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Phage-antibiotic synergy to combat multidrug resistant strains of Gram-negative ESKAPE pathogens

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Bacteriophage-antibiotic-synergy (PAS) was investigated to target *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter cloacae*. Whole genome sequencing indicated that bacteriophage KPW17 targeting *K. pneumoniae*, clustered with genus *Webervirus*, ECSR5 targeting *E. cloacae* clustered with *Eclunavirus*, PAW33 targeting *P. aeruginosa* clustered with *Bruynoghevirus*, while ABTW1 targeting *A. baumannii* clustered with *Vieuvirus*. PAS analysis showed that the combination of ciprofloxacin (CIP) and levofloxacin (LEV) with PAW33 resulted in the synergistic eradication of all tested *P. aeruginosa* strains. Similarly, the combined use of doripenem (DOR) and LEV with KPW17 resulted in the synergistic eradication of the environmental and clinical *K. pneumoniae* strains, while the combined use of DOR and gentamicin (CN) with ECSR5 was synergistic against the clinical *E. cloacae* NCTC 13406. Gentamicin with ECSR5, however, only exhibited an additive effect for *E. cloacae* 4L, while ABTW1 with piperacillin-tazobactam (TZP) and imipenem (IPM) resulted in an indifferent interaction between the bacteriophage and tested antibiotics against the clinical *A. baumannii* AB3, i.e., the activity of the combination is equal to the activity of most active agent. Thus, while the observed PAS may offer an opportunity for the re-introduction or more efficient application of certain antibiotics to combat antibiotic resistance, extensive research is required to determine the optimal phage-antibiotic combinations, dosages and treatment regiments.

Keywords Bacteriophages, Antibiotics, Pathogens, Synergy, ESKAPE, Combination therapies

Pseudomonas aeruginosa (*P. aeruginosa*), *Acinetobacter baumannii* (*A. baumannii*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Enterobacter cloacae* (*E. cloacae*) are commonly associated with life-threatening hospital-acquired infections, such as septicaemia, urinary tract infections, respiratory tract infections, surgical wound infections, and endocarditis[1–3]. These bacteria form part of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.), which are classified as highly virulent bacteria resistant to many first line and last-resort antibiotics[4]. While multiple antibiotics have been used to treat these infections, these opportunistic pathogens display resistance to a variety of antibiotic classes, including carbapenems, polymyxins, cephalosporins and β-lactams[4–7]. These resistance mechanisms can be classified as either intrinsic, acquired, or adaptive resistance, where intrinsic resistance may include decreased membrane permeability, increased expression of efflux pumps (transport antibiotics out of the cell) or the production of antibiotic-inactivating enzymes[6]. Acquired resistance is achieved through the horizontal gene transfer of resistance genes or mutational changes, while adaptive resistance may be attributed to the formation of biofilms or persister cells, where antibiotic access to the bacterial cells is limited and antibiotic resistance may develop due to sub-optimal antibiotic treatment concentrations[6].

It is however, concerning to note that the increased occurrence of these opportunistic pathogens in extra-hospital reservoirs, i.e., soil, food, surface water and wastewater, has been linked to community-acquired infections (infections contracted outside of hospital/clinical settings)[8–10]. For example, Wang et al.[11] reported on a case of community-acquired *P. aeruginosa* pneumonia that progressed to septic shock and multiple organ dysfunction syndrome. Additionally, the opportunistic pathogen rapidly developed resistance to carbapenems and the treatment regime had to be adapted. The appearance and spread of antibiotic resistant *P. aeruginosa, A. baumannii, K. pneumoniae* and *E. cloacae* in the environment thus represents a new challenge and treatment

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interventions that are effective within clinical settings and within the broader natural environment (e.g., water industry, food industry, agricultural industry) need to be investigated. Moreover, as it is essential that antimicrobial resistance be targeted in a sustainable and environmentally friendly manner[12], the use of biological control agents, defined as "the use of natural or modified organisms, genes, or gene products, to reduce the effects of undesirable organisms"[13], has received renewed interest. One such biological control agent that has been shown to be effective at targeting bacterial pathogens in multiple industries and sample types and displays potential to be used in combination with other physical or chemical treatment strategies, is bacteriophages[14,15].

Lytic bacteriophages (bacterial viruses which infect, replicate within, and subsequently kill the infected bacterial host cell during progeny release)[16] have been used to target food-borne pathogens (food safety)[17], for the treatment of infectious diseases (agricultural crops, livestock and human)[17,18] and in bioremediation strategies for the selective removal of various bacterial species from water sources[19–21]. Bacteriophages can also penetrate and disrupt bacterial biofilms; a primary survival mechanism of bacteria in clinical settings and the natural environment[22–24]. Moreover, bacteriophages have been reported to work synergistically when applied in combination with antibiotics, a phenomenon termed phage-antibiotic synergy (PAS). Synergistic antimicrobial activity can subsequently be obtained when (i) antibiotics increase the susceptibility of bacteria to bacteriophages by weakening the bacterial population, or (ii) bacteriophages that interact with bacterial drug efflux systems, could restore antibiotic sensitivity in resistant target bacterial populations[25,26]. Altamirano et al.[27] studied the combination of the bacteriophage øFG02 with ceftazidime against a multidrug resistant (MDR) clinical *A. baumannii* strain, which was resistant to this antibiotic. A checkerboard assay was conducted with varying concentrations of ceftazidime and øFG02 and was monitored for 16 h. Results indicated that bacteriophage resistant mutants emerged in all bacteriophage-only controls, while the combination treatments resulted in a sustained suppression of *A. baumannii* growth, even at subinhibitory concentrations, indicating that there was a synergistic interaction between øFG02 and ceftazidime[27].

As alternative strategies for the treatment of MDR and extensively drug resistant (XDR) *P. aeruginosa, A. baumannii, K. pneumoniae* and *E. cloacae* infections are urgently required and antibiotic resistant bacteria are increasingly detected in the environment, the current study aimed to investigate the potential of PAS to target environmental and clinical isolates of *P. aeruginosa, A. baumannii, K. pneumoniae* and *E. cloacae*. To achieve this aim, bacteriophages effective against these Gram-negative ESKAPE pathogens were isolated from various water sources, sequenced, annotated, and phylogenetically and morphologically characterised. An antibiotic disc diffusion assay was subsequently used to determine which bacteriophage-antibiotic combinations potentially displayed PAS, whereafter checkerboard assays were conducted to identify which antibiotic classes could be used in combination treatment interventions.

Results

Isolation, host range determination and characterisation of bacteriophages

In total, 17 presumptive *K. pneumoniae* bacteriophages were isolated from the wastewater samples collected; four using the reference *K. pneumoniae* ATCC 10031 as the target bacterial host, and 13 using the environmental *K. pneumoniae* S1 43 as the target bacterial host and were designated as KPW1-KPW17. The host range of the isolated bacteriophages was assessed using the spot test assay (Fig. S1, Supplementary Information). Overall, isolate KPW17 displayed the highest activity against the *Klebsiella* spp. isolates, with activity recorded against 73% ($n = 8/11$) of the *K. pneumoniae* strains and 43% ($n = 6/14$) of the *Klebsiella oxytoca* (*K. oxytoca*) strains. Notably, activity was recorded against reference (ATCC 13383, 10031, and 333305), environmental (506/e and S1 43) and clinical (P2, P3 and MCC3) *K. pneumoniae* strains. Additionally, no activity was observed against the 73 non-target bacterial strains analysed (Table S1; Supplementary Information). KPW17 formed clear plaques approximately 5 mm in diameter surrounded by a turbid halo.

Two presumptive *E. cloacae* bacteriophages were isolated from surface run-off using the reference *E. cloacae* NCTC 13406 as the target bacterial host and were assigned code identifiers ECSR5 and ECM5. The host range analysis of the two bacteriophages indicated that ECSR5 displayed lytic activity against 18% ($n = 4/23$) of the reference, environmental and clinical *Enterobacter* spp. tested (Fig. S1), similarly, ECM5 displayed lytic activity against 18% ($n = 4/23$) of the reference, environmental and clinical *Enterobacter* spp. tested (results not shown) with no growth inhibition observed for both bacteriophages against the 27 non-target bacterial species analysed (Table S1; Supplementary Information). ECSR5 formed clear plaques approximately 2 mm in diameter.

Based on the results of the host range determination and plaque morphology, bacteriophage isolates KPW17 and ECSR5 were morphologically characterised using transmission electron microscopy (TEM) (results summarised in the Supplementary Information). Briefly, morphological features indicated that KPW17 belongs to the myovirus morphological group while ECSR5 was classified in the siphovirus morphological group, both in the class *Caudoviricetes*. Bacteriophage vB_PaeP_PAW33 (hereafter referred to as PAW33) was isolated from wastewater and partially characterised as a podovirus by Reyneke et al.[14]. Additionally, the bacteriophage vB_AbaS_ABTW1 (hereafter referred to as ABTW1) was isolated from hospital wastewater and was characterised as siphovirus by Morris et al.[28].

It should be noted that the efficiency of the bacteriophages against *P. aeruginosa, A. baumannii, K. pneumoniae* and *E. cloacae* planktonic cells and biofilms was also investigated, with the methods and results outlined in the Supplementary Information. PAW33 displayed activity against the reference strain *P. aeruginosa* ATCC 27853, the environmental *P. aeruginosa* S4 12 strain, and the clinical *P. aeruginosa* PAO1 strain. KPW17 showed activity against the reference *K. pneumoniae* ATCC 13383 strain, the environmental *K. pneumoniae* S1 43 strain, and the clinical *K. pneumoniae* P3 strain. ECSR5 displayed activity against the reference *E. cloacae* NCTC 13406 strain and environmental *E. cloacae* 4L strain however, no lytic activity was observed against the tested clinical *E. cloacae* strains, while ABTW1 displayed activity against the clinical *A. baumannii* AB3 strain and no lytic activity was observed against the tested reference and environmental *A. baumannii* strains.

Bacteriophage genome sequencing and annotation

Genomic features of PAW33

Whole genome sequencing (WGS) and assembly indicated that the genome of PAW33 was 45 269 bp with a GC content of 53%. PhageTerm analysis showed that the PAW33 genome is circularly permutated with headful packaging, where a terminase initiates packaging on the phage concatemer at specific *pac* sites, and cuts are subsequently made at variable positions once the bacteriophage head becomes full[29]. Annotation with Pharokka identified a total of 82 coding domain sequences (CDS) of which 26 were functionally annotated, displaying homology with bacteriophage proteins available in the PHROGs database, while 56 CDS were annotated as bacteriophage hypothetical proteins (Fig. 1A). Additionally, no CDS with homology to virulence factor genes or antibiotic resistance genes were identified. Functionally annotated CDS identified genes related to transcriptional regulation, DNA/RNA and nucleotide metabolism, lysis, moron (nonessential genes), auxiliary metabolic and host takeover, head and packaging and tail structure. Four tRNA sequences were also identified, and the coding density was calculated as 96.6%.

