 | MOI = 100.0
Absorbance
Hour
Well
G07
G08
G09
G10
G11
G12
H07
H08
H09
H10
H11
H12

Figure 4.6 **Panels showing growth curves of *P. aeruginosa* PAO1-QUB in the presence of *Pseudomonas* phage K9-6.**

P. aeruginosa PAO1-QUB was grown in a 96 well microtiter plate containing LB and 2,3,5-Triphenyltetrazolium chloride in the presence of Pseudomonas phage K9-6. Each row shows a biological repeat and each line a line within a graph a technical repeat or individual well. Columns indicate an increasing MOI starting with a phage only control in the first column.

4.2.2.4 Restriction digestions

Restriction digestions were performed to identify identical bacteriophage prior to genome assembly. Initially, this was done using three restriction endonucleases (§2.12). Analysis of the digestion patterns found nine unique banding patterns, 37% (7/19) of the bacteriophage had unique banding patterns whist 16% (3/19) shared the additional pattern. Digestions with EcoRI-HF, NdeI and HindIII failed to separate all of the bacteriophage genomic DNA extracts, the remaining 47% (9/19) of the genomes were not cut by the enzymes (Figure 4.7; Figure 8.5).

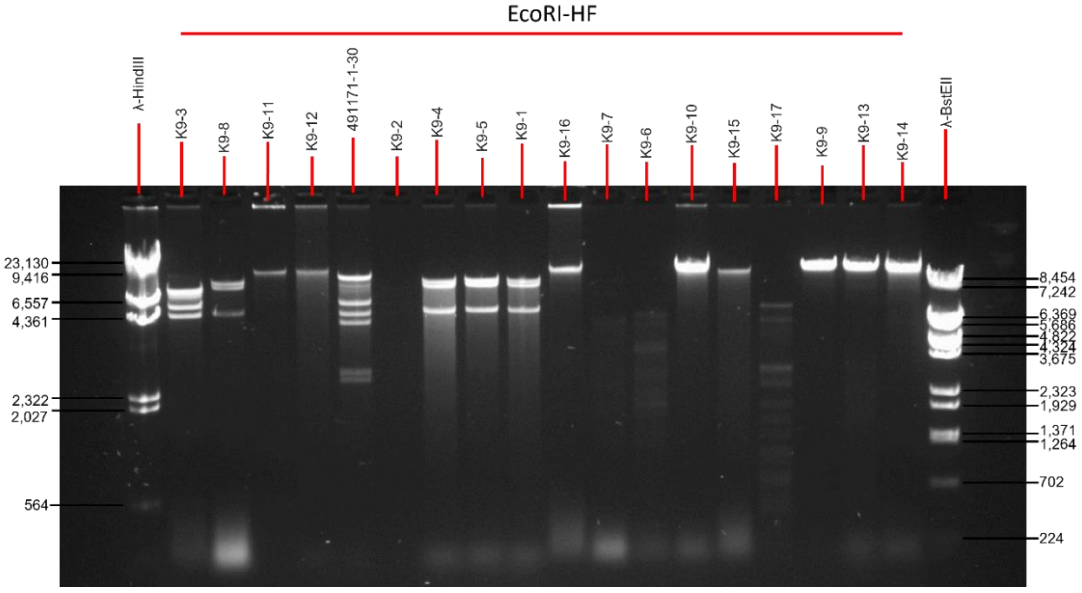


Figure 4.7 **Ethidium Bromide agarose gel image of bacteriophage restriction digestion with EcoRI-HF.**

Restriction digestions of genomic DNA extracts from 18 *P. aeruginosa* bacteriophage were performed using EcoRI-HF as described in §2.12. Subsequently, the digestions were run on an agarose gel containing Ethidium Bromide (§2.4). Ladder sizes are shown in bp.

To resolve the remaining bacteriophage, the genomes of *Pseudomonas* phage K9-9,10,11,12,13,14,15,16 and 18 were digested using HincII and XbaI (Figure 4.8). This identified another pattern shared by *Pseudomonas* phage K9-13 and 14. Where no restriction patterns were seen or the same restriction pattern was seen, the host range profile was used to separate the bacteriophage for sequencing.

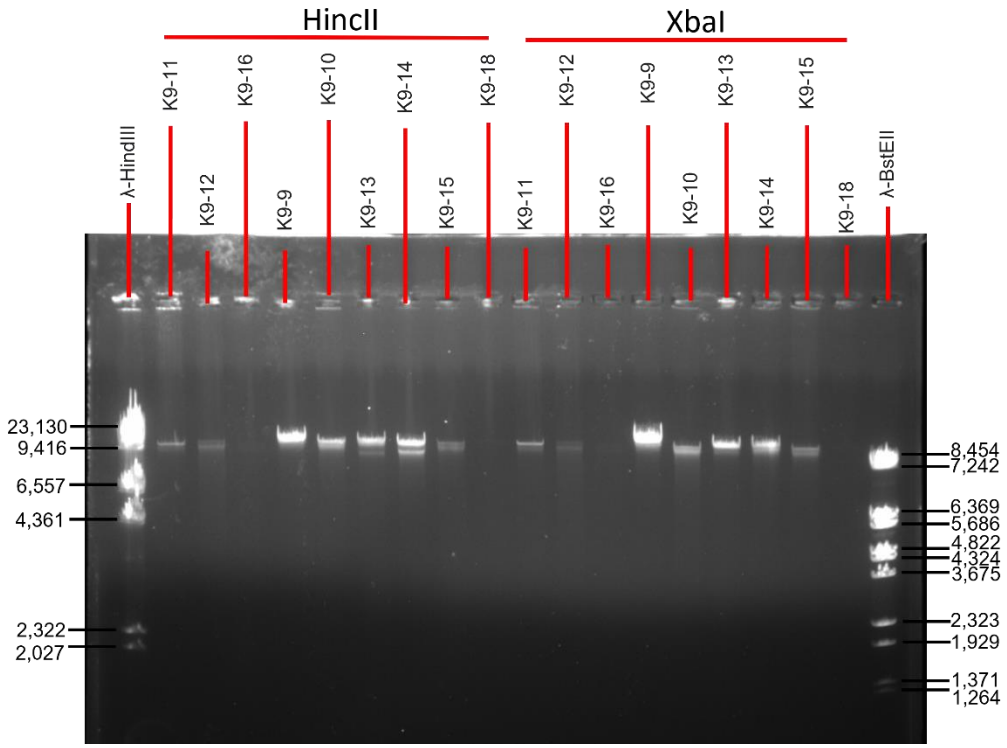


Figure 4.8 **Ethidium Bromide agarose gel image of bacteriophage restriction digestion with HincII and XbaI.**

Restriction digestions of genomic DNA extracts from nine *P. aeruginosa* bacteriophage were performed using HincII and XbaI as described in §2.12. Subsequently, the digestions were run on an agarose gel containing Ethidium Bromide (§2.4). Ladder sizes are shown in bp.

4.2.2.5 Analysis of *P. aeruginosa* genomes for factors implicated in bacteriophage insensitivity

The genome sequences of 35 isolates of *P. aeruginosa* from clinical cases of canine OE were determined as described in §3.2.6. A selection of these were sequenced as part of a different project and due to evidence of lysogeny were not included in bacteriophage host range analysis. Of the remaining 29 isolates, susceptibility to bacteriophage in the host range assay was investigated (Figure 4.9). Analysis of the genome sequences was performed to determine differences in susceptibility to the bacteriophage isolated as part of the present study.

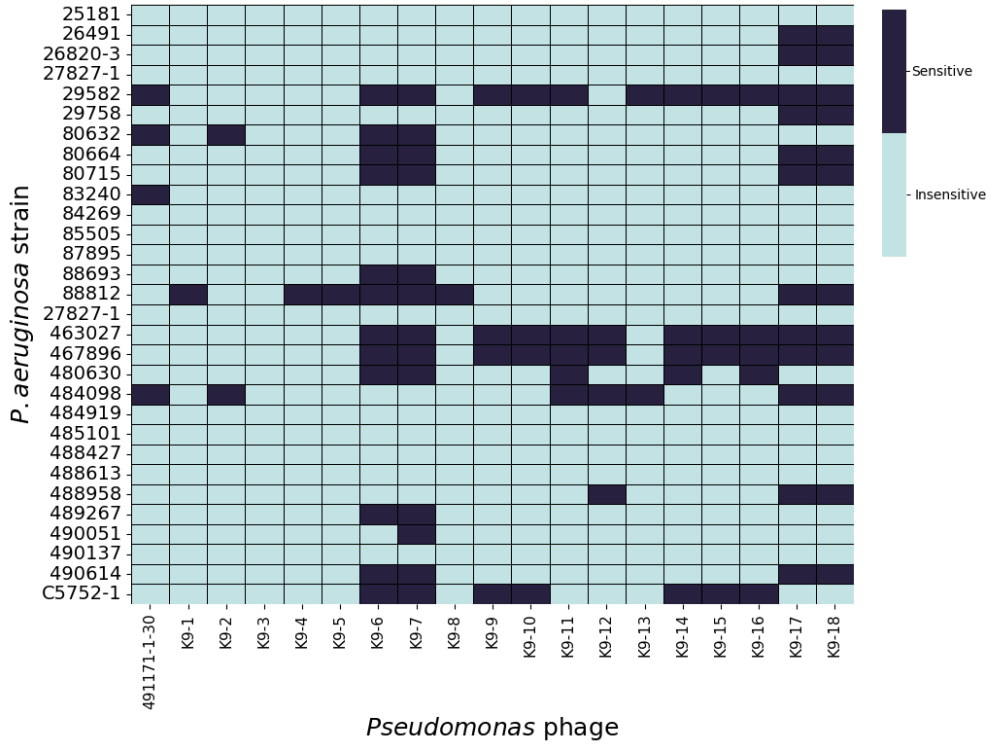


Figure 4.9 **Host range of 29 isolates of *P. aeruginosa* from clinical cases of canine otitis externa.**

Susceptibility of *P. aeruginosa* isolates to 19 bacteriophage was determined using a host range assay (§4.2.2.2). Genome sequencing had been performed for 35 *P. aeruginosa* isolates, 29 of which were assessed for susceptibility to bacteriophage infection. Insensitivity was assessed by the absence of clearing, or a score of 0 in a host range assay.

4.2.2.6 Identification of CRISPR-Cas systems in *P. aeruginosa* genomes

CRISPR-Cas systems can recognise foreign DNA, such as bacteriophage genomes and degrade it protecting the host from infection (Labrie, Samson & Moineau, 2010). Therefore, the presence of CRISPR arrays and Cas proteins was assessed in the genome sequences of the *P. aeruginosa* clinical isolates (§2.22.6). This found that 38% (10/26) of the sequenced isolates contained a CRISPR-Cas system, of those, 100% (10/10) contained Cas type IF and a further 30% (3/10) contained more than one type of Cas protein (Table 4.2).

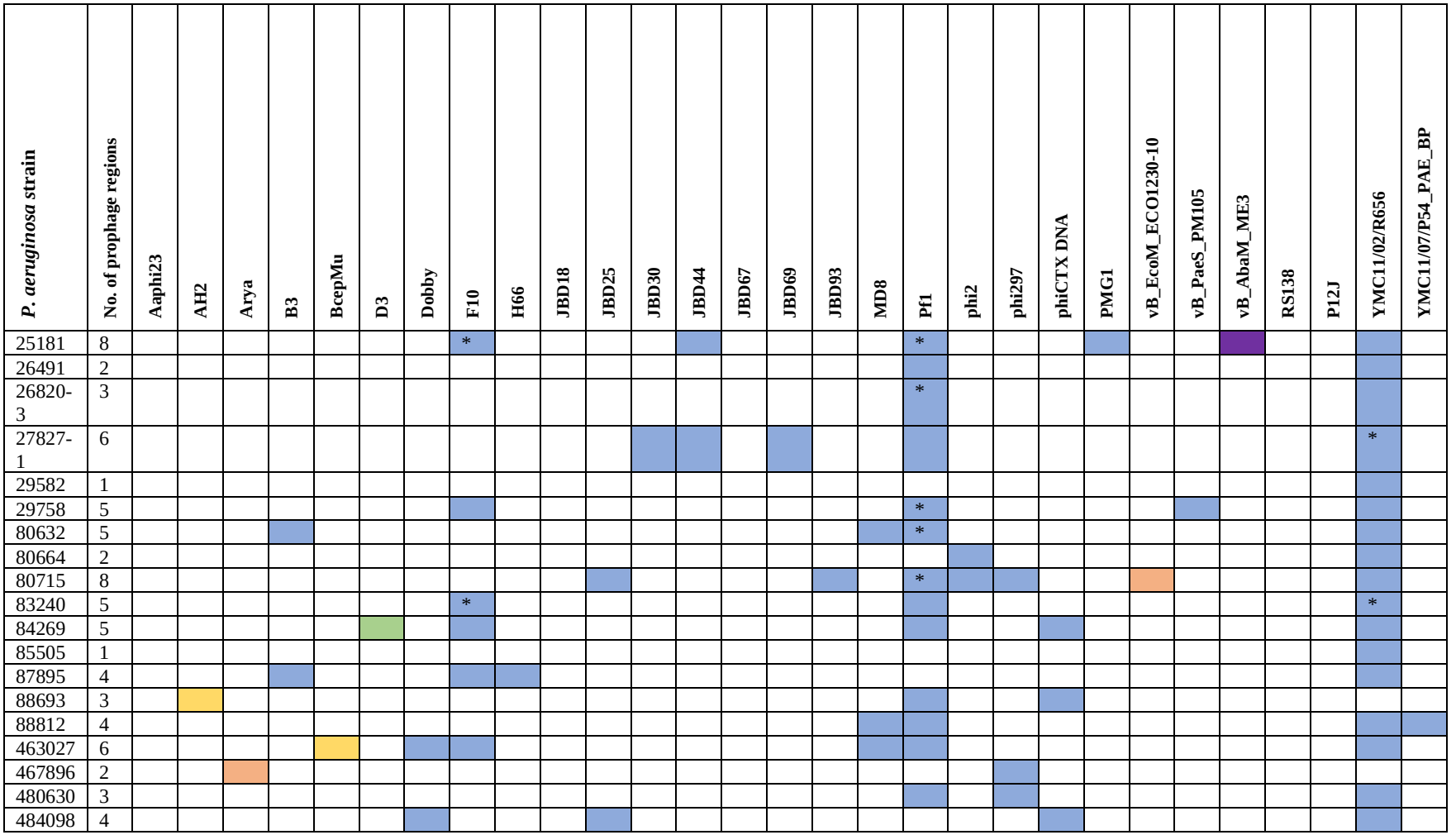
***P. aeruginosa* strain**	**CRISPR-Cas identified (Y/N)**	**Cas Type**
25181	Y	IF
26491	Y	IF
26820-3	Y	IF
27827-1	N	-
29582	N	-
29758	Y	IF
80632	Y	IF
80664	Y	IF and IE
80715	Y	IC
83240	Y	IF
84269	N	-
85505	Y	IF
87895	N	-
88693	N	-
88812	Y	IF
27827-1	N	-
463027	N	-
467896	Y	IF
480630	Y	IF and IC
484098	N	-
484919	N	-
485101	N	-
488427	N	-
488613	N	-
488958	N	-
489267	Y	IF
490051	N	-
490137	N	-
490614	Y	IF and IE
C5752-1	N	-

Table 4.2 **Summary of CRISPR-Cas presence in clinical *P. aeruginosa* genome sequences**.

Results from CRISPRCasFinder detailing the presence of CRISPR arrays and Cas proteins in the genome sequences of *P. aeruginosa* clinical isolates.

4.2.2.7 Prediction of prophage regions in *P. aeruginosa* genome sequences

Prophage maintained within the genomes of their host can contain genes that encode for superinfection exclusion systems, these proteins can prevent bacteriophage DNA from entering the host and therefore confer immunity to infection by other bacteriophage (Labrie, Samson & Moineau, 2010). Predicted prophage regions within the genomes were assessed using PHASTEST (§2.22.7). This identified that prophage sequences were abundant within the genomes of clinical *P. aeruginosa* with as many as 10 prophage regions being identified in a single *P. aeruginosa* genome (Table 4.3). Notably, not all of these were found to be complete, completeness was determined by PHASTEST using criteria such as the number of coding sequences, and coding sequences containing bacteriophage related keywords such as ‘capsid’ (Table 8.2).


P. aeruginosa strain
No. of prophage regions
Aaphi23
AH2
Arya
B3
BcepMu
D3
Dobby
F10
H66
JBD18
JBD25
JBD30
JBD44
JBD67
JBD69
JBD93
MD8
Pf1
phi2
phi297
phiCTX DNA
PMG1
vB_EcoM_ECO1230-10
vB_PaeS_PM105
vB_AbaM_ME3
RS138
P12J
YMC11/02/R656
YMC11/07/P54_PAE_BP
25181 8
26491 2
26820-3 3
27827-1 6
29582 1
29758 5
80632 5
80664 2
80715 8
83240 5
84269 5
85505 1
87895 4
88693 3
88812 4
463027 6
467896 2
480630 3
484098 4

484919	3																		*											
485101	7																												*	
488427	7																				*									
488613	10							*			*								*											
488958	3																		*											
489267	6																													
490051	7								*																				*	
490137	7																		*											
490614	3																													
C5752-1	4																				*									

Table 4.3 **Predicted prophage regions in the genome sequences of 29 *P. aeruginosa* isolates.**

PHASTEST was used to screen the genome sequences of 29 isolates of *P. aeruginosa* from clinical cases of canine otitis externa for evidence of prophage regions. Where a prophage region was identified, the coding sequences were compared, and the closest related was identified. Colours prefix of the closest relative; Blue – *Pseudomonas* phage, Orange – Enterobacter phage, Gold – *Burkholderia* phage, Red – *Ralstonia* phage, Green – Bacteriophage, Purple – Acinetobacter. * - A region with similarity to this bacteriophage was identified more than once.

4.2.3 Bacteriophage genome analysis

Genomic DNA extractions of the 19 isolated bacteriophage were performed as described in §2.11.

4.2.3.1 Bacteriophage genome assembly

Bacteriophage genome assembly was performed as described in §2.21.2. Two bacteriophage, *Pseudomonas* phage K9-8 and *Pseudomonas* phage K9-18 were unable to be recovered from the sequencing reads.

Subsampling of reads removed contigs with very low coverage (Figure 8.6), as a result, subsampled reads did not always produce the same assembly. One example of this is after subsampling the contig contained the genome assembly for *Pseudomonas* phage K9-5 had been lost. Interestingly, assembly of *Pseudomonas* phage K9-16 appeared to assemble two different phage genomes which shared no similarity as determined by an alignment using nucleotide BLAST.

The genome of *Pseudomonas* phage K9-18 was unable to be resolved using either the complete set of reads or after subsampling. Further analysis of the assembled reads indicated host DNA contamination. To resolve this, trimmed reads were aligned to the genome of *P. aeruginosa* 484098, which had been used to propagate the bacteriophage, and the unmapped reads used for assembly, this resulted in a fragmented genome assembly.

The completeness of the draft genomes was determined by comparing similarity and size to closely related bacteriophage as determined by PhageClouds. These genomes were then screened to determine the termini using PhageTerm (§2.21.2). PhageTerm identified the termini in 16% (3/19) of the bacteriophage

genomes. Where defined termini could not be identified manual genome reordering was performed using the closest relative as a reference, although this was not possible for some fragmented assemblies such as *Pseudomonas* phage K9-18. In some cases, a close relative could not be identified so the terminase large subunit (terL) was used (Table 4.4).

***Pseudomonas* bacteriophage**	**DNA packaging mechanism**	**Closest *Pseudomonas* phage relative**	**Accession**
491171-1-30	Headful (pac)	NA	-
K9-1	Headful (pac)	NA	-
K9-2	Headful (pac)	NA	-
K9-3	Unknown	Fc22	NC_074742.1
K9-4	Unknown	MD8	NC_031091.1
K9-5	Unknown	MD8	NC_031091.1
K9-6	Unknown	DL68	NC_028971.1
K9-7	Unknown	shane	MT119368.1
K9-9	New	AN14	KX198613.2
K9-10	Unknown	AN14	KX198613.2
K9-11	New	YuA	NC_010116.1
K9-12	New	vB PaeS PAO1 Ab19	NC_042115.1
K9-13	Unknown	vB PaeS PAO1 Ab19	NC_042115.1
K9-14	New	PAE1	NC_028980.1
K9-15	New	AN14	KX198613.2
K9-16 large	Unknown	AN14	KX198613.2
K9-16 small	Unknown		
K9-17	New	vB PaeS C1	NC_069749.1
K9-18	Unknown	Fc22	NC_074742.1

Table 4.4 **Summary of the method used for genome reordering for each bacteriophage**.

Draft bacteriophage genome assemblies were screened to determine the termini using PhageTerm, where the termini were identified, the genome was reordered. Where defined termini could not be identified manual genome reordering was performed using the closest relative as a reference which is reported here.

The reordered bacteriophage genomes were then annotated using Pharokka, each bacteriophage was compared with the reference genome where that existed using Clinker (Figure 4.10). This was used as a final stage of quality control to ensure no large regions of the genome was missing.

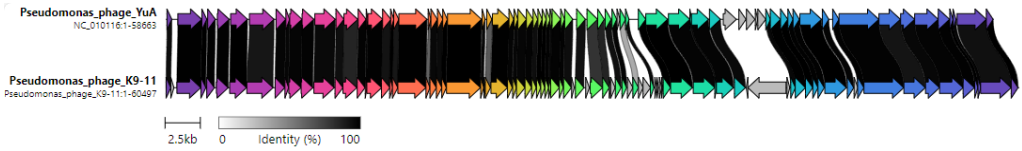


Figure 4.10 **Clinker alignment of *Pseudomonas* phage K9-11 and closest relative *Pseudomonas* phage YuA.**

Complete bacteriophage genomes were annotated using Pharokka and the Genbank outputs were aligned to the closest relative using Clinker. The results were analysed to ensure no large regions of the genome was missing.

4.2.3.2 *Pseudomonas* bacteriophage genomic variation

A summary of the bacteriophage genomes assembled as part of the present study are shown in Table 4.5. Classification of the bacteriophage at the genus and species level was determined using taxmyPHAGE (§2.23), *Pseudomonas* phage K9-18 was unable to be classified due to fragmentation of the genome. A majority (67%, 12/18) of the bacteriophage could be assigned to an existing genus. Of these 50% (6/12) were classified as *Yuavirus* spp. Interestingly, some of the isolated bacteriophage (3/18) were classified within an existing species. The remaining 33% (6/18) were identified as new genera.

***Pseudomonas* bacteriophage**	**Length (bp)**	**GC (%)**	**CDS**	**Coding Density (%)**	**Genus**	**Species**
491171-1-30	66,222	63.71	77	98.05	Hollowayvirus	New Species
K9-1	38,977	62.05	66	97.27	New Genus	-
K9-2	40,250	63.01	60	98.27	New Genus	-
K9-3	39,481	62.77	60	99.61	New Genus	-
K9-4	38,957	62.05	65	97.32	New Genus	-
K9-5	38,935	62.04	65	97.38	New Genus	-
K9-6	62,494	55.65	97	95.95	Pbunavirus	New Species
K9-7	66,528	55.55	107	94.67	Pbunavirus	LMA2
K9-9	62,159	64.51	96	96.69	Yuavirus	New Species
K9-10	62,159	64.52	96	96.68	Yuavirus	New Species
K9-11	60,497	64.15	88	96.85	Yuavirus	Pseudomonas virus Yua
K9-12	57,640	63.07	88	95.28	Abidjanvirus	New Species
Pseudomonas phage K9-13	57,718	63.05	89	95.61	Abidjanvirus	New Species
K9-14	61,866	64.20	93	96.39	Yuavirus	New Species
K9-15	62,159	64.52	97	96.66	Yuavirus	New Species
K9-16 large	61,759	64.53	94	97.04	Yuavirus	Pseudomonas virus M6
K9-16 small	38,977	62.05	65	97.24	New Genus	-
K9-17	43,056	53.61	57	97.41	Septimatrevirus	New Species
K9-18	62,121	60.85	57	~98.69		

Table 4.5 **Summary of bacteriophage genome features.**

The number of coding sequences and the percentage coding density was calculated by Pharokka. Genus and species were classified using taxmyPHAGE.

The genome size of the investigated bacteriophages appears to fall into two groups, a smaller group with genomes ranging from 38,935 to 43,056bp and a larger group with a range of 57,640 to 66,528bp. This pattern was mirrored when investigating the number of coding sequences, the smaller group encoded between 57-65 proteins while the larger group encoded 88-107. A linear regression model identified a correlation between the size of the genome and the number of coding sequences (Figure 4.11). There was a clear outlier and further investigation revealed that this was *Pseudomonas* phage K9-18, due to the difficulties assembling this genome and the number of contig it is likely that this assembly is not complete.

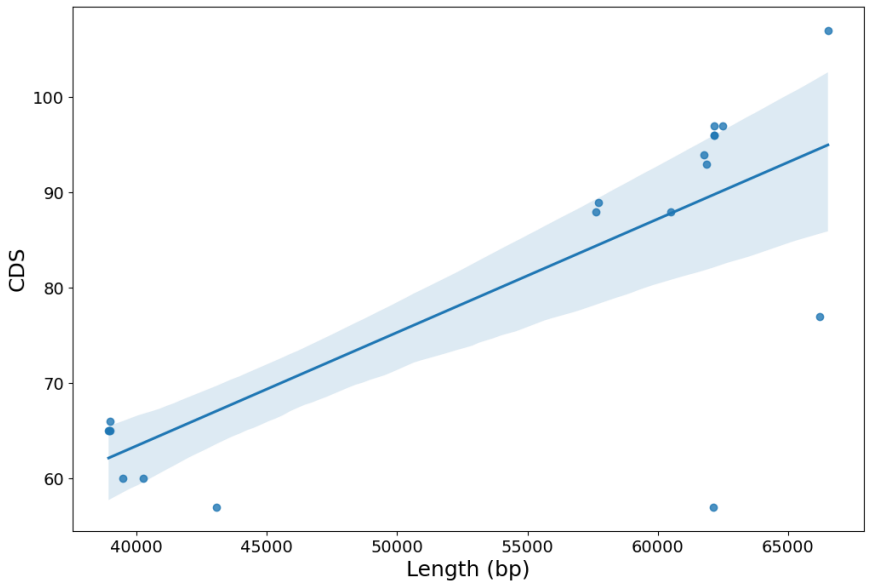


Figure 4.11 **Comparison of genome length and the number of coding sequences predicted by Pharokka.**

A line plot showing the relationship between bacteriophage genome length and the number of coding sequences (CDS). A linear regression model was plotted using seaborn's regplot.

4.2.3.3 Bacteriophage lifestyle prediction

Bacteriophage can be classified as either virulent or temperate, this is determined by their lifestyle. Generally, when considering the use of bacteriophage as a therapeutic, virulent bacteriophage are preferred due to their lytic life cycle. To this end, three tools were used to assess the lifestyle of the bacteriophage based on the genome sequences (§2.23). This identified differences between the tools, 40% (8/20) of the bacteriophage were given the same lifestyle prediction across the three tools while the remaining 60% (12/20) received at least one different prediction from one of the other tools (Table 4.6).

	Lifestyle Prediction		
***Pseudomonas* bacteriophage**	**PhaTYP**	**BACPHLIP**	**PhageScope**
491171-1-30	Temperate	Temperate	Temperate
K9-1	Temperate	Temperate	Temperate
K9-2	Virulent	Virulent	Temperate
K9-3	Temperate	Virulent	Temperate
K9-4	Temperate	Temperate	Temperate
K9-5	Temperate	Temperate	Temperate
K9-6	Virulent	Virulent	Virulent
K9-7	Virulent	Virulent	Virulent
K9-9	Temperate	Virulent	Temperate
K9-10	Temperate	Virulent	Temperate
K9-11	Temperate	Virulent	Temperate
K9-12	Temperate	Virulent	Temperate
K9-13	Temperate	Virulent	Temperate
K9-14	Temperate	Virulent	Temperate
K9-15	Temperate	Virulent	Temperate
K9-16 long	Temperate	Virulent	Temperate
K9-16 short	Temperate	Temperate	Temperate
K9-17	Virulent	Virulent	Virulent
K9-18	Not tested	Not tested	Not tested

Table 4.6 **Comparison of three tools used to identify bacteriophage lifestyles, PhaTYP, BACPHLIP and PhageScope.**

Pseudomonas phage K9-18 was not tested in this work due to a requirement of the tools being a complete genome assembly.

In an attempt to finalise the classification, the annotated genomes were investigated for the presence of genes with a function in host genome integration and excision (Table 4.7). This highlighted that where an integrase was identified all three programs correctly classified the lifestyle as temperate. However, where a transposase was present the results were more variable. Finally, Pharokka failed to identify any coding sequences with an integration or excision function for *Pseudomonas* phage K9-9 to *Pseudomonas* phage K9-16 long however 2/3 of the lifestyle predictors identified these as temperate bacteriophage.

***Pseudomonas* bacteriophage**	**Presence of a gene with integration or excision function**
491171-1-30	Excisionase and transcriptional regulator
K9-1	Integrase + excisionase
K9-2	DNA transposition protein + transposase
K9-3	Transposase + DNA transposition protein
K9-4	Integrase + excisionase
K9-5	Excisionase + integrase
K9-6	
K9-7	
K9-9	
K9-10	
K9-11	
K9-12	
K9-13	
K9-14	
K9-15	
K9-16 long	
K9-16 short	Excisionase + integrase
K9-17	
K9-18	DNA transposition protein + transposase + excisionase and transcriptional regulator

Table 4.7 **Presence of a gene with integration or excision function within the bacteriophage genomes.**

Genome annotations using Pharokka were screened to identify genes with a function in integration or excision that would suggest a temperate lifestyle.

