ISOLATION OF BACTERIOPHAGE AGAINST *PSEUDOMONAS AUREGINOSA* FROM MALE URINARY TRACT INFECTION (UTI) specimen.

Abstract

Bacteriophages were isolated from raw sewage and were then selected base on the size and clarity of plaque that were formed. The selected phages were further enriched and purified. The isolated bacteriophages were found to be able to lyse Pseudomonas aeruginosa gotten from raw sewage.

Keywords*:* *Pseudomonas aeruginosa*; PCR; sewage water; virulent bacteriophages.

Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans throughout their life span due to the uncontrollable factors of lead cause by humans activities, in which the infections target the kidneys, bladder, ureters, and urethra. Symptoms of urinary tract infections include: pain during urination (dysuria), frequent urination (frequency), blood in urine (hematuria), turbid or cloudy urine, fever and abdominal pain.

The organisms most commonly responsible for urinary tract infections (UTIs) are *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus faecalis (CDC 2021).*

Bacteriophages, also known as phages, are viruses that infect and replicate only in bacterial cells (Ackermann, H.W 2006). They are ubiquitous in the nature and are recognized as the most abundant microorganisms on earth (Ackermann, H.W 2004). They are extremely diverse in size, morphology, and genomic organization (Aksyuk *et al* 2009). However, all consist of a nucleic acid genome encased in a shell of phage-encoded capsid proteins, which protect the genetic material and mediate its delivery into the next host cell (Batinovic *et al* 2019). Phages replicate

within the bacterium following the injection of their genome into its cytoplasm (McGrath 2007). Electron microscopy has allowed the detailed visualization of hundreds of phage types, some of which appear to have "heads," "legs", and "tails". Despite this appearance, phages are non-motile and depend upon Brownian motion to reach their targets. (Simmonds, 2018). Like all viruses, bacteriophages are very species-specific with regard to their hosts and usually only infect a single bacterial species or even specific strains within a species (Guan, L *et al* 2016). Once a bacteriophage attaches to a susceptible host, it pursues one of two replication strategies: lytic or lysogenic (Bishop-Lilly *et al*, 2019). During a lytic replication cycle, a phage attaches to a susceptible host bacterium, introduces its genome into the host cell cytoplasm, and utilizes the ribosomes of the host to manufacture its proteins (Hampton, H. G *et al* 2020). The host cell resources are rapidly converted to viral genomes and capsid proteins, which assemble into multiple copies of the original phage. As the host cell dies, it is either actively or passively lysed, releasing the new bacteriophage to infect another host cell. In the lysogenic replication cycle, the phage also attaches to a susceptible host bacterium and introduces its genome into the host cell cytoplasm (Oechslin, F. 2018). However, the phage genome is instead integrated into the bacterial cell chromosome or maintained as an episomal element where, in both cases, it is replicated and passed on to daughter bacterial cells without killing them (McGrath S *et al* 2007). Integrated phage genomes are termed prophages, and the bacteria containing them are termed lysogens (Mangas, E. L *et al* 2019). Prophages can convert back to a lytic replication cycle and kill their host, most often in response to changing environmental conditions (Ptashne, 2006).

Phages were first used as antibacterial agents by the former Soviet Republic of Georgia during the 1920s and 1930s for treating bacterial infections. They had widespread use, including treatment of soldiers in the Red Army. However, they were abandoned for general use in the West for several reasons:

- Antibiotics were discovered and marketed widely. They were easier to make, store, and to prescribe.
- Medical trials of phages were carried out, but a basic lack of understanding raised questions about the validity of these trials.
- Publication of research in the Soviet Union was mainly in the Russian or Georgian languages and for many years, was not followed internationally.

The use of phages has continued since the end of the Cold War in Russia, Georgia and elsewhere in Central and Eastern Europe. The first regulated, randomized, double-blind clinical trial was reported in the *Journal of Wound Care* in June 2009, which evaluated the safety and efficacy of a bacteriophage cocktail to treat infected venous ulcers of the leg in human patients. The FDA approved the study as a Phase I clinical trial. The study's results demonstrated the safety of therapeutic application of bacteriophages, but did not show efficacy. The authors explained that the use of certain chemicals that are part of standard wound care (e.g. lactoferrin or silver) may have interfered with bacteriophage viability. Shortly after that, another controlled clinical trial in Western Europe (treatment of ear infections caused by *Pseudomonas aeruginosa*) was reported in the journal *Clinical Otolaryngology* in August 2009. The study concludes that bacteriophage preparations were safe and effective for treatment of chronic ear infections in humans. Additionally, there have been numerous animal and other experimental clinical trials evaluating the efficacy of bacteriophages for various diseases, such as infected burns and wounds, and cystic fibrosis associated lung infections, among others (Kutter, 2010).