A total of 95 *Pseudomonas* bacteriophage genomes were included in the VIRIDIC analysis, where the percentage identity of the first BLAST result was 97.43% with a query coverage of 97%. The VIRIDIC analysis clustered PAW33 with 33 other *Pseudomonas* bacteriophages within the genus *Bruynoghevirus*, including *Pseudomonas* phage Epa4 (Accession number: MT118288.1), which PAW33 shared the highest intergenomic similarity with of 92.395%. Phyologenetic analysis using a hypothetical protein of the core genes clustered PAW33 with seven *Pseudomonas* bacteriophages with a bootstrap value of 83 (Fig. 2A). Thus, based on the

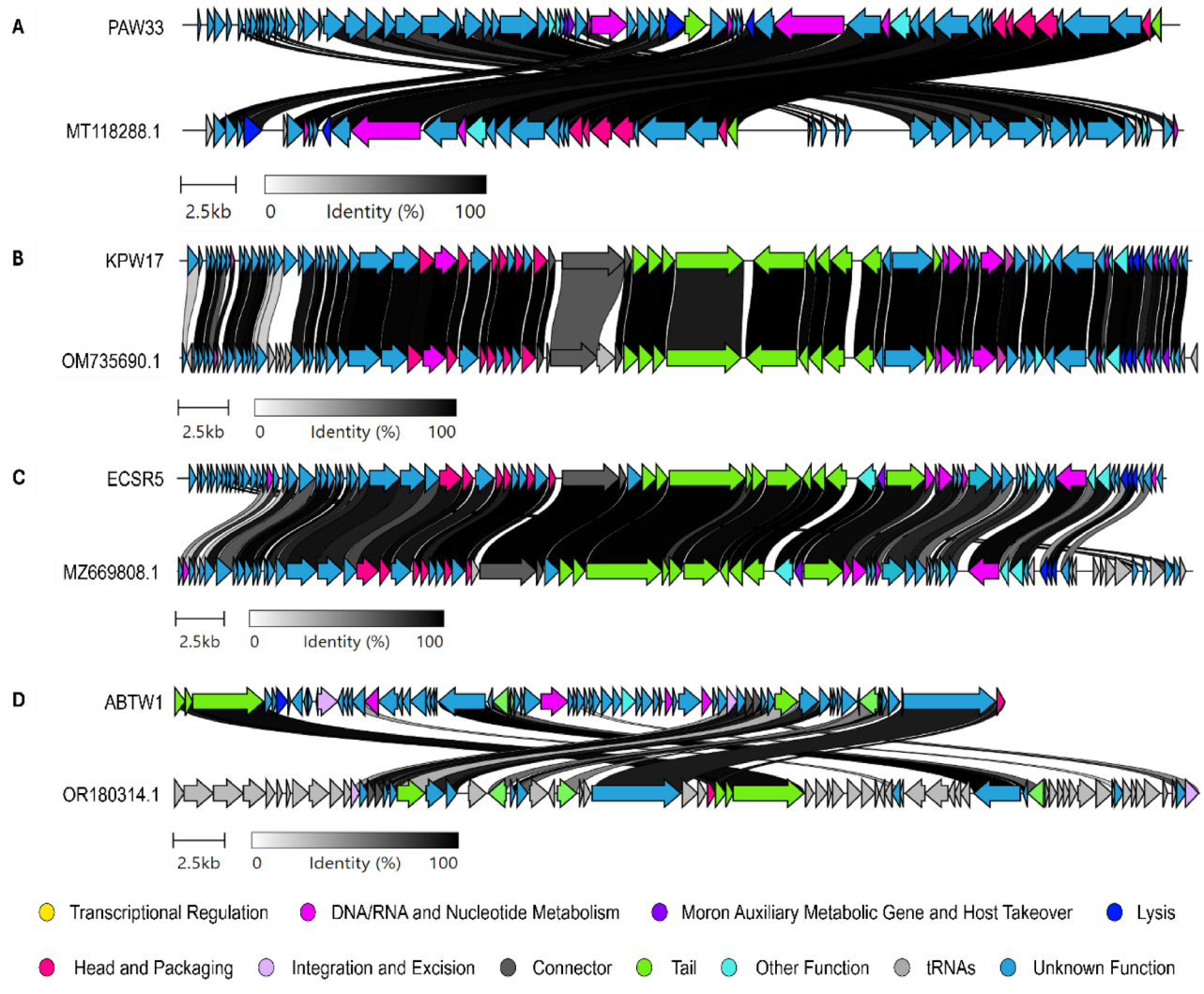

Fig. 1. Complete genome map of bacteriophages (**A**) PAW33, (**B**) KPW17, (**C**) ECSR5, (**D**) ABTW1 and their homology with their closest relative visualised with clinker. Coding domain sequences (CDS) annotated by Pharokka are represented by arrows, where the arrowheads denote the orientation of the respective CDS. Colour coding for the various CDS functions are presented in the key at the bottom of the figure. The lines connecting the genomes represent gene homology with the value of the line representing the percentage identity.

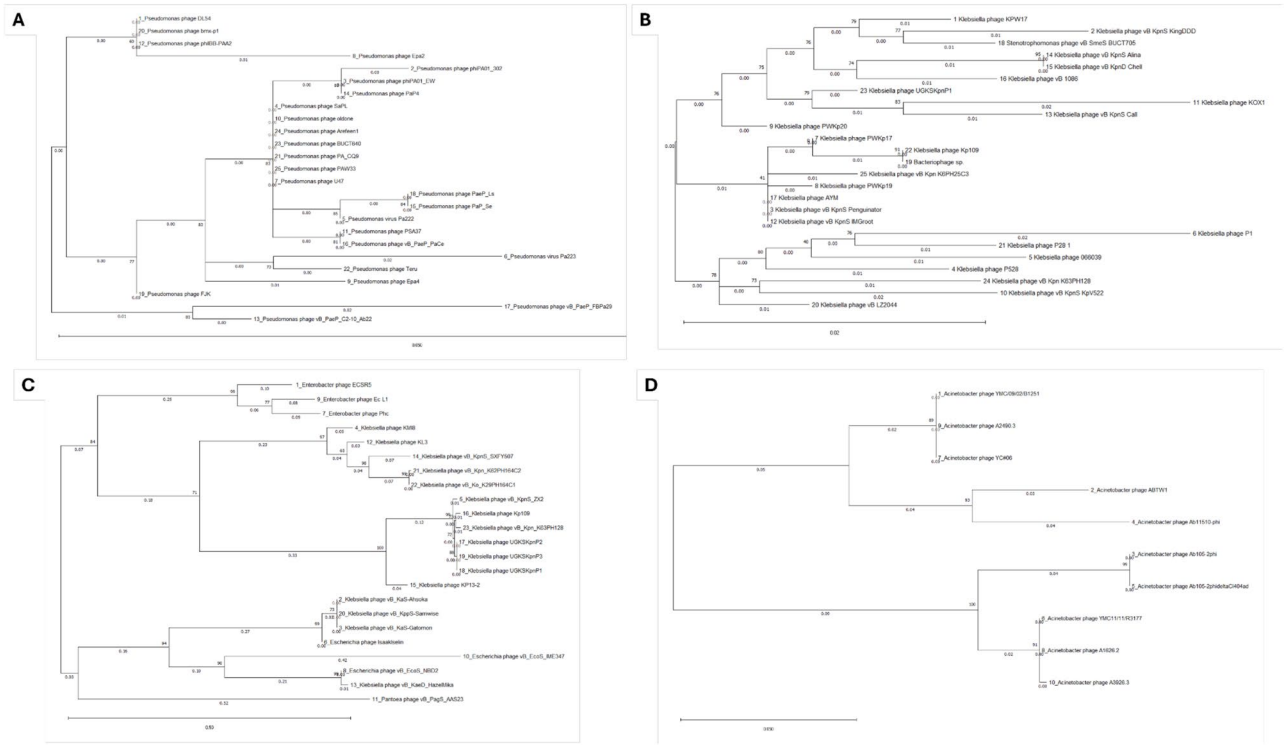

Fig. 2. Phylogenetic analysis of the core genes of (**A**) PAW33, (**B**) KPW17, (**C**) ECSR5 and (**D**) ABTW1 as identified with GET_HOMOLOGUES and GET_PHYLOMARKERS.

genomic data PAW33 was renamed *Bruynoghevirus PAA2* PAW33 according to the updated nomenclature of Turner et al.[30].

Genomic features of KPW17

The WGS analysis indicated that the KPW17 genome was 50 416 bp with a GC content of 51%. PhageTerm analysis indicated that the KPW17 genome is circularly permutated with headful packaging[29]. Annotation analysis with identified a total of 88 CDS (Fig. 1B). The functional annotation of the 36 CDS, identified genes related to transcriptional regulation, DNA/RNA and nucleotide metabolism, lysis, moron, auxiliary metabolic and host takeover, head and packaging, connector and tail structure, while 52 CDS were identified as hypothetical bacteriophage proteins. Additionally, no tRNAs or genes associated with virulence factors or antibiotic resistance were identified. Overall, the coding density of the genome was 92.19%.

The VIRIDIC analysis utilised a total of 98 bacteriophage genomes, including bacteriophages infecting *Klebsiella* spp, with the percentage identity of 99.23% and query coverage of 96% for the top BLAST result. This then indicated that KPW17 and the *Stenotrophomonas* bacteriophage vB_SmeS_BUCT705 (Accession number: OM735690.1) shared the same species cluster within the genus *Webervirus*, with a nucleotide identity of 95.43%. This was confirmed by phylogenetic analysis with a hypothetical protein where KPW17 clustered with vB_SmeS_BUCT705 with high bootstrap value of 79 (Fig. 2B). KPW17 was subsequently named *Webervirus BUCT705* KPW17.

Genomic features of ECSR5

The genome of ECSR5 was 51 128 bp with a GC content of 49%. The ECSR5 genome is circularly permutated with headful packaging[29]. A total of 94 CDS were identified, of which 35 were functionally annotated, while 59 CDS were identified as bacteriophage hypothetical proteins (Fig. 1C). The functional annotation of the CDS, identified genes related to transcriptional regulation, DNA/RNA and nucleotide metabolism, lysis, moron, auxiliary metabolic and host takeover, head and packaging, connector and tail structure. Additionally, no tRNAs or genes associated with virulence factors or antibiotic resistance were identified. Overall, the coding density of the genome was 93.46%.

Pairwise intergenomic similarity analysis implemented a total of 91 bacteriophage genomes, including those infecting *Enterobacter* spp. and the first BLAST result had a percentage identity of 94.30% with a query coverage of 89%. The VIRIDIC analysis indicated that ECSR5 shared a genus cluster with two *Enterobacter* bacteriophages, Phc (Accession number: MZ669808.1) and Ec_L1 (Accession number: NC_042122.1), in the genus *Eclunavirus*, with a nucleotide identity with ECSR5 of 83.85% and 74.41%, respectively. This was confirmed by phylogenetic analysis using a hypothetical protein where ECSR5 clustered with Phc and Ec_L1 with high bootstrap values of 99 (Fig. 2C). ECSR5 was then named *Eclunavirus ecsr5*.