4.2.3.4 Presence of resistance or virulence genes

Due to the prediction of some of the isolated bacteriophage having a temperate lifestyle, the presence of antimicrobial resistance and virulence genes was investigated. Similarly, some bacteriophage are able to transfer AMR genes through transduction (Brown-Jaque, Calero-Cáceres & Muniesa, 2015).

As part of the annotation pipeline, Pharokka screens for antimicrobial resistance genes using the CARD database and virulence genes using the virulence factor database. Analysis of these results did not identify and antibiotic resistance or virulence genes in any of the bacteriophage sequences.

4.2.3.5 Intergenomic similarities

Pairwise intergenomic similarities between 18 bacteriophage genomes were calculated using VIRIDIC, *Pseudomonas* phage K9-18 was excluded to the fragmented nature of the genome assembly (Figure 4.12).

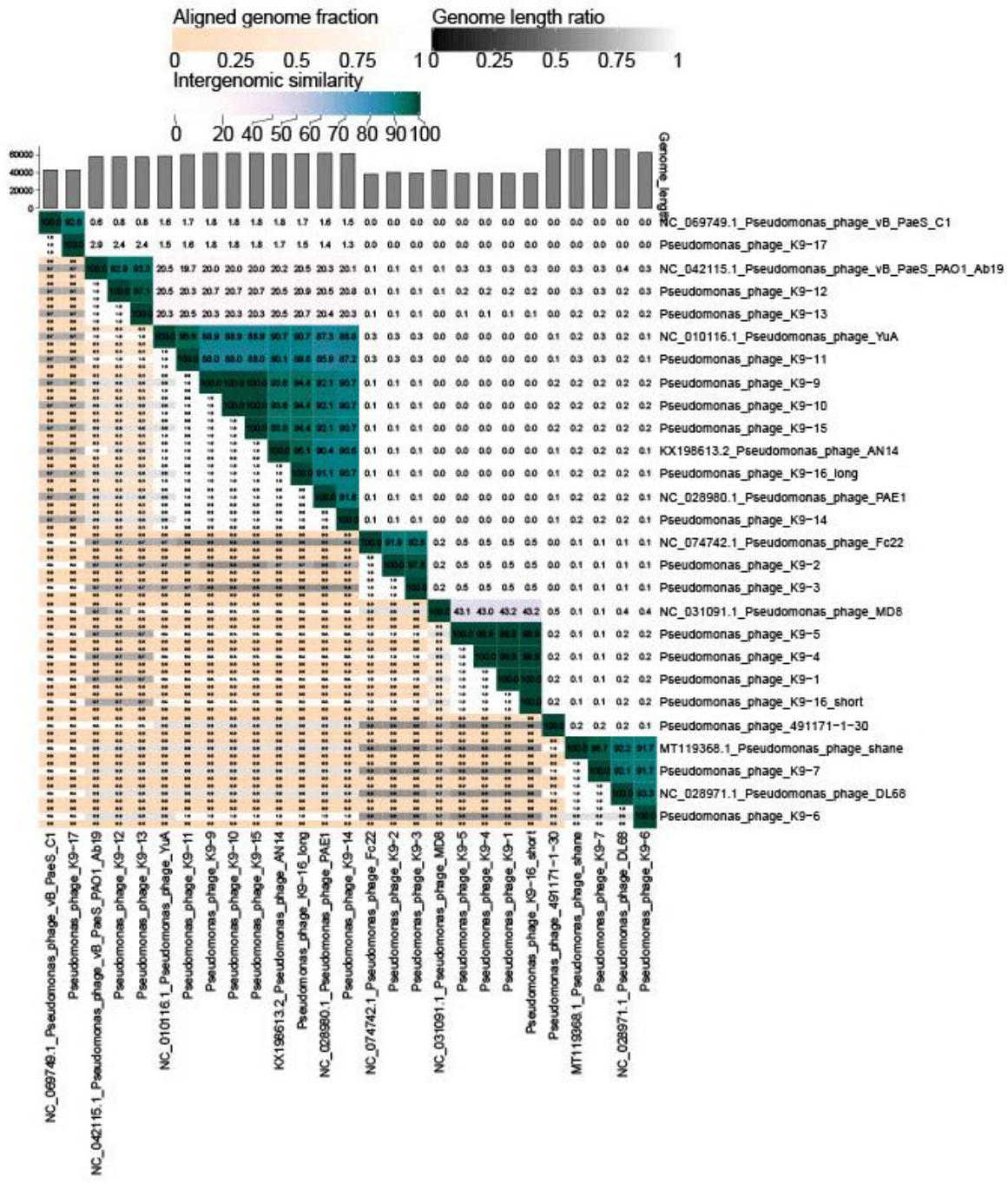


Figure 4.12 **Intergenomic similarities calculated by VIRIDIC.**

Heatmap generated by VIRIDIC of 19 bacteriophage genomes identified in this work in addition to closely related reference genomes identified using BLAST, *Pseudomonas* phage K9-18 was removed from the analysis due to the fragmented genome. The upper right half is a heatmap sorted by hierarchical clustering based on intergenomic similarity. The bottom left portion of the graph shows the aligned genome fractions and genome length ratio. Finally, at the top of the graph is a histogram of the bacteriophage genome lengths.

VIRIDIC identified 8 genus and 17 species clusters (Table 4.8), this identified a large genus cluster containing *Pseudomonas* phage K9-11, K9-14, K9-16 long, K9-9, K9-10 and K9-15. Interestingly, this large cluster had ~20% similarity to *Pseudomonas* phage K9-12 and K9-13 identifying similarities between these two genera of bacteriophage. Finally, *Pseudomonas* phage 491171-30-1 and K9-17 were identified as singletons when compared to the other bacteriophage from this work.

***Pseudomonas* bacteriophage**	**Genus Cluster**	**Species Cluster**
491171-1-30	7	10
K9-1	8	11
K9-2	6	16
K9-3	6	16
K9-4	8	11
K9-5	8	11
K9-6	2	17
K9-7	2	2
K9-9	1	12
K9-10	1	12
K9-11	1	3
K9-12	4	13
K9-13	4	13
K9-14	1	14
K9-15	1	12
K9-16 long	1	1
K9-16 short	8	11
K9-17	5	15

Table 4.8 **Table of assigned genus and species clusters.**

Findings from VIRIDIC genus and species clustering of 18 bacteriophage genomes.

4.2.3.6 Proteomic phylogenetic tree

To assess the relationship between the bacteriophage isolated as part of the present study and related bacteriophage. A proteomic tree was constructed using 100 closely related bacteriophage genomes (§2.23). The bacteriophage from the present study were found on two main branches. The results from the proteomic tree were in agreement with VIRIDIC. Interestingly, *Pseudomonas* phage K9-6 and K9-7 were located on a distinct branch to the other isolates from the present study.

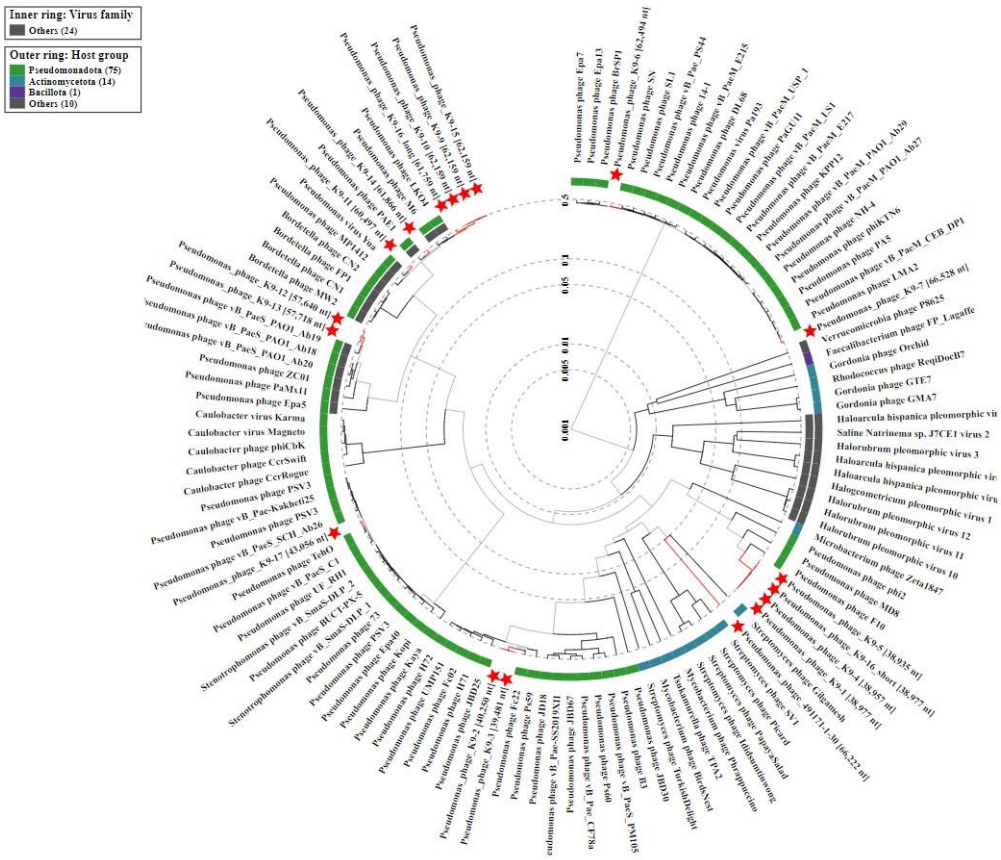


Figure 4.13 **Proteomic phylogenetic tree generated using ViPTree.**

Circular phylogenetic tree generated using ViPTree including 18 bacteriophage isolated as part of the present study, labelled using red stars and 100 closely related bacteriophage.

4.2.3.7 Genomic similarities of prophage regions

To assess the similarity of prophage sequences identified in §4.2.2.7, the prophage regions were extracted using PHASTEST and pairwise intergenomic similarities calculated using VIRIDIC between prophage and the bacteriophage isolated in the present study (Figure 8.7). This identified that the prophage regions were largely unrelated to the bacteriophage isolated as part of the present study. However, *Pseudomonas* phage K9-5, K9-4, K9-1 and K9-16 short were found to be part of the same genes cluster as a prophage region from *P. aeruginosa* 84269. Additionally, *Pseudomonas* phage K9-2, K9-3 were part of the same genus cluster as prophage regions from *P. aeruginosa* 80715, 490051 and 484098 (Table 8.3). Similarly, when a proteomic tree was constructed using the bacteriophage and prophage the majority of bacteriophage isolates were on distinct branches, however *Pseudomonas* phage K9-2, K9-3, K9-5, K9-4, K9-1 and K9-16 were on branches alongside prophage isolates (Figure 4.14).

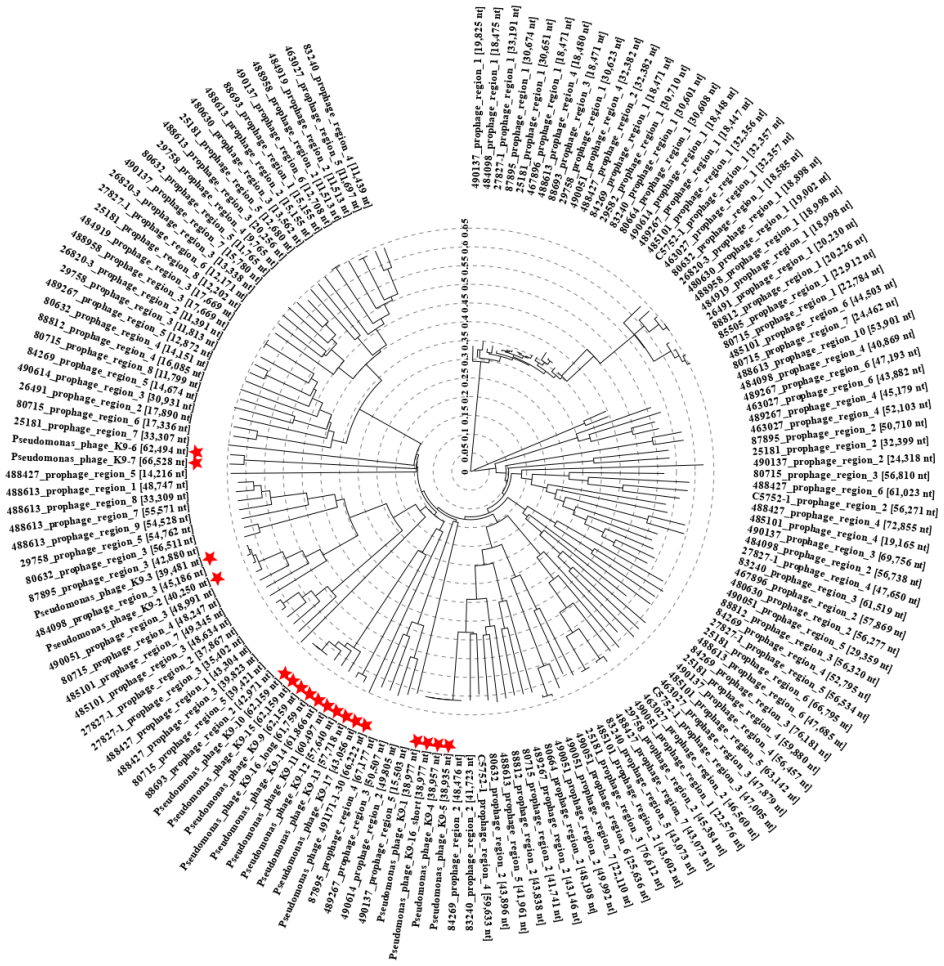


Figure 4.14 **Proteomic phylogenetic tree generated using ViPTree.**

Circular phylogenetic tree generated using ViPTree including 18 bacteriophage isolated as part of the present study, labelled using red stars and prophage regions identified by PASTEST.

4.3 Discussion

Bacteriophage are the most abundant biological entities in the biosphere with an estimated population of 10^{31}, many of these exhibiting large genomic diversity (Comeau et al., 2008). The rate of bacteriophage discovery and classification is high (Ackermann & Prangishvili, 2012), as of 2022 the International Committee of Taxonomy of Viruses (ICTV) has established 11,273 species of bacteriophage (Turner et al., 2023). Not all work investing bacteriophage will be focused on

the search of alternative antimicrobials, however a better understanding of bacteriophage biology could aid in future therapeutic bacteriophage research.

It has been suggested that *P. aeruginosa* isolated from canine otitis externa infections originate from the environment (Morris et al., 2017; Vives-Flórez & Garnica, 2006). Findings from the present study support this notion (§3.2.6). Wastewater is known to be an important source of environmental bacteriophage (Ballesté et al., 2022), including bacteriophage that infect *P. aeruginosa* (Akremi et al., 2022; Aghaee et al., 2021), as such, samples collected in this study were focused on wastewater effluent and bodies of water near WWTPs. No bacteriophage were found in the environmental water samples. This indicates that samples contaminated with faecal material contain a higher titre of bacteriophage than environmental water samples, this is in line with other reports that sewage contaminated sites harboured more bacteriophage than uncontaminated sources (Aghaee et al., 2021). Although *P. aeruginosa* is commonly quoted as ubiquitous in soil and aqueous environments, it is more frequently isolated from soil and water sources linked to human activity including sewage (Crone et al., 2020). Given that bacteriophage will primarily be found in an environment with a host, this likely explains the results from the present study.

As the aim of this study was to isolate bacteriophage that could be used therapeutically to treat canine otitis externa with chronic *P. aeruginosa* infection. When evaluating a bacteriophage for therapeutic use, it is important to consider characteristics that would be beneficial in this role. Virulent bacteriophage can only undergo the lytic lifecycle, therefore these bacteriophage are generally preferred for this application (Fernández et al., 2019). A tentative assessment of

lifestyle can be assessed by the appearance of bacteriophage clearing on a double layer agar plate. Clear plaques indicate lytic bacteriophage while cloudy plaques suggest a lysogenic (temperate) lifestyle (Jurczak-Kurek et al., 2016). Initial characterisation was performed by determining the host range of the isolated bacteriophage against a panel of 80 isolates of *P. aeruginosa* from canine OE in addition to *P. aeruginosa* PAO1-QUB and 484098. As part of this experiment, lysis was characterised between 0-4 (no clearing – complete clearing). This highlighted that there was evidence of temperate bacteriophage amongst the collection.

Although plaque morphology can give an indication as to the lifestyle of the bacteriophage, temperature bacteriophage can enter the lytic lifecycle depending on a number of factors (Howard-Varona et al., 2017). Analysis of the bacteriophage genomes revealed that 15% (3/20) were predicted to be lytic and 25% (5/20) were predicted to be temperate. No consensus was reached for the lifestyle of the other bacteriophage, therefore manual investigation was performed to identify evidence suggesting a temperate lifestyle, this showed that where Pharokka had identified an integrase a temperate lifestyle could accurately be determined by any of the tools. All of the tools tested employ machine learning methods to predict bacteriophage lifestyle however, PhageScope also uses a large database of bacteriophage genomes. BACPHLIP had a default prediction of a lytic genome unless contrary evidence is detected. This is most likely the reason why *Pseudomonas* phage K9-9 to *Pseudomonas* phage K9-16 long were identified as temperate by two of the tools but lytic using BACPHLIP, this notion is supported by Pharoka also being unable to identify any CDS that have a function in integration or excision. Of these bacteriophage, taxmyPHAGE

classified them within two genera, *Yuavirus* and *Abidjanvirus*, the lifecycle of bacteriophage within these genera has not been determined and therefore more work is required before a definitive conclusion can be made (Campbell et al., 2021; Ceyssens et al., 2008). Interestingly, VIRIDIC analysis of the bacteriophage isolated in the present study and prophage sequences recognised using PHASTEST identified that *Pseudomonas* phage K9-9 to *Pseudomonas* phage K9-16 did not cluster amongst the prophage sequences, this might suggest that these bacteriophage are lytic.

Host range is another important characteristic when considering bacteriophage for use as a therapeutic. Bacteriophage that have a host range limited to a single species are desirable as they will not infect the microbiota of the patient, in contrast, within a species, bacteriophage that infect many strains increases the chances that a given bacteriophage can be used for any infection (Fernández et al., 2019). Other *Pseudomonas* spp. were not investigated as part of the present study however, the microbiota of the canine ear generally consists of Gram-positive organisms and yeast (Shaw, 2016; Fraser, 1961; Lyskova, Vydrzalova & Mazurova, 2007), at the time of writing no bacteriophage has been reported with a host range that includes Gram-positive and Gram-negative bacteria. Analysis of the host range results found that 61% (50/82) of the isolates were susceptible to at least one of the bacteriophage. *Pseudomonas* phage K9-7 and *Pseudomonas* phage K9-6 were found to have the largest host range of the bacteriophage isolates as part of the present study, infecting 40% and 34% of the *P. aeruginosa* isolates respectively.

Two studies isolated bacteriophage against *P. aeruginosa* and investigated host range using a panel of 121 cystic fibrosis and 140 clinical and environmental

isolates respectively. The largest host range reported by each study was 68% and 33% (Olszak et al., 2015; Akremi et al., 2022).

Overall, this indicates that the majority bacteriophage isolated in the present study have a narrow host range. Despite screening the environmental samples with clinical *P. aeruginosa* isolates, the majority of bacteriophage were isolated using PAO1-QUB, future work could focus on the isolation of bacteriophage using only clinical *P. aeruginosa* from OE to isolated bacteriophage with a larger host range. Moreover, it is possible to widen the host range of *P. aeruginosa* bacteriophage using phage training. This approach takes advantage of bacteriophage evolution by co-incubating a mixture of bacteriophage and host strains for multiple passages. This had already been demonstrated using *P. aeruginosa* bacteriophage and is another area that could be explored in the future (Mapes et al., 2016).

Efficiency of plating (EOP) was performed after removing *P. aeruginosa* determined insensitive to bacteriophage from the host range assay, despite this, 10% (5/50) were found to be insensitive to all the tested bacteriophage. In the host range assay, a spot containing 1×10^8 PFU/mL was applied to the bacterial overlay. Lysis from without is bacterial lysis that does not rely on bacteriophage infection and can generally occurs when bacteriophage concentrations of are high (Abedon, 2011).

For remaining isolates EOP was calculated as a percentage of PFU/mL on the target *P. aeruginosa* over the PFU/mL on the propagation *P. aeruginosa* (PAO1-QUB and 484098). Analysis of these results revealed that the isolated bacteriophage were able to infect *P. aeruginosa* PAO1-QUB more readily than

other susceptible isolates, notably *Pseudomonas* phage K9-18 was isolated using *P. aeruginosa* 484098 but was able to productively infect PAO1-QUB with a higher efficiency. Only 22% (11/50) of host isolates displayed an increased sensitivity measured as an EOP value greater than 100%, to a given bacteriophage. Moreover, EOP scores were ranked as previously described, this identified that where a bacteriophage was able to infect a given host strain, it did so with high efficiency. Suggesting that despite these differences, infection efficiency was similar to that of the propagation strain.

Resistance or reduced susceptibility of infection can be achieved by mutation or modifications of the receptor, this can influence the number and structure of the receptor in addition to the expression of a protein that can bind to and subsequently block attachment of the phage to a receptor. Another mechanism is the production of extracellular matrix to block receptors or finally the production of receptor inhibitors (Labrie, Samson & Moineau, 2010). Similarly, intracellular factors can also impact EOP, this includes DNA degradation through systems such as restriction-modification and CRISPR-Cas systems in addition to prophage mediated protection such as superinfection exclusion (Labrie, Samson & Moineau, 2010).

Estimation of CRISPR-Cas arrays identified that 38% (10/26) of the sequenced isolates contained a CRISPR-Cas system and 100% (10/10) of these contained Cas type IF but Cas types IE and IC were also identified. One study performed analysis of 672 *P. aeruginosa* genomes, largely from human origin, and identified that 45% contained intact CRISPR-Cas arrays (van Belkum et al., 2015). An intact system is defined as a system with an intact CRISPR locus with a complete set of *cas* genes with no obvious mutations (Bondy-Denomy &

Davidson, 2014) Moreover, they identified that IF subtypes most commonly, followed by IE and IC (van Belkum et al., 2015). Another study analysed 275 genomes of *Pseudomonas* spp. and identified CRISPR structures in 19.6%, the majority of these positive genomes belonging to *P. aeruginosa* (Parra-Sánchez et al., 2023). This is in line with what has been described in the present study. Resistance against bacteriophage infection mediated by CRISPR-Cas has been identified in *P. aeruginosa* (Cady et al., 2012). It is worth noting that an intact CRISPR-Cas system does not signify an active one, further analysis of the CRISPR spacers could determine this (Bondy-Denomy & Davidson, 2014).

The presence of prophage regions in the genome sequences of 26 clinical *P. aeruginosa* was estimated. This found that 100% (26/26) contained at least one prophage sequence additionally, up to 10 prophage regions could be identified within a single *P. aeruginosa* genome. Prophage regions can include genes that encode proteins to resist superinfection. One such mechanism is superinfection exclusion, where DNA of a closely related bacteriophage is prevented from entering the host (Labrie, Samson & Moineau, 2010). The intergenomic similarity of the bacteriophage isolated as part of the present study and prophage regions extracted using PHASTEST was calculated and identified that some of the bacteriophage belonged to the same genus cluster as one or more prophage. Unfortunately, these bacteriophage were largely ineffective at infecting any of the hosts including host strains containing closely related prophage so it is therefore not possible to ascertain if superinfection exclusion was involved. Despite this, a variety of prophage mediated resistance mechanisms have been classified in *P. aeruginosa* which largely function by altering receptors such as the type IV pilus and O-antigen (Bondy-Denomy et al., 2016). This mechanism

would function more broadly as they would prevent infection from any bacteriophage that could recognise the receptor.

The final assay used to characterise the bacteriophage isolates was the indirect measurement of lysis by monitoring the growth of *P. aeruginosa* PAO1 in the presence of 2,3,5-Triphenyltetrazolium chloride (TTC) using an OmniLog plate reader assay. In this case MOI is referred to as the ratio of bacteriophage particles to bacterial cells. Analysis of the bacterial growth identified that MOIs lower than 100 were ineffective at controlling the growth of *P. aeruginosa* PAO1-QUB for any of the tested bacteriophage. Looking specifically at MOI 100, only 32% (6/19) of the bacteriophage were able to reduce the total growth in comparison to the control. Two of these, *Pseudomonas* phage K9-6 and K9-7, appeared to significantly disrupt the growth of *P. aeruginosa* PAO1-QUB.

Bacterial cells growing under different conditions i.e. sessile within top layer agar or planktonic in liquid culture likely have significantly different expression of genes. One group identified a similar phenotype when investigating bacteriophage against *Curvibacter* spp. AEP1.3. The bacteriophage was able to infect and produce clear plaques in double-layer agar, however growth in liquid media showed no difference between the phage and control samples. Further investigation identified differences in expression patterns between the states which likely explains this phenotype (Ulrich et al., 2022). This is one explanation for the lack of lytic activity observed in the present study.

Alternatively, the activity of some bacteriophage may not immediately be observed (Magin, Garrec & Andrés, 2019). One limitation of the assay used in the present study is the use of TTC which crosses bacterial cell membranes and

is reduced within the cell from the electron transport chain producing formazan which is red in colour (Defez, Andreozzi & Bianco, 2017). As a result, after ~15 h a reduction would not be recorded. Future work could use bacteriophage counts to get a more accurate understanding of bacteriophage growth however this method could only be performed on a small number of samples at once as it would be time consuming.

The error bars of *Pseudomonas* phage K9-6, shown as 95% confidence interval, displayed a large amount of variation. To investigate this, individual technical and biological repeats were plotted. This highlighted spontaneous regrowth of the host in individual technical repeats. The rapid nature of the development of resistance, between 10-15 h, hinted that a subpopulation of the *P. aeruginosa* was resistant to infection from this bacteriophage. These subpopulations carry preexisting mutations, likely in the bacteriophage receptor, and can therefore grow in the presence of the bacteriophage. To confirm this, a Luria & Delbrück (1943) experiment could be performed. Rapid regrowth of bacteriophage resistant subpopulations has been previously reported in *P. aeruginosa*. Analysis of these mutants identified a host fitness cost (Li et al., 2022). Similarly, one study identified a lytic bacteriophage, OMKO1, that recognised outer membrane porin M (OprM) as a receptor-binding site. Development of resistance to OMKO1 resulted in the *P. aeruginosa* host becoming more susceptible to a panel of four antibiotics from different classes (Chan et al., 2016). This example demonstrates that utilising the selective pressure of a bacteriophage could result in improved clinical outcomes if used in combination with antibiotics. Further testing of these resistant isolates could provide an insight into similar effects. Both *Pseudomonas* phage K9-6 and K9-7 were classified as *Pbunavirus* spp. by

taxmyPHAGE with *Pseudomonas* phage K9-7 classified as *Pbunavirus* LMA2. Work from another study has identified that multiple *Pbunavirus Pseudomonas virus PB1* like bacteriophage recognised LPS as a receptor (Garbe et al., 2010) with two other studies noting the receptors specifically as the O antigen (Li et al., 2023; Uchiyama et al., 2016). Moreover, one of the bacteriophage was able to infect *P. aeruginosa* isolates where the O5 antigen was not present, eluding to multiple receptors being recognised.

Finally, the use of bacteriophage cocktails is known to improve the therapeutic potential of bacteriophage. Bacteriophage cocktails can be used to reduce the development of resistance to the virus (Abedon, Danis-Wlodarczyk & Wozniak, 2021). Future work to determine the specific host receptor for each bacteriophage could be used to create cocktails of bacteriophage that target different receptors. This could overcome the regrowth that was observed in the present study. Assessing combinations of these bacteriophage using the Omnilog would potentially highlight a greater impact on bacterial growth than when any of the bacteriophage were administered alone.

For some samples, multiple bacteriophage were isolated, restriction digest using 5 restriction endonucleases were performed to produce restriction fragments for each of the bacteriophage to identify if duplicates of bacteriophage were identified. This identified 9 banding patterns, some of which were shared amongst more than one bacteriophage. Additionally, no digestion was observed using any of the enzymes for 7 of the bacteriophage. Of these 5 were classified as *Yuavirus* spp., *Yuavirus Pseudomonas phage YuA* has been previously shown to be insensitive to 10/13 restriction enzymes including EcoRI and HindIII tested in the present study (Ceyssens et al., 2008).

Bacteriophage genomic DNA extractions were sequenced using Illumina (short read) sequencing. Two bacteriophage genomes were unable to be recovered from the assemblies including *Pseudomonas* phage K9-8. When the coverage of the assembled genomes was assessed, it was clear that each bacteriophage was not equally represented within the reads. During Illumina library preparation, DNA fragments are amplified using PCR, this step is known to introduce bias especially for sequences with a high GC content (>65%) (Aird et al., 2011). This notion does not explain the low coverage of all the *Pseudomonas* bacteriophage genomes as *Pseudomonas* phage K9-6 and K9-7 were found to have a GC content ~55%, this implicates another factor. One potential factor was host DNA contamination leading to an inflated DNA concentration value. A final explanation can be attributed to modification of bases. Difficulties were identified in the PCR amplification of *Yuavirus Pseudomonas phage YuA* for genome sequencing (Ceyssens et al., 2008), if similar base substitution or modifications were present here that could result in an under representation of these reads in the sequencing library.

Despite this, assemblies were resolved for 17/19 of the bacteriophage and a partial genome of *Pseudomonas* phage K9-18 was resolved after some additional processing. Interestingly, two distinct genomes were assembled from the reads of *Pseudomonas* phage K9-16 with one of these being 1.5× bigger than the other.

PhageTerm was used to identify the termini of the bacteriophage genomes although it was unable to determine the termini of 84% (16/19) of the genomes. Confirmation of circular permutation of the genome was attempted using (a) (p)erfect (c)ircle? (Fass, 2015) but this was unsuccessful. After the genomes had been reordered using the closet relative as a reference the genomes were

annotated using Pharokka. A limitation of annotation tools for use in bacteriophage is that they often miss coding sequences, an example of this is the o-spanin gene which is embedded within the i-spanin gene (Kongari et al., 2018). Manual curation of phage genomes can sometimes improve annotation beyond that obtained by automated tools such as Pharokka and future work could focus on this aspect of improve the genomes prior to submission to online databases such as GenBank. This highlights that bacteriophage genome assemblies require a large amount of manual curation to get a good result and some key areas of bacteriophage genome assembly where more research is needed.