Materials And Method

Sample collection

50ml of raw sewage samples was collected from two septic tanks at the Department of works, Akwa Ibom State University. The samples were analyzed in the Microbiology Laboratory of Akwa Ibom State University, Ikot Akpaden, Mkpat Enin.

Study Population and Sample

Two samples were used for the research project. The first sample involved sewage collection from the Works Department of Akwa Ibom State University. The sewage was used for isolation of phages because it is known that sewage is one of the natural reservoirs for numerous bacteriophages. Secondly, urine samples infected with UTI were collected from Emmanuel Hospital, Eket, Akwa Ibom State. The aim of the urine collection was to isolate *Pseudomonas aeruginosa* which in turn were used together with the sewage for phage development.

Isolation of *Pseudomonas aeruginosa* from raw sewage

Five milliliters (5 ml) of sewage samples were cultured on CLED agar plates without dilution and incubated at 37 ^{o}C for 48 h. A single colony of each isolate was inoculated on MacConkey agar and incubated at 37 ^{o}C for 48 h.

Characterization of *Pseudomonas* species

Isolates forming round, flat, and colorless colonies on MacConkey agar indicated that the organism is a lactose non-fermenter were suspected to be *Pseudomonas* species. The isolates were characterized using Gram staining alongside biochemical tests such as catalase test, citrate test, coagulase test, nitrate test, and oxidase test.

Gram staining

This is a staining technique that helps to differentiate bacteria into Gram-positive and Gram-negative cells. A grease free dry slide was taken. The inoculating loop was sterilized on a flame of a basin burner, and a loopful of the test organism was transferred by sterile loop and a smear was made at the center. The smear was allowed to air – dry and the smear was heat fixed by passing the slide 3-4 times through the flame quickly with the smear slide facing up. The slide was placed on the staining rack and covered with crystal violet. This was left for 1min and carefully rinsed under running tap water. The slide was flooded with Gram's iodine solution and allowed for 1 min. the slide was rinsed in a gentle running water tap and then flooded with decolorizing agent (ethyl alcohol) for 20-30 sec0nds. The slide was rinsed gently under running tap water and drain completely. The smear was counter-stained with safranin and was allow to stand for about 30 seconds to 1min. the slide was rinsed under running tap water. It was air-dried and observed under microscope using X100 objective lens. Gram-positive cells were stained purple under the microscope while Gram-negative were stained red as described by Cheesebrough (2002).

Catalase test

This test was carried out to identify the various isolates with the ability to produce catalase enzymes that breakdown hydrogen peroxide into water and oxygen. A small amount of bacteria colony was transferred to a suface of clean, dry glass slide using a wire loop or sterile wooden stick. A drop of 3% H_2O_2 was placed on to the slide and mixed. A positive result is rapid evolution of oxygen (within 5-10sec.) as evidenced by bubbling.

Citrate test

Two (2) grams of Sodium Citrate, 5g Sodium Chloride, 1g Dipotassium Phosphate, 1g Ammonium Dihydrogen Phosphate, 0.08g Bromothymol Blue, 0.2g Magnesium Sulphate and 15g agar was mixed together, and 1000ml sterile distilled water was dispensed in the same mixture. The pH was adjusted to 6.9 and gentle heat was applied to dissolve agar. About 3-4 ml was collected in test tubes and sterilized at 121^oC for 15 minutes in an autoclave. The tubes were inoculated and incubated at 37^0C for 24-48 hours. The color changes in the medium that was observed. Color change of medium to blue indicate citrate positive. No color change of the medium indicates citrate negative.

Coagulase test

This test was performed to indicate the presence of various isolates with the ability to produced enzyme known as “Coagulase” capable of coagulating human blood plasma. The slide method

was used (Cheesebrough, 2002). Some drop of normal saline was placed on a clean grease free slide. The test organism was added to it and mixed properly, followed by a drop of plasma and mixed gently. Climping/agglutination reaction within 5-10 seconds indicated a positive Coagulase test, while no agglutination indicated Coagulase negative.