Genomic features of ABTW1

The WGS analysis indicated that the ABTW1 genome was 40 312 bp with a GC content of 40% and no identified termini[28,29]. A total of 74 CDS were identified (Fig. 1D). The functionally annotated 18 CDS identified genes were related to transcriptional regulation, DNA/RNA and nucleotide metabolism, lysis, integration and excision, head and packaging, connector and tail structure, while 56 CDS were identified as hypothetical bacteriophage proteins. Additionally, 1 tRNA was identified and no genes associated with virulence factors or antibiotic resistance were identified. Overall, the coding density of the genome was 95.43%.

The VIRIDIC analysis utilised a total of 79 *Acinetobacter* bacteriophage genomes where the top BLAST result had percentage identity of 96.96% and query coverage of 42%. Subsequently, the VIRIDIC analysis indicated that ABTW1 did not share a species or genus cluster with the analysed genomes. The bacteriophage with the highest nucleotide identity of 41.029% was *Acinetobacter* bacteriophage A3926.3 (Accession number: OR180314.1) in the genus *Vieuvirus*. Phylogenetic analysis using a minor tail protein indicated that ABTW1 clustered with Ab11510-phi (Accession number: MT361972.1) with a bootstrap value of 93 (Fig. 2D and Fig. S4). The nucleotide identity of 41.029% suggests that ABTW1 may belong to a novel genus and species.

Identification of potential bacteriophage-antibiotic combinations

To identify bacteriophage-antibiotic combinations with synergistic potential, the Kirby-Bauer disc diffusion assay was used to test the susceptibility of the *K. pneumoniae*, *P. aeruginosa, A. baumannii* and *E. cloacae* strains to a series of antibiotics (Table S2), before and after exposure to bacteriophages KPW17, PAW33, ABTW1 and ECSR5 for 24 h, respectively, using bacterial challenge tests (Supplementary Information, Sect. 1.1). Bacteriophage susceptibility testing then indicated that *P. aeruginosa* S4 12, *K. pneumoniae* ATCC 13383, *E. cloacae* NCTC 13406, *E. cloacae* 4L, and *A. baumannii* AB3 were resistant to their respective bacteriophages after the 24 h. Additionally, bacterial isolates resistant to more than three classes of antibiotics were classified as MDR, while those resistant to at least one antimicrobial agent in all but two classes or less were classified as XDR.

Kirby-Bauer disc diffusion assays following exposure to PAW33

Classification of the measured zones of inhibition according to the EUCAST[31] guidelines, indicated that before exposure to PAW33, the *P. aeruginosa* ATCC 27583 and PAO1 strains were susceptible, while the *P. aeruginosa* S4 12 environmental strain was classified as XDR (Fig. 3A). Similarly, according to the CLSI[32] guidelines, prior to exposure to PAW33 the *P. aeruginosa* ATCC 27583 and clinical *P. aeruginosa* PAO1 strains did not display

A

PAW33												
EUCAST							CLSI					
ATCC		S4 12		PAO1		Antibiotics	ATCC		S4 12		PAO1	
0 h	24 h	0 h	24 h	0 h	24 h		0 h	24 h	0 h	24 h	0 h	24 h
Aminoglycosides												
S	S	S	R	S	S	AK	S	S	S	I	S	S
IE	IE	IE	IE	IE	IE	CN	S	S	S	I	S	S
Carbapenems												
I	R	R	R	I	I	IPM	S	I	I	I	S	S
S	S	R	R	S	S	MEM	S	S	R	R	S	S
Cephalosporins												
I	R	R	R	R	I	FEP	S	S	R	I	S	S
I	I	I	I	I	I	CAZ	S	S	S	S	I	S
Fluoroquinolones												
I	I	R	I	I	I	CIP	S	S	I	S	S	S
I	I	R	I	I	I	LEV	S	S	R	I	I	S
Monobactams												
R	I	R	I	I	I	ATM	I	S	I	S	S	S
Penicillins												
I	I	I	I	I	I	TZP	S	S	S	S	S	S

B

KPW17												
EUCAST							CLSI					
ATCC		S1 43		P3		Antibiotics	ATCC		S1 43		P3	
0 h	24 h	0 h	24 h	0 h	24 h		0 h	24 h	0 h	24 h	0 h	24 h
Aminoglycosides												
S	S	S	S	S	S	AK	S	S	S	S	S	S
S	S	S	S	R	R	CN	S	S	S	S	R	R
S	S	S	S	R	R	TOB	S	S	S	S	R	R
Carbapenems												
S	S	S	S	I	S	DOR	S	S	S	S	I	S
S	S	S	S	I	S	IPM	S	S	S	S	I	S
S	S	S	S	S	S	MEM	S	S	S	S	S	S
Cephalosporins												
S	S	S	S	R	R	CAZ	S	S	S	S	R	R
S	S	S	S	R	R	CRO	S	S	S	S	R	R
Fluoroquinolones												
S	S	S	S	R	R	CIP	S	S	S	S	R	R
S	S	S	S	R	I	LEV	S	S	S	S	R	I
Monobactams												
S	S	S	S	R	R	ATM	S	S	S	S	R	R
Penicillins												
R	R	R	R	R	R	AMP	R	R	R	R	R	R
I	I	I	I	R	R	SAM	S	S	S	S	R	R
S	S	R	S	R	R	PRL						
S	S	S	S	R	R	TZP	S	S	I	S	R	R
Tetracyclines												
						DO	S	S	S	S	R	R
						TE	S	S	S	S	R	R
Miscellaneous												
S	S	S	S	R	R	SXT						
S	S	S	S	R	R	W						

Fig. 3. Antibiotic susceptibility profiles of the (**A**) *P. aeruginosa* reference (ATCC 27583), environmental (S4 12), and clinical (PAO1) strains as determined by disc diffusion assays before (0 h) and after (24 h) exposure to PAW33 during the bacterial challenge tests; and (**B**) *K. pneumoniae* reference (ATCC 13383), environmental (S1 43), and clinical (P3) strains as determined by disc diffusion assays before (0 h) and after (24 h) exposure to KPW17 during the bacterial challenge tests. Results were classified according to the EUCAST (2022) and CLSI (2022) guidelines as Resistant (R; Red); Intermediate (I; Orange); Susceptible (S; Green). Grey Blocks = Classification data not available. Amikacin (AK), Ampicillin (AMP), Ampicillin/Sulbactam (SAM), Aztreonam (ATM), Cefepime (FEP), Ceftazidime (CAZ), Ceftriaxone (CRO), Ciprofloxacin (CIP), Doripenem (DOR), Doxycycline (DO), Gentamicin (CN), Imipenem (IPM), Levofloxacin (LEV), Meropenem (MEM), Piperacillin (PRL), Piperacillin-tazobactam (TZP), Sulphamethoxazole/Trimethoprim (SXT), Tobramycin (TOB), Tetracycline (TE), Trimethoprim (W).

resistance to any of the tested antibiotics, while the *P. aeruginosa* S4 12 environmental strain was confirmed as XDR (Fig. 3A).

While exposure to PAW33 for 24 h during the bacterial challenge test did not change the overall classification of the *P. aeruginosa* test strains based on the EUCAST[31] and CLSI[32] guidelines, notable differences with regards to the effectiveness of specific antibiotics were noted for all test strains. For example, according to both the EUCAST[31] and CLSI[32] guidelines, the reference *P. aeruginosa* ATCC 27583 strain displayed increased resistance to imipenem (IPM) but became more susceptible to aztreonam (ATM) (Fig. 3A). Additionally, the environmental *P. aeruginosa* S4 12 strain showed increased resistance to amikacin (AK), while an increase in susceptibility to ciprofloxacin (CIP), levofloxacin (LEV) and ATM was observed (Fig. 3A). In comparison, according to the CLSI[32] guidelines, the clinical *P. aeruginosa* PAO1 strain displayed increased susceptibility to LEV and ceftazidime (CAZ) (Fig. 3A). Thus, ATM, CIP, LEV, and TZP (based on the millimetre increase of the inhibition zone of the bacteriophage treated *P. aeruginosa*; Table S3) were included in the PAS investigation as an increase in susceptibility was observed for individual *P. aeruginosa* strains.

Kirby-Bauer disc diffusion assays following exposure to KPW17

Classification of the measured zones of inhibition according to the EUCAST[31] guidelines, indicated that before exposure to KPW17, the *K. pneumoniae* ATCC 13383 and S1 43 strains were susceptible, while the *K. pneumoniae* P3 clinical strain was classified as XDR (Fig. 3B). Correspondingly, according to the CLSI[32] guidelines subsequently confirmed that the *K. pneumoniae* ATCC 13383 reference strain and the *K. pneumoniae* S1 43 environmental strain were susceptible (Fig. 3B), while the *K. pneumoniae* P3 clinical strain was confirmed as XDR (Fig. 3B).

While exposure to KPW17 for 24 h during the bacterial challenge test did not change the overall classification of the *K. pneumoniae* test strains based on the EUCAST[31] and CLSI[32] guidelines, differences with regards to the effectiveness of specific antibiotics were noted for all test strains, except for the antibiogram of the reference *K. pneumoniae* ATCC 13383 strain, which remained unchanged. According to both the EUCAST[31] and CLSI[32] guidelines, the clinical *K. pneumoniae* P3 displayed an increase in susceptibility to LEV, doripenem (DOR) and IPM (Fig. 3B). Additionally, the environmental *K. pneumoniae* S1 43 became more susceptible to PRL and TZP, according to the EUCAST[31] and CLSI[32] guidelines, respectively (Fig. 3B). Thus, DOR, LEV, PRL and TZP were included in the PAS investigation as an increase in susceptibility was observed for the *K. pneumoniae* environmental and clinical test strains.

Kirby-Bauer disc diffusion assays following exposure to ECSR5

Classification of the measured zones of inhibition according to the EUCAST[31] and CLSI[32] guidelines, indicated that prior to bacteriophage exposure, the *E. cloacae* NCTC 13406 reference strain was classified as MDR, while the *E. cloacae* 4L environmental strain did not display resistance to any of the tested antibiotics (Fig. 4A).

Exposure to bacteriophage ECSR5 did not change the overall classification or the antibiogram of the *E. cloacae* test strains based on the EUCAST[31] and CLSI[32] guidelines. However, the environmental *E. cloacae* 4L strain became more resistant to tobramycin (TOB) according to the EUCAST[31] guidelines (Fig. 4A). Furthermore, the greatest increase in the measured zone of inhibition (Table S5) was recorded for DOR for the reference *E. cloacae* NCTC 13406 strain, while CN displayed the greatest increase for the environmental *E. cloacae* 4L, however this did not result in a change in classification according to the EUCAST[31] or CLSI[32] guidelines. Thus, based on the millimetre increase in zone diameter of the bacteriophage treated *E. cloacae* (Table S5), DOR and CN were included in the PAS investigation.