Taxonomic classification of the bacteriophage using taxmyPHAGE identified that the isolated bacteriophage belonged to 5 known genera while 6/19 of the bacteriophage were identified as belonging to a new genus, taxmyPHAGE uses ICTV cutoffs for genus and species. Intergenomic similarities were calculated using VIRIDIC, this identified 7 genus clusters, separating the new genera into two new genera. VIRIDIC separated the bacteriophage classified as new genus into two genus clusters, containing 4 and 2 bacteriophage respectively. Using default settings, VIRIDIC determines genus using 70% similarity and species as 95% whereas ICTV uses 50% and 95%. *Pseudomonas* phage K9-4 and K9-5, part of the larger new genus cluster were identified as having *Pseudomonas* phage MD8 as a close relative, VIRIDIC analysis highlights only a 40% intergenomic similarity with this bacteriophage supporting the notion of a new genus. Additionally, *Pseudomonas* phage K9-2 and K9-3 which made up the smaller genome cluster had a high intergenomic similarity (~92%) with *Pseudomonas* phage Fc22 (Carballo-Ontiveros et al., 2020), this bacteriophage has not been classified to the genus level again supporting this idea that these

bacteriophage belong to a novel genus. Future work could involve the proposal of a new genus to the ICTV (Adriaenssens & Brister, 2017).

To complement work done assessing host range, bacteriophage lifestyle was predicted using three tools as discussed above, this identified multiple temperate bacteriophage. Generally, temperate bacteriophage are not considered suitable candidates for therapeutic use due to their ability to transfer antimicrobial resistance and virulence genes (Fernández et al., 2019) in addition to their unpredictable lifestyles. Despite this, temperate bacteriophage have been shown to reduce mortality in murine and *Drosophila* infection models with *P. aeruginosa* PA14 from inhibiting motility rather than lysis of the host (Chung, Sim & Cho, 2012). None of the bacteriophage genomes assessed in this work were found to contain antimicrobial resistance or virulence genes, and some promising results were identified in the EOP and Omnilog assays for temperate bacteriophage *Pseudomonas* phage 491171-1-30. Further work could be performed to identify if similar physiological effects are identified with the bacteriophage isolated in this study.

A final important consideration when assessing bacteriophage for use as a therapeutic is stability (Fernández et al., 2019). Improper storage or low stability can result in the application of reduced titre bacteriophage suspensions (Wdowiak, Paczesny & Raza, 2022). Factors affecting bacteriophage stability include temperature and pH (Jończyk-Matysiak et al., 2019). SM is frequently supplemented with gelatin; this helps to stabilise the bacteriophage particles (Sambrook & Russell, 2001). Similarly, calcium and magnesium salts are often included in the media when isolating novel bacteriophage. This is due to the requirement of Ca^{2+} or Mg^{2+} divalent ions for attachment or intracellular growth

of many bacteriophage (Van Twest & Kropinski, 2009). These steps were omitted during the isolation process in an attempt to select for non-fastidious bacteriophage. Unfortunately, the stability of the isolated bacteriophage was not assessed in this work, this will be an important step in the future characterisation of these isolates.

In this study, 18 bacteriophage isolated from the environment and an additional bacteriophage from an existing collection were characterised and assessed for their use as an alternative to antibiotics for the treatment of *P. aeruginosa* infection for cases of canine otitis externa. Host range and efficiency of plating highlighted that the isolated bacteriophage had a relatively low efficacy on the clinical isolates. Similarly, most of the bacteriophage were unable to control the growth of *P. aeruginosa* over a 24-hour period at multiple MOIs. Two of the isolated bacteriophage did stand out as having a good therapeutic potential, namely *Pseudomonas* phage K9-6 and K9-7, future work could involve a cocktail of these bacteriophage and *in vivo* assessment using *Galleria mellonella*. Assessment of the genomes of *P. aeruginosa* isolates from canine otitis externa (§3.2.6) identified the diverse nature of this pathogen, this likely explains the limited host range of many of the bacteriophage. Future work could also involve further isolation of bacteriophage against these clinical isolates so that potential future treatments could be personalised based on susceptibility to a cocktail of bacteriophage.

Chapter 5 Biological Control of *Pseudomonas aeruginosa* Using *Bdellovibrio bacteriovorus*

5.1 Introduction

Bdellovibrio bacteriovorus can prey upon a variety of Gram-negative bacteria via a biphasic predatory lifestyle. *B. bacteriovorus* has been isolated from soil, fresh water, and marine environments (Klein & Casida, 1967; Chu & Zhu, 2010; Taylor et al., 1974). *B. bacteriovorus* has also been isolated from the gastrointestinal tract of humans and animals highlighting their ability to survive in this environment but more importantly their presence in the gut has been linked to a heathier state (Schwudke et al., 2001; Iebba et al., 2013). Principally, environmental *Bdellovibrio* have been more extensively characterised, namely *B. bacteriovorus* HD100 and 109J were both isolated from soil (Stolp & Starr, 1963; Rendulic et al., 2004; Rittenberg, 1972). During its motile attack phase, *B. bacteriovorus* is propelled via a single polar sheathed flagellum (Thomashow and Rittenberg, 1985). When a prey cell is encountered, there is a reversible attachment, followed by irreversible attachment and entry to the prey periplasm mediated by the formation of a small pore. When inside the periplasm, the host rounds into an osmotically stable bdelloplast, at this point the prey cell is killed and the predator begins to replicate, releasing enzymes to degrade macromolecules from the cytoplasm of the host (Negus et al., 2017, Lambert et al., 2011)

B. bacteriovorus was originally isolated as an accidental discovery while trying to isolate bacteriophage (Stolp & Petzold, 1962). This highlights the similarity in the cultivation of these organisms with both invading another prey or host cell and utilising the cell contents to produce progeny ultimately killing the host/prey measured by the formation of clearing or individual plaques in a bacterial lawn.

In comparison to bacteriophage, which generally have small host ranges, *B. bacteriovorus* is able to prey upon on a wide range of Gram-negative bacteria, with one study identifying 68 different strains from 18 separate genera successfully preyed upon, including pathogens such as *E. coli* and *K. pneumoniae* (Dashiff et al., 2011). Other studies investigating the susceptibility of *P. aeruginosa* to *B. bacteriovorus* highlighted more variable results (Dashiff et al., 2011; Markelova, 2010; Waso, Khan & Khan, 2019; Shanks et al., 2013). Due to this, in the present study, *E. coli* S17-1 was included in all assays as a positive control for predation as it is used for the laboratory maintenance of *B. bacteriovorus* (Lambert & Sockett, 2008).

The rise in the number of antimicrobial resistant infections has prompted research in the search for alternative treatments in both human and animals (O'Neill, 2016). One such application is the treatment of secondary infections for cases of canine otitis externa. *P. aeruginosa* are the most common bacteria isolated from chronic otitis, with up to 35% of cases affected within the literature (Nuttall & Cole, 2007; Petrov et al., 2019). As part of the present study, a collection of 253 clinical isolates from cases of canine OE were characterised (Chapter 3). This identified that antimicrobial resistance and biofilm formation was common amongst the clinical strains.

Further to this, 19 bacteriophage were isolated and characterised, two of which showed promise as candidates for the treatment of *P. aeruginosa* infections in cases of canine OE (Chapter 4).

5.1.1 Aims and Hypothesis

The aim of this chapter was to evaluate *B. bacteriovorus* as an alternative therapy for *P. aeruginosa* infections in cases of canine otitis externa.

1. Hypothesis: ***Bdellovibrio bacteriovorus* is able to prey upon clinical *Pseudomonas aeruginosa*.** Experiments performed *in vitro* will demonstrate the ability of *B. bacteriovorus* HD100 to infect and kill *P. aeruginosa* isolated from clinical cases of canine otitis externa.

5.2 Results

5.2.1 Predation on YPSC double layer agar plates

Successful predation can be measured by the formation of clearing or individual plaques (§5.1) in a bacterial lawn in double later agar plates (Figure 5.1).

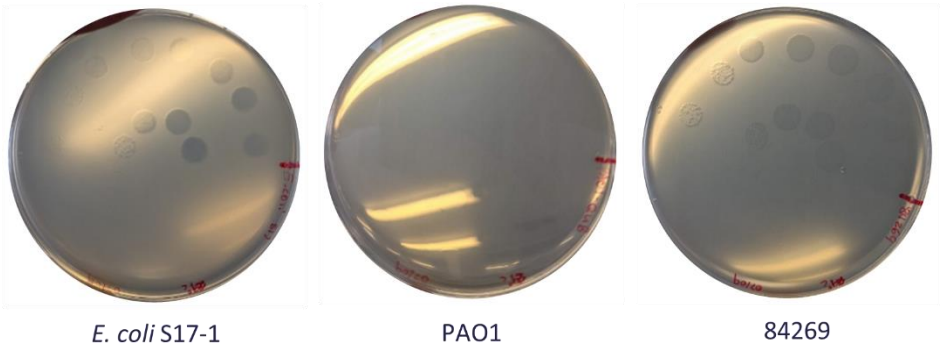


Figure 5.1 **Double layer YPSC agar plates highlighting positive and negative predation results.**

Predation by *B. bacteriovorus* as shown above by zones of cleaning of lawns of prey bacteria (*E. coli* S17-1 and *P. aeruginosa* 84269), the absence of these zones indicates no predation as seen for PAO1. Isolates were grown in YPSC double layer agar for 120 h at 29°C in the presence of a serial dilution of *B. bacteriovorus* HD100.

Initial assessment of susceptibility was performed by spotting a serial dilution of *B. bacteriovorus* predatory culture onto lawns of the 253 clinical isolates at two temperatures, 29° C and 37° C (§2.14).

A minority (16%, 40/253) of the clinical isolates were susceptible to predation at either of the tested temperatures (Table 5.1). Of the susceptible isolates 88% (35/40) and 75% (30/40) were preyed upon at 29° C and 37° C respectively. Additionally, none of the PAO1 sublines were susceptible to predation by *B. bacteriovorus* at either temperature.

	Temperature	
***P. aeruginosa* Strain**	**29º C**	**37º C**
80664	Y	Y
84381	Y	Y
80632	Y	Y
84269	Y	Y
88033	Y	N
88812	Y	N
468424R	Y	Y
484098	Y	Y
489267	Y	N
490137	Y	Y
490051	Y	Y
484919	Y	Y
487280	Y	Y
485101	Y	N
495165	Y	Y
491136	Y	Y
488958	Y	Y
467896	Y	N
26758	Y	N
22406	Y	N
26820-3	Y	Y
26777	Y	Y
26491	Y	Y
29582	Y	Y
240682-1	Y	Y
488613	Y	Y
498492	Y	N
485015	Y	Y
28472	Y	N
84142(2)	N	Y
C-3501	N	Y
487632	N	Y
27660	N	Y
25966	N	Y
30084	Y	N
28279	Y	N
28415	Y	Y
2776	Y	Y
26697-1	Y	Y
26633	Y	Y
PAO1-QUB	N	N
PAO1-N	N	N
PAO1-L	N	N
PAO1-UW	N	N

Table 5.1 **Summary of isolates that were susceptibility to *B. bacteriovorus* predation.**

The ability of *B. bacteriovorus* to prey upon 253 clinical *P. aeruginosa* isolates in addition to four sublines of *P. aeruginosa* was first assessed by spotting dilutions on the predator onto lawns of *P. aeruginosa* using YPSC double layer agar at two temperatures. The results represent three biological repeats. Y – indicates the appearance of clearing and therefore predation. N – the absence of clearing suggesting no predation.

5.2.2 Predation in liquid culture

Subsequently, a selection of 81 clinical *P. aeruginosa* isolates were tested for their susceptibility to predation by *B. bacteriovorus* in liquid YPSC culture by monitoring OD_{600} for 72 h (§ 2.16). The 81 isolates were selected on the bases of evidence of lysogeny (§4.2.2.1).

Out of the 81 isolates tested, four appeared to have a temperate phage that impacted the results, noted as a decrease in OD_{600} in both the test and control samples in addition to the absence of *B. bacteriovorus* when viewed on the microscope. Additionally, bacterial debris was noted which is common during bacteriophage infection (Rabinovitch, Aviram & Zaritsky, 2003). These isolates were subsequently removed from further analysis. Of the remaining 77 isolates, visualisation of the growth curves highlighted two different growth patterns for *P. aeruginosa* isolates (Figure 5.2).

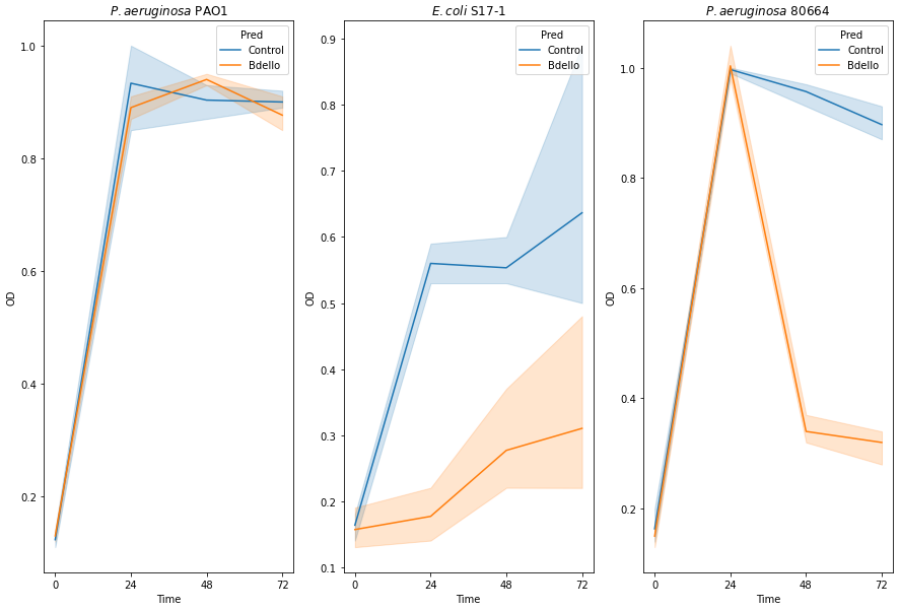


Figure 5.2 **Growth curves of *P. aeruginosa* isolates in the presence if *B. bacteriovorus* in liquid culture.**

The ability of *B. bacteriovorus* to prey upon clinical *P. aeruginosa* isolates in vitro using YPSC broth was tested. The values represented here are the mean with 95% confidence interval of three biological repeats.

The first pattern, highlighted with PAO1, is indicative of no predation, within the first 24 h an increase in OD_{600} was observed for both the control and *B. bacteriovorus* positive samples, the OD_{600} then remained the same for the duration of the experiment. The majority of isolates tested (86%, 66/77) exhibited this pattern and were classed as non-sensitive to predation. The second pattern contains the isolates sensitive to *B. bacteriovorus,* shown using 80664 as an example. A minority (14%, 11/77) were found to have this patten and classified as sensitive to *B. bacteriovorus* predation. The first 24 h appear the same as pattern one with an initial increase in OD_{600} observed for both *B. bacteriovorus* treated and untreated samples. However, between 24 and 48 h there is a large reduction in OD_{600} suggesting predation by *B. bacteriovorus* which was confirmed by an increase in *B. bacteriovorus* observed using a microscope, the OD_{600} was then stable for the final 24 h.

No initial growth of *E. coli* S17-1 was observed suggesting that *B. bacteriovorus* was able to prevent growth of S17-1 despite being in YPSC broth. The pattern was not observed for any of the clinical *P. aeruginosa*.

5.2.3 Microscopy

To confirm that intraperiplasmic predation of *P. aeruginosa* by *B. bacteriovorus* was taking place, microscopy experiments were performed by Dr J. Tyson (§2.17) using a selection of nine clinical *P. aeruginosa* isolates in addition to *P. aeruginosa* PAO1-QUB and *E. coli* S17-1 (Figure 5.3). Analysis of the images identified that bdelloplasts were present in all the samples. This included PAO1, 80842, 80610, 84556, and 2943 which were all identified as non-sensitive to predation by *B. bacteriovorus* when considering double layer agar predation (§5.2.1).

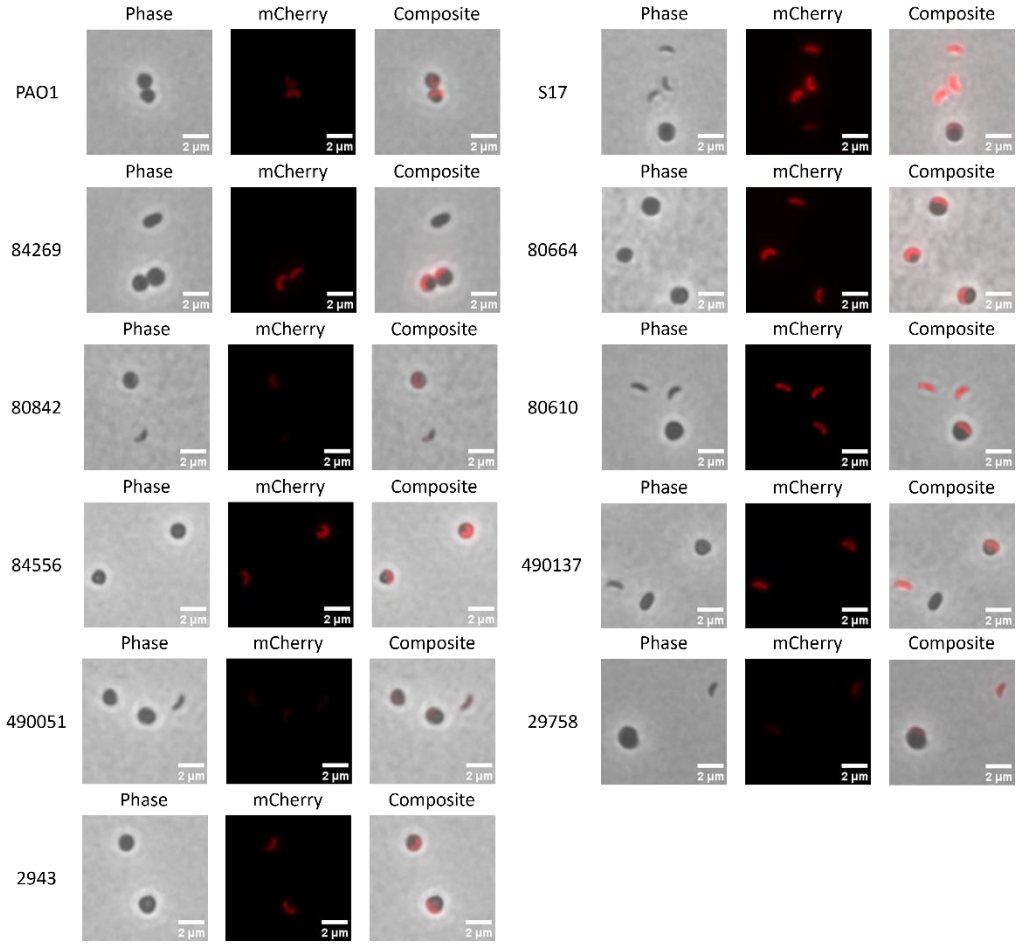


Figure 5.3 **Formation of bdelloplasts in clinical *P. aeruginosa* isolates following *Bdellovibrio* exposure**.

Fluorescence microscopy images of *B. bacteriovorus* HD100:Bd0064mCherry up to two hours post inoculation with nine *P. aeruginosa* isolates from cases of canine otitis externa in addition to *P. aeruginosa* PAO1 and *E. coli* S17-1. Images represent one biological repeat. Scale bar is equivalent to 2 µm.

5.2.4 Predation of pre-formed biofilm

During the analysis of 253 *P. aeruginosa* from cases of canine OE it was found that 82% (207/253) of strains produced strong levels of biofilm and only 4% (10/253) produced no quantifiable biofilm under the tested conditions (§3.2.3). To consider *B. bacteriovorus* as an alternative therapy its ability to prey upon cells within a biofilm was assessed by exposing pre-formed *P. aeruginosa* biofilms to *B. bacteriovorus* (§2.18). This assay was only performed using seven

clinical *P. aeruginosa*, however, these results showed that PAO1 and 86% (6/7) of strains' pre-formed biofilms were significantly reduced in the presence of *B. bacteriovorus* at 29° C, while at 37° C there was no significant reduction in the levels of biofilm for PAO1, and only 71% (5/7) of the strains still showed a reduction. Interestingly, strain 467523 had no significant reduction in biofilm at 29° C but did at 37° C (Figure 5.4).

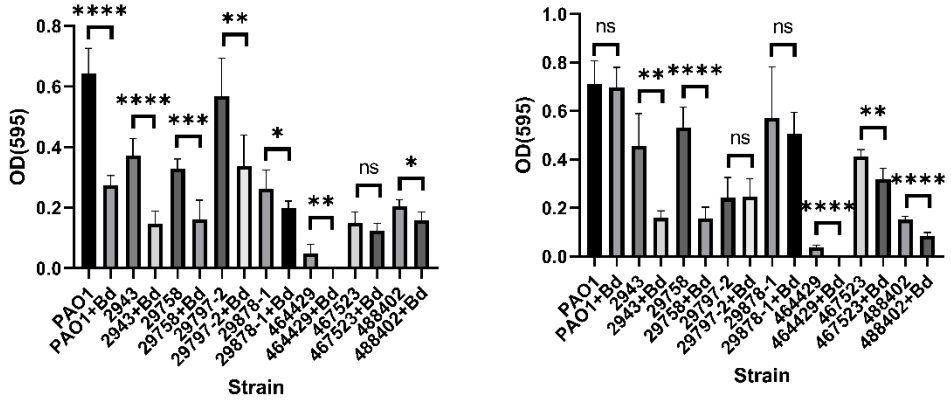


Figure 5.4 **Level of pre-formed biofilms in the presence of *B. bacteriovorus***.

Pre-formed biofilms of seven *P. aeruginosa* isolates from canine otitis externa were exposed and PAO1 were exposed to *B. bacteriovorus* and incubated at 29° C (left) and 37° C (right). Values shown represent one biological and 6 technical repeats, statistical significance was calculated using Welch's t-test. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

5.2.5 Effect of ear cleaner on the viability of *B. bacteriovorus*

The use of an ear cleaner is common in cases of canine OE as they aid in the removal of debris from the ear canal (Swinney et al., 2008). As these products alone have been shown have some effect against clinical *P. aeruginosa* (Swinney et al., 2008; Steen & Paterson, 2012) the effect of the ear cleaner on the viability of *B. bacteriovorus* was investigated. Briefly this was performed by combining attack phase *B. bacteriovorus* with either Ca/HEPES or a commercial ear wash and monitoring recoverable *B. bacteriovorus* (§2.19).

This identified that after two hours of exposure the PFU/mL recorded was 1.7×10^7 and 1.7×10^8 for the ear wash and Ca/HEPES solutions respectively. However, after 24 h the mean PFU/mL in the ear wash had been reduced to only 34 PFU/mL while the recoverable number of predators in Ca/HEPES remained high (5.2×10^7) (Figure 5.5). This result highlighted that the presence of the ear wash had a significant impact ($p<0.0001$) on the viability of *B. bacteriovorus* after 24 h while in the attack phase when compared Ca/HEPES using Welch's t test.

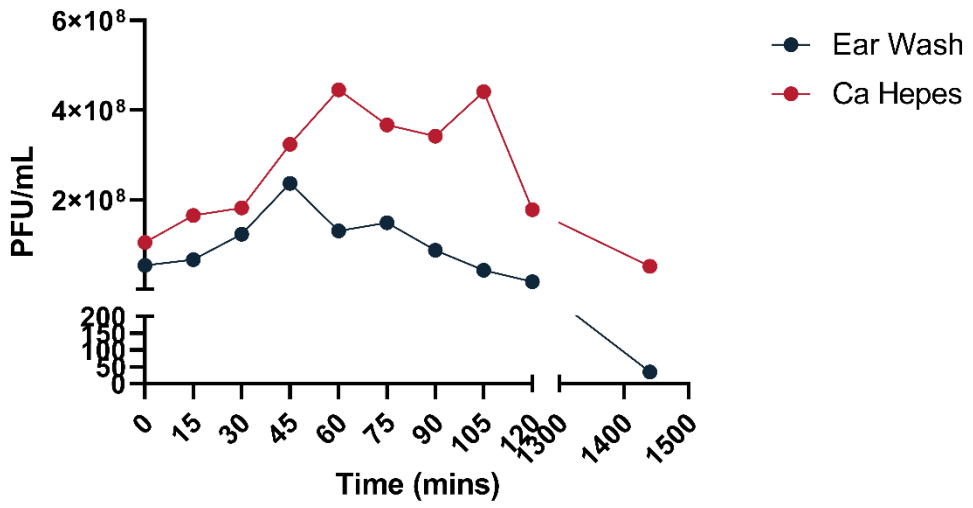


Figure 5.5 **Viability of *B. bacteriovorus* in the presence of commercially available ear wash**.

B. bacteriovorus was combined with Ca/HEPES buffer and ear wash at 29° C. Viability was calculated by enumerating the *B. bacteriovorus* cultures every 15 min for 2 h and then again after 24 h. Values shown represent the mean of three biological repeats.

5.2.6 Detection of secreted predation-inhibitory products

Due to the large proportion of clinical *P. aeruginosa* that were identified as non-sensitive to predation by *B. bacteriovorus*, the presence of secreted inhibitory products was investigated by inoculating top agar containing a mixture of *B.*

bacteriovorus and *E. coli* S17-1 with a spot of *P. aeruginosa* (§2.15). The predator:prey ratio in the overlay should allow complete clearing of the lawn as a result, any growth of the *E. coli* within the lawn around the *P. aeruginosa* suggests the inhibition of predation by *B. bacteriovorus* (Figure 5.6). Of the 253 clinical *P. aeruginosa* isolates that were tested, a zone of inhibition was reported for 58% (147/253) of the isolates.

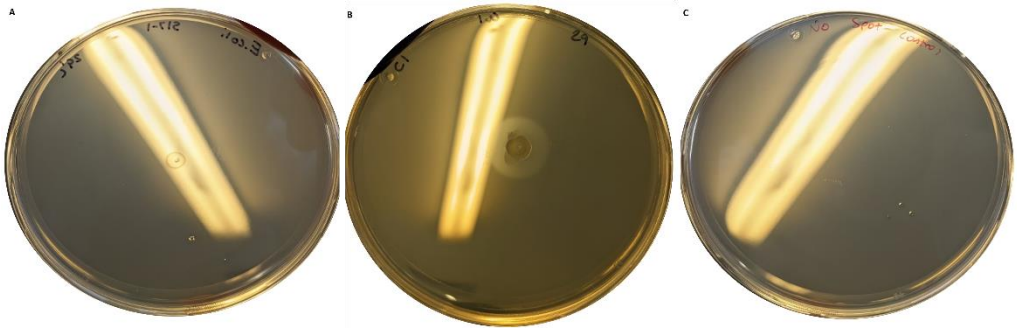


Figure 5.6 **Agar plate photographs investigating the presence of products that inhibit predation.**

B. bacteriovorus HD100 was combined with *E. coli* S17-1 in YPSC top agar, once dry the agar was inoculated with a spot of *P. aeruginosa* which was then incubated statically at 29° C and 37° C for 5 days. A. Shows an example prey where no zone of inhibition was identified. B. *P. aeruginosa* C5752-1 growth in the centre of the plate with a zone of turbidity as a result of *E. coli* S-17 growth within the top agar due to inhibition of *B. bacteriovorus*. C. A completely clear lawn of with no spot.

5.2.7 Identification of genes associated with sensitivity to *B. bacteriovorus*

Pan-genome analysis was performed using GFF3-files produced by Bakta combination with Roary (§2.22.8). The pan-genome of the 35 *P. aeruginosa* clinical isolates contained a total of 14,938 genes. These were divided between core genes (n=4699), soft core genes (n=245), shell genes (n=1367) and finally cloud/accessory genes (n=8627).

The presence and absence of genes known to encode for inhibitory products was investigated (Figure 5.7), however no correlation was identified between the presence of these genes and non-susceptibility to predation.