Indole Test

Peptone broth was prepared by weighing (15) ml of peptone broth in a test tube and sterilized in an autoclave at 121^{o}C for 15 minutes at 15lbs pressure. A loop full of bacteria culture was inoculated in broth and incubated for 24-48 hours at 37^{o}C. Two (2) drops of Kovac's reagent were dispensed into the test tubes and mixed together after sterilization. (Ajiboye and Emmanuel, 2021).

Methyl Red Test:

The bacteria culture was inoculated into a fresh sterile broth medium and incubated at 37^{o}C for 48 hours. A sterile pipette was used to dispense 5 drops of Methyl red reagent into the broth culture and colour change was observed (Ajiboye and Emmanuel, 2021).

Oxidase test

This test was carried out to identify the various isolates with the ability to produce the enzyme "oxidase". The filter method was employed. The test organism was picked out and smeared on a filter paper, and then some drops of tetramethyl-P-Phenylenediaminedihydrochloride was placed

on the filter paper. A change in colour to sleep purple or blue indicated a positive test, and no colour change indicated a negative test.

Voges- Proskauer Test

Preparation of 5 % α – naphthol in ethanol and 40 % Sodium hydroxide in deionized water was done. MR-VP broth was also prepared and 5 ml dispensed in different test tubes and sterilization was done at 121^{o}C for 15 minutes using an autoclave. The medium was allowed to cool to room temperature. Inoculum from fresh culture media was introduced in different test tube and this was incubated together with the control at 37^{o}C for 48 hours. About 2.5 ml of culture was dispensed in a sterile cultures tube and 5 drops of methyl red reagent was added. The test organism was also compared with the control and colour change was observed (Ajiboye and Emmanuel, 2021).

Molecular identification of *Pseudomonas* isolate

DNA Extraction

PCR Amplification

16S rDNA amplification was carried out using universal primers, 27F (AGAGTTTGATCCTGGCTCAG) and 1392R (GGTTACCTTGTTACGACTT). The reaction mix was prepared in a final volume of 20 µL containing 4 µL Taq buffer (5×), 1.2 µL of MgCl2 4 µL of dNTPs, 0.5 µL of each primer, 0.2 µL of Taq polymerase, 2 µL of extracted DNA and 6.6 µL of nuclease free H2O.

DNA Sequencing and Phylogenetic Analysis

Sanger sequencing was performed at the Center of Innovation (USMBA, Fez-Morocco) using a ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).Preliminary identifications were performed based on sequence assembly and by search in the NCBI database. Strains were attributed to a particular genus when the sequence similarity with a strain type was at least 98% and to a given species when sequence similarity was at least 99.5%.

Phage Development

45ml of raw sewage was measured out into a graduated measuring cylinder and decanted into a 250ml Erlenmeyer flask containing 5ml of decabroth and 5ml of cultured *Pseudomonas aeruginosa*. The mixture was then incubated at 37 ^{o} C for 24 hrs to the allow development of bacteriophages. After incubation, the sewage culture was centrifuged at 2500 rpm for 10min and the supernatant was filtered through a 0.45micro membrane filter to separate the lysed bacteria from the phage sample. The phages remained in the filtrate due to its considerable lower size.

Spot Test

The spot test was carried out to determine the presence of phages in the filtrate obtained after filtration.

Plaque Assay

Soft agar overlay method (Double layer agar method) was followed to determine the number of phage particles in the lysate. Normal Nutrient agar was melted and cooled to 45^0c. This agar was poured into petri dishes and allowed to solidify. Soft Nutrient agar was melted, 3ml of the agar was placed into each of the 3 tubes and held at 45^0c. 0.1ml of *Pseudomonas aeruginosa* culture was added into each tube and labeled as 1, 2, 3. To the first test tube, 10ul of phage suspension was added from step 2 above, then mixed and spread on one of the normal agar plate prepared earlier. The procedure was repeated with second test tube using 50ul of phage suspension. The content of the third tube was spread without adding phage suspension. This plate was served as control. The soft agar was allowed to solidify in all the plates. When the soft agar solidified, the plates was inverted and incubated at 37^0c. Development of plaques (clear zones where viruses have infected and lysed the bacterial cells) was then examined periodically for growth.

Results

Sample Processing and Isolation

The sample was processed (i.e shaken vigorously to mix) and was prepared for the serial dilution. Of 10^{-1} to 10^{-5} was carried out for the samples; after which 1ml from dilution factors 10^{-3} to 10^{-5} was poured aseptically into the petri dishes (using pour plating method/technique) and appropriate medium (Nutrient agar) was poured into the petri dishes