Kirby-Bauer disc diffusion assays following exposure to ABTW1

Classification of the measured zones of inhibition according to the EUCAST[31] and CLSI[32] guidelines, indicated that the *A. baumannii* AB3 clinical strain was classified as XDR (Fig. 4B).

Based on the EUCAST[31] and CLSI[32] guidelines, exposure to ABTW1 for 24 h during the bacterial challenge test did not change the overall classification of the *A. baumannii* strain tested (Fig. 4B). However, an increase in the measured zone of inhibition (Table S6) was recorded for IPM and TZP against the clinical *A. baumannii* AB3 strain. Thus, based on the millimetre increase in zone diameter of the bacteriophage treated *A. baumannii* (Table S6), IPM and TZP were included in the PAS investigation.

Bacteriophage-antibiotic combination treatment analysis

Broth microdilution checkerboard assays to monitor for PAW33-antibiotic-synergy

Results indicated that PAW33 had an MIC [multiplicity of infection (MOI) value] of 0.01, 1, and 0.1, against the reference *P. aeruginosa* ATCC 27853, environmental *P. aeruginosa* S4 12, and clinical *P. aeruginosa* PAO1 strains, respectively (Table 1). An MIC of 8 µg/mL was then recorded for ATM, against the reference *P. aeruginosa* ATCC 27853 and clinical *P. aeruginosa* PAO1 strains, while an MIC of 4 µg/mL was recorded for the environmental *P. aeruginosa* S4 12 (Table 1). Combination of ATM with PAW33 then resulted in a synergistic effect (FIC = 0.25) for the eradication of the environmental *P. aeruginosa* S4 12 strain, as the required ATM and PAW33 concentrations decreased to 1 µg/mL and an MOI of 0.001, respectively (Table 1). A partial synergistic effect was then observed for the combination treatment against the reference *P. aeruginosa* ATCC 27853 (FIC = 0.60) and clinical *P. aeruginosa* PAO1 (FIC = 0.60) strains, with the required ATM and PAW33 concentrations decreasing to 4 µg/mL and MOI's of 0.001 and 0.01, respectively (Table 1).

For CIP, the reference *P. aeruginosa* ATCC 27853, environmental *P. aeruginosa* S4 12, and clinical *P. aeruginosa* PAO1 strains had MICs of 0.5 µg/mL, 2 µg/mL, and 0.25 µg/mL, respectively (Table 1). Combination of CIP with PAW33 then resulted in a synergistic effect for the eradication of all the *P. aeruginosa* test strains, with FICs of 0.14 to 0.35 recorded (Table 1). Overall, when used in combination with PAW33, the required CIP concentration

A

ECSR5								
EUCAST					CLSI			
NCTC 13406		4L		Antibiotics	NCTC 13406		4L	
0 h	24 h	0 h	24 h		0 h	24 h	0 h	24 h
Aminoglycosides								
S	S	S	S	AK	S	S	S	S
S	S	S	S	CN	S	S	S	S
S	S	S	R	TOB	S	S	S	S
Carbapenems								
S	S	S	S	DOR	S	S	S	S
S	S	S	S	IPM	S	S	S	S
S	S	S	S	MEM	S	S	S	S
Cephalosporins								
R	R	S	S	FEP	SDD	SDD	S	S
R	R	S	S	CAZ	R	R	S	S
Fluoroquinolones								
S	S	S	S	CIP	S	S	S	S
S	S	S	S	LEV	S	S	S	S
Monobactams								
R	R	S	S	ATM	R	R	S	S
Penicillins								
R	R	S	S	TZP	R	R	S	S

B

ABTW1				
EUCAST			CLSI	
AB3		Antibiotics	AB3	
0 h	24 h		0 h	24 h
Aminoglycosides				
R	R	AK	R	R
R	R	CN	R	R
R	R	TOB	R	R
Carbapenems				
R	R	DOR	R	R
R	R	IPM	R	R
R	R	MEM	R	R
Cephalosporins				
		FEP	R	R
		CAZ	R	R
Fluoroquinolones				
R	R	CIP	R	R
R	R	LEV	I	I
Penicillins				
		TZP	R	R

Fig. 4. Antibiotic susceptibility profiles of the (**A**) *E. cloacae* reference (NCTC 13406) and environmental (4L) strains as determined by disc diffusion assays before (0 h) and after (24 h) exposure to ECSR5 during the bacterial challenge tests; and (**B**) *A. baumannii* clinical (AB3) strain as determined by disc diffusion assays before (0 h) and after (24 h) exposure to ABTW1 during the bacterial challenge tests. Results were classified according to the EUCAST (2022) and CLSI (2022) guidelines as Resistant (R; Red); Intermediate (I; Orange); Susceptible, dose dependent (SSD, Yellow); Susceptible (S; Green). Grey Blocks = Classification data not available. Amikacin (AK), Aztreonam (ATM), Cefepime (FEP), Ceftazidime (CAZ), Ciprofloxacin (CIP), Doripenem (DOR), Gentamicin (CN), Imipenem (IPM), Levofloxacin (LEV), Meropenem (MEM), Piperacillin-tazobactam (TZP), Tobramycin (TOB).

decreased to 0.125 µg/mL for the reference *P. aeruginosa* ATCC 27853; for the environmental *P. aeruginosa* S4 12, the concentration decreased to 0.25 µg/mL, while the concentration decreased to 0.063 µg/mL for the clinical *P. aeruginosa* PAO1 strain (Table 1). Correspondingly, the required PAW33 concentration decreased ten-fold for the reference *P. aeruginosa* ATCC 27853 (MOI = 0.001) and clinical *P. aeruginosa* PAO1 (MOI = 0.01) strains, and 100-fold for the environmental *P. aeruginosa* S4 12 (MOI = 0.01) strain when used in combination with CIP (Table 1).

For LEV, the environmental *P. aeruginosa* S4 12 strain had an MIC of 4 µg/mL, while an MIC of 2 µg/mL was recorded for the reference *P. aeruginosa* ATCC 27853 and clinical *P. aeruginosa* PAO1 strains, respectively (Table 1). Combination of LEV with PAW33 then resulted in a synergistic effect for the eradication of the reference *P. aeruginosa* ATCC 27853 (FIC = 0.35), environmental *P. aeruginosa* S4 12 (FIC = 0.23), and clinical *P. aeruginosa* PAO1 (FIC = 0.14) strains (Table 1). Overall, when used in combination with PAW33, the LEV concentrations decreased to 0.5 µg/mL for the reference *P. aeruginosa* ATCC 27853 and environmental *P. aeruginosa* S4 12 strains, and to 0.25 µg/mL for the clinical *P. aeruginosa* PAO1. Correspondingly, the required PAW33 concentrations decreased to MOI of 0.001 for the reference *P. aeruginosa* ATCC 27853 and clinical *P. aeruginosa* PAO1, and to 0.1 for the environmental *P. aeruginosa* S4 12 (Table 1).

The broth microdilution assays indicated that TZP had a MIC of 32 µg/mL for the environmental *P. aeruginosa* S4 12 and clinical *P. aeruginosa* PAO1 strains, while an MIC of 64 µg/mL was recorded for the reference *P. aeruginosa* ATCC 27853 strain (Table 1). Combination of TZP with PAW33 then resulted in a synergistic effect for the eradication of the environmental *P. aeruginosa* S4 12 (FIC = 0.13) strain, with partial synergism observed against the reference *P. aeruginosa* ATCC 27853 (FIC = 0.60) and clinical *P. aeruginosa* PAO1 (FIC = 0.60) strains (Table 1). Overall, when used in combination with PAW33, the TZP concentrations for the reference *P. aeruginosa* ATCC 27853, environmental *P. aeruginosa* S4 12 and clinical *P. aeruginosa* PAO1 decreased to 32 µg/mL, 4 µg/mL, and 16 µg/mL, respectively, while the PAW33 concentration (MOI value) decreased to 0.001 for the reference and environmental *P. aeruginosa* strains ATCC 27853 and S4 12, and to 0.01 for the clinical *P. aeruginosa* PAO1 (Table 1).

Broth microdilution checkerboard assays to monitor for KPW17-antibiotic-synergy

Based on the results obtained for the preliminary antibiotic screening using the Kirby-Bauer disc diffusion assays, the combination of KPW17 with DOR, LEV, PRL, and TZP to target the reference *K. pneumoniae* ATCC 13383, environmental *K. pneumoniae* S1 43, and clinical *K. pneumoniae* P3 strains, were subsequently investigated for potential PAS.

Results from the KPW17 MIC determination, subsequently indicated that KPW17 had an MIC (MOI value) of 1, 0.01, and 10, against the reference *K. pneumoniae* ATCC 13383, environmental *K. pneumoniae* S1 43, and

Phage	Antibiotic	Isolate	$MIC_{Antibiotic}$ (μg/mL) alone	MIC_{Phage} (MOI) alone	$MIC_{Antibiotic}$ (μg/mL) combined	MIC_{Phage} (MOI) combined	FIC	Classification
PAW33	Aztreonam (ATM)	ATCC	8	0.01	4	0.001	0.60	Partial Synergy
		S4 12	4	1	1	0.001	0.25	Synergy
		PAO1	8	0.1	4	0.01	0.60	Partial Synergy
	Ciprofloxacin (CIP)	ATCC	0.5	0.01	0.125	0.001	0.35	Synergy
		S4 12	2	1	0.25	0.01	0.14	Synergy
		PAO1	0.25	0.1	0.063	0.01	0.35	Synergy
	Levofloxacin (LEV)	ATCC	2	0.01	0.5	0.001	0.35	Synergy
		S4 12	4	1	0.5	0.1	0.23	Synergy
		PAO1	2	0.1	0.25	0.001	0.14	Synergy
	Piperacillin/ tazobactam (TZP)	ATCC	64	0.01	32	0.001	0.60	Partial Synergy
		S4 12	32	1	4	0.001	0.13	Synergy
		PAO1	32	0.1	16	0.01	0.60	Partial Synergy
KPW17	Doripenem (DOR)	ATCC	< 0.0156	1	ND[a]	ND[a]	ND[a]	ND[a]
		S1 43	2	0.01	0.5	0.001	0.35	Synergy
		P3	2	10	0.5	0.0001	0.25	Synergy
	Levofloxacin (LEV)	ATCC	1	1	0.125	0.001	0.13	Synergy
		S1 43	4	0.01	1	0.001	0.35	Synergy
		P3	1	10	0.5	0.001	0.50	Synergy
	Piperacillin/ tazobactam (TZP)	ATCC	32	1	4	0.01	0.14	Synergy
		S1 43	512	0.01	64	0.001	0.23	Synergy
		P3	128	10	256	10	3.00	Antagonism
	Piperacillin (PRL)	ATCC	2	1	0.125	0.1	0.16	Synergy
		S1 43	64	0.01	> 128	> 1	> 3.00	Antagonism
		P3	> 512	10	ND[a]	ND[a]	ND[a]	ND[a]
ECSR5	Doripenem (DOR)	NCTC	0.25	10^4	0.063	0.001	0.24	Synergy
		4L	0.25	10^4	> 0.5	$> 10^4$	> 3.00	Antagonism
	Gentamicin (CN)	NCTC	0.5	10^4	0.25	0.0001	0.5	Synergy
		4L	2	10^4	2	0.0001	1.00	Additive
ABTW1	Imipenem (IPM)	AB3	64	10^4	128	0.0001	2.00	Indifference
	Piperacillin/ tazobactam (TZP)	AB3	512	10^4	512	10^4	1.10	Indifference