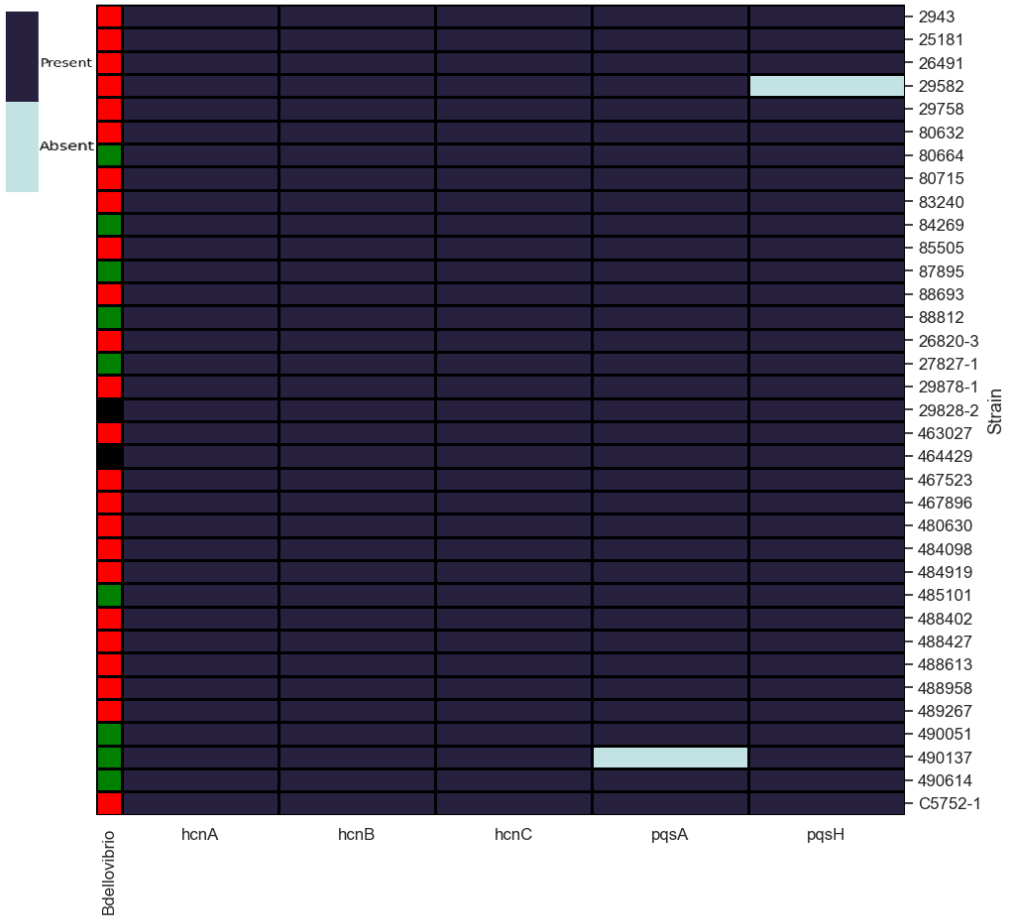


Figure 5.7 **Presence and absence of genes known to produce predation-inhibitory products.**

Heatmap displaying presence and absence of *hcnABC* and *psqAH* in the genomes of 35 *P. aeruginosa* canine otitis externa isolates. Additionally, susceptibility to predation by *B. bacteriovorus* in liquid culture is shown Green – susceptibility to predation - Red – Not susceptibility to predation - Black – Not tested.

Subsequently, Scoary was performed to identify genes from the clinical *P. aeruginosa* isolates associated with sensitivity to *B. bacteriovorus* predation. Two of the sequenced *P. aeruginosa* strains, 29828-2 and 464429, were not

included in the liquid predation assay and were therefore not included in the Scoary analysis.

Scoary revealed 100 genes associated with susceptibility to *B. bacteriovorus* ($p<0.05$). Of the 100 genes, 6% (6/100) were duplicates and 2% (2/100) were hypothetical proteins and were subsequently removed from further analysis. The top 10 most associated genes are shown in Table 5.2

Gene	**Function**	**P Value**
	Phosphopantetheine-binding protein	0.000531
	ATP-dependent helicase	0.003079
zorA	Anti-phage defense ZorAB system ZorA	0.003079
motB	Chemotaxis protein MotB	0.003079
amrR	TetR family transcriptional regulator AmrR	0.013272
	TraG-N domain-containing protein	0.013272
	DUF2158 domain-containing protein	0.013272
	DUF3077 domain-containing protein	0.015369
ivlB	thiamine pyrophosphate-requiring protein	0.015369
	DUF4142 domain-containing protein	0.015369

Table 5.2 **Summary of the top 10 results from Scoary analysis.**

Roary analysis was paired with a trait table containing sensitivity to predation in the liquid predation assay and used to calculated genes that were overrepresented in the groups with Scoary.

5.2.8 *P. aeruginosa* phylogeny and *B. bacteriovorus* susceptibility

A phylogenetic tree was constructed containing 35 isolates from the present study in addition to 70 isolates listed in the PGBD (§3.2.6.4) and visualised containing susceptibility to *B. bacteriovorus* predation in a liquid culture assay (Figure 5.8). This identified that isolates that were susceptible to predation by *B. bacteriovorus* were not closely related and could be found in all three groups on the tree.

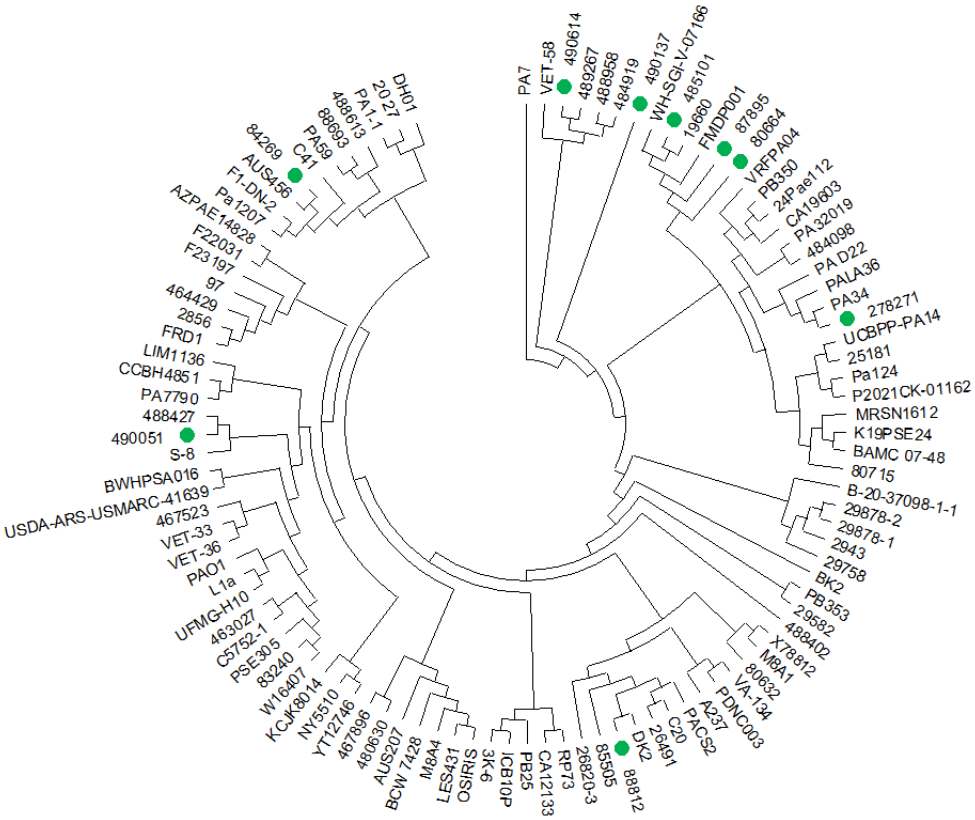


Figure 5.8 **Phylogenetic tree of *P. aeruginosa* strains and relationship to susceptibility to *B. bacteriovorus*.**

Mashtree with bootstrapping (100 replicates) was used to calculate the phylogenetic distances of the *P. aeruginosa* isolates from the present study in addition to 70 isolates from the *Pseudomonas* database to create a neighbour joining tree which was visualised using MEGA. Susceptibility to predation by *B. bacteriovorus* in liquid culture is shown in green.

5.3 Discussion

P. aeruginosa is not part of the microbiota of the canine ear but is frequently isolated from cases of chronic OE, and the nature of this pathogen often makes treatment difficult. Biofilm formation and antimicrobial resistance highlight the need for alternative treatments. In this study, *B. bacteriovorus*, a predatory bacterium with a unique biphasic lifestyle, was investigated to this end.

The ability of *B. bacteriovorus* to prey upon a lawn of clinical *P. aeruginosa* isolates was first investigated using YPSC double layer agar. This was performed at two temperatures; 29° C which is commonly used for laboratory maintenance of *B. bacteriovorus* (Lambert & Sockett, 2008) while 37° C was included as it represents a clinically relevant temperature (Grono, 1970a). This found that 16% (40/253) of the clinical isolates were preyed upon by *B. bacteriovorus* with a larger proportion susceptible only at 29° C, which is in line with the optimum temperature of *B. bacteriovorus* as described above. Multiple sublines of *P. aeruginosa* PAO1 have been identified, four of which, namely PAO1-QUB, PAO1-N, PAO1-L and PAO1-UW, were assessed as part of the present study and shown to have different physiological characteristics (Chapter 3). As such, it seemed appropriate to determine differences in *B. bacteriovorus* susceptibility, this identified that all of the sublines were non-susceptible to predation by *B. bacteriovorus*.

Further experiments using liquid co-culture found a similar number of clinical isolates susceptible to predation (14%;11/77). The formation of a bdelloplast, and therefore active predation by *B. bacteriovorus*, was confirmed using fluorescence microscopy. Interestingly, bdelloplast formation was observed

even where strains were reported as resistant to both predation assays such as *P. aeruginosa* PAO1-QUB.

Studies investigating the sensitivity of *P. aeruginosa* to predation by *B. bacteriovorus* are conflicting. Initial studies using *B. bacteriovorus* 109J, found that the tested *P. aeruginosa* strains were largely non-susceptible using liquid and top agar methods (Dashiff et al., 2011). In contrast, other groups have investigated the susceptibility of clinical *P. aeruginosa* strains to predation by *B. bacteriovorus* 109J and identified that predation was seen for a number of clinical isolates (Sun et al., 2017; Saralegui et al., 2022). Accurate comparisons cannot be made due to these groups using a different strain of *B. bacteriovorus* in comparison to the current study.

Shanks et al., (2013) tested a collection of *P. aeruginosa* isolates from ocular infections and found that 100% (10/10) of the isolates were susceptible to *B. bacteriovorus* HD100, using liquid co-culture only. Despite the difference in sample size, this is a higher susceptibility than has been reported in the present study. Other researchers investigating *P. aeruginosa* susceptibility to *B. bacteriovorus* HD100 have only used one host when demonstrating predation, therefore accurately assessing the prey range of *B. bacteriovorus* HD100 within *P. aeruginosa* strains is difficult (Iebba et al., 2014; Pantanella et al., 2018; Liu et al., 2023a). At the time of writing, the present study represents the largest panel of clinical *P. aeruginosa* strains that has been assessed for susceptibility to predation by *B. bacteriovorus*.

Interestingly, Tajabadi and colleagues (2023) identified a statistically significant difference between the CFU/mL of *P. aeruginosa* PAO1 when grown in the

presence of *B. bacteriovorus* HD100. Between 24 h and 168 h there was ~1 $\log_{10}$ CFU/mL reduction of *P. aeruginosa* PAO1 in the presence of *B. bacteriovorus*. These results are similar to what was reported in the present study, as the presence of *Bdellovibrio* did not prevent the growth of *P. aeruginosa* PAO1. However, a difference in OD_{600} when *P. aeruginosa* PAO1 was grown in YPSC broth in the presence of *B. bacteriovorus* was not observed in the present study. This could be explained by differences that are known to exist between *P. aeruginosa* sublines. For instance, PAO1-UW has a 2.2 Mb inversion when compared to the original PAO1, moreover PAO1-DSM was found to outnumber other sublines during stationary phase, suggesting enhanced survival under these conditions (Klockgether et al., 2010). It is possible that differences between sublines could manifest as sensitivity to predation by *B bacteriovorus*. Alternatively, fluorescence microscopy performed as part of the present study identified bdelloplast, indicative of predation, for all the samples including *P. aeruginosa* PAO1. This means that some predation is taking place that is not observable with the methods used in this study. This likely means that observing OD_{600} alone is not enough to get an accurate understanding of the predatory-prey dynamics. The findings presented by Tajabadi et al., (2023) in combination with the findings of bdelloplast using fluorescence microscopy in the present study are interesting as it suggests that a subpopulation *P. aeruginosa* PAO1 is susceptible to predation. The host range of bacteriophage can be widened by co-incubating a mixture of bacteriophage and host strains for multiple passages (Mapes et al., 2016). Evolution studies using *Bdellovibrio* spp. NC01 cocultured with *Pseudomonas* spp. NC02 for 2880 h found that although mutations did

occur, they did not significantly improve prey range or predation efficiency (Mulvey et al., 2023).

The formation of biofilms allows *P. aeruginosa* to better survive in its environment and significantly reduces the effectiveness of treatment in the case of OE (Fusconi et al., 2011; Pye, Yu & Weese, 2013; Robinson et al., 2019; Chan et al., 2019a), this can result in prolonged chronic infections (Pye, 2018). The high proportion of strong biofilm formers in the population of *P. aeruginosa* from cases of canine OE has been discussed previously (§3.2.3). Previous studies have shown that *B. bacteriovorus* 109J is able to remove pre-formed *E. coli* biofilm (Kadouri & O'Toole, 2005), however, more recently it has been shown that under conditions where biofilm is still growing, *B. bacteriovorus* can become immobilised in the matrix of the biofilm and therefore unable to reach prey cells (Wucher et al., 2021). Preliminary experiments investigating the removal of pre-formed *P. aeruginosa* biofilms highlighted some promising results at both 29° C and 37° C. This is in line with reports from other researchers (Iebba et al., 2014). Notably, after the preformed biofilm had been exposed to Ca/HEPES only for 24 h at 37° C, the OD_{595} of the crystal violet stain was 0.71, this is a large reduction compared to OD_{595} 2.46 after the first 24 h in LB. This suggests that the biofilm of *P. aeruginosa* is unstable when exposed to Ca/HEPES. A high concentration of calcium ions has been demonstrated to promote biofilm formation in *Vibrio cholerae* and result in a thicker biofilm for mucoid *P. aeruginosa* isolates (Sarkisova et al., 2005). Starvation, such as when in a nutrient depleted environment such as Ca/HEPES, can induce biofilm dispersal in multiple species including *P. aeruginosa* (Huynh et al., 2012), this

is one explanation for the reduction in preformed biofilm that was found in the present work.

Ca/HEPES and dNB (dilute nutrient broth) or ddNB (double dilute nutrient broth) are used interchangeably by different groups investigating *B. bacteriovorus* (Lambert & Sockett, 2008; Dashiff et al., 2011; Kadouri & O'Toole, 2005). One group noted that the use of ddNB could maintain preformed biofilms on the surface of microtiter wells for up to 120 h (Kadouri & O'Toole, 2005). As biofilms seemed unstable when submerged in Ca/HEPES buffer, future investigation would require the optimisation of the method exhibited in the present study, this could start by investigating methods used by other groups (Kadouri & O'Toole, 2005; Iebba et al., 2014).

The potential for the use of *B. bacteriovorus* as a novel therapeutic is clear when considering other bacterial species (Atterbury et al., 2011; Willis et al., 2016). However, 88% of clinical *P. aeruginosa* isolates were identified as non-sensitive in the agar predation assay in the present study. Unlike bacteriophage, which recognise very specific receptor sites, *B. bacteriovorus* is able to prey upon multiple genera of Gram-negative bacteria (Dashiff et al., 2011). Until recently the mechanism by which *B. bacteriovorus* recognised its prey was unknown. Caulton et al., (2024) identified a mosaic adhesive trimer (MAT) superfamily, a family of homologous proteins, localised at various points on the predator surface. These were found to be diverse, are thought to be responsible for the wide prey range observed in *B. bacteriovorus*. Six MAT constructs were screened for adhesion to an O-antigen array consisting of 26 glycans from 11 bacteria and one (Bd2439) showed strong binding to *Proteus mirabilis* OXK glycan and significant binding to glycans from *E. coli* K-235 and *Serratia*

marcescens. The O-antigen of *P. aeruginosa* is highly variable with the International Antigenic Typing Scheme (IATS) recognising 20 different serotypes (Liu et al., 1983; Liu & Wang, 1990). This could explain the difference in sensitivity to *B. bacteriovorus* that was found in the present study however, deletion of Bd2439 showed no defect in the predation (Caulton et al., 2024) highlighting that prey recognition is more complex than this and more work is needed to further understand predatory-prey interactions.

The differences between isolates may not be due to a defect in recognition, it could be attachment, invasion or another part of the life cycle. In fact, *B. bacteriovorus* has been shown to overcome LPS modification, capsules, and S-layers (Negus et al., 2017). To explore this idea, the presence of secreted inhibitory products was investigated. This found that 58% of the clinical *P. aeruginosa* isolates were identified to secrete inhibitory products. *P. aeruginosa* is known to produce three types of pyocins which are bacteriocins, and could be important when considering predation, as the *Bdellovibrio* may be sensitive to such bacteriocins (Michel-Briand & Baysse, 2002). Additionally, Mun and coworkers showed that *Chromobacterium piscinae* could produce cyanide up to concentrations of 800 μM, although just 200 μM was enough to significantly reduce the swimming speeds of *B. bacteriovorus* HD100, which in turn reduced the predatory efficiency of the *Bdellovibrio* (Mun et al., 2017). *P. aeruginosa* is also able to produce cyanide via the *hcnABC* genes (Blumer & Haas, 2000). Similarly, *P. aeruginosa* PA14 mutants have been used to detect inhibitors *B. bacteriovorus* 109J and found that quinolone compounds produced by the *pqs* quorum sensing system could inhibit predation of *E. coli* in the presence of these compounds (Hoshiko et al., 2021). The presence of indole has also been found

to inhibit *Bdellovibrio* predation when present in 2 mM concentrations (Dwidar, Nam & Mitchell, 2015). Indole is not produced by *P. aeruginosa* (Lee, Jayaraman & Wood, 2007) so this factor was not assessed in the present study. Investigation into the presence and absence of *hcnABC, pqsA* and *pqsH* did not correlate with sensitivity to predation by *B. bacteriovorus*. Hoshiko et al., (2021) found that even in the presence of *P. aeruginosa pqsA* or *pqsH* mutants, *B. bacteriovorus* was unable to prey upon the *Pseudomonas* and some inhibition was still seen, implicating another unknown factor in resistance to predation. Pan-genome analysis was performed using Scoary which identified 100 genes associated with sensitivity to *B. bacteriovorus*. Neither *hncABC* nor *pqsAH* were present in the Scoary analysis and so future work could focus on the results from Scoary to identify potential factors that could be involved in the resistance of predation by *B. bacteriovorus*.

When evaluating *B. bacteriovorus* as a therapeutic option for canine OE, it is also important to consider factors within the ear that could reduce the efficacy of predation. In addition to the factors discussed above, environmental factors can also have an influence on predation. One such factor that will be important to consider in the case of otitis is viscosity from excess debris in the ear canal, as it has been shown that as viscosity increases predation is slowed until it is almost completely inhibited, most likely as a result of a loss of motility (Im et al., 2019). This debris also prevents the proper function of some antimicrobials (Nuttall, 2016). Due to this, dogs with canine OE can be prescribed with ear cleaners, these are commercially available and often used alongside antibiotics. When the effect of a commercial ear cleaner on the viability of *B. bacteriovorus*, a 7 $\log_{10}$ PFU/mL drop was reported after 24 h. The composition of ear clearers

varies but generally include cerumenolytics, surfactants, astringents, antimicrobials, and anti-inflammatories (Swinney et al., 2008). In this work, only one product was evaluated, TrizAural, which contains disodium EDTA dihydrate in addition to five other ingredients (Dechra, 2024). Tris-EDTA has been shown to have an antimicrobial effect against pathogens isolated from cases of canine OE (Wooley & Jones, 1983; Steen & Paterson, 2012; Swinney et al., 2008). It is therefore likely that the disodium EDTA dihydrate was responsible for the reduction in *B. bacteriovorus* viability. This also highlights that other products used in the ear could have a similar effect and will need to be investigated. Notably this will include antibiotics that are used to treat the infection as gentamicin and ciprofloxacin can inhibit *B. bacteriovorus* at 0.06 and 0.31 µg/mL respectively (Marine et al., 2020).

In the present study, *B. bacteriovorus* was assessed for its use as an alternative to antibiotics for the treatment of *P. aeruginosa*. Specifically, clinical isolates from cases of canine OE. A large proportion of the population were non-sensitive to predation however some sensitive isolates were also identified which highlights the potential of the unique predatory bacteria. Preliminary results were promising for the removal of pre-formed biofilm. Similarly, results identified the production of products that inhibit predation, further work investigating these products would give researchers a better understanding of how prey cells can resist predation and potentially uncover mechanisms to overcome these.

Chapter 6 General Discussion

The work described in this thesis has highlighted the potential for the use of novel therapies, namely bacteriophage and *B. bacteriovorus*, as alternatives to antibiotics for the treatment of *P. aeruginosa* infections in cases of canine OE. Adoption of a novel therapy for *P. aeruginosa* infections in canine OE could result in significantly improved clinical outcomes for patients with chronic antimicrobial resistant *P. aeruginosa* infections by reducing or eliminating the need for surgery. Additionally, the use of novel therapies may reduce the development of antimicrobial resistant isolates from this infection. This could reduce the burden of antimicrobial resistant infections seen in veterinary practices or extend the life of existing antibiotics currently used in practice. Some success has already been reported for the use of bacteriophage therapy to *P. aeruginosa* OE in both humans and dogs. Despite this, more research is needed investigating factors such as the effect of bacteriophage treatment on the ear microbiota and how the characteristics of the bacteriophage effect treatment. Additionally, multiple studies have shown successful applications of *B. bacteriovorus in vivo* although the use of this predator for treating OE has not been described previously. Moreover, the safety and capability of each of these alternative therapies has been demonstrated in humans and animals which supports the idea that they can be used for cases of OE as was the basis for the present study.

P. aeruginosa is an important nosocomial pathogen, in addition to being frequently isolated from patients with cystic fibrosis. Due to this, previous studies have focused on isolates from these sources and less is known about this pathogen's role in canine OE. To this end, the first part of the present study involved the characterisation of *P. aeruginosa* strains isolated from cases of

canine OE (Chapter 3). Acute OE is commonly associated with overgrowth of the natural ear microbiota, initially dominated by Gram-positive bacteria and yeast, then superseded by *P. aeruginosa* which commonly results in severe chronic infections. Two factors which are important in such chronic infections include antimicrobial resistance and biofilm formation, it was therefore important to assess these factors for the collection of clinical isolates. A majority of clinical isolates in the present study produced strong levels of biofilm. Additionally, resistance to fluoroquinolones was also common, while resistance to other classes was relatively low. This finding supports evidence from the literature that these factors are involved in pathogenicity (Pye, 2018). The latter point is particularly important as fluoroquinolones are a common component of combined steroid, antibiotic, and antifungal products used to treat canine OE.

An important part of the present study was the use of whole genome sequencing to perform genomic analysis of 35 *P. aeruginosa* isolates. At the time of writing, this is the largest study using whole genome sequencing to investigate *P. aeruginosa* from canine OE sources. Previous studies have used sequencing of selected genes to identify mutations that confer resistance (Arais et al., 2016; Park et al., 2020), while others have performed whole genome sequencing of *P. aeruginosa* from canine sources that included some otitis samples but that was not the main objective of the work (Scott et al., 2019). The work presented by Hawkins et al., (2010) identified the potential for the use of bacteriophage to treat *P. aeruginosa* infections with a focus on clinical outcomes. Analysis of the assembled genomes can build upon this work widening our understanding of the host so that bacteriophage treatment can be better utilised for this type of infection in the future. An example of this was the identification of predicted

prophage regions in the genomes of the clinical *P. aeruginosa* which can prevent superinfection.

P. aeruginosa isolated from human infections are largely non-clonal with some highly successful epidemic clones and clonal complexes (Maatallah et al., 2011). MLST analysis of the OE isolates identified that a majority of isolates from the present study (80%) belonged to a unique sequence type. Interestingly, one of the canine isolates was found to belong to ST111 which is associated with antimicrobial resistant nosocomial infections (Oliver et al., 2015). Without more information it is impossible to speculate too much about this result, however the zooanthroponotic transmission of VIM-2 producing *P. aeruinogsa* has already been demonstrated by other researchers (Fernandes et al., 2018). Moreover, contact with a symptomatic dog can result in *Pseudomonas* transmission between dogs and people (Santaniello et al., 2020). Considering this, the significance of these infections from a one health perspective is highly important.

Phylogenetic analysis of canine isolates from the present study clustered along with human, animal and environmental isolates from other sources, suggesting that the isolates from dog ears were genomically diverse which supports the notion of non-clonal isolates from infection. Perhaps the most important finding from the sequencing was the identification of mutations that resulted in amino acid substitution and deletions that have been shown to confer resistance to fluoroquinolones. Hypermutable strains of *P. aeruginosa* have been isolated from chronic lung infections in humans facilitating the development of resistance, and mutations that confer resistance to almost all classes of antibiotics have been identified (López-Causapé et al., 2018). Consequently, it seems likely

that even with the development of a new antibiotic, resistance would develop, which highlights the need for alternative treatment strategies.

P. aeruginosa isolates implicated in canine OE may originate from the environment (Morris et al., 2017). Additionally, environmental isolates showed no statistically significant difference in pathogenesis when compared to clinical isolates (Vives-Flórez & Garnica, 2006). The presence and absence of virulence genes was investigated using the 35 clinical *P. aeruginosa* isolates from canine OE and compared using hierarchical clustering to other human, animal and environmental isolates. This highlighted that there is no specific virulence profile that predisposed an isolate for causing canine OE infections and supports the notion that these strains could have originated from the environment. As described in Chapter 4, it therefore seemed appropriate to isolate bacteriophage that originate from the same environment using environmental samples. To that end, samples from 19 sources, primarily wastewater but also other environmental water samples and samples from a dairy farm slurry, were collected.

In order to maximise its therapeutic potential, a given bacteriophage should have specific properties. Namely a strictly lytic lifecycle, broad host range and stability under a variety of environmental conditions (Fernández et al., 2019). Two bacteriophage with a predicted lytic lifecycle, a wide host range in regards to the clinical *P. aeruginosa* isolates and the ability to control *P. aeruginosa* PAO1 in liquid culture were isolated as part of the present study. This also highlighted the limitations of isolating bacteriophage from the environment as a large proportion of the bacteriophage were predicted to have a lysogenic lifestyle. Another limitation of the bacteriophage isolated in the present study

was identified when the growth kinetics of *P. aeruginosa* PAO1-QUB was monitored in the presence of different bacteriophage isolated as part of this work. It was noted that for one of these, spontaneous regrowth was seen, this was most likely regrowth of subpopulations. Further characterisation of this finding would be an important next step.

Chapter 5 investigated the ability of *B. bacteriovorus* to prey upon clinical *P. aeruginosa* from cases of canine OE. The present study showed that *B. bacteriovorus* was able to prey upon a number of clinical *P. aeruginosa* isolates *in vitro*. Interestingly, fluorescence microscopy highlighted bdelloplast even for strains that appeared non-sensitive to predation in the other assays suggesting that some predation was taking place. This finding warrants further experimentation, as populations may initially appear non-susceptible to predation, however, over an extended period of time, or when growth is slower such as within a biofilm, come under control. This could also explain why pre-formed biofilms were significantly reduced in the presence of *B. bacteriovorus* even for non-susceptible isolates. This could also be important in infections where the host immune system is present. Experiments performed using zebrafish larvae have shown that *B. bacteriovorus* can act synergistically with the host immune cells, specifically neutrophils and macrophages (Willis et al., 2016). During severe cases of canine OE where ulceration is present, macrophages may be found in the ear canal. Ultimately, this could mean that even where a drastic reduction in *P. aeruginosa* is not seen from *B. bacteriovorus* predation, a positive outcome could still occur. Even in the absence of interaction with the immune system, *B. bacteriovorus* may suppress *P. aeruginosa* enough

to result in reduced clinical symptoms, although more work would be required to confirm this.

Different bacteriophage have been used in therapeutic cocktails, and also in combination with antibiotics (Abedon, Danis-Wlodarczyk & Wozniak, 2021; Osman et al., 2023). Bacteriophage cocktails can be used to reduce the development of resistance to the virus, typically by targeting different host receptors while simultaneously increasing the number of strains that can be treated (Abedon, Danis-Wlodarczyk & Wozniak, 2021). Under certain circumstances, the use of a cocktail could reduce the host range due to competition between the viruses (Molina et al., 2022). Similarly, combining bacteriophage and antibiotics can have a synergistic effect but again could be antagonistic if an inappropriate combination is selected (Osman et al., 2023). *B. bacteriovorus* has been used in combination with antibiotics to evaluate synergistic interactions. As *Bdellovibrio* preys upon on Gram-negative bacteria, the presence of violacein, an antibiotic effective against Gram-positives, was assessed in co-cultures containing both Gram-positive and negative pathogens. Neither were effective at controlling the other target organisms. When used in combination the presence of both antimicrobial agents did not impact each other and in a coculture and both Gram-positive and negative were reduced (Im et al., 2017).

The future direction of this work could focus on the use of a combination of *B. bacteriovorus* and bacteriophage, as has already been demonstrated by two groups (Hobley et al., 2020; Morris et al., 2024). Hobley *et al*., (2020) investigated the combined action of *B. bacteriovorus* and a bacteriophage that was reported to produce halo plaques on a lawn of *E. coli* prey. Through

experimental and mathematical studies, it was shown that within liquid cultures containing *E. coli* prey and both predations, despite the phage infecting the prey much more quickly, the rapid development of a phage resistant prey population allowed *B. bacteriovorus* to grow and together the two predators ultimately reduced the prey population to undetectable levels, something nether could do alone (Hobley et al., 2020). Similarly, Morris et al., (2024) identified that a combination of *B. bacteriovorus* and bacteriophage followed by solar disinfection could reduced the numbers of drug resistant *Acinetobacter baumannii* to bellow the limit of detection.

If a combined therapy was found to be more effective, the efficacy of the treatment could be tested in an *in vivo Galleria mellonella* insect model. This model is preferred by researchers due to the lack of restrictive ethical rules, low cost, ease of use and finally the presence of an innate immune system (Ménard et al., 2021). As a result, the ability of bacteriophage to kill clinical *P. aeruginosa* isolates from cystic fibrosis and canine OE has been assessed using this model (Olszak et al., 2015; Antoine et al., 2021). Moreover, no effect was seen when *G. mellonella* was injected with *B. bacteriovorus* (Shanks et al., 2013). A long-term aim of the present study would be to test a combination therapy for a case of canine OE that had reached a therapeutic end point and measure factors such as a reduction in *P. aeruginosa* and increase in *B. bacteriovorus* or bacteriophage or changes in tissues.