Table 1. Minimum inhibitory concentration and combined inhibitory effects of the tested antibiotics and bacteriophages against the respective reference, environmental and clinical *P. aeruginosa, K. pneumoniae, E. cloacae* and *A. baumannii* strains. [a]ND – not determined due to out-of-range MIC.

clinical *K. pneumoniae* P3 strains, respectively (Table 1). For DOR, the environmental *K. pneumoniae* S1 43, and clinical *K. pneumoniae* P3 strains had an MIC of 2 μg/mL, while the reference *K. pneumoniae* ATCC 13383 strain had an MIC of < 0.0156 μg/mL (Table 1). A checkerboard assay was thus not conducted for *K. pneumoniae* ATCC 13383 as the MIC was below typical MIC test ranges[33]. Combination of DOR with KPW17 then resulted in a synergistic effect for the eradication of the environmental and clinical *K. pneumoniae* strains, with FICs of 0.35 and 0.25 recorded, respectively (Table 1). Correspondingly, the required KPW17 concentration decreased to an MOI of 0.001 for the environmental *K. pneumoniae* S1 43, and to 0.0001 for the clinical *K. pneumoniae* P3 strain, while the DOR concentration decreased to 0.5 μg/mL for both the environmental *K. pneumoniae* S1 43 and clinical *K. pneumoniae* P3 strains (Table 1).

For LEV, the reference *K. pneumoniae* ATCC 13383 and clinical *K. pneumoniae* P3 strains had an MIC of 1 μg/mL, while an MIC of 4 μg/mL was recorded for the environmental *K. pneumoniae* S1 43 strain (Table 1). Combination of LEV with KPW17 then resulted in a synergistic effect for the eradication of the reference *K. pneumoniae* ATCC 13383 (FIC = 0.13), environmental *K. pneumoniae* S1 43 (FIC = 0.35), and clinical *K. pneumoniae* P3 (FIC = 0.50) strains (Table 1). Overall, when used in combination with KPW17, the LEV concentration decreased to 0.125 μg/mL for the reference *K. pneumoniae* ATCC 13383, 1 μg/mL for the environmental *K. pneumoniae* S1 43 strain and to 0.5 μg/mL for the clinical *K. pneumoniae* P3 strain (Table 1). Correspondingly, the required KPW17 concentration decreased to an MOI of 0.001 for all three *K. pneumoniae* strains (Table 1).

An MIC of 512 μg/mL was recorded for TZP, against the environmental *K. pneumoniae* S1 43 strain, while an MIC of 128 μg/mL and 32 μg/mL was recorded for the clinical *K. pneumoniae* P3 and reference *K. pneumoniae* ATCC 13383 strains, respectively (Table 1). Combination of TZP with KPW17 then resulted in a synergistic effect for the eradication of the reference *K. pneumoniae* ATCC 13383 (FIC = 0.14) and environmental *K. pneumoniae* S1 43 (FIC = 0.23) strains (Table 1). An antagonistic effect (FIC = 3.00), where the combination

treatment results in a lower activity than either agent alone, was however, observed for the combination treatment when targeting the clinical *K. pneumoniae* P3 (Table 1). Overall, when used in combination with KPW17, the TZP concentration decreased to 4 µg/mL and 64 µg/mL for the reference *K. pneumoniae* ATCC 13383 and environmental *K. pneumoniae* S1 43 strains, respectively, and increased to 256 µg/mL for the clinical *K. pneumoniae* P3 strain (Table 1). Correspondingly, the KPW17 concentration (MOI value) decreased to 0.01 and 0.001 for the reference *K. pneumoniae* ATCC 13383 and environmental *K. pneumoniae* S1 43 strains, respectively, while the MOI remained at 10 for the clinical *K. pneumoniae* P3 strain (Table 1).

Finally, for PRL, the clinical *K. pneumoniae* P3 strain had an MIC of > 512 µg/mL, while an MIC of 2 µg/mL and 64 µg/mL was recorded for the reference *K. pneumoniae* ATCC 13383 and the environmental *K. pneumoniae* S1 43 strains, respectively (Table 1). As the MIC of the clinical *K. pneumoniae* P3 strain was above the typical MIC test range[33], a checkerboard assay was not conducted (Table 1). Combination of PRL with KPW17 then resulted in a synergistic effect for the eradication of the reference *K. pneumoniae* ATCC 13383 (FIC = 0.16), with the PRL MIC decreasing to 0.125 µg/mL (Table 1). However, an antagonistic effect was observed for the environmental *K. pneumoniae* S1 43 (FIC > 3.00) strain, as the required PRL concentration increased to greater than 2 × MIC (128 µg/mL) when used in combination with KPW17 (Table 1). Correspondingly, the required KPW17 concentration decreased for the reference *K. pneumoniae* ATCC 13383 strain (MOI = 0.1) and increased for the environmental *K. pneumoniae* S1 43 strain (MOI > 1), respectively, when used in combination with PRL (Table 1).

Broth microdilution checkerboard assays to monitor for ECSR5-antibiotic-synergy

Based on the results obtained for the preliminary antibiotic screening using the Kirby-Bauer disc diffusion assays, the combination of ECSR5 with DOR and CN to target the reference *E. cloacae* NCTC 13406 and environmental *E. cloacae* 4L strains, were subsequently investigated for potential PAS.

Results from the ECSR5 MIC determination, subsequently indicated that ECSR5 had an MIC (MOI value) of 10^4 against the reference *E. cloacae* NCTC 13406 and environmental *E. cloacae* 4L (Table 1). For DOR, the reference *E. cloacae* NCTC 13406 and environmental *E. cloacae* 4L strains had an MIC of 0.25 µg/mL (Table 1). Subsequently, the combination of ECSR5 and DOR resulted in synergistic effect for the reference *E. cloacae* NCTC 13406 with an FIC of 0.24 recorded (Table 1). The MIC of DOR then decreased to 0.063 µg/mL, while the MOI of ECSR5 decreased to 0.001 (Table 1). For the environmental *E. cloacae* 4L strain, the MIC increased to above 0.5 µg/mL (2 × MIC), and the MOI also increased to above an MOI = 10^4. The FIC was thus reported as > 3.00 indicating that the combination of DOR and ECSR5 had an antagonistic effect on the environmental *E. cloacae* 4L (Table 1).

For CN, the reference *E. cloacae* NCTC 13406 strain had an MIC of 0.5 µg/mL, while an MIC of 2 µg/mL was recorded for the environmental *E. cloacae* 4L strain. Combination of CN with ECSR5 then resulted in a synergistic effect for the reference *E. cloacae* NCTC 13406 strain (FIC = 0.5), with the CN MIC decreasing to 0.25 µg/mL and the required bacteriophage concentration (MOI value) decreasing to 0.0001 (Table 1). For the environmental *E. cloacae* 4L strain, an additive effect was recorded (FIC = 1.00), with the MIC for CN remaining at 2 µg/mL, while the required bacteriophage concentration (MOI value) decreased to 0.0001 (Table 1).

Broth microdilution checkerboard assays to monitor for ABTW1-antibiotic-synergy

Based on the results obtained from the Kirby-Bauer disc diffusion assays, ABTW1 was investigated in combination with IPM and TZP against the clinical *A. baumannii* AB3 for PAS. Subsequently, the MOI of ABTW1 was recorded as 10^4 for the clinical *A. baumannii* AB3. For IPM, the MIC was recorded as 64 µg/mL, while for TZP an MIC of 512 mg/L was recorded (Table 1). The combination of ABTW1 with IPM or TZP then resulted in an indifferent effect with FIC indices of 2.00 and 1.10 recorded, respectively, for the clinical *A. baumannii* AB3 strain (Table 1). The MIC of IPM increased to 128 µg/mL, while the MOI of ABTW1 decreased to 0.0001 (Table 1). The combination of ABTW1 did not however, result in any change to the MIC of TZP or the MIC (MOI value) of ABTW1 (Table 1).

Discussion

The World Health Organisation (WHO) classified the Gram-negative ESKAPE pathogens *P. aeruginosa, A. baumannii, K. pneumoniae* and *Enterobacter* spp. as "Priority 1: Critical" and "Priority 2: High" pathogens for the research and development of novel antimicrobials, due to (1) their significant contribution to the burden of disease in both developed and developing countries, and (2) their increased resistance to multiple classes of first line and last-resort antibiotics[34]. Alarmingly, the detection of antibiotic resistant environmental and clinical *P. aeruginosa, A. baumannii, K. pneumoniae* and *Enterobacter* spp. strains in environmental or extra-hospital reservoirs has increased, with a corresponding increase in the occurrence of community-acquired outbreaks linked to these opportunistic pathogens[8]. Accordingly, the risk posed by antibiotic resistant organisms is no longer limited to clinical environments, and it is essential that treatment interventions be investigated that effectively target strains originating from clinical and natural ecosystem environments. While the use of lytic bacteriophages has subsequently been recommended as an alternative treatment strategy[17,23,35] it is important to firstly characterise the individual bacteriophages to ensure maximum potential in controlling the target organism[22].

As indicated, the PAW33 bacteriophage was previously isolated and partially characterised by Reyneke et al.[14] and displayed activity against 92% ($n = 11/12$) of the *P. aeruginosa* strains tested using spot test assays (Supplementary Information, Sect. 2.1). In the current study, WGS identified PAW33 as a member of the genus *Bruynoghevirus* which includes other *Pseudomonas* spp. bacteriophages[36]. Additionally, ABTW1 was isolated and partially characterised by Morris et al.[28]. This bacteriophage displayed no activity against the 23 non-target species analysed and displayed lytic activity against 29% ($n = 6/21$) of the *A. baumannii* strains tested, indicating

a broad host range (Supplementary Information, Sect. 2.2). Furthermore, ABTW1's closest related genus was identified as *Vieuvirus* which consists of siphovirus bacteriophages with activity against *A. baumannii*[28]. In the current study, ABTW1 only displayed 41.029% intergenomic similarity with other bacteriophages, suggesting that this may be a novel genus and species. However, genes related to integration and excision were identified in ABTW1, which may indicate a lysogenic life cycle.

There was a high isolation incidence of bacteriophages targeting *K. pneumoniae* with seventeen bacteriophages isolated from 6 samples. The KPW17 bacteriophage was isolated in the current study from wastewater and characterised as a myovirus bacteriophage with high genetic similarity to the family Drexlerviridae and genus *Webervirus* which contains bacteriophages effective against *Klebsiella* spp. and other Enterobacteriaceae. KPW17 was most closely related to a *Stenotrophomonas* bacteriophage, and this bacterial genus and the *Klebsiella* genus are in the class Gammaproteobacteria. Various bacteriophages have been isolated using *K. pneumoniae* as isolation host, with these bacteriophages classified as having a narrow (i.e., activity limited to a specific strain) or broad (i.e., activity limited to a specific spp. or genus) host range, or being polyvalent bacteriophages (i.e., activity against various genera). For example, Horváth et al.[37] reported on the isolation of a vB_KpnS_Kp13 bacteriophage that only displayed activity against K24 capsular type *K. pneumoniae* isolates, while Askoura et al.[38] isolated polyvalent bacteriophages using *K. pneumoniae* that displayed activity against *K. pneumoniae* and *E. coli* strains. Bacteriophage KPW17 was subsequently classified as having a broad host range against *Klebsiella* spp. as lytic activity was observed against various strains of *K. pneumoniae* and *K. oxytoca*. The ability of KPW17 to infect multiple *Klebsiella* spp. may thus be due to the host receptor. For example, the presence of outer membrane proteins or LPS may act as bacteriophage binding sites allowing for differential infection.

A lower isolation incidence was observed for *E. cloacae*, with only two bacteriophages isolated from 5 samples. The ECSR5 bacteriophage was also isolated in the current study from surface runoff collected from Sir Lowry's Pass Village urban informal settlement (Cape Town, South Africa) and was morphologically identified as a siphovirus in the family Drexlerviridae and genus *Eclunavirus.* This genus consists of other bacteriophages effective against *Enterobacter* spp.[36]. Additionally, ECSR5 only displayed 83.850% intergenomic similarity with other bacteriophages, suggesting that this may be a novel species. ECSR5 also exhibited a broad host range, as all strains susceptible to ECSR5 are included in the *Enterobacter cloacae* complex (i.e., *E. cloacae* and *Enterobacter hormaechei*). This infection of multiple species may be due to shared virulence factors such as biofilm formation, which produces LPS that may act as a bacteriophage receptor[39]. Notably, WGS and annotation indicated that the four bacteriophages PAW33, KPW17, ECSR5 and ABTW1, did not contain any coding regions identified as virulence factors or antibiotic resistance genes, indicating that these bacteriophages may be suitable for application as biological control agents.

We then explored a method for the preliminary screening of antibiotics for PAS by performing Kirby-Bauer assays before and after bacteriophage treatment. While changes in the antibiogram of bacteriophage resistant isolates have been explored using the Kirby-Bauer disc diffusion assay[40], this has not been explored as a preliminary assay to detect PAS. Generally, PAS studies have focused on plaque assays, planktonic cell time kill assays and biofilm assays for the in vitro detection of PAS, selecting commonly used antibiotics[26]. Subsequently, the Kirby-Bauer assays performed in the current study, were able to screen multiple antibiotic classes for multiple target strains and narrow down the antibiotics used in checkerboard assay to further explore PAS. Thus, the preliminary Kirby-Bauer disc diffusion assay indicated that PAW33 may potentially work in a synergistic manner with penicillins (TZP), monobactams (ATM), and fluoroquinolones (CIP and LEV), while KPW17 displayed promise for use with penicillins (PRL and TZP), carbapenems (DOR), and fluoroquinolones (LEV), ECSR5 may work synergistically with carbapenems (DOR) and aminoglycosides (CN), while PAS of ABTW1 with penicillins (TZP) and carbapenems (IPM) was investigated. Overall, an increase in zone diameter (inhibition zone) observed in the bacteriophage treated isolates in comparison to the untreated isolates corresponded to a synergistic to indifferent interaction during the checkerboard assays. Furthermore, these interactions were strain specific, suggesting that the Kirby-Bauer disc diffusion assay in combination with bacterial challenge tests could be an easy method to screen for PAS and identify antibiotics or antibiotic classes for more comprehensive PAS testing. Moreover, this could be applied in medical testing, to identify effective bacteriophage-antibiotic combinations for the specific infective strain.

While multiple studies have investigated the potential of PAS to combat antibiotic resistant bacteria, several mechanisms or a combination of mechanisms may potentially explain synergistic interactions. These include the (1) re-sensitisation of antibiotic resistant bacteria, (2) targeting of different bacterial sites, (3) changes in bacterial cell morphology, (4) antibiotic-induced bacteriophage production, and (5) bacteriophage-mediated penetration of antibiotics into biofilms[41]. Aztreonam is a monocyclic β-lactam antibiotic that displays antibacterial activity against Gram-negative bacteria through the inhibition of bacterial cell wall synthesis by blocking peptidoglycan crosslinking, whereafter cell lysis may be mediated by bacterial cell wall autolytic enzymes[42]. The checkerboard assays then indicated that ATM in combination with PAW33 resulted in partial synergy and synergy for the *P. aeruginosa* strains. Similarly, while investigating the potential of ATM in combination with bacteriophage E79 to target *P. aeruginosa* PAO1, Davis et al.[43] reported an increase in plaque size and a decreased latency period, representing increased bacteriophage lytic activity. Additionally, the authors reported that sub-inhibitory ATM significantly increased the biofilm eradication ability of E79. Aztreonam is only approved for use in humans, and is thus, regarded as a critically important antibiotic[44]. However, resistance to ATM commonly includes mutations that result in the over-expression of the chromosomal bla_{AmpC} gene which encodes for a wide-spectrum class C β-lactamase, or the over-expression of multidrug efflux pumps (e.g., MexAB-OprM) and deficiencies in porins[42,43,45,46]. Davis et al.[43] then showed that *P. aeruginosa* displays a filamentous morphology and are unable to complete cell division in the presence of sub-inhibitory concentrations of ATM, and thus may be more sensitive to bacteriophage lysis due to an increase in protein-synthesising machinery in the elongated cells resulting in increased bacteriophage production. A similar interaction may thus have occurred between ATM and PAW33

in the current study, which can potentially reduce the effects of resistance mechanisms exhibited by clinical and environmental *P. aeruginosa* strains.

For all of the potential PAS strategies that have been investigated, the synergistic potential of bacteriophages in combination with CIP has been increasingly reported[26]. Ciprofloxacin is a fluoroquinolone that displays antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria[47]. The antibacterial action of CIP is mediated through the inhibition of bacterial DNA gyrase and topoisomerase IV, thus preventing DNA replication[47]. Notably, the checkerboard assays indicated that the combination of PAW33 with CIP exhibited synergy for all three target *P. aeruginosa* strains. Similarly, Oechslin et al.[48] reported that the use of a 12-bacteriophage cocktail in combination with CIP resulted in a synergistic effect in vitro while targeting the *P. aeruginosa* CHA strain, with the combination treatment also preventing the establishment of bacteriophage-resistant variants. Additionally, the authors demonstrated that the combination of the bacteriophage cocktail with CIP could treat an experimental endocarditis model in rats, with the combination treatment resulting in a 64% survival rate[48]. Ciprofloxacin is a commonly prescribed antibiotic for various infections, including gastrointestinal and lower respiratory tract infections[49]. Furthermore, *P. aeruginosa* infections are commonly treated with CIP in cystic fibrosis patients, where increased resistance to CIP may be detrimental[49]. This resistance may be acquired through mutations in genes encoding the target proteins and regulators of efflux pumps[47]. The interaction between CIP and bacteriophages has subsequently been attributed to various mechanisms, including changes in cell morphology, bacteriophage-mediated penetration into biofilms and evolutionary trade-offs[41,49]. For example, a case study by Chan et al.[50] outlined how a patient who underwent aortic arch replacement surgery, was infected with *P. aeruginosa* on the aortic graft. The patient was treated with CIP and CAZ antibiotics. After several reoccurrences of the *P. aeruginosa* infection over a period of four years (2012 to 2015), the FDA approved the use of bacteriophages as an emergency investigational new drug therapy[50]. The *P. aeruginosa* bacteriophage (OMKO1) was then applied directly to the infection site in combination with CAZ and CIP. The *P. aeruginosa* infection subsequently resolved with no signs of reinfection for 4 weeks post treatment, suggesting that PAS combinations with CIP may be effective in clinical settings[50].

Levofloxacin is a fluoroquinolone antibiotic which also exhibits activity against Gram-positive and Gram-negative bacteria[51]. The checkerboard assays then indicated that LEV in combination with PAW33 and KPW17 resulted in synergy for the *P. aeruginosa* and *K. pneumoniae* strains tested, respectively. While investigating the potential of PAS to target the *Burkholderia cepacia* complex using nine antibiotics and two bacteriophages, Kamal and Dennis[52] reported that the assessed fluoroquinolone antibiotics (LEV and CIP) displayed PAS with bacteriophages KS12 and KS14. Using a plaque assay, it was shown that the plaque size of both bacteriophage KS12 and KS14 on *Burkholderia cenocepacia* K56-2 and *Burkholderia cenocepacia* C6433, respectively, increased in the presence of LEV (½ × MIC)[52]. Resistance to LEV may, however, be acquired through mutations in the genes encoding for target proteins or efflux pump as well as in DNA gyrase or topoisomerase IV[47,53]. Severe adverse effects have also been noted, including photosensitivity, diarrhoea, seizures and peripheral neuropathy, while prolonged exposure to LEV is linked to fungal and bacterial superinfection[54]. Thus, the potential for a combined LEV-bacteriophage treatment could reduce LEV dosages and exposure times, thereby minimizing adverse effects and superinfections.