In conclusion, the work presented in this thesis has shown that *P. aeruginosa* isolated from cases of canine OE are genotypically distinct, with no particular virulence genes associated with this disease. Moreover, antimicrobial resistance, particularly to fluroquinolones and a strong level of biofilm formation is

common amongst *P. aeruginosa* isolates from this disease. Development of resistance through the acquisition of mutations was common amongst the clinical isolates and this highlights the need for alternatives to antibiotics. The work in this thesis has demonstrated that bacteriophage that infect and kill clinical *P. aeruginosa* can be readily isolated from the environment. Moreover, *B. bacteriovorus* was shown to prey upon several of the clinical isolates in multiple assays. The results highlight the potential of alternative therapies for the treatment of *P. aeruginosa* infections and the potential for reduced antibiotic use and in future work this could feed into an *in vivo* assessment which could significantly improve the outcomes of canine OE and other *P. aeruginosa* infections.

Chapter 7 References

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Chapter 8 Appendix

8.1 Supplementary material

8.1.1 Complete canine otitis externa strain collection

***P. aeruginosa* strain**	**Origin**	**Original Isolation**
80842	Royal Veterinary College	Unknown
80664	Royal Veterinary College	Unknown
84556	Royal Veterinary College	Unknown
80610	Royal Veterinary College	Unknown
84181	Royal Veterinary College	Unknown
84381	Royal Veterinary College	Unknown
83240	Royal Veterinary College	Unknown
84653	Royal Veterinary College	Unknown
84142	Royal Veterinary College	Unknown
84750	Royal Veterinary College	Unknown
84331	Royal Veterinary College	Unknown
84480	Royal Veterinary College	Unknown
84987	Royal Veterinary College	Unknown
83386	Royal Veterinary College	Unknown
85566	Royal Veterinary College	Unknown
85571	Royal Veterinary College	Unknown
85552	Royal Veterinary College	Unknown
80632	Royal Veterinary College	Unknown
83304	Royal Veterinary College	Unknown
80715	Royal Veterinary College	Unknown
84269	Royal Veterinary College	Unknown
84456	Royal Veterinary College	Unknown
85115	Royal Veterinary College	Unknown
87870	Royal Veterinary College	Unknown
85505	Royal Veterinary College	Unknown
84654	Royal Veterinary College	Unknown
84496	Royal Veterinary College	Unknown
85546	Royal Veterinary College	Unknown
88693	Royal Veterinary College	Unknown
85344	Royal Veterinary College	Unknown
88033	Royal Veterinary College	Unknown
87895	Royal Veterinary College	Unknown
88812	Royal Veterinary College	Unknown
88549	Royal Veterinary College	Unknown
88023	Royal Veterinary College	Unknown
89011 (2)	Royal Veterinary College	Unknown
88030	Royal Veterinary College	Unknown
89022	Royal Veterinary College	Unknown
88440	Royal Veterinary College	Unknown
83213	Royal Veterinary College	Unknown
83719	Royal Veterinary College	Unknown
83635	Royal Veterinary College	Unknown
83389	Royal Veterinary College	Unknown

83693	Royal Veterinary College	Unknown
84688	Royal Veterinary College	Unknown
85300	Royal Veterinary College	Unknown
491804	NationWide Laboratories	Blandford Forum
464009 (1)	NationWide Laboratories	Stamford
467884	NationWide Laboratories	Rugeley
462080	NationWide Laboratories	Mansfield
495200	NationWide Laboratories	Biddulph
487390	NationWide Laboratories	St Helens
488519	NationWide Laboratories	Malta
464429	NationWide Laboratories	Northampton
467523	NationWide Laboratories	Edinburgh
494759	NationWide Laboratories	Penrith
489935	NationWide Laboratories	Blandford Forum
467153	NationWide Laboratories	Congleton
490559?	NationWide Laboratories	Northampton
464570	NationWide Laboratories	Northwich
490142	NationWide Laboratories	Lichfield
490055	NationWide Laboratories	Glasgow
488363	NationWide Laboratories	Northampton
468424R	NationWide Laboratories	Brighton and Hove
468424L	Unknown	Unknown
484098	NationWide Laboratories	Blandford Forum
467985	NationWide Laboratories	Romford
468512	NationWide Laboratories	Thornton-Cleveleys
483253	NationWide Laboratories	Winsford
469525	NationWide Laboratories	Nottingham
491810	NationWide Laboratories	Northwich
491809	NationWide Laboratories	Chalfont Saint Giles
489267	NationWide Laboratories	Manchester
491019	NationWide Laboratories	Lichfield
491171	NationWide Laboratories	Swadlincote
490614	NationWide Laboratories	Bramhall
491708(1)	NationWide Laboratories	Royal Leamington Spa
494920	NationWide Laboratories	Unknown
486412	NationWide Laboratories	Glasgow
462206	NationWide Laboratories	North Walsham
491243	NationWide Laboratories	Henley-on-Thames
490471	NationWide Laboratories	Ashton-under-Lyne
490219	NationWide Laboratories	Unknown
490290	Unknown	Unknown
486414	NationWide Laboratories	St Helens
491037	NationWide Laboratories	Stoke-on-Trent
490137	NationWide Laboratories	Penrith
490594	NationWide Laboratories	St Helens
462969	NationWide Laboratories	London
469154	NationWide Laboratories	Liverpool
489748	NationWide Laboratories	St Helens

453344	NationWide Laboratories	Chatham
485418	NationWide Laboratories	St Helens
488666	NationWide Laboratories	St Helens
488401	NationWide Laboratories	NN4 6FF
491041	NationWide Laboratories	Dubai
490051	NationWide Laboratories	Blandford Forum
488402	NationWide Laboratories	Barnstaple
464016	NationWide Laboratories	Ulverston
489179	NationWide Laboratories	Easingwold
467565R	NationWide Laboratories	Thornton-Cleveleys
463027	NationWide Laboratories	Tipton
488427	NationWide Laboratories	Axminster
487845	NationWide Laboratories	Barnstaple
491808	NationWide Laboratories	Bexhill
485366	NationWide Laboratories	Wolverhampton
484919	NationWide Laboratories	Dubai
487280	NationWide Laboratories	Maidstone
485101	NationWide Laboratories	St Helens
495165	NationWide Laboratories	Easingwold
468424	NationWide Laboratories	Thornton-Cleveleys
468571	NationWide Laboratories	Blackburn
491136	NationWide Laboratories	St Helens
488517	NationWide Laboratories	Reading
491245	NationWide Laboratories	Blandford Forum
462188	NationWide Laboratories	Royston
480630	NationWide Laboratories	Barnstaple
467565 (1)	NationWide Laboratories	Nottingham
488521	NationWide Laboratories	Welwyn
488958	NationWide Laboratories	Cupar
462781	NationWide Laboratories	Hexham
484916	NationWide Laboratories	St Helens
467565 (2)	Unknown	Unknown
464591(1)	NationWide Laboratories	Ross-on-Wye
491610	NationWide Laboratories	St Helens
467896	NationWide Laboratories	Buntingford
464009 (2)	Unknown	Unknown
27827-1	University of Copenhagen	Unknown
27039	University of Copenhagen	Unknown
28495	University of Copenhagen	Unknown
29797-1	University of Copenhagen	Unknown
29712	University of Copenhagen	Unknown
29775	University of Copenhagen	Unknown
29825	University of Copenhagen	Unknown
28045-2	University of Copenhagen	Unknown
27786	University of Copenhagen	Unknown
27207	University of Copenhagen	Unknown
2848	University of Copenhagen	Unknown
26758	University of Copenhagen	Unknown

10435-2	University of Copenhagen	Unknown
5660-2	University of Copenhagen	Unknown
C3683	University of Copenhagen	Unknown
25181	University of Copenhagen	Unknown
5690	University of Copenhagen	Unknown
26228	University of Copenhagen	Unknown
22406	University of Copenhagen	Unknown
C37291	University of Copenhagen	Unknown
26712	University of Copenhagen	Unknown
C5752-1	University of Copenhagen	Unknown
26277	University of Copenhagen	Unknown
C3524	University of Copenhagen	Unknown
27003	University of Copenhagen	Unknown
C5768-2	University of Copenhagen	Unknown
26675-2	University of Copenhagen	Unknown
26820-2	University of Copenhagen	Unknown
29885	University of Copenhagen	Unknown
26737-1	University of Copenhagen	Unknown
26551	University of Copenhagen	Unknown
29264	University of Copenhagen	Unknown
26820-3	University of Copenhagen	Unknown
26820-3	University of Copenhagen	Unknown
28504	University of Copenhagen	Unknown
26740	University of Copenhagen	Unknown
28093	University of Copenhagen	Unknown
27473	University of Copenhagen	Unknown
28260	University of Copenhagen	Unknown
23180	University of Copenhagen	Unknown
25395	University of Copenhagen	Unknown
27267	University of Copenhagen	Unknown
29570-2-2	University of Copenhagen	Unknown
28517	University of Copenhagen	Unknown
40910-1	University of Copenhagen	Unknown
29130	University of Copenhagen	Unknown
27197	University of Copenhagen	Unknown
28272	University of Copenhagen	Unknown
26777	University of Copenhagen	Unknown
27174	University of Copenhagen	Unknown
26992	University of Copenhagen	Unknown
26491	University of Copenhagen	Unknown
27321	University of Copenhagen	Unknown
27995	University of Copenhagen	Unknown
26853	University of Copenhagen	Unknown
26768	University of Copenhagen	Unknown
29582	University of Copenhagen	Unknown
28728	University of Copenhagen	Unknown
27360	University of Copenhagen	Unknown
240682-1	University of Copenhagen	Unknown

469375	NationWide Laboratories	Wootton Bridge
488613	NationWide Laboratories	Swadlincote
498491	NationWide Laboratories	St Helens
498492	NationWide Laboratories	Maidstone
488518	NationWide Laboratories	St Helens
453191	NationWide Laboratories	Snodland
464591(2)	Unknown	Unknown
467139	NationWide Laboratories	Watford
483186	NationWide Laboratories	Northwich
485015	NationWide Laboratories	Smethwick
472819	NationWide Laboratories	Fleetwood
467505	NationWide Laboratories	Bootle
488188	NationWide Laboratories	Wolverhampton
473025	NationWide Laboratories	Shirley
491708(2)	Unknown	Unknown
493691	NationWide Laboratories	Lichfield
464127	NationWide Laboratories	Ulverston
28045-1	University of Copenhagen	Unknown
25467	University of Copenhagen	Unknown
28712	University of Copenhagen	Unknown
22814	University of Copenhagen	Unknown
29051	University of Copenhagen	Unknown
28472	University of Copenhagen	Unknown
10476-1	University of Copenhagen	Unknown
23275	University of Copenhagen	Unknown
29893-1	University of Copenhagen	Unknown
28494	University of Copenhagen	Unknown
84142(2)	Unknown	Unknown
C-3501	University of Copenhagen	Unknown
487632	NationWide Laboratories	Harlow
27660	University of Copenhagen	Unknown
25966	University of Copenhagen	Unknown
30084	University of Copenhagen	Unknown
29663	University of Copenhagen	Unknown
28279	University of Copenhagen	Unknown
23276	University of Copenhagen	Unknown
27889-1	University of Copenhagen	Unknown
28415	University of Copenhagen	Unknown
29878-2	University of Copenhagen	Unknown
29569	University of Copenhagen	Unknown
2782	University of Copenhagen	Unknown
26667	University of Copenhagen	Unknown
29405	University of Copenhagen	Unknown
25837	University of Copenhagen	Unknown
25131	University of Copenhagen	Unknown
2776	University of Copenhagen	Unknown
26982	University of Copenhagen	Unknown
26392-2	University of Copenhagen	Unknown

2943	University of Copenhagen	Unknown
29844-1	University of Copenhagen	Unknown
29843-2	University of Copenhagen	Unknown
26907	University of Copenhagen	Unknown
27523-1	University of Copenhagen	Unknown
C-3499	Unknown	Unknown
89011-2	Unknown	Unknown
26697-1	University of Copenhagen	Unknown
26633	University of Copenhagen	Unknown
27744	University of Copenhagen	Unknown
29878-1	University of Copenhagen	Unknown
29758	University of Copenhagen	Unknown
29797-2	University of Copenhagen	Unknown
29844-2	University of Copenhagen	Unknown
27660-2	University of Copenhagen	Unknown
94987	Unknown	Unknown
27983	University of Copenhagen	Unknown
29570-2-1	University of Copenhagen	Unknown

Table 8.1 **Complete collection of clinical *P. aeruginosa* used in this work.**

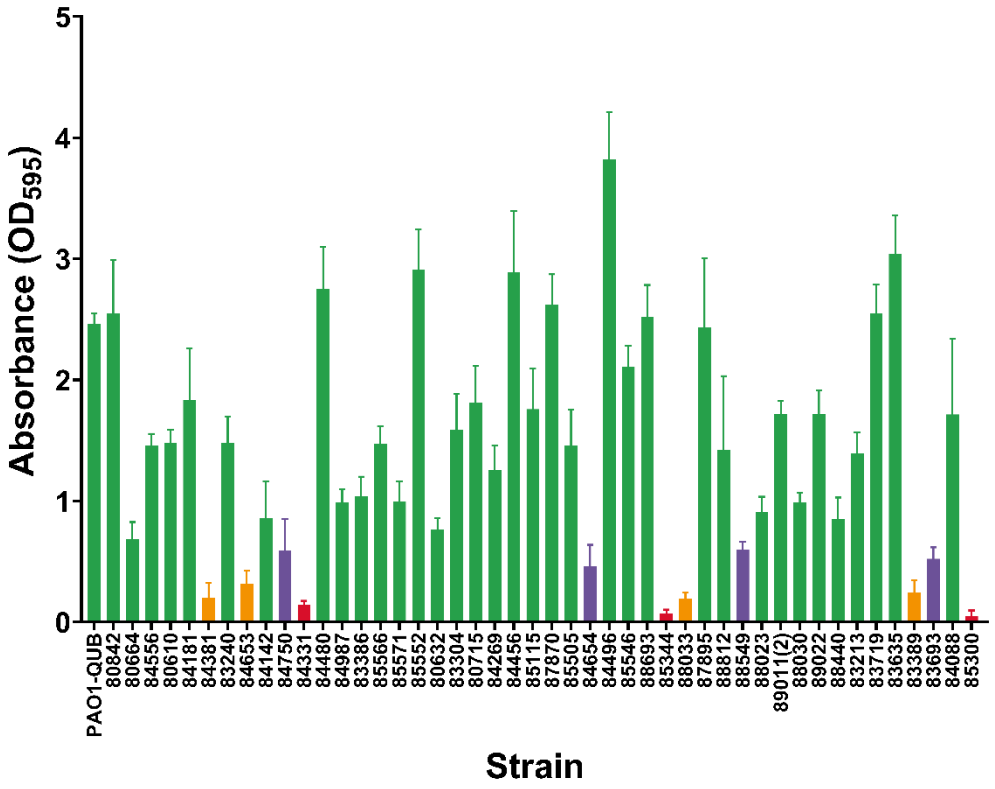
Royal Veterinary College (n = 46), NationWide Laboratories (n = 93) and an EU Collection from the University of Copenhagen, School of Veterinary Medicine (n = 104). The source of the remaining (n = 10) isolates is unknown.

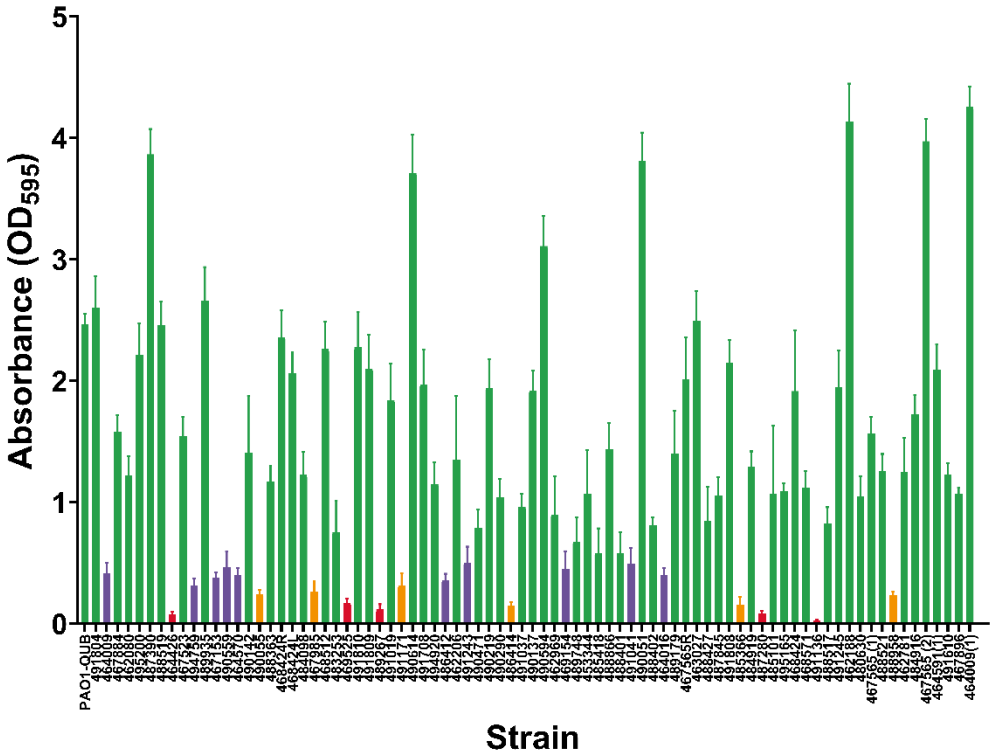
8.1.2 Extracting amino acid sequences for genes of interest

```
from Bio import SeqIO
import os

genes_of_interest = {"": [], "": [], "": [], "": [], "": [], "": []}

def gene_of_interest(GOI):
    fasta_list = []
    for f in gen_files:
        for rec in SeqIO.parse(f, "genbank"):
            if rec.features:
                for feature in rec.features:
                    if feature.type == "CDS":
                        if "gene" in feature.qualifiers:
                            if GOI in feature.qualifiers["gene"][0]:
                                seq = feature.qualifiers["translation"]
                                id = ">{}:{} {}".format(rec.id,
feature.qualifiers["gene"][0], rec.description)
                                fasta_list.append("{}\n{}".format(id, *seq))
    return fasta_list

def save_fasta(GOI, GOI_info):
    with open('{}.fasta'.format(GOI), 'w') as fp:
        for item in GOI_info:
        # write each item on a new line
            fp.write("%s\n" % item)
```

Figure 8.1 **Python script used to extract the amino acid sequences of genes of interest.**

8.1.3 Complete biofilm formation ability of *P. aeruginosa* isolates





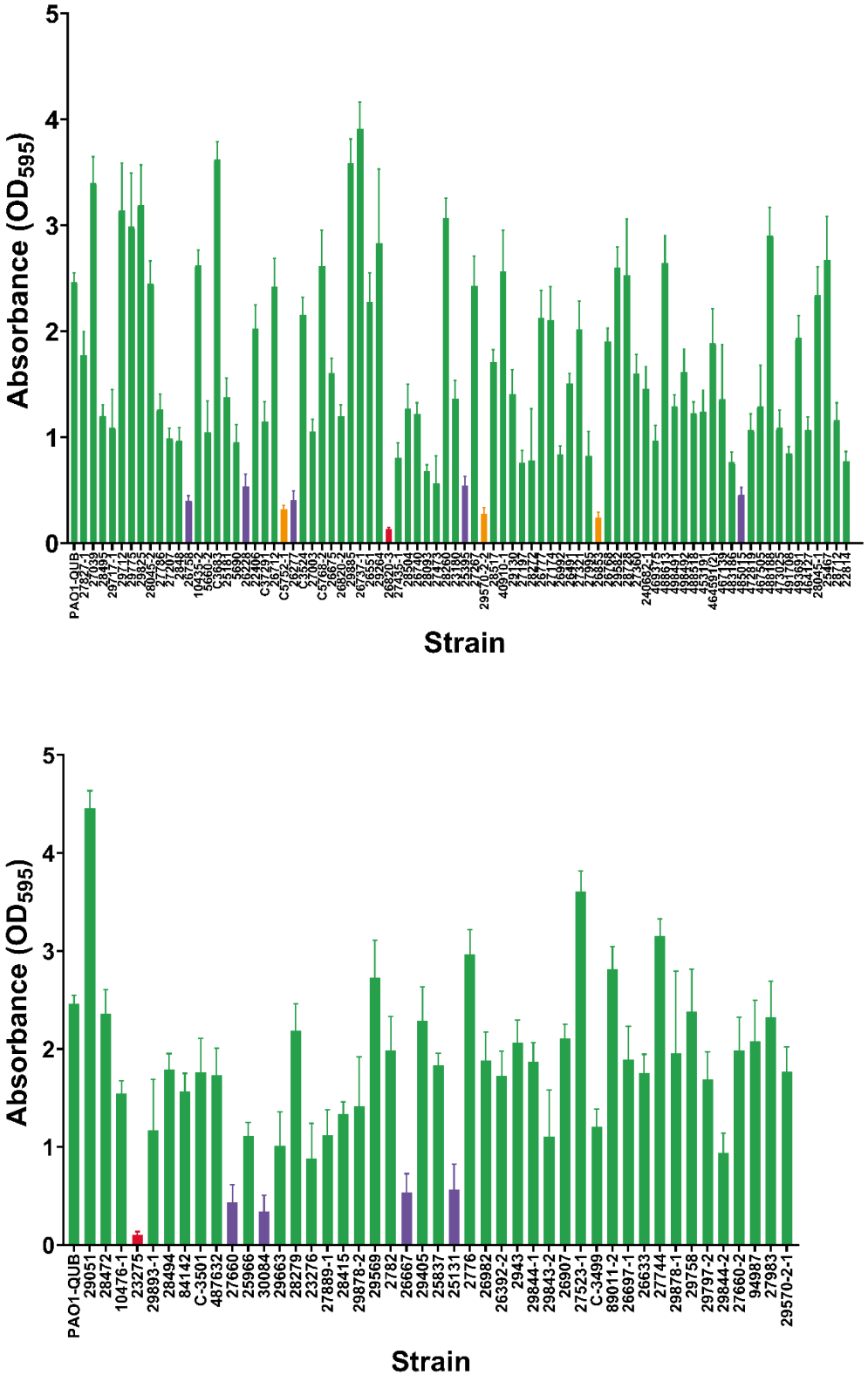


Figure 8.2 **Biofilm formation of P. aeruginosa from canine otitis externa.**

The biofilm forming ability of 253 *P. aeruginosa* strains was tested in a crystal violet, 96 well plate assay. Green – Strong biofilm producing - Purple – Moderate biofilm producing - Orange – Weak biofilm producing - Red – No quantifiable biofilm. The values here are from three biological and six technical repeats represented by the mean with 95% confidence interval.

8.1.4 Comparison of biofilm formation of PAO1 sublines

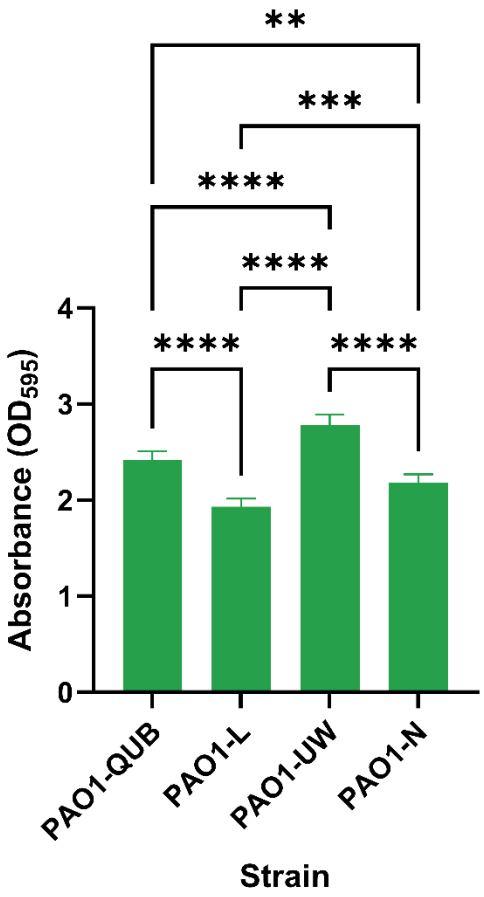
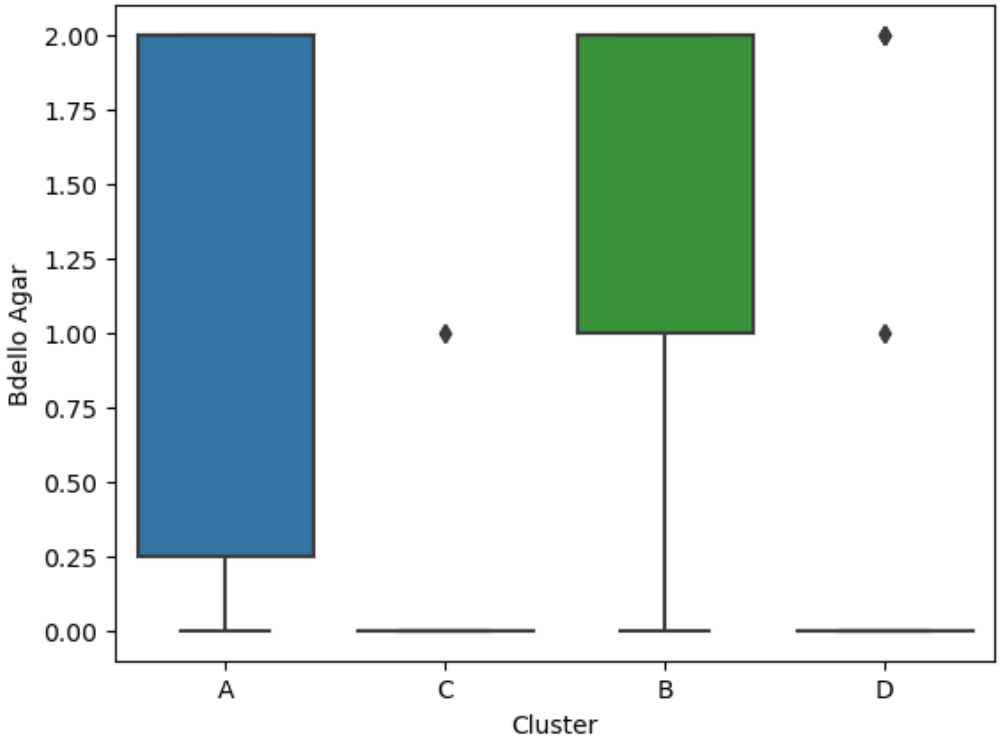
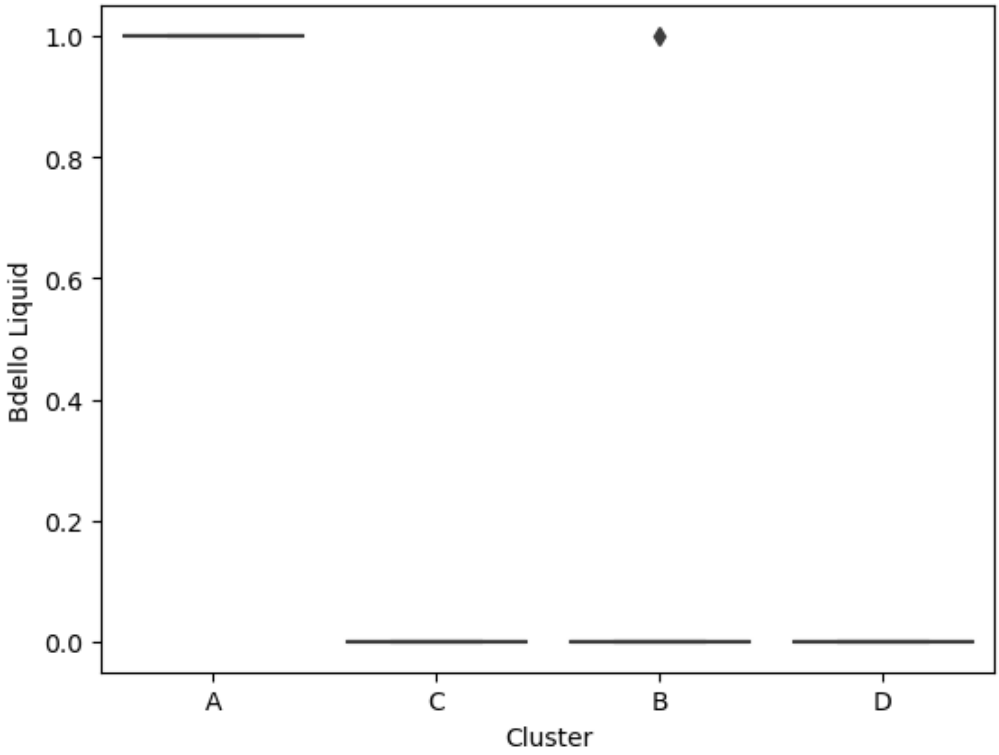