Piperacillin, TZP, DOR and IPM are β-lactam antibiotics that act on the cell wall by binding to penicillin-binding proteins, whereafter cell lysis is mediated by bacterial cell wall autolytic enzymes. Overall, it has been noted that PAS is predominantly recorded for cell wall synthesis inhibiting antibiotics (e.g., CAZ and PRL) as the bacteriophage and antibiotic target the bacterial cell wall with different modes of action[55]. Results from the checkerboard assay in the current study then indicated that synergy (ATCC 13383 and S1 43) and antagonism (P3) were obtained when combining PRL and KPW17 for the removal of the *K. pneumoniae* strains. Similarly, Stachurska et al.[56] observed potential PAS when combining PRL with bacteriophage $T4_5$ against *E. coli* K-12 C600, using a plaque formation assay, where an increase in plaque size was observed. Uchiyama et al.[55] then reported on synergy between bacteriophage KPP22 and PRL when targeting *P. aeruginosa* strains (PAO1, PA4, PA23 and PA29). At present the combination of TZP has not been extensively assessed for PAS, however, as stated cell wall acting antibiotics often interact synergistically with bacteriophages. Accordingly, in the current study, synergy (S4 12) and partial synergy (ATCC 27853 and PAO1) was obtained when combining TZP and PAW33 for the eradication of the *P. aeruginosa* strains, while the combination of TZP and KPW17 for the eradication of the *K. pneumoniae* strains resulted in a synergistic (ATCC 13383 and S1 43) and antagonistic (P3) effect. Piperacillin and TZP are only approved for use in humans, and are thus, regarded as critically important antibiotics with a high AMR risk[44]. These antibiotics are often regarded as last-resort treatments for severe bacterial infections, thus, a synergistic interaction with bacteriophages may slow the development of resistance to these antibiotics. Beta-lactam antibiotics can cause filamentation resulting in disruptions to the peptidoglycan layer, increasing sensitivity to bacteriophages due to bacteriophage enzymes such as holins and endolysins[57]. Furthermore, PAS may be due to bacteriophage depolymerases, that degrade bacterial polysaccharides and capsular polysaccharides. This can allow the antibiotic to penetrate biofilms or the cells themselves[57]. The isolated bacteriophages (PAW33 and KPW17) are effective against their host biofilms (Fig. S7, Supplementary Information), suggesting that this could contribute to the PAS observed in the current study. Additionally, the combination of TZP and ABTW1 for the removal of the clinical *A. baumannii* AB3 strain resulted in indifference. Similarly, Vashisth et al.[58], reported on synergy between bacteriophage φAB182 and several antibiotics (CAZ, cefotaxime, colistin, polymixin B) to target *A. baumannii*, while no synergy was identified for the tested penicillin AMP. This suggests that other antibiotics such as colistin should be tested for PAS against *A. baumannii*[58].

Doripenem falls into the carbapenem class of antibiotics. This class binds to penicillin-binding proteins thereby inhibiting cell wall synthesis[59]. Results from the checkerboard assay indicated that synergy (S1 43 and P3) was then obtained when combining DOR and KPW17 for the removal of the *K. pneumoniae* strains, while synergy (NCTC 13406) and antagonism (4L) were obtained when combining DOR and ECSR5 for the removal

of *E. cloacae* strains. While studies investigating PAS with DOR are limited, some bacteriophages produce peptidoglycan degrading enzymes such as endolysins and murein synthesis inhibitors[60]. These enzymes can then work synergistically with cell wall synthesis inhibiting antibiotics, thus increasing the bacteria's susceptibility to the antibiotics. Whole genome analysis of KPW17 and ECSR5 indicated the presence of peptidoglycan degrading enzymes (i.e., endolysins, holins), thus this may contribute to the synergy observed. The antagonistic effect seen against specifically the environmental *E. cloacae* 4L was however, not unexpected as a decrease in inhibition zone (diameter) was observed post-ECSR5 treatment (Table S5). While research into antagonistic bacteriophage antibiotics interactions is limited, Ma et al.[61] found that a polyvalent bacteriophage SaP7 in combination with amoxicillin or FEP displayed an antagonistic effect against *Salmonella* sp. The authors then hypothesised that antagonism may thus occur if antibiotics interfere with bacteriophage production. For example, the antibiotic may induce stress in the target bacteria resulting in cell death before signification bacteriophage production occurs[61]. It is thus hypothesised that a similar scenario may have occurred when targeting the environmental *E. cloacae* 4L. This is supported by the results in the bacterial challenge tests (Supplementary Information, Sect. 2.3) where ECSR5 was unable to effectively inhibit the bacterial growth.

Imipenem is also a β-lactam antibiotic, in the subgroup carbapenem and is effective against both Gram-negative and Gram-positive bacteria[62]. Imipenem inhibits cell wall synthesis by binding to penicillin-binding proteins, preventing the addition of peptidoglycan to the cell wall[62]. Results from the checkerboard assay indicated that the combination of ABTW1 and IPM resulted in an indifferent effect against the clinical *A. baumannii* AB3 strain. Phage-antibiotic synergy with IPM against *A. baumannii* has however, been reported by Luo et al.[63]. Briefly, the bacteriophage dosage (i.e., MOI of 100, 1 and 0.01) of bacteriophage YC#06 in combination with IPM was investigated against a clinical *A. baumannii* strain. Results indicated that there was a synergistic interaction between IPM and YC#06, with the greatest synergistic effect observed at a bacteriophage MOI of 1 and 0.01[63]. Similarly, Hu et al.[64] suggested that an efflux pump may be important in IPM resistance, and it is thus hypothesised that the bacteriophage ABTW1 may recognise an efflux pump for adsorption as the bacteriophage resistant *A. baumannii* strains exhibited increased susceptibility to IPM. Imipenem is generally used in combination with other antibiotics such as cilastatin and relebactam, to treat hospital-acquired infections[62]. Synergy of IPM with bacteriophages thus provides an alternative treatment combination that may have diminished side effects (i.e., diarrhoea, increased alanine, increased aspartate transferase) in comparison to the three antibiotic combination[62,65]. Resistance to IPM in *A. baumannii* is commonly due to hydrolysing oxacillinase-encoding genes (e.g., *oxa23, oxa24, oxa58*) that hydrolyse carbapenems weakly and can be poorly expressed[66]. Carbapenem resistance can also be caused by efflux pumps and outer membrane proteins with multiple genes and mechanisms contributing to carbapenem resistance in *A. baumannii*, suggesting that a bacteriophage-antibiotic combination treatment may be more effective.

Gentamicin is an aminoglycoside antibiotic commonly used for the treatment of Gram-negative bacteria[67]. It confers its bactericidal activity by binding to the 16S rRNA of the ribosome thereby inhibiting translation[67]. Results from the checkerboard assay indicated that a synergistic (NCTC 13406) and additive (4L) effect was obtained when combining CN and ECSR5 for the removal of *E. cloacae* strains. While PAS with CN has not been extensively investigated for *E. cloacae*, several studies have investigated CN PAS for *P. aeruginosa* with mixed results. For example, Engeman et al.[68] investigated a cocktail (PAM2H) of five bacteriophages, namely, EPa5, EPa11, EPa15, EPa43 and EPa43, in combination with CN against *P. aeruginosa*, with a synergistic effect observed. In contrast, Nikolic et al.[69], investigated the interaction between bacteriophages JG024 and SES43300 and CN against *P. aeruginosa* and *S. aureus*. Results indicated that the combination of JG024 and CN was not effective against *P. aeruginosa* with an FIC of 1.00 to 1.83 recorded, while SES43300 and CN resulted in an additive effect[69]. Gentamicin is approved for both human and animal use and is classified as a critically important antimicrobial as (1) this antibiotic class is one of limited available therapies to treat bacterial infection in humans and (2) the antimicrobial class is used to treat bacterial infections possibly transmitted from non-human sources or with resistance genes from non-human sources[44]. Thus, the PAS demonstrated in the current study provides a combination treatment that could reduce the transmission of antibiotic resistance.

In conclusion, the use of bacteriophages in combination with antibiotics, may offer an opportunity to increase and/or prolong the use of antibiotics in the context of a rise in global antimicrobial resistance; specifically, by re-sensitising bacteria to antibiotics that have become ineffective or increasing the therapeutic potential of antibiotics that are not recommended for use in monotherapy. Additionally, this study shows that PAS may be strain specific, resulting in vastly different interactions (synergy vs antagonism) when applied against different strains. Future research should thus focus on identifying the underlying mechanisms that facilitate PAS, such as the bacteriophage binding receptors or capsule degrading enzymes, as this information may not only be used to develop standardised bacteriophage-antibiotic combination treatment protocols but may also be utilised in the development of genetically engineered bacteriophages that could increase the treatment efficiency of PAS. Additionally, the combined use of bacteriophage-derived enzymes in combination with antibiotics should be investigated, as the use of bacteriophage enzymes may negate the establishment of resistant sub-populations.

Materials and methods

Bacterial strains, bacteriophages and growth conditions

Klebsiella pneumoniae ATCC 10031 (reference strain), *K. pneumoniae* S1 43 (environmental strain) and *K. pneumoniae* MCC3 (clinical strain) were used for the isolation, propagation, and characterisation of *K. pneumoniae* bacteriophages, while the reference strain *E. cloacae* NCTC 13406 was used for the isolation, propagation and characterisation of *Enterobacter* spp. bacteriophages. The bacterial strains (target and non-target bacterial species) utilised for the host range determination of the isolated bacteriophages are indicated in Table S1 (Supplementary Information).

Bacteriophage PAW33 was isolated from wastewater and partially characterised by Reyneke et al.[14]. Whole genome sequencing of PAW33 was not previously conducted and was included in the current study. Additionally, the bacteriophage ABTW1 was isolated from hospital wastewater and was characterised by Morris et al.[28]. The genome annotation of ABTW1 was not included in the Morris et al.[28] study and was thus included in the current study.

The reference isolates *K. pneumoniae* ATCC 13383, *E. cloacae* NCTC 13406 and *P. aeruginosa* ATCC 27853, environmental isolates *K. pneumoniae* S1 43, *E. cloacae* 4L and *P. aeruginosa* S4 12, and clinical isolates *K. pneumoniae* P3, *P. aeruginosa* PAO1, and *A. baumannii* AB3 were used for the bacterial challenge tests (Supplementary Information; Sect. 1.1), biofilm eradication assays (Supplementary Information; Sect. 1.2), and bacteriophage-antibiotic combination treatment strategies. All strains were obtained from the Water Resource Laboratory Culture Collection (Department of Microbiology, Stellenbosch University) and were cultured at 30 or 37 °C in tryptic soy broth (TSB; Biolab, Merck, Wadeville, South Africa) or on tryptic soy agar (TSA; Biolab, Merck). For the double-layer plaque assays (double-layer overlays), the TSA medium contained 1.2% (w/v) Agar Bacteriological (Biolab, Merck) in the bottom layer and 0.6% agar (w/v) in the soft top layer[70].

Isolation and characterisation of *K. pneumoniae* and *E. cloacae* bacteriophages

The *K. pneumoniae* and *E. cloacae* bacteriophages were isolated by screening six 1 L samples of influent wastewater collected from the Stellenbosch Wastewater Treatment Plant (GPS co-ordinates: – 33.943505, 18.824584). Additionally, five 1 L surface run-off samples were collected from two sites at Sir Lowry's Pass Village urban informal settlement in Cape Town, South Africa (GPS co-ordinates: -34.152741; 18.908422) and river water from one site at the Plankenbrug River (GPS co-ordinates: – 33.927761, 18.850544).

Enrichment for *K. pneumoniae* and *E. cloacae* bacteriophages was performed as outlined by Reyneke et al.[14], whereafter five repeated rounds of plaque purification and re-infection were performed for the presumptive bacteriophage samples. The bacteriophages were selected for further studies based on their initial lysis profiles during bacteriophage purification (i.e., number and consistency of plaque formation, plaque clarity and plaque size). The purified bacteriophages were used to prepare concentrated bacteriophage stock solutions [$> 10^8$ plaque forming units (PFU)/mL] for use in subsequent experiments using the modified small-scale liquid culture method outlined in Reyneke et al.[14] or the plate lysis assay described by Bonilla and Barr[71]. The concentrated samples were stored at 4 °C until further use.

The host range of the isolated *K. pneumoniae* and *E. cloacae* bacteriophages was determined by spotting 10 µL of the concentrated bacteriophage stock solutions (10^6 to 10^7 PFU/mL) onto TSA with 5 mL freshly prepared soft top agar, which had been inoculated with 50 µL of the strain to be tested and incubating the plates at 30 °C (*K. pneumoniae*) or 37 °C (*E. cloacae*) for 18 h[70]. The host range for each bacteriophage was determined in triplicate and consisted of screening 11 K. *pneumoniae* strains, 22 *Enterobacter* spp., and 73 non-target bacterial strains representative of 14 bacterial genera (Table S1; Supplementary Information). Reference, environmental and clinical isolates of both the target and non-target bacterial strains were included in the host range determination analysis. The *K. pneumoniae* and *E. cloacae* bacteriophages with the greatest host range and largest plaque morphology were then selected for further characterisation and analysis and were subsequently referred to as KPW17 (isolated from wastewater) and ECSR5 (isolated from surface run-off), respectively. While the lytic activity of ECSR5 and ABTW1 was tested against reference, environmental and clinical strains of *E. cloacae* and *A. baumannii*, respectively, no lytic activity (ECSR5) was observed against the tested clinical *E. cloacae* strains, while no lytic activity (ABTW1) was observed against the tested reference and environmental *A. baumannii* strains, and these strains were excluded from further analysis.

Following host range determination, KPW17 and ECSR5 were morphologically characterised using transmission electron microscopy. Briefly, carbon-coated Formvar grids (Agar Scientific, Essex, UK) were treated using glow discharge to increase the hydrophilicity of the surface. Concentrated bacteriophage samples of $> 10^{10}$ PFU/ml were loaded onto the grids and stained with 2% uranyl acetate. The samples were visualised with a Field Electron and Ion Company T20 TEM (FEI Company, Hillsboro, Oregon, USA) at the Electron Microscope Unit of the Aaron Klug Centre for Imaging and Analysis at the University of Cape Town. The isolated bacteriophages were morphologically characterised as described by the International Committee on Taxonomy of Viruses[36].

Genome sequencing and phylogenetic analysis

The DNA of bacteriophages KPW17, PAW33, ECSR5 and ABTW1 was extracted as outlined by Reyneke et al.[14]. Quality control was performed using the Tapestation system (Agilent Technologies, United States) for fragment analysis and Qubit HS assay (Thermo Fisher Scientific, South Africa) for quantification by Inqaba Biotechnical Industries (Pretoria, South Africa). Library preparation and sequencing of the extracted bacteriophage DNA, was performed by Inqaba Biotechnical Industries using the New England Biolabs Next protocol (Massachusetts, United States), followed by sequencing using the Illumina NextSeq500 platform (Illumina, Inc., United States). Quality control of the reads was assessed with FastQC[72], where adapters and overrepresented sequences were removed, and poor-quality bases trimmed. The bacteriophage genomes were then assembled de novo using SPAdes[73] as outlined by Shen and Millard[74]. The genomes were compared to the NCBI virus database using Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify highly similar bacteriophage sequences, limiting the search to viruses (taxid: 10239). Additionally, PhageTerm was used to identify bacteriophage termini and packaging mechanisms[29]. The pairwise intergenomic similarity of the viruses was calculated using VIRIDIC which implements the algorithm used by the ICTV[75]. The bacteriophage genomes were identified through a BLAST search, with all hits to partial genomes excluded from the analysis. The bacteriophage genomes were annotated and screened for the presence of virulence factors and antibiotic resistance genes against the Virulence Factor Database and Comprehensive Antibiotics Resistance Database, respectively, using Pharokka[76]. Phylogenetic analysis of the core genes for each isolated bacteriophage as well

as the bacteriophage sequences with the highest intergenomic similarity and shared gene clusters (as identified by VIRIDIC) was constructed using GET_HOMOLOGUES and GET_PHYLOMARKERS[77,78]. The annotated genome sequences were submitted to GenBank and were assigned accession numbers PQ305824 (PAW33), PQ305823 (KPW17), PQ305822 (ECSR5) and PQ305821 (ABTW1). The sequences were placed under embargo and will be released to the public on 1 September 2025.

Bacteriophage-antibiotic combination treatment strategies

Kirby-Bauer disc diffusion assays

To identify potential synergistic bacteriophage-antibiotic combination treatments, the susceptibility of the *P. aeruginosa* strains (ATCC 27853, S4 12 and PAO1), *K. pneumoniae* strains (ATCC 13383, S1 43 and P3), *E. cloacae* strains (NCTC 13406 and 4L), and clinical *A. baumannii* strain AB3 to a series of antibiotics (before and after exposure to PAW33, KPW17, ECSR5, and ABTW1, respectively), was determined using Kirby-Bauer disc diffusion assays. The respective host bacterial strains used for the inoculation of the bacterial challenge test flasks and the bacterial cells harvested after the 24-h bacterial challenge tests (Supplementary Information, Sect. 1.1) were resuspended in 0.85% saline. The OD_{625nm} of the bacterial suspension was measured using a GENESYS™ 20 Visible Spectrophotometer (Thermo Fisher Scientific) and subsequently adjusted to 0.08–0.13, which corresponds to approximately 1.5×10^8 colony forming units (CFU)/mL[31]. One hundred microliters (100 µL) of the adjusted bacterial suspension was spread plated onto Mueller–Hinton agar (MHA; Biolab, Merck). Commercially prepared, fixed concentration, antibiotic discs (Oxoid, Basingstoke, UK) [19 antibiotics for *K. pneumoniae*, 10 antibiotics for *P. aeruginosa*, 12 antibiotics for *E. cloacae* and 11 antibiotics for *A. baumannii* outlined in Table S2 (Supplementary Information)] were placed onto the surface of the agar in triplicate and the plates were incubated at 37 °C for 18 to 24 h[32]. A negative control (6 mm blank disc; Oxoid) was included and *P. aeruginosa* ATCC 27853 was included as quality control. The diameter of the zone of inhibition around each antibiotic disc was measured to the closest millimetre and compared to the clinical breakpoints as outlined in the EUCAST[31] and CLSI[32] guidelines (Tables S3 to S6, Supplementary Information).

Broth microdilution checkerboard assays

The MIC of the antibiotics, identified by the disc diffusion assays [*K. pneumoniae*—DOR (Sigma-Aldrich, St. Louis, USA), LEV (Sigma-Aldrich), TZP (Sigma-Aldrich), and PRL (Sigma-Aldrich); *P. aeruginosa*—ATM (Sigma-Aldrich), CIP (Fluka Chemicals, North Carolina, USA), LEV (Sigma-Aldrich), and TZP (Sigma-Aldrich); *E. cloacae*—DOR (Sigma-Aldrich) and CN (Melford Laboratories Ltd, UK); *A. baumannii*—IPM (Sigma-Aldrich) and TZP (Sigma-Aldrich)], were determined using the broth microdilution method. The stock solutions of the respective antibiotics were serially diluted to the suggested MIC determination ranges for *K. pneumoniae, E. cloacae, A. baumannii* and *P. aeruginosa* as outlined for each antibiotic by Andrews[33]. Cell suspensions of the *K. pneumoniae* strains (ATCC 13383, S1 43 and P3), *P. aeruginosa* strains (ATCC 27853, S4 12 and PAO1), *E. cloacae* strains (NCTC 13406 and 4L), and clinical *A. baumannii strain* AB3 were prepared in Mueller–Hinton Broth (MHB; Biolab, Merck) as outlined in "Kirby-Bauer disc diffusion assays" section[31]. Ninety microliters (90 µL) of MHB (Biolab, Merck) and 100 µL of each of the antibiotic dilutions were transferred into the wells of a Greiner CELLSTAR 96-well culture plate (Merck, Johannesburg, South Africa), whereafter 10 µL of the adjusted OD_{625nm} bacterial inoculums were added to the respective wells. A positive control (Gram-negative ESKAPE organism in MHB without antibiotic) and a sterility control (MHB) were included for each MIC assay. The 96-well plates were incubated at 37 °C for 24 h and the susceptibility of the *K. pneumoniae, P. aeruginosa, E. cloacae* and *A. baumannii* strains was determined by measuring the OD_{595nm} before (0 h) and after incubation (24 h) using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Sandton, South Africa). The MIC was assigned as the lowest concentration of each antibiotic that reduced the target strains ≥ 90%[79].

Following MIC determination, interactions between KPW17, ECSR5, PAW33 or ABTW1 and the selected antibiotics were assessed against the *K. pneumoniae, P. aeruginosa, E. cloacae* or *A. baumannii* strains, respectively, using the checkerboard broth microdilution method as previously described by Thummeepak et al.[80]. Briefly, analysis was performed in MHB with a final inoculum of 5×10^5 CFU/mL of the respective bacterial strains included per well of the Greiner CELLSTAR 96-well culture plate (Merck). The selected antibiotic and corresponding bacteriophage were diluted two-fold horizontally (antibiotic) and ten-fold vertically (bacteriophage), respectively. The concentrations for the bacteriophages ranged from an MOI of 0.001 to 100, while the antibiotic concentrations ranged from 1/16 × MIC to 2 × MIC. The 96-well microtiter plates were subsequently incubated at 37 °C overnight. The effect of the bacteriophage-antibiotic concentration on the *K. pneumoniae, P. aeruginosa, E. cloacae* and *A. baumannii* strains was determined by measuring the OD_{595nm} and calculating the MIC as outlined Rautenbach et al.[79]. In order to evaluate the activity of the combination treatment strategy, FIC indices were calculated using Eq. (1) as outlined by Samadi et al.[81], where an FIC ≤ 0.5 indicates synergy, FIC > 0.5 but < 1 indicates partial synergy, FIC = 1.0 indicates an additive effect, FIC > 1.0 but ≤ 2.0 indicates indifference, and FIC > 2.0 indicates an antagonistic effect[80].

$$FICindices = \left(\frac{MIC_{Phage}incombination}{MIC_{Phage}alone}\right) + \left(\frac{MIC_{Antibiotic}incombination}{MIC_{Antibiotic}alone}\right) \quad (1)$$

Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials. The datasets generated and/or analysed during the current study are available in the NCBI repository, https://www.ncbi.nlm.nih.gov/ (under embargo till 1 September 2025).

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Author contributions

Conceived and designed the experiments: BR, TCM and WK. Performed the experiments: BR and TCM. Analysed the data: BR and TCM. Contributed reagents/materials/analysis tools: WK and SK. Compiled and edited the manuscript: BR, TCM, SK and WK.

Declarations

Ethical clearance

Ethical clearance was obtained for the collection and processing of environmental water samples through the Research Ethics Committee: Biosafety and Environmental Ethics at Stellenbosch University (Ethics Ref No. BEE-2019-9466).

Competing interests

The authors declare no competing interests.

Additional information

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