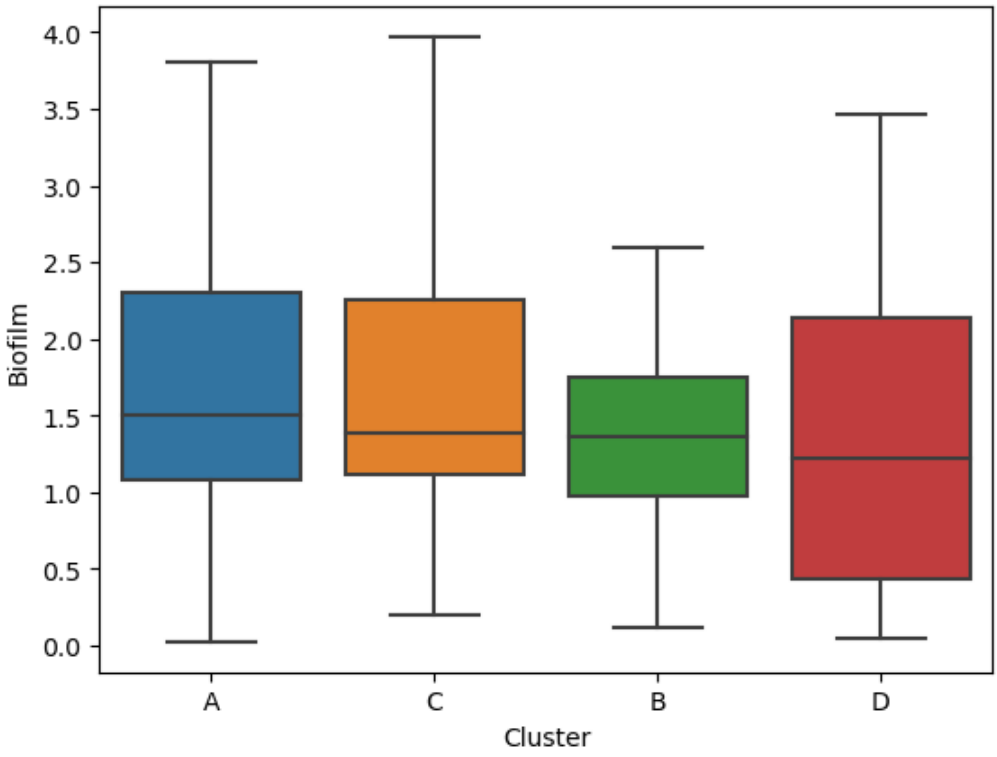
Figure 8.3 **Comparison of biofilm formation between *P. aeruginosa* sublines.**

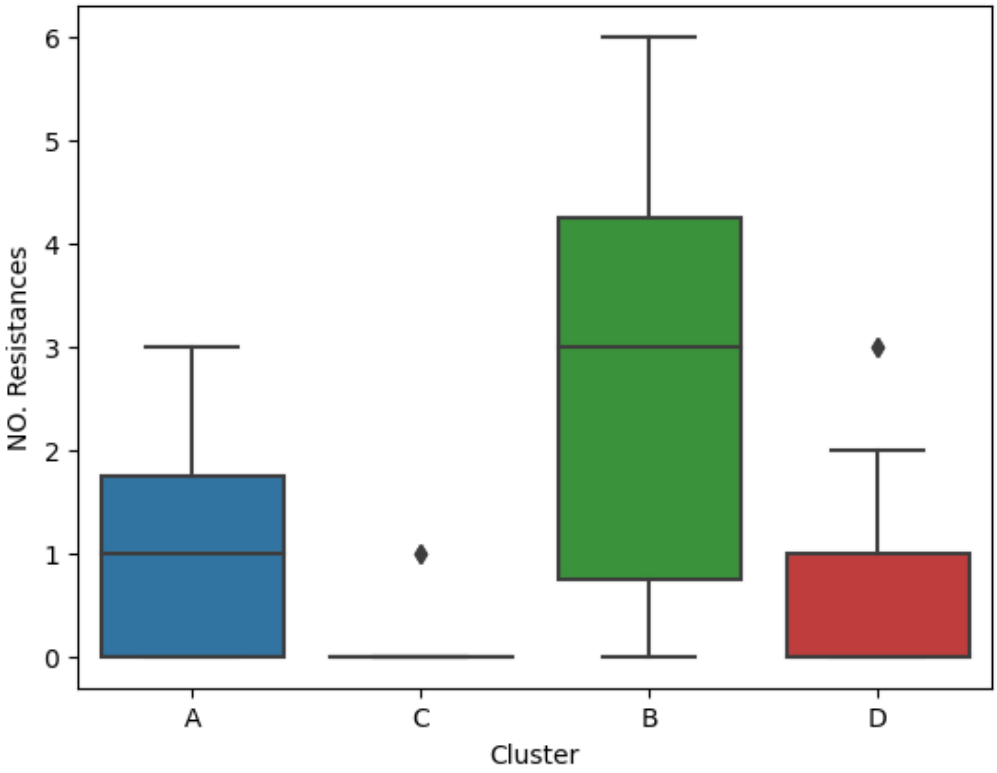
The ability of three common *P. aeruginosa* PAO1 sublines, PAO1-L originally from the Dieter Haas laboratory, PAO1-UW, maintained at the University of Washington, and PAO1-N from the University of Nottingham to form biofilm in vitro was compared using a 96 well microtiter plate assay to PAO1-QUB from the present study. Green – Strong biofilm producing. Welch's ANOVA test revealed a statistically significant difference in biofilm formation ($p<0.0001$). Dunnett's T3 multiple comparisons test found that all the groups were statistically significantly different. Three biological repeats with 24 technical repeats are represented by the mean with 95% confidence interval. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$

8.1.5 Cluster analysis






Biofilm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
C
B
D
Cluster


NO. Resistances
6
5
4
3
2
1
0
A
C
B
D
Cluster

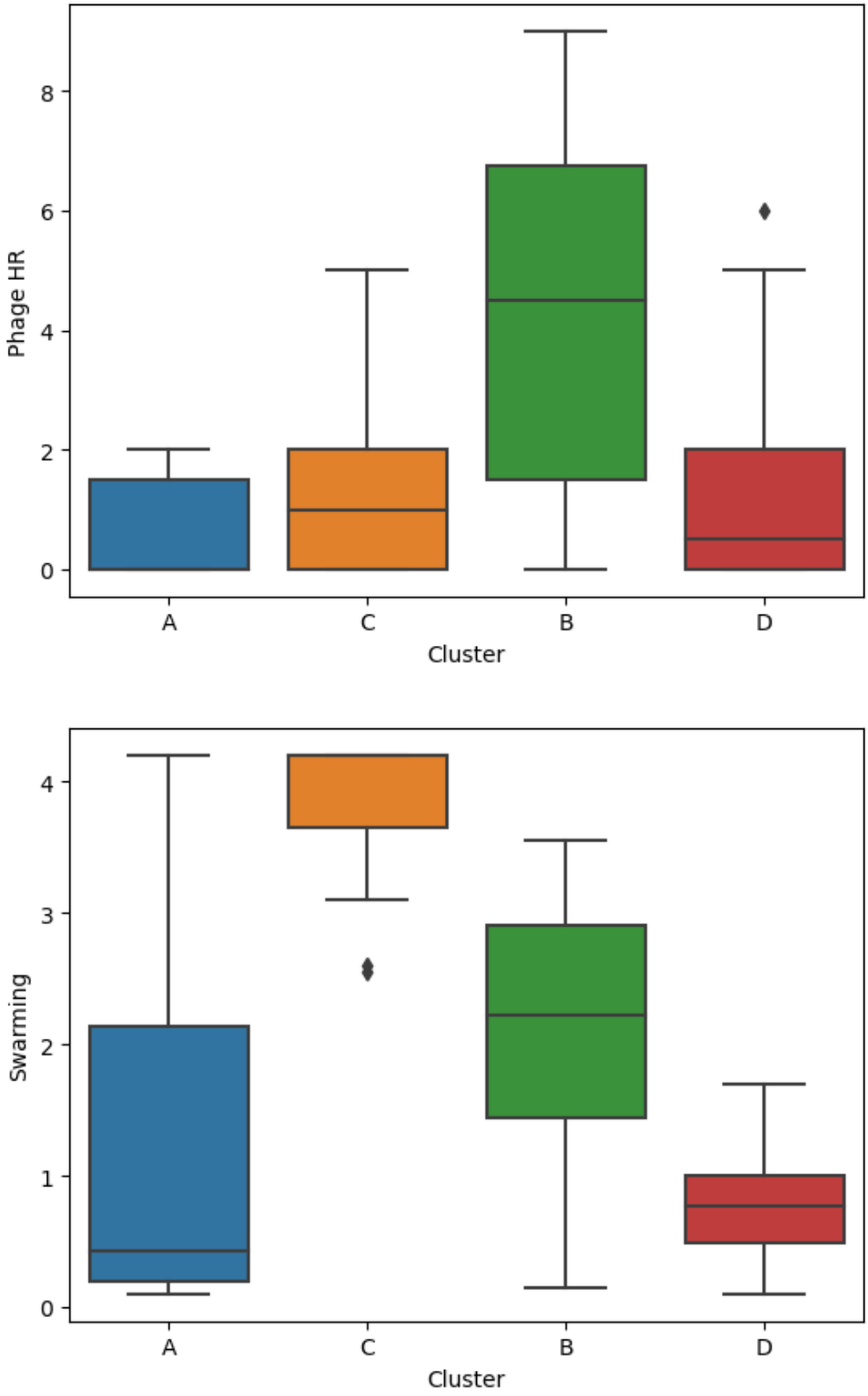
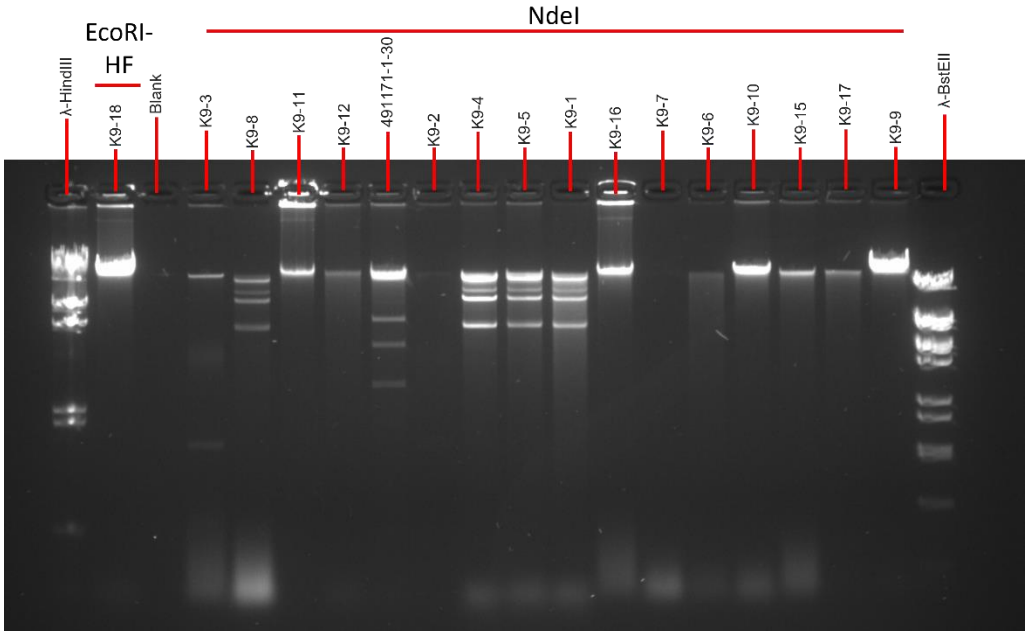


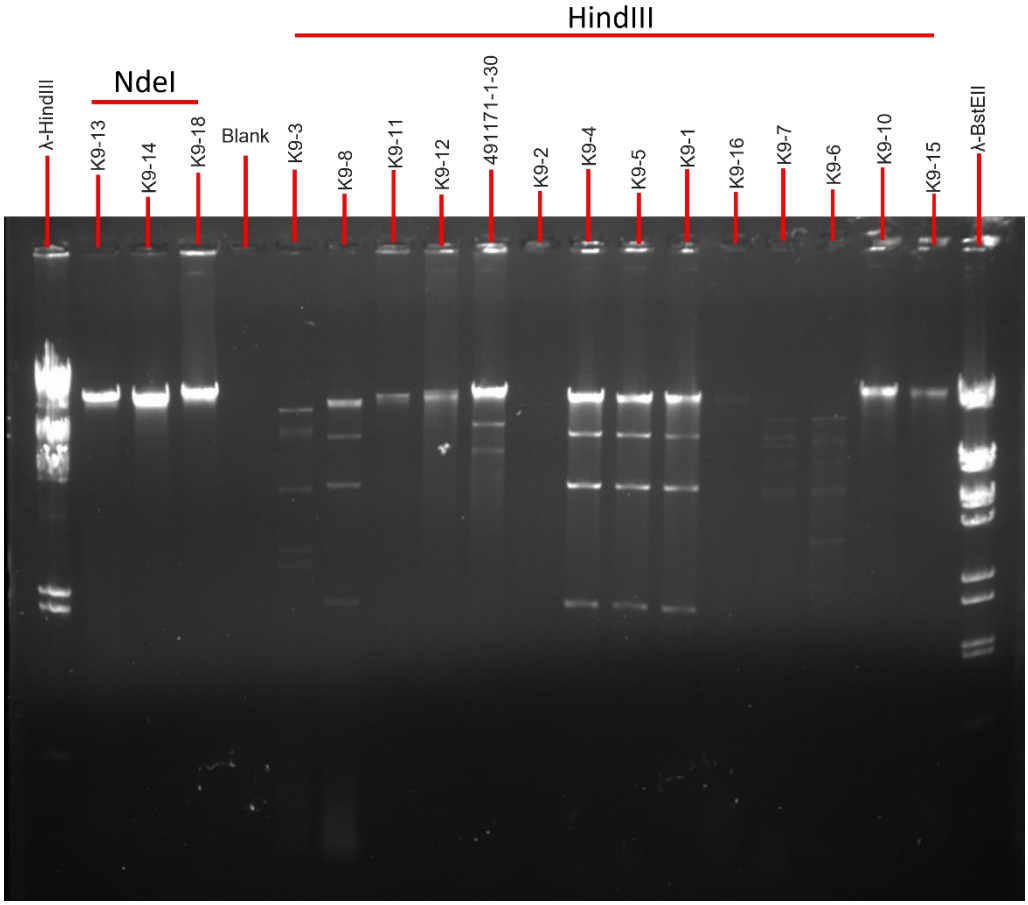
Figure 8.4 **Breakdown of hierarchical clustering results.**

Hierarchical clustering using Euclidean distance with Ward linkage was performed using the results from six experiments, namely the crystal violet biofilm and swarming motility assays, the total number of resistances to the tested antibiotics, predation by B. bacteriovorus on YPSC agar

and in YPSC broth and finally bacteriophage host range. Above shows the distribution of results in each assay for each of the clusters.

8.1.6 Bacteriophage restriction digestion gels





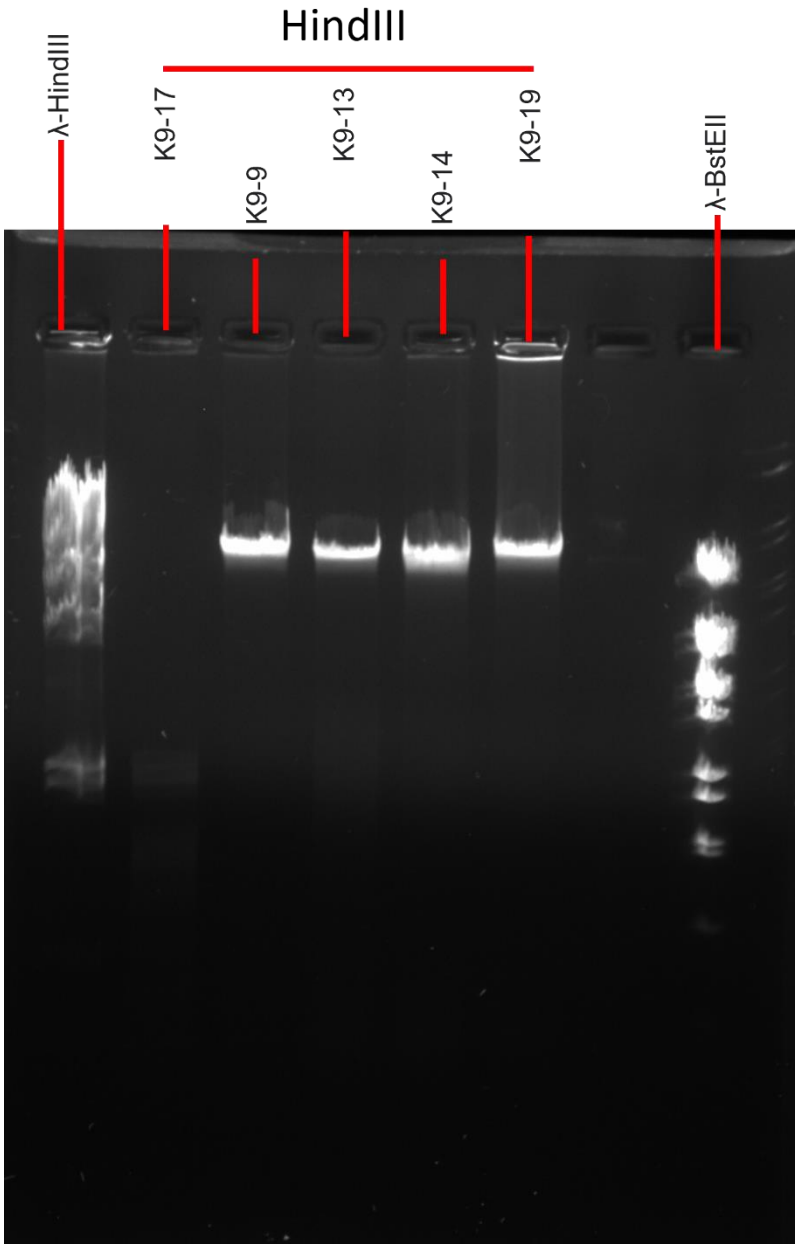


Figure 8.5 **Ethidium Bromide agarose gel image of bacteriophage restriction digestion with three restriction endonucleases**.

Restriction digestions of genomic DNA extracts from 19 bacteriophage were performed using EcoRI-HF, NdeI and HindIII as described in §2.12. Subsequently, the digestions were run on an agarose gel containing Ethidium Bromide (§2.4).

8.1.7 Complete prophage analysis

Strain		Number of prophage regions	
25181		8	
Length (bp)	Completeness	CDS	Closest Relative
30,600	Intact	37	Pseudomonas phage YMC11/02/R656
32,300	Intact	41	Pseudomonas phage F10
76,600	Intact	79	Pseudomonas phage F10
59,800	Intact	90	Pseudomonas phage PMG1
12,600	Intact	18	Pseudomonas phage Pf1
66,700	Intact	77	Pseudomonas phage JBD44
33,300	Intact	30	Acinetobacter phage vB_AbaM_ME3
12,200	Intact	17	Pseudomonas phage Pf1
26491		2	
20,200	Intact	27	Pseudomonas phage YMC11/02/R656
17,800	Questionable	17	Pseudomonas phage Pf1
26820-3		3	
18,800	Questionable	23	Pseudomonas phage YMC11/02/R656
11,300	Questionable	11	Pseudomonas phage Pf1
13,300	Intact	15	Pseudomonas phage Pf1
27827-1		6	
33,100	Intact	40	Pseudomonas phage YMC11/02/R656
37,800	Intact	53	Pseudomonas phage JBD30
35,400	Intact	51	Pseudomonas phage JBD69
47,600	Intact	47	Pseudomonas phage YMC11/02/R656
56,500	Questionable	78	Pseudomonas phage JBD44
12,100	Intact	14	Pseudomonas phage Pf1
29582		1	
30,700	Intact	37	Pseudomonas phage YMC11/02/R656
29758		5	
30,600	Intact	38	Pseudomonas phage YMC11/02/R656
45,300	Intact	46	Pseudomonas phage F10
11,800	Questionable	11	Pseudomonas phage Pf1
9,700	Intact	12	Pseudomonas phage Pf1
54,700	Intact	61	Pseudomonas phage vB_PaeS_PM105
80632		5	
18,500	Questionable	24	Pseudomonas phage YMC11/02/R656
43,800	Intact	61	Pseudomonas phage MD8
56,500	Intact	53	Pseudomonas phage B3

14,100	Intact	15	Pseudomonas phage Pf1
11,700	Intact	14	Pseudomonas phage Pf1
80664		2	
30,600	Intact	36	Pseudomonas phage YMC11/02/R656
48,100	Intact	65	Pseudomonas phage phi2
80715		8	
22,700	Intact	29	Pseudomonas phage YMC11/02/R656
41,700	Intact	59	Pseudomonas phage phi2
56,800	Intact	70	Pseudomonas phage phi297
48,200	Intact	49	Pseudomonas phage JBD25
39,400	Intact	53	Pseudomonas phage JBD93
17,300	Questionable	10	Pseudomonas phage Pf1
24,400	Intact	29	Escherichia phage vB_EcoM_ECO1230-10
11,700	Questionable	11	Pseudomonas phage Pf1
83240		5	
30,600	Intact	37	Pseudomonas phage YMC11/02/R656
41,700	Intact	59	Pseudomonas phage F10
61,500	Intact	76	Pseudomonas phage YMC11/02/R656
11,400	Intact	11	Pseudomonas phage Pf1
43,000	Intact	45	Pseudomonas phage F10
84269		5	
18,400	Intact	25	Pseudomonas phage phiCTX DNA
48,400	Intact	57	Pseudomonas phage F10
76,100	Intact	94	Bacteriophage D3
52,700	Questionable	65	Pseudomonas phage YMC11/07/P54_PAE_BP
14,600	Intact	18	Pseudomonas phage Pf1
85505		1	
22,900	Intact	29	Pseudomonas phage YMC11/02/R656
87895		4	
30,600	Intact	38	Pseudomonas phage YMC11/02/R656
50,700	Intact	59	Pseudomonas phage F10
42,800	Intact	52	Pseudomonas phage B3
67,100	Intact	69	Pseudomonas virus H66
88693		3	
15,100	Intact	14	Pseudomonas phage Pf1
42,900	Intact	54	Burkholderia phage AH2
18,400	Intact	25	Pseudomonas phage phiCTX DNA
88812		4	

20,200	Intact	27	Pseudomonas phage YMC11/02/R656
43,800	Intact	58	Pseudomonas phage MD8
56,300	Intact	57	Pseudomonas phage YMC11/07/P54_PAE_BP
16,000	Questionable	11	Pseudomonas phage Pf1
463027		6	
32,300	Intact	40	Pseudomonas phage YMC11/02/R656
46,500	Intact	51	Pseudomonas phage F10
47,800	Intact	53	Pseudomonas phage MD8
52,100	Intact	52	Burkholderia cenocepacia phage BcepMu
11,600	Intact	12	Pseudomonas phage Pf1
43,800	Intact	52	Pseudomonas phage Dobby
467896		2	
18,400	Intact	24	Enterobacter phage Arya
57,800	Intact	67	Pseudomonas phage phi297
480630		3	
19,000	Questionable	23	Pseudomonas phage YMC11/02/R656
56,200	Intact	53	Pseudomonas phage phi297
13,500	Intact	11	Pseudomonas phage Pf1
484098		4	
18,400	Intact	25	Pseudomonas phage phiCTX DNA
56,700	Intact	49	Pseudomonas phage YMC11/02/R656
45,100	Intact	48	Pseudomonas phage JBD25
40,800	Intact	48	Pseudomonas phage Dobby
484919		3	
18,900	Questionable	22	Pseudomonas phage YMC11/02/R656
11,500	Intact	15	Pseudomonas phage Pf1
17,600	Questionable	14	Pseudomonas phage Pf1
485101		7	
32,300	Intact	40	Pseudomonas phage YMC11/02/R656
43,600	Intact	64	Pseudomonas phage F10
48,600	Intact	56	Pseudomonas phage JBD25
19,100	Incomplete	24	Pseudomonas phage YMC11/02/R656
63,100	Intact	98	Bacteriophage D3
44,500	Intact	51	Escherichia phage vB_EcoM_ECO1230-10
49,300	Intact	48	Pseudomonas phage JBD67
488427		7	
43,300	Intact	62	Pseudomonas phage JBD93

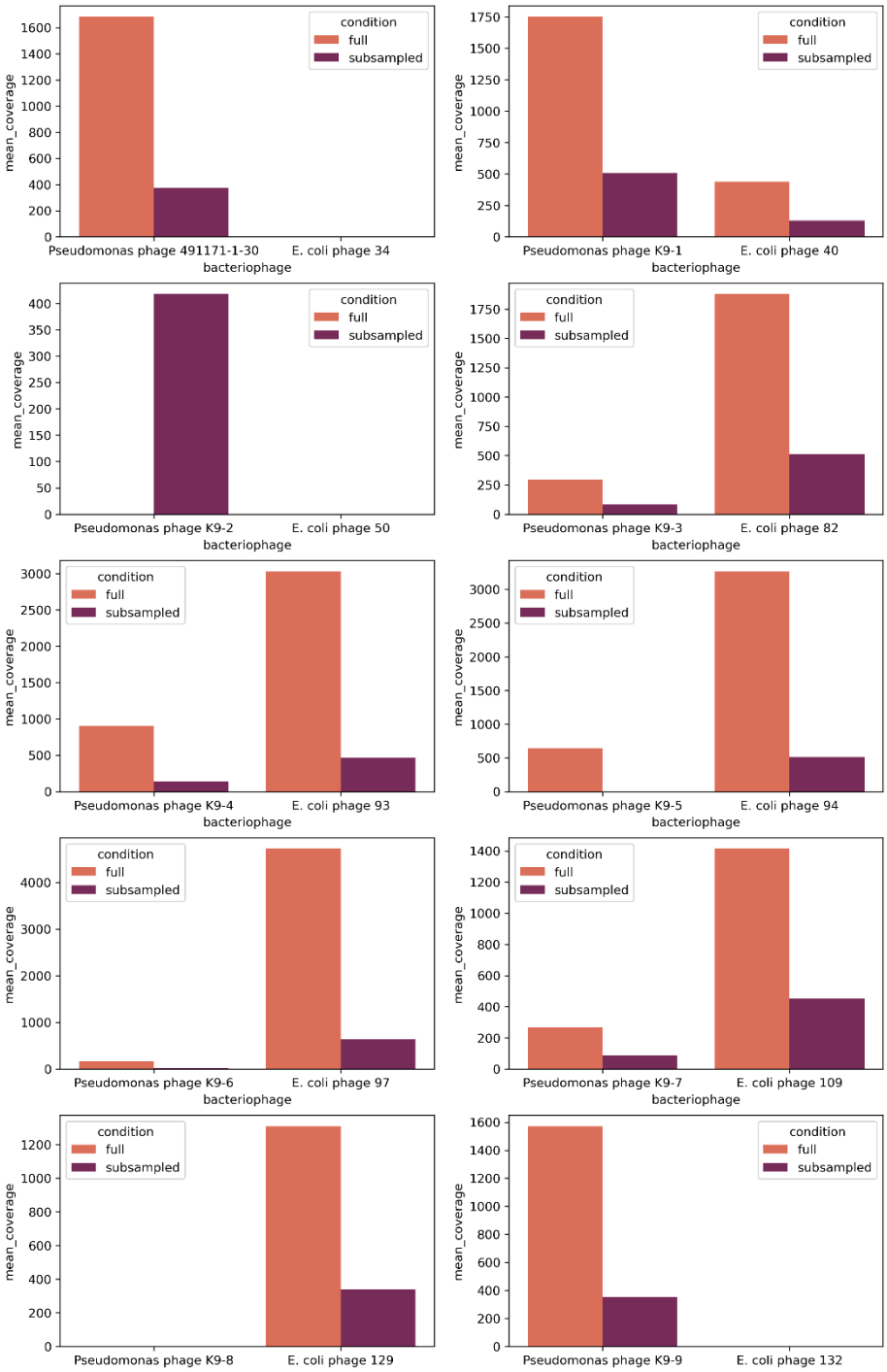
32,300	Intact	40	Pseudomonas phage YMC11/02/R656
39,800	Intact	57	Pseudomonas phage JBD30
72,800	Intact	62	Pseudomonas phage phi297
14,200	Questionable	20	Ralstonia phage p12J
61,000	Intact	69	Pseudomonas phage phi297
43,000	Intact	45	Pseudomonas phage F10
488613		10	
48,700	Intact	52	Pseudomonas phage JBD18
15,100	Intact	14	Pseudomonas phage Pf1
20,200	Intact	16	Pseudomonas phage Pf1
18,400	Intact	25	Pseudomonas phage Dobby
41,900	Intact	55	Pseudomonas phage MD8
47,600	Intact	59	Pseudomonas phage PMG1
55,500	Intact	57	Pseudomonas phage JBD18
33,300	Intact	40	Pseudomonas phage vB_PaeS_PM105
54,500	Intact	52	Pseudomonas phage JBD18
53,900	Intact	53	Pseudomonas phage Dobby
488958		3	
18,900	Questionable	22	Pseudomonas phage YMC11/02/R656
11,500	Intact	15	Pseudomonas phage Pf1
17,600	Questionable	14	Pseudomonas phage Pf1
489267		6	
18,400	Intact	23	Escherichia phage vB_EcoM_ECO1230-10
43,100	Intact	61	Pseudomonas phage phi2
50,500	Intact	57	Pseudomonas virus H66
45,100	Intact	51	Burkholderia cenocepacia phage BcepMu
12,800	Questionable	11	Pseudomonas phage Pf1
47,100	Intact	52	Pseudomonas phage Dobby
490051		7	
22,500	Intact	20	Ralstonia phage RS138
49,900	Incomplete	65	Pseudomonas phage F10
48,900	Intact	55	Pseudomonas phage JBD25
32,300	Intact	40	Pseudomonas phage YMC11/02/R656
29,300	Intact	34	Pseudomonas phage YMC11/02/R656
25,600	Incomplete	42	Pseudomonas phage F10
22,100	Incomplete	29	Pseudomonas phage F10
490137		7	
19,800	Intact	25	Pseudomonas phage phiCTX DNA
24,300	Questionable	23	Pseudomonas phage F10
69,700	Intact	82	Pseudomonas phage YMC11/02/R656

56,400	Intact	77	Pseudomonas phage PMG1
15,500	Incomplete	21	Bacteriophage Aaphi23
12,700	Questionable	10	Pseudomonas phage Pf1
15,700	Intact	18	Pseudomonas phage Pf1
490614		3	
18,400	Intact	23	Pseudomonas phage Dobby
49,800	Intact	58	Pseudomonas virus H66
30,900	Intact	14	Pseudomonas phage Pf1
C5752-1		4	
32,300	Intact	40	Pseudomonas phage YMC11/02/R656
56,200	Intact	62	Pseudomonas phage phi297
47,000	Intact	47	Pseudomonas phage phi297
59,600	Intact	61	Pseudomonas phage F10

Table 8.2 **Results from PHASTEST analysis of clinical *P. aeruginosa* genome sequences.**

The prediction of prophage sequences was performed using PHASTEST (§2.22.7). Presented for each *P. aeruginosa* isolate is the number of predicted prophage regions, in addition to the length of that region (bp), the completeness of the prophage sequence, the number of coding sequences and finally the closest relative.

8.1.8 Coverage of bacteriophage reads before and after subsampling



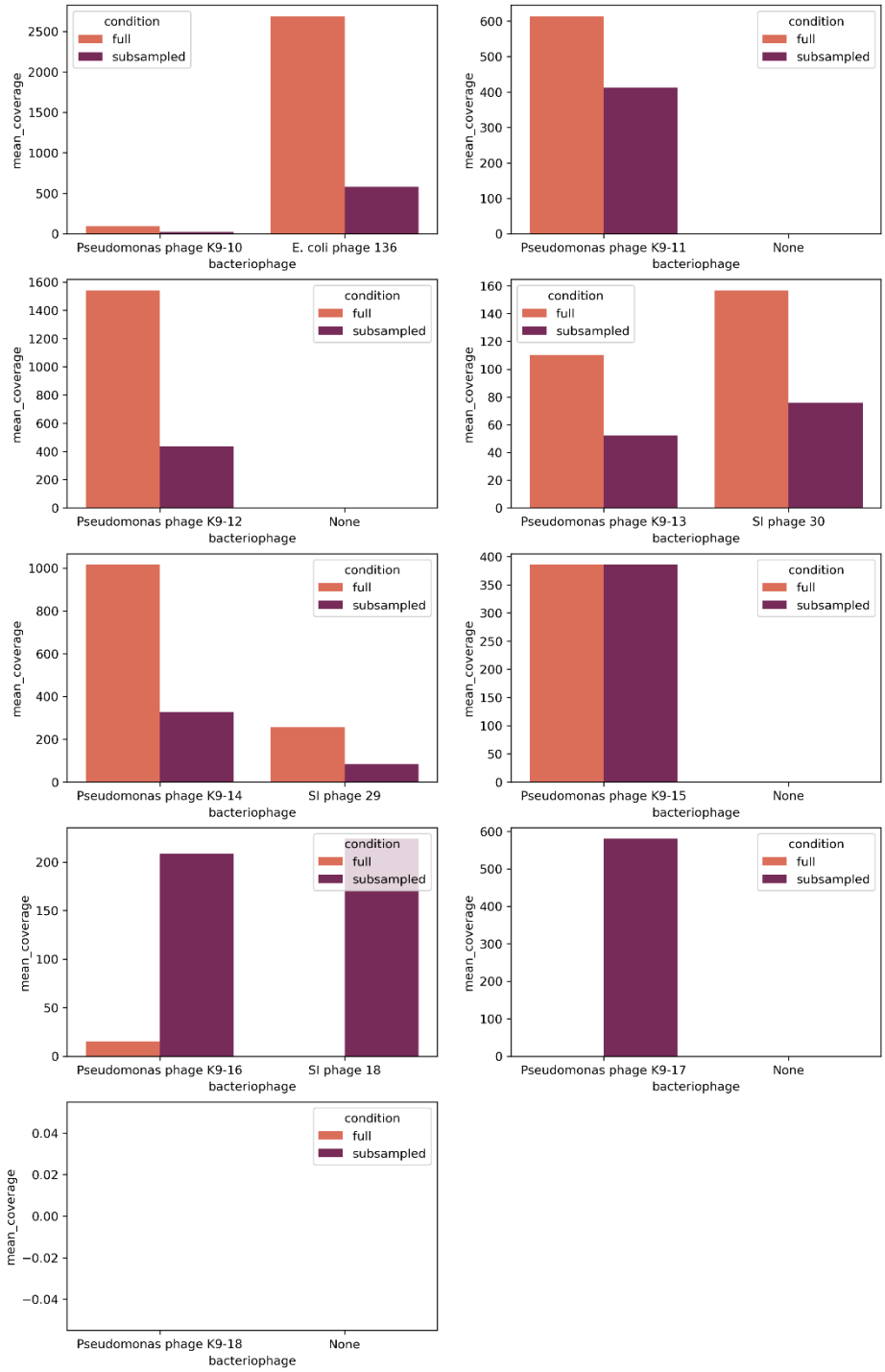


Figure 8.6 **Mean coverage of bacteriophage genomes assembled from co-sequenced reads.**

Up to two bacteriophage genome samples were combined and co-sequenced in one Illumina library preparation. Coverage was calculated using the complete set of trimmed reads and then repeated using a subsample of reads. A coverage of 0 indicates that a contig representing an assembly of the bacteriophage could not be identified.

8.1.9 VIRIDIC heatmap containing prophage regions

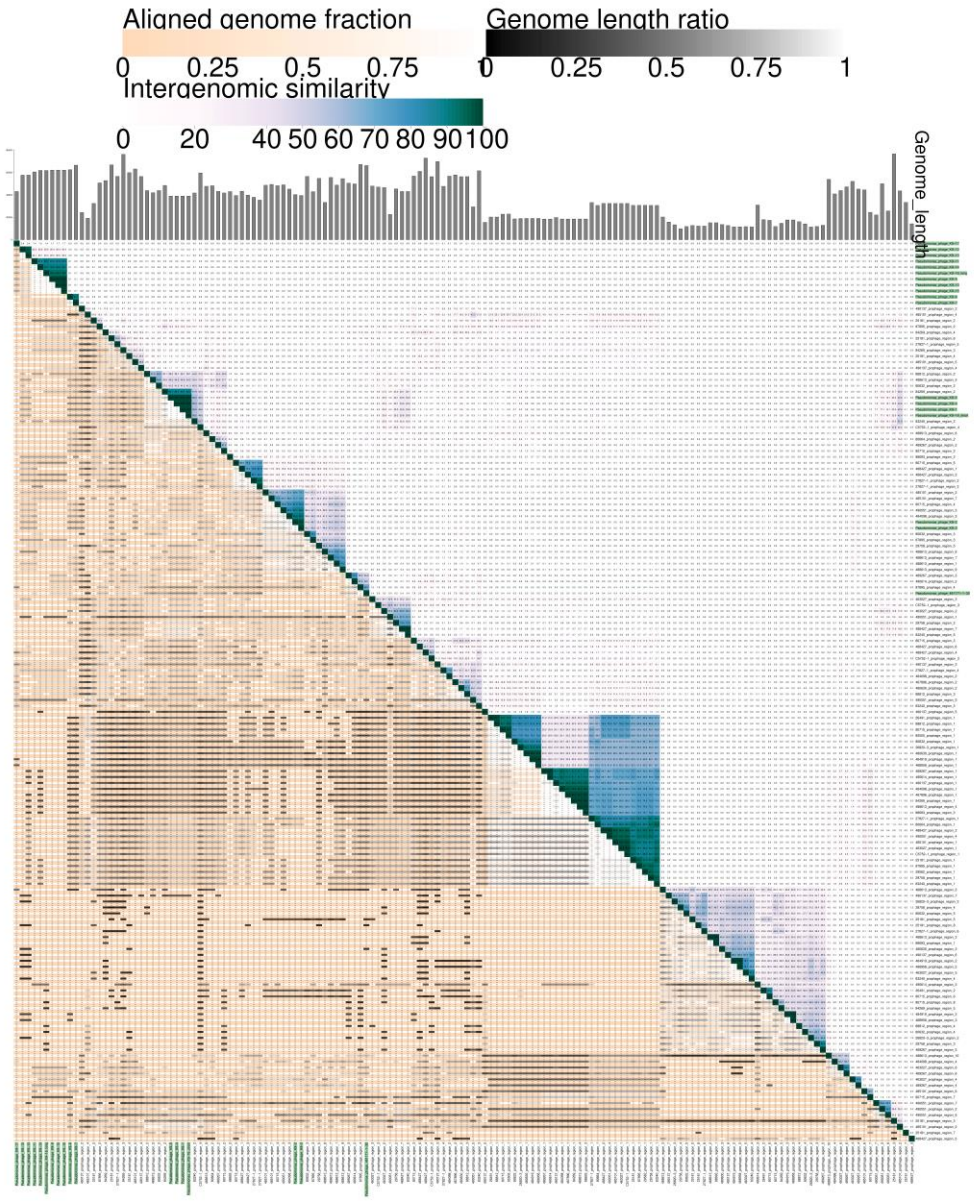


Figure 8.7 **Heatmap showing intergenomic similarities calculated by VIRIDIC.**

Heatmap generated by VIRIDIC of 19 bacteriophage genomes identified in this work in addition to prophage regions extracted using PHASTEST (§4.2.2.7). *Pseudomonas* phage K9-18 was removed from the analysis due to the fragmented genome. The upper right half is a heatmap sorted by hierarchical clustering based on intergenomic similarity. The bottom left portion of the graph shows the aligned genome fractions and genome length ratio. Finally, at the top of the graph is a histogram of the bacteriophage genome lengths. Green – *Pseudomonas* bacteriophage isolated in the present study.

8.1.10 VIRIDIC genus and species clustering

Genome	Species cluster	Genus cluster
Pseudomonas phage K9-17	123	89
Pseudomonas phage K9-12	120	88
Pseudomonas phage K9-13	120	88
Pseudomonas phage K9-11	119	87
Pseudomonas phage K9-14	121	87
Pseudomonas phage K9-16 long	122	87
Pseudomonas phage K9-9	118	87
Pseudomonas phage K9-10	118	87
Pseudomonas phage K9-15	118	87
Pseudomonas phage K9-6	125	90
Pseudomonas phage K9-7	126	90
490137 prophage region 2	76	56
485101 prophage region 4	44	36
25181 prophage region 2	2	2
87895 prophage region 2	106	78
84269 prophage region 4	104	76
25181 prophage region 6	6	6
27827-1 prophage region 5	18	15
84269 prophage region 3	103	75
25181 prophage region 4	4	4
485101 prophage region 5	45	37
490137 prophage region 4	78	58
88812 prophage region 2	110	81

488613 prophage region 5	58	47
80632 prophage region 2	85	63
84269 prophage region 2	102	74
Pseudomonas phage K9-5	117	74
Pseudomonas phage K9-4	117	74
Pseudomonas phage K9-1	117	74
Pseudomonas phage K9-16 short	117	74
83240 prophage region 2	99	72
C5752-1 prophage region 4	115	85
488613 prophage region 6	59	48
80664 prophage region 2	90	66
489267 prophage region 2	64	50
80715 prophage region 2	92	67
88693 prophage region 2	109	80
80715 prophage region 5	95	69
488427 prophage region 1	48	13
488427 prophage region 3	49	13
27827-1 prophage region 2	15	13
27827-1 prophage region 3	16	13
485101 prophage region 3	43	35
485101 prophage region 7	47	39
80715 prophage region 4	94	30
490051 prophage region 3	71	30
484098 prophage region 3	38	30

Pseudomonas phage K9-2	124	30
Pseudomonas phage K9-3	124	30
80632 prophage region 3	86	64
87895 prophage region 3	107	64
29758 prophage region 5	25	20
488613 prophage region 8	61	49
488613 prophage region 7	60	43
488613 prophage region 1	54	43
488613 prophage region 9	62	43
489267 prophage region 3	65	51
490614 prophage region 2	82	51
87895 prophage region 4	108	79
Pseudomonas phage 491171-1-30	116	86
463027 prophage region 3	28	22
C5752-1 prophage region 3	114	84
463027 prophage region 2	27	21
490051 prophage region 1	69	52
29758 prophage region 2	22	17
488427 prophage region 7	53	17
83240 prophage region 5	53	17
80715 prophage region 3	93	68
488427 prophage region 6	52	42
488427 prophage region 4	50	40
C5752-1 prophage region 2	113	83

490137 prophage region 3	77	57
27827-1 prophage region 4	17	14
484098 prophage region 2	37	14
467896 prophage region 2	33	27
480630 prophage region 2	35	28
88812 prophage region 3	111	28
490051 prophage region 5	72	54
83240 prophage region 3	100	73
490137 prophage region 5	79	59
26491 prophage region 1	9	9
88812 prophage region 1	9	9
80715 prophage region 1	91	9
85505 prophage region 1	91	9
80632 prophage region 1	84	9
26820-3 prophage region 1	11	9
480630 prophage region 1	34	9
484919 prophage region 1	34	9
488958 prophage region 1	34	9
489267 prophage region 1	63	26
490614 prophage region 1	63	26
490137 prophage region 1	75	26
484098 prophage region 1	32	26
467896 prophage region 1	32	26
84269 prophage region 1	32	26

488613 prophage region 4	32	26
88693 prophage region 3	32	26
27827-1 prophage region 1	14	1
80664 prophage region 1	89	1
488427 prophage region 2	26	1
490051 prophage region 4	26	1
485101 prophage region 1	26	1
463027 prophage region 1	26	1
C5752-1 prophage region 1	26	1
25181 prophage region 1	1	1
87895 prophage region 1	1	1
29582 prophage region 1	20	1
29758 prophage region 1	21	1
83240 prophage region 1	21	1
488613 prophage region 3	57	46
490137 prophage region 7	81	61
26820-3 prophage region 3	13	12
29758 prophage region 4	24	19
80632 prophage region 5	88	19
25181 prophage region 5	5	5
25181 prophage region 8	8	8
27827-1 prophage region 6	19	16
488613 prophage region 2	56	45
88693 prophage region 1	56	45

480630 prophage region 3	36	29
490137 prophage region 6	80	60
484919 prophage region 2	40	32
488958 prophage region 2	40	32
463027 prophage region 5	30	24
83240 prophage region 4	101	24
490614 prophage region 3	83	62
26491 prophage region 2	10	10
80715 prophage region 6	96	10
80715 prophage region 8	98	71
84269 prophage region 5	105	77
484919 prophage region 3	41	33
488958 prophage region 3	41	33
88812 prophage region 4	112	82
80632 prophage region 4	87	65
26820-3 prophage region 2	12	11
29758 prophage region 3	23	18
489267 prophage region 5	67	18
488613 prophage region 10	55	44
484098 prophage region 4	39	31
463027 prophage region 6	31	25
489267 prophage region 6	68	25
463027 prophage region 4	29	23
489267 prophage region 4	66	23

485101 prophage region 6	46	38
80715 prophage region 7	97	70
490051 prophage region 7	74	55
490051 prophage region 2	70	53
490051 prophage region 6	73	53
25181 prophage region 3	3	3
485101 prophage region 2	42	34
25181 prophage region 7	7	7
488427 prophage region 5	51	41

Table 8.3 **Table of assigned genus and species clusters.**

Findings from VIRIDIC genus and species clustering of 18 bacteriophage genomes from the present study and prophage regions identified using PHASTEST (§4.2.2.7).

8.2 PIPS reflective statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

A requirement of my PhD program was the completion of a three-month professional internship for PhD students. To satisfy this requirement, I was hosted by Dr Frieda Jorgensen within food, water, and environmental microbiology at UKHSA Porton Down. My duties included three research projects: "Metagenomic analysis of contaminated chicken nuggets", "Using qPCR for the rapid quantification of Campylobacter spp" and "Isolation of *E. albertii* or EPEC from supermarket chicken". The later project was a particular success and I continued to work on the project with Dr Jorgensen after my PIPS had finished.

The microbiology lab at UKHSA was ISO 17025 accredited, which gave me experience working in an ISO accredited environment and familiarised me with working safely within SOPs to perform DNA extractions using a Qiagen EZ Advanced XL platform and using existing qPCR methods to identify bacteria.

This work also involved the enrichment of samples using selective methods in addition and identification of isolates MALDI-TOF methods.

While on my placement I had the opportunity to visit the Quadram institute as part of an open day. While there I got to hear about the research that was being performed there as well as a tour of the facilities. This trip was a highlight of my PIPS.

This placement was the first time I had worked in microbiology outside of a university and helped me realise I would enjoy a career in public health microbiology, which is something I will look at pursuing after my PhD.

8.3 Publications from this work

microorganisms

MDPI

Review

Pseudomonas spp. in Canine Otitis Externa

Bailey Secker [1,2], Stephen Shaw [1] and Robert J. Atterbury [1,*]

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Abstract: Canine otitis externa (OE) is a commonly diagnosed condition seen in veterinary practice worldwide. In this review, we discuss the mechanisms of the disease, with a particular focus on the biological characteristics of *Pseudomonas aeruginosa* and the impact that antibiotic resistance has on successful recovery from OE. We also consider potential alternatives to antimicrobial chemotherapy for the treatment of recalcitrant infections. *P. aeruginosa* is not a typical constituent of the canine ear microbiota, but is frequently isolated from cases of chronic OE, and the nature of this pathogen often makes treatment difficult. Biofilm formation is identified in 40–95% of *P. aeruginosa* from cases of OE and intrinsic and acquired antibiotic resistance, especially resistance to clinically important antibiotics, highlights the need for alternative treatments. The role of other virulence factors in OE remains relatively unexplored and further work is needed. The studies described in this work highlight several potential alternative treatments, including the use of bacteriophages. This review provides a summary of the aetiology of OE with particular reference to the dysbiosis that leads to colonisation by *P. aeruginosa* and highlights the need for novel treatments for the future management of *P. aeruginosa* otitis.

Keywords: *Pseudomonas*; canine; otitis externa; dog; companion animal

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1. Introduction

1.1. Canine Otitis Externa

Otitis externa (OE) is defined as inflammation of the external ear canal that may affect the entire length, or any part, of the canal from the tympanic membrane (TM) to the outer meatus, and is often associated with concurrent pinnal changes. The disease can be painful and/or pruritic, acute, or chronic, and may affect one or both ears. Chronic OE is defined variably, but is usually considered to be disease of over three months duration [1], or which returns within three months and/or for the ear to demonstrate changes consistent with chronic disease [2].

OE is a very common condition in dogs presenting to veterinary clinics worldwide [3]. Secondary infections, particularly those involving *Pseudomonas* spp., most commonly *Pseudomonas aeruginosa*, are key in disease progression, morbidity, and treatment failure. Tissue changes, such as swelling and glandular hyperplasia, may result from the primary cause or secondary infection and further complicate the disease [4]. In severe cases, medical management may fail, requiring surgical intervention and resulting in hearing loss and risk of continued infection [5].

1.2. Normal Ear Structure and Function

The long cylindrical nature of the ear canal requires a delicate balance of the epidermal microbiota and a specialised function to maintain health [6]. Epidermal cells of the drum and ear wall are constantly being renewed. Corneocytes, shed from the surface, mix with sebaceous and ceruminous secretions to form cerumen (ear wax) that acts as a vehicle to

carry material, including microorganisms, out of the ear. The underlying epidermis moves centrifugally towards the outer meatus to facilitate this [7].

A higher density of ceruminous glands has been associated with the development of OE in dogs predisposed to otitis [8,9]. These secretions largely determine the acidity of the canine ear—at around pH 6—providing a buffered environment that regulates pH changes [10]. Acute ear infections can be accompanied by a reduction in the pH of the canal wall, whereas chronic disease may be more associated with an alkaline shift. Acidity may act to control infection [11], and stronger acids—particularly acetic acid—can be used as an otic disinfectant in people as well as dogs [12,13].

The microbiota of the healthy canine ear is maintained through a complex combination of mechanical, chemical, immunological, and microbial interactions, similar to those described for the human ear [14]. Innate and specific immune responses mediated by host defence peptides, such as β-defensin (cBD3)-like and cathelicidin (cCath), and the secretion of IgA, IgM, and (particularly) IgG in cerumen contribute to this [15]. Bacterial and epidermal antimicrobial peptides may act to limit bacterial growth and alter the immunosurveillance of commensal organisms. The disruption of the microbiota may lead to sustained dysbiosis and subsequent secondary infections. A further understanding of this may assist in the development of more effective treatments for OE.

1.3. Normal Ear Microbiota

Microorganisms in the ear are characterised using three main techniques: cytological examination, bacterial culture, and deep sequencing [16–18]. The cytological examination of otic discharge/cerumen is mostly used in the clinical setting as a quick, convenient test. However, this test is not sensitive and may fail to identify low or normal numbers of bacteria and yeasts [19], resulting in a binary interpretation of either normality on the one hand or infection and inflammation on the other; for instance, in *P. aeruginosa* infection, short rods are seen (Figure 1). Bacterial culture is also used in clinics, with or without the benefit of cytology, and will often identify bacteria and the non-lipid–dependent yeast *Malassezia pachydermatis*, which will grow on non-selective agar plates, regardless of whether they are in excess. When commensals are cultured, the clinical context, preferably also supported by cytology, is key to the interpretation of the laboratory results.

The organisms present in the normal canine ear have been investigated in only a few studies [16,20–22]. Traditional culturing has revealed the predominance of *Staphylococcus pseudintermedius*, *Bacillus* spp., coagulase-negative *Staphylococcus* spp., *Micrococcus* spp., as well as *Malassezia* spp., with smaller numbers of *Corynebacterium* spp. and Gram-negative rods [19,23,24]. Recently the microbiota of the middle ears was assessed in six healthy Beagle dogs and found to be similar to those of the external ear canal [25]. In OE, these organisms are over-represented, but the results of culture often reveal less commonly isolated organisms, such as *Staphylococcus schleiferi*, that are likely part of the normal microbiota [26]. However, as the disease progresses, transient organisms not adapted to the epidermis can invade and become established.

More recently, deep sequencing and metagenomics have revealed a richer population of bacteria and yeasts that characterise the microbiome of normal ears. They also provide evidence of changes in the microbiota that precede and follow the invasion of environmental organisms, such as *P. aeruginosa*. A metagenomic study of 257 ear swabs from 89 different dog breeds in the US (n = 256) and UK (n = 1) found *Staphylococcus pseudintermedius*, *Malassezia* spp., and *Streptococcus* spp., as would be expected from cytology and traditional culture, but also reported *Cutibacterium acnes* as commonly as *Staphylococcus pseudintermedius* [27]. Separate studies of dogs in the US, Belgium, and France found that *Corynebacterium*, *Streptococcus*, and *Staphylococcus* were frequently reported, although there was considerable variation in the microbiome between individual animals [27–29]. The reader is referred to other texts for a more detailed discussion of the normal microbiota of the ear [27,30].

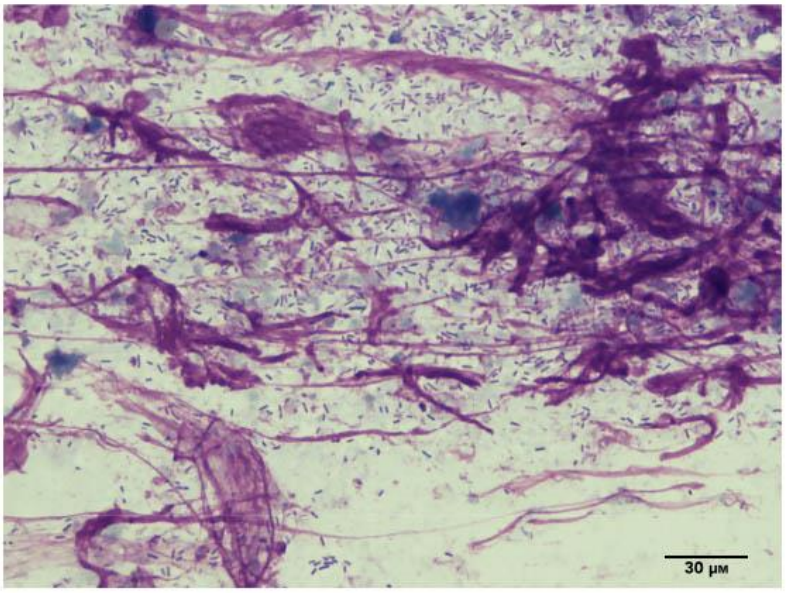

Figure 1. Cytology showing rods and nuclear streaming indicative of an active infection with *Pseudomonas aeruginosa*—modified Romanovsky stain (×1000).

Dysbiosis of the ear may be characterised by an overgrowth of *Malassezia* or bacterial populations, but there is not a consistent pattern of change from normal to infected ears, and this may also vary according to breed [31]. Dysbiosis often makes dogs more vulnerable to ear infections with *Pseudomonas*, particularly when accompanied by other factors, such as neglect. *P. aeruginosa* is not an obligate pathogen, and studies investigating the source of these infections often highlight environmental sources—particularly water [32], although nosocomial infections in veterinary surgery are possible [33].

2. Clinical Framework

August (1988) [4] devised a useful clinical framework that separated the factors involved in the development of canine OE into primary disease, predisposing factors, and perpetuating factors. This allowed a structured approach to diagnosis and treatment. More recently, the perpetuating factors have been separated into physical changes and secondary infections resulting in the PSPP (primary, secondary, perpetuating, and predisposing factors) system that is widely taught and used (Figure 2). However, this separation, though useful, is artificial and, for instance, otic inflammation and dysbiosis are intimately connected. Beyond these divisions that might be used to describe otitis, one group developed the OTIS-3 scale to describe the severity of OE using a composite score of erythema, oedema/swelling, erosion/ulceration, and exudate, but this is used infrequently [34].

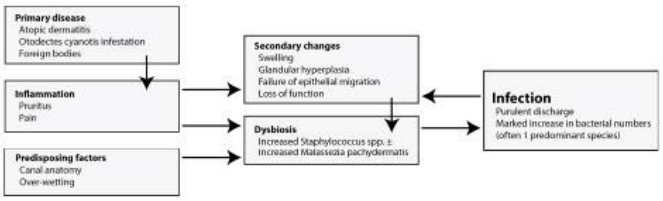

Figure 2. Diagram showing the influences and progress of ear disease from primary disease to infection.

2.1. *Primary Factors*

These describe the causes of initial inflammation in the ear. The most common are allergy, presence of foreign bodies, and infestations with *Otodectes cynotis*. Between 70 and 80% of dogs suffering from allergic skin disease (food and environmental allergy) have OE [35,36]. In particular, OE has been increasingly linked with canine atopic dermatitis (cAD) over the last few decades. In the 1980s, between 5 and 17% of dogs with cAD had OE [37,38], but by the early 2000s, this was reported to be up to 43% by Saridomichelakis et al. [39] and then later 50% in a large study of cAD by Favrot [40] However, this difference could be explained by a broadening of the definition of otitis to include inflammatory, as well as infected, OE [35]. It is important to note that primary factors are not identified in some cases [39].

The association between atopic dermatitis and OE complicates the PSPP system, as allergy causes inflammation and increases the probability of secondary infection. One study compared the microbiota of dogs suffering from atopic dermatitis with control animals using linear discriminant effect size analysis (LEfSe). Significantly greater numbers ($p < 0.05$) of some microbial species were found in the ears of dogs suffering from cAD compared with unaffected control animals. This was especially evident for *Staphylococcus*, which was present in 43.53% of cAD cases and 5.12% of controls. Populations of *Ralstonia*, *Methylotenera*, and *Lactobacillus* have also been reported as higher in dogs suffering from cAD [28]. Conversely, Apostolopoulos et al. [41] found lower abundances of *Brevibacterium* and *Macrococcus* ($p \leq 0.05$) in the ear canal of German shepherd dogs with allergy, although no difference in *Staphylococcus* colonisation was reported.

The type of primary disease may influence the subsequent bacterial and yeast infections. Zur, Lifshitz, and Bdolah-Abram [42] reported *Malassezia* and rods more commonly in allergy and endocrine disease, respectively. In contrast, Paterson and Matyskiewicz [35] reported that allergy was a common cause of *Pseudomonas* infections in dogs (70%).

Despite the presence of primary disease, many dogs only present to the clinic upon the subsequent development of secondary infections with both normal skin commensals as well as environmental organisms, such as *Pseudomonas*.

2.2. *Secondary Infections*

Secondary infection usually causes a marked increase in the clinical signs experienced (increased discharge with a purulent exudate, malodour, and irritation or pain) in canine OE, and many patients are not presented until this is noted. The normal canine ear is not sterile, and depending on the primary disease, some dysbiosis may be seen in the absence of clinical infection, blurring the line between traditionally binary infected versus non-infected ears. Inflamed ears are often dysbiotic, with an overgrowth of *Staphylococcus pseudintermedius* and *Malassezia pachydermatis* [28]. In the absence of perpetuating factors (below), reducing inflammation may be sufficient to resolve the overgrowth [43].

Table 1 summarises the microbial species found in OE using culture-based and metagenomic methods. *Staphylococcus* spp. (2.97–58.5%) and *P. aeruginosa* (5.83–35.5%) are both frequently isolated using culture, along with the fungus *Malassezia pachydermatis* (8.75–30.9%).

Shotgun metagenomics has further identified the obligate anaerobes *Peptostreptococcus canis* and *Porphyromonas cangingivalis* in 5.52% and 4.38% of OE cases respectively [27]. The anaerobic nature of these organisms means that they have not yet been implicated in cases of OE; however, they have both been identified in the canine mouth [44,45].

Table 1. Bacteria and fungi commonly isolated from cases of canine otitis externa using both culture-based and metagenomic methods.

Genus	Species	Prevalence Using Traditional Culture	Prevalence Using Metagenomics
Staphylococcus	*S. intermedius*, coagulase-positive Staphylococci, *S. pseudintermedius*, *S. schleiferi*, *S. schleiferi* spp. Coagulans, coagulase-negative Staphylococci	58.5% + 6.2% [24]; 36.83% + 2.97% [46]; 24.30% [47]	22% [29]; 11.25% + 8.54% [27]
Pseudomonas	*P. aeruginosa*	35.5% [47]; 16.24% [46]; 7.2% [24]	18.6% [29]; 5.83% [27]
Malassezia	*M. pachydermatis*	30.9% [24]; 30.01% [46]	8.75% [27]
Streptococcus	*S. canis*, β-haemolytic Streptococci, non-haemolytic Streptococci, *S. halichoeri*, *S. agalactiae*	29.9% + 4.1% [24]; 6.2% [47]; 2.97% [46]	5.42% + 3.80% + 0.83% [27]; 2.2% [29]
Proteus	*P.* spp., *P. mirabilis*	14.4% [24]; 6.8% [47]; 3.56% [46]	5.60% [29]; 2.29% [27]
Escherichia	*E. coli*	10.30% [24]; 4.2% [47]; 3.17% [46]	
Corynebacterium	*C. auriscanis*, *C. freneyi*, *C.* spp.	0.79% [46]	7.08% + 4.38% [27]; 5.4% [29]
Finegoldia	*F. magna*		5.83% [27]
Peptostreptococcus	*P. canis*		5.52% [27]
Lactobacillus			5.5% [29]
Enterococcus	*E. faecium*, *E. faecalis*	5.2% [24]	2.30% [29]; 1.04% [27]
Enterobacteriaceae (Unknown genus)			4.9% [29]
Porphyrimonas	*P. cangingivalis*		4.5% [29]; 4.38% [27]
Arcanobacterium	*A. canis*		4% [27]
Peptoniphilus	*P. harei*		2.29% [27]
Candida	*C.* spp.	2.38% [46]	
Bacillus	*B.* spp.	0.99% [46]	
14 Others			2.08–0.63% [27]

Corynebacterium spp. Was isolated from 0.79% of canine OE patients in Bulgaria [46]. However, metagenomic sequencing suggests that this could be an underestimate, as its prevalence could be up to 5.4–7.08% [27,29]. This is in line with other studies showing the greater sensitivity of 16S rRNA sequencing compared with traditional culture methods [18]. However, the results of DNA sequence analysis should be interpreted with caution, as they do not distinguish between viable and dead cells.

Given the microbial diversity of healthy ears and the range of environmental and physical exposures that can initiate infection, it is unsurprising that the relative abundance of pathogens in canine OE also varies considerably between studies. However, despite this diversity, *Pseudomonas* spp. are the most common bacteria in chronic otitis, with up to 35%

of cases affected (Figure 3) [48]. Typically, the time from the onset of primary disease to the reporting of *Pseudomonas* infection is between 10 and 28 months [35].

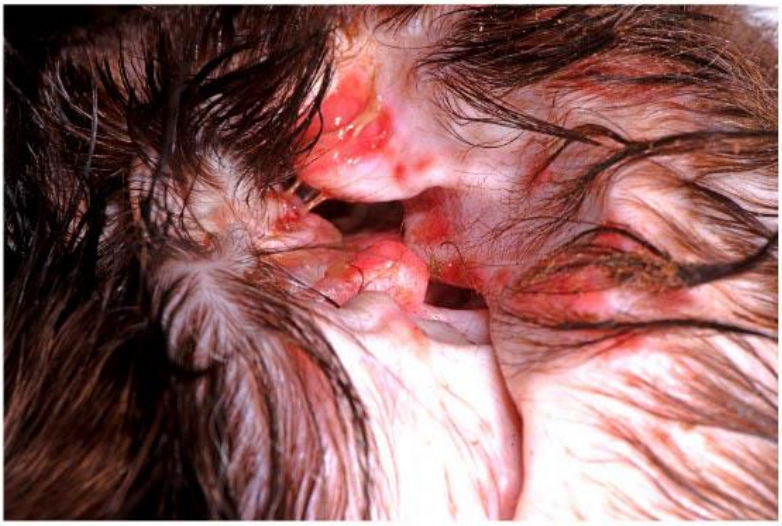

Figure 3. An example of the clinical appearance of *Pseudomonas* otitis in a Cocker spaniel.

2.3. Perpetuating Factors

Perpetuating factors represent the tissue changes in the ear canal that develop as a result of disease and that prevent the resolution of OE and make repeated infection more likely [4]. Swelling and hyperplasia of the canal wall are followed by the enlargement of the ceruminous glands (modified sweat glands) and, finally, hidradenitis. Once the disease has progressed to this stage, it is often irreversible, and with increased surface area and ineffective physical and mechanical defences, bacteria such as *Pseudomonas* thrive. Bacterial toxins and physical accumulation of discharge can cause the rupture of the tympanic membrane, allowing infection to spread to the middle ear, resulting in otitis media (OM), which can then act as a perpetuating reservoir of infection [49]. Ear canal stenosis (38%) and OM (25%) were identified as the most prevalent perpetuating factors in Greece among 100 dogs of varying breeds [39].

2.4. Predisposing Factors

Predisposing factors are not causative of inflammation, but increase the probability of developing otitis. Addressing these rarely results in the complete alleviation or resolution of OE once it has developed. Predisposing factors include the physical traits of the dogs such as long, pendulous, hairy, or V-shaped drop pinna, as well as narrow external canals. These traits may lead to higher levels of moisture within the ear canal, which favour bacterial growth/survival. External factors, such as swimming and over-treatment with aqueous ear cleaners, may similarly disrupt normal functioning by increasing moisture within the ear canal, disrupting the normal microbiota and predisposing to OE [39,50].

2.5. Prevalence

Estimates of canine OE prevalence range between 5–20% [4]. In the UK, canine OE was a frequent diagnosis (7.3%) in a random sample of 22,000 dogs attending primary care practices over a one-year period in 2016, which was second only to periodontal disease (12.52%) [51]. Further investigation revealed that certain breeds were predisposed to this disease, namely Basset Hounds, Chinese Shar Pei, and Labradoodles, whereas Chihuahuas were the least susceptible [50]. An earlier study in South-Eastern England found that OE was the most prevalent diagnosis for dogs (10.2%), followed by periodontal disease (9.3%) [52]. A similar prevalence has been reported in South Korea (6.3%) [53], the US (13%) [54], and New Zealand (7.5%) [55].

2.6. Treatment

Treatment for OE has three aims: reduce clinical signs, restore normal numbers and distribution of microbial organisms, and restore normal ear function. The PSPP system directs the clinician to consider the primary cause. In cases where there are minimal perpetuating changes and mild dysbiosis, removing the primary disease may be sufficient to restore ear health. Steroids are often included in the treatment of canine OE; these aid in the management of inflammation of the ear canal and are administered orally, parenterally, or topically. Although steroids do not have a direct effect on the ear microbiota [56], their use in infections, including *Pseudomonas*, is controversial as they may reduce useful inflammation and immune responses; however, severe swelling is often a marked impediment to successful treatment and they are widely used. Negative effects are offset as steroids are usually part of combined steroid, antibiotic, antifungal products (cSAA products)—see Table 2 below.

Table 2. Otic treatments containing antibiotics in the UK.

Steroid	Antibiotic	Antifungal
Dexamethasone (as acetate) 0.9 mg/mL	Marbofloxacin 3 mg/mL	Clotrimazole 10 mg/mL
Prednisolone 2.5 mg/mL	Diethanolamine fusidate 5.0 mg/mL Framycetin sulphate 5.0 mg/mL	Nystatin 100,000 iu/mL
Hydrocortisone aceponate 1.11 mg/mL	Gentamicin sulfate 1505 iu/mL	Miconazole nitrate 15.1 mg/mL
Betamethasone valerate 0.88 mg/mL	Gentamicin sulfate 2640 iu/mL	Clotrimazole 8.80 mg/mL
Mometasone furoate 0.9 mg/mL	Orbifloxacin 8.5 mg/mL	Posaconazole 0.9 mg/mL
Prednisolone acetate 5 mg/mL	Polymyxin B sulfate 0.5293 mg/mL	Miconazole nitrate 23 mg/mL
Mometasone furoate 2.2 mg/dose	Florfenicol 16.7 mg/dose	Terbinafine 14.9 mg/dose
Betamethasone acetate 1 mg/dose	Florfenicol 10 mg/dose	Terbinifine 10 mg/dose

Note: no products have marketing authorisation for use in the middle ear.

Topical antimicrobial therapy is generally preferred by clinicians for treating canine OE. This is because otic preparations provide a much higher local drug concentration than systemic treatments [57]. The concentration used is such that culture and susceptibility data must be considered carefully as the antibiotic is being used at many times the MIC. For this reason, and following cytological examination, antibiotics are often used empirically [48]. Bacterial resistance is a common feature of recurrent OE and constitutive resistance is a feature of *Pseudomonas* infections. For cases of *P. aeruginosa* OE, three classes of antibiotics are often used. These are fluoroquinolones, such as marbofloxacin, and aminoglycosides, particularly gentamicin and polymyxin B. Topical otic treatments containing antibiotics

with marketing authorisation in the UK are shown in Table 2. In addition, some clinicians prepare ad hoc solutions using injectable solutions of antibiotics when resistance is seen or suspected [58] e.g., ticarcillin–clavulanic acid [59], amikacin [60], ceftazidime [61], and enrofloxacin [62]. There is a perception that such mixtures may be less ototoxic in the case of a ruptured tympanic membrane, but this is not substantiated in the literature [63].

Dogs with canine OE may be prescribed ear cleaners, usually as an adjunctive to antibiotics. This aids in the removal of debris from the ear canal, which is important for the proper function of some antimicrobials, namely aminoglycosides and polymyxin B, which have reduced efficiency in the presence of pus [57,64]. The specific composition varies between products; however, they generally include cerumenolytics, surfactants, astringents, antimicrobials, and anti-inflammatories [13], as well contain ingredients that disrupt biofilms [65]. When products with antimicrobial action are used, this can have a marked effect on the success of therapy. Such disinfectant cleaners can have a faster rate of action than antibiotics, and by providing an alternative mechanism to kill bacteria, make the development of resistance by mutant selection less likely. For fluoroquinolones, the mutant prevention concentration for *Pseudomonas* is many times the minimum inhibitory concentration and, in infections where high numbers of organisms are present, additional products are essential [66,67].

The efficacy of ear cleaners in inhibiting clinical *P. aeruginosa* from canine OE varies based on the composition. Multiple studies have failed to identify key components or properties of ear cleaners, e.g., pH. However, the combination of Tris-EDTA plus 0.15% chlorhexidine has shown some effect against *P. aeruginosa* [13,68], and products containing this formulation have been widely adopted for this reason.

In some cases of chronic OE, the disease may progress to a point where medical treatment will be unsuccessful in controlling infection. In such cases, marked canal hyperplasia, secondary OM, and biofilm production in the presence of a multi-drug-resistant *Pseudomonas* infection are often evident. At this stage, surgery is necessary, including total ear canal ablation with or without bulla osteotomy. This procedure involves the removal of the infected ear canal and bulla contents, resulting in the resolution of disease in most cases [69].

2.7. Environmental Prevalence

P. aeruginosa is often described as ubiquitous in soil and aqueous environments. However, it is more frequently isolated from soil and water sources linked to human activity, such as those contaminated with oil hydrocarbons or pesticides [70]. Urban rivers have been linked to an increased prevalence of *P. aeruginosa* [70]. In an outbreak of human OE in the Netherlands involving *P. aeruginosa*, 83% of cases were linked to swimming in freshwater lakes, even though the water met quality standards [71].

2.8. Pseudomonas in the Veterinary Environment

The contamination of the veterinary clinical environment has been reported in the findings of surveillance studies [72–74] and has been linked to life-threatening infections following cardiac and orthopaedic open surgery [75,76]. In ear disease, the contamination of otic speculums is a particular risk, chiefly in practices with poor speculum availability and high case volumes where disinfection between patients is inadequate [33,77]. This is also a problem in human ear clinics [78], although this can be addressed by using disposable speculums. There is a need for further studies to examine other parts of the otoscope system, such as handles and trailing wires, as these may pose a considerable infection risk. The impact of the accidental inoculation of *Pseudomonas* may be increased by the strategic use of narrow-spectrum antibiotic treatments containing florfenicol targeting *Staphylococcus* spp., rather than broad-spectrum products.

2.9. In the Home

P. aeruginosa has been isolated from multiple sites in the home, such as surfaces, water supplies, and dishwasher rubber seals. Several studies have identified household drains as important reservoirs of *P. aeruginosa* [79–81]. Clonal isolates to those causing OE in dogs have been found in the oral cavity of affected dogs and other animals in the home, as well as water bowls and taps [32], although the directionality of transmission could not be established. Clonal *P. aeruginosa* isolates have been found in household taps used to fill water bowls [32]. The same study used statistical modelling to link swimming in pools and visiting dog parks to a 64% higher prevalence of *Pseudomonas* otitis [32]. This makes sense given the occurrence of *P. aeruginosa* in environmental water samples and even in commercial swimming pools [82]. Finally, grooming products in the home and professional salons have been found to harbour *P. aeruginosa* [83]. *P. aeruginosa* can also be isolated from the ears of apparently healthy dogs, suggesting an unknown history of OE [84] or contamination from the environment. This could result in the contamination of the home environment and subsequent reinfection or infection of other animals/humans in the household.

2.10. Isolates

The population structure of *P. aeruginosa* causing clinical infections in animals and people is largely non-clonal, i.e., the strains involved exhibit a high degree of diversity, with little or no association between groups and diseases [85]. Very few studies have examined the genomic profiles of *P. aeruginosa* strains causing canine otitis specifically. Whole-genome MLST has revealed a high diversity of *P. aeruginosa*, with 45 different sequence types (STs) identified from 80 isolates in one study [86] and 27 STs from 29 isolates in another [87]. This mirrors studies of *Pseudomonas* from human infections, which also found a largely non-clonal population structure punctuated by highly successful epidemic clones/clonal complexes [88]. There are reports of *Pseudomonas* transmission between dogs and people, especially following contact with symptomatic dogs [89]. However, the directionality of transmission is difficult to establish, as the zooanthroponotic transmission of *P. aeruginosa* has also been reported [90].

P. aeruginosa isolates from canine otitis have been found to exhibit an intermediate or strong ability to form biofilms compared with isolates from other sites [91], which may contribute to their increased antimicrobial resistance (AMR) [92]. Multidrug resistance appears to be more common in isolates from dogs (35%) than from other animal species, namely horses and cows (0%), particularly those isolated from cases of otitis [85]. However, stable clonal variants of *P. aeruginosa* that exhibit either enhanced biofilm formation or accelerated detachment may coexist in the same clinical sample. Greater production of extracellular polysaccharides has been found to positively correlate with the intracellular levels of the secondary messenger cyclic di-GMP in clinical *P. aeruginosa* canine otitis isolates [93]. The coexistence and cooperation of these variants may enhance bacterial persistence overall in what has been described as the "insurance hypothesis" [94].

3. Toxin Production/Virulence Factors

Few studies have investigated virulence factors specific to the development of canine OE. While it is widely accepted that biofilm formation and antibiotic resistance contribute to *P. aeruginosa* infection in canine OE [57], other virulence factors are relatively unexplored. In insect and plant infection models, clinical (hospital) and environmental (soil and water) isolates showed no statistically significant difference in pathogenesis [95], supporting the notion of infections originating from exposure to environmental reservoirs.

Biofilm formation is likely the most important virulence factor involved in chronic *P. aeruginosa* canine OE infections and is associated with poor treatment outcomes [92,96,97]. Biofilms provide protection from antimicrobials and the immune system, and facilitate dispersion to other environments (for a detailed review, see [98]). In cases of canine OE, this often presents as the development of OM, which can subsequently re-infect the ear

canal [1]. Bacteria form biofilms by attaching to a surface and producing extracellular polymeric substances comprising exopolysaccharides, extracellular DNA, and proteins. Biofilms are produced by 40–95% of *P. aeruginosa* isolates from canine OE [92,96,97] and 92% of human patients with chronic OE, but only 20% from those with acute otitis [99]. *Pseudomonas* isolates from human OE showed increased adherence to guinea pig epithelial cell lines compared with isolates from other sites of infection [100]. The removal of the biofilm using novel therapies, such as chemical ear peeling, significantly reduced the recurrence of symptoms compared with ciprofloxacin/hydrocortisone antibiotic treatment in humans [99].

Hattab et al. [91] investigated the presence of five virulence genes in *P. aeruginosa* isolates from dogs, including from the ear canal. Three genes, *lasB* (elastase A), *aprA* (alkaline protease), and *plcH* (haemolytic phospholipase C), were present in all of the tested isolates, while *exoS* (bi-functional type-III cytotoxin) and *toxA* (Exotoxin A) were present in 87.5% and 91.7%, respectively [91]. Similarly, *P. aeruginosa* isolated from human OM patients in Egypt was positively amplified between two and ten of the quorum sensing (*rhlR*, *rhlI*, *lasR*, *lasI*) and virulence genes (*lasB*, *toxA*, *aprA*, *algD*, *exoS*, and *plcH*) [101]. While these findings may help to identify areas for further investigation, we need to determine if/when these genes are expressed to establish their role in disease. Details of *P. aeruginosa* virulence and secretion systems have been reviewed elsewhere [102] and this review will concentrate on those factors with specific relevance to canine OE.

Bacterial proteases are a major contributor to disease. *P. aeruginosa* secretes several proteases, including alkaline protease (AprA), Elastase A and B (LasA and LasB), protease IV (PrpL), small protease (PASP), large exoprotease (LepA), and others [103]. Protease activity, measured by the degradation of azocasein, from *P. aeruginosa* infection has been identified in humans with OM [104]. The total protease activity of *P. aeruginosa* isolates from canine OE is more variable than that from other animal sources ($p < 0.0001$), but is the same on average ($p = 0.7538$) when assessed by the hide powder azure absorbance assay [105]. The treatment of a chinchilla model of *P. aeruginosa* OM with bacterial protease inhibitor (GM 6001) resulted in higher survival (66%) compared with the control, gentamicin and gentamicin + GM 6001 groups, although this was not significant ($p = 0.2674$) [106].

P. aeruginosa isolates from chronic cases of canine OE have been found to have a reduced mean elastase activity compared with other animal isolates ($p < 0.0001$); moreover, some strains produce a stable elastase-negative phenotype due to deficiencies in the *rhl* quorum sensing system. Interestingly, the *rhl* phenotype was observed in an isolate with wild-type elastase activity, implicating an unknown constituent of quorum sensing being important in chronic otitis infections [105,107].

Haemolytic phospholipase C (*plcH*) is an exoenzyme that is able to lyse red blood cells [108] in addition to components of eukaryotic cell membranes [109]. No specific studies have been performed investigating the role of *plcH* in cases of OE. The intradermal inoculation of the protein in an in vivo mouse model resulted in a concentration-dependent response showing either no change, erythema, or dermonecrosis [110].

Bacterial secretion systems are important for the virulence of Gram-negative bacteria as they allow for the transport of proteins across the two membranes and, in some cases, directly into a target cell. Exotoxin A (ToxA) is exported via the SEC-dependent type II secretion system. The toxin is part of the AB toxin family, where the A subunit has enzymatic activity and the B subunit facilitates attachment to CD91 (also known as alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein α2MR/LRP). Once inside the host cytoplasm, the protease furin cleaves the protein and it is transported to the trans Golgi network, where it inhibits the eukaryotic elongation factor-2 by ADP-ribosylation, inducing apoptosis. This allows the toxin to bind to and kill cells from many different tissues [111,112]. Increased transcription of exotoxin A in human OE patients has been associated with patients suffering from more severe symptoms compared with those with 'mild to moderate' symptoms [113]. Additionally, when applied to the middle ear, the toxin causes the apoptosis of epithelial cells and can penetrate the inner ear, causing

damage to the cochlea in multiple animal studies [114–116]. Whether the toxin is able to cross the tympanic membrane during OE and have this effect has not been investigated.

ExoS is part of the type III secretion system of *P. aeruginosa*. It possesses an N-terminal GTPase-activating protein region that triggers actin cytoskeleton disruption, and a C-terminal adenosine diphosphate ribosyl transferase, which is a major cause of host apoptosis [117]. The presence of ExoS is commonly mutually exclusive with ExoU, although the reason for this and its role in infection are unclear [118,119]. *P. aeruginosa* isolates from human chronic OM cases were significantly more likely to be *exoU*+ than *exoS*+ compared with a control group of isolates from blood and respiratory infections [120]. In human cases of OE, *P. aeruginosa* isolates have been shown to produce significantly less pyocyanin and alginate than isolates from other sites of infection, in addition to increased deoxyribonuclease production [100]. Whether differences in these factors are seen in canine OE and their clinical significance remains to be elucidated.

4. Antibiotic, Disinfectant, and Biological Control

In addition to biofilm formation, the outer membrane of *P. aeruginosa* has limited permeability, which confers some intrinsic resistance to antimicrobials and allows specific antibiotic resistance mechanisms to act more effectively. Resistance can also arise from efflux pumps and the expression of a chromosomal β-lactamase [121].

AMR, especially for Gram-negative rods, has been recognised as a problem in canine otitis for many years [122], with numerous studies documenting resistance profiles for *P. aeruginosa* isolates. Fluoroquinolone resistance has frequently been reported, particularly with respect to enrofloxacin (27–68%), orbifloxacin (55–82%), and marbofloxacin (33–35%). Gentamicin resistance seems to vary greatly depending on the study, with resistance reported in 3–43% of isolates [46,48,87,123–125]. Multidrug resistance, which is defined as resistance to at least one antibiotic from three or more classes [126], was recorded in 13–35% of isolates [87,123]. Non-susceptibility to carbapenem antibiotics has also been reported in 15–23% of *P. aeruginosa* isolates from canine otitis [86,123]. This is important as the World Health Organisation identified a critical need for new antibiotics against carbapenem-resistant *P. aeruginosa* [127]. Carbapenem resistance can arise in *P. aeruginosa* by either the production of carbapenemase, efflux pump over-expression, or reduced outer-membrane permeability [128]. Clinical canine otitis *P. aeruginosa* isolates producing metallo-β-lactamase VIM-2 have been identified in Korea [86].

Petrov and co-workers [46] monitored AMR patterns in dogs suffering from both Gram-positive and Gram-negative infections in OE between 2007–2011, and again in 2013–2017. They found that, for most antibiotics tested against *P. aeruginosa* between these time points, resistance had increased. Notably, gentamicin resistance increased from 2% to 15%, as did resistance to tobramycin (20% to 26%), amikacin (0% to 18%), lincomycin/spectinomycin (40% to 93%), and polymyxin B (0% to 50%). In contrast, enrofloxacin resistance decreased (38% to 27%). In France, the investigation of *P. aeruginosa* resistance profiles from canine OE surveillance databases found that resistance to gentamicin remained unchanged over a 5-year period, while resistance to enrofloxacin first increased and then slowly decreased over the following years. Worryingly, resistance to both enrofloxacin and gentamycin was reported in 19.4% of isolates. The decrease in resistance to enrofloxacin was likely due to the decreased exposure of companion animals to antibiotics over the study period [125].

Park and colleagues [84] investigated the antibiotic susceptibility of *P. aeruginosa* isolates from various body sites of healthy and diseased dogs. While antibiotic resistance was more prevalent in diseased samples, resistance was still present in isolates from otherwise healthy dogs. Specifically, resistance to ciprofloxacin (10.5%) was the most common, while ciprofloxacin–gentamicin–tobramycin, gentamicin, and tobramycin all showed the same level of resistance (2.6%).

Potential Alternative Treatments

Increasing AMR, particularly among the ESKAPE pathogens, including *Pseudomonas*, has driven research into enhancing the efficacy of current therapeutic options and the development of novel alternatives. Prior to 2000, there was very little research on alternative treatments for *Pseudomonas* OE. One study investigated the use of new antibiotic, ticarcillin in treatment-resistant cases, resulting in the initial resolution of 11 out of 12 cases, although two of these later relapsed [129]. More recently, synergy and partial synergy were reported when a combination of polymyxin B/miconazole and marbofloxacin/gentamicin was tested on *P. aeruginosa* canine otitis isolates [130,131].

Ethylenediaminetetraacetic acid (EDTA), specifically Tris-EDTA, has been studied as an adjuvant in combination with antimicrobials in relation to OE for many years [132–134], and is now a common component of many commercially available ear cleaners [68]. When used alone, Tris-EDTA typically produces a bacteriostatic effect on *P. aeruginosa*, unless applied in excess [97]. However, it has been used to eradicate biofilms produced by clinical *P. aeruginosa* isolates [97,135] or reduce the MIC of certain antibiotics for *P. aeruginosa* present in biofilms [65].

Tris-EDTA has been found to act synergistically with amikacin (fractional inhibitory concentration, FIC = 0.1994) and neomycin (FIC = 0.1646) against *P. aeruginosa* isolates from canine OE [133]. It has also been found to complement the action of enrofloxacin [136], marbofloxacin, and gentamicin [135] when used against AMR *P. aeruginosa* from cases of OE.

N-acetylcysteine (NAC) is another candidate being explored as an adjuvant for use in canine OE. Similarly to Tris-EDTA, it has been shown to inhibit the growth of *P. aeruginosa*, including in canine otitis clinical isolates [97,137,138]. Synergistic interactions between NAC and enrofloxacin or gentamicin have been observed for one *P. aeruginosa* isolate, but were indifferent or antagonistic at the concentrations tested in vitro for most isolates [137]. NAC is also able to remove biofilms formed by clinical *P. aeruginosa* (20,000–80,000 µg/mL minimum biofilm eradication concentration (MBEC)) isolates; however, it is important to note that this was at potentially ototoxic concentrations (<20,000 µg/mL) [97].

Narasin and monensin were unable to inhibit clinical *P. aeruginosa* and other Gram-negative otitis pathogens at the tested concentrations when used alone, but an additive effect for narasin (but not monensin) was seen in combination with Tris-EDTA [139,140]. However, monensin was found to significantly reduce, but not eradicate, *P. aeruginosa* biofilm development [97].

A combination of enrofloxacin plus silver sulfadiazine demonstrated increased antimicrobial susceptibility, with the mean MIC decreasing from 12.97 µg/mL to 1.52/3.05 µg/mL [141]. Similarly, clinical canine OE *P. aeruginosa* isolates challenged with silver sulfadiazine alone were all found to be susceptible, with 80% of isolates having a mean MIC of <10 µg/mL. It was noted that a commercially available product should be effective in vivo, as it has a concentration much higher than the MICs seen in the study [142].

Phytochemicals have been investigated for *P. aeruginosa* inhibition. An antimicrobial effect has been reported for cinnamon oil, cinnamaldehyde [143], oregano oil, carvacrol, thyme oil, thymol [144], basil oil, rosemary oil, clary sage oil [145], and *Harungana madagascariensis* extract [146] in vitro. In all cases, *P. aeruginosa* required a higher MIC than other common otitis pathogens, specifically Gram-positive organisms, such as *S. pseudintermedius*. One group tested an EDTA combination, and a synergistic interaction was observed for cinnamon oil (FIC = 0.27) and cinnamaldehyde (FIC = 0.26) [143]. Song and colleagues [147] showed that, when applied alone, *P. aeruginosa* was resistant to manuka oil (MIC > 8% *v*/*v*). However, when combined with Tris-EDTA, the MIC was reduced to 0.5% or less. This was also true for multidrug-resistant isolates. When investigating the use of a commercially available essential oil blend in vivo for cases of acute OE, it was reported that 33.3% of dogs were "cured" and a further 20.9% showed strong improvements. Importantly, rods were not present in the cytology; therefore, more work is needed to assess its use in chronic cases of OE involving *P. aeruginosa*. Despite this, the blend did inhibit a clinical strain of *P. aeruginosa* when tested in vitro [148].

Another promising alternative treatment is the use of antimicrobial photodynamic therapy. The use of tetra-cationic porphyrins to inactivate multidrug-resistant *P. aeruginosa* isolates from canine OE and other infection sites has been demonstrated in vitro [149,150]. Antimicrobial photodynamic therapy has been successfully used in vivo for a case of OE caused by a VIM-2 Metallo-β-lactamase-producing *P. aeruginosa* that had been unresponsive to treatment with enrofloxacin [151].

The effect of cold atmospheric microwave plasma was investigated using primarily Gram-positive canine otitis isolates, but the study did include an ATCC *P. aeruginosa*. Gram-negative bacteria, including the ATCC *P. aeruginosa*, were more susceptible to cold atmospheric microwave plasma than other isolates. For example, after 10 s of exposure at a plasma intensity of 30 W, *P. aeruginosa* survival was only 22.9%, while that of *S. aureus* was 50.3%. However, this has yet to be tested on canine OE isolates [152].

The safety and capability of in vivo bacteriophage therapy have already been assessed against antibiotic-resistant *P. aeruginosa* causing chronic otitis in humans and dogs. In humans, twenty-four patients were selected, of which twelve received bacteriophage. Those who were treated with the phage reported clinical improvement and no adverse effects, with three cases seeming to be cured after a single treatment [153]. In the canine study, ten dogs suffering from antibiotic-resistant *P. aeruginosa* otitis were treated once with a cocktail of six bacteriophages and monitored. After 48 h there was a 67% reduction in the number of *P. aeruginosa*. After 18 months, three of the animals had recovered from the disease and another three had no detectable *P. aeruginosa*; again, no side effects were reported [154]. Although these results are promising, bacteriophages are often strain-specific; therefore, a cocktail of phages targeting different receptors is required. Additionally, phage resistance may be seen, although this was not investigated in the above studies. It is also potentially important to note that the chronic nature of *P. aeruginosa* OE might require multiple rounds of phage treatment, further increasing the risk of developing resistance and therefore potentially requiring multiple phage cocktails per treatment.

5. Conclusions

Canine OE is a common disease in veterinary practices, with the most recent study from the UK reporting a prevalence of 7.3%. Cases are often only seen after an increase in clinical signs from secondary infection. When addressing this disease, it is important to consider the primary, secondary, perpetuating, and predisposing factors. Certain breeds are predisposed to this disease due to factors such as long, pendulous, hairy, or V-shaped drop pinna, and allergy is the most common primary factor. Advances in sequencing technologies have provided a better understanding of the natural microbiota of the canine ear and how allergies can lead to dysbiosis of the ear canal. This potentially helps us to understand why allergy is such a prevalent primary factor. Despite this, *P. aeruginosa* is still commonly isolated from cases of canine OE and, due to widely reported antimicrobial resistance and biofilm formation, still poses a major issue for clinicians. Research investigating alternative treatments has seen promising results in vitro, with only a few being tested in vivo. Further work and in vivo experiments will be required in order to provide a better prognosis for the future.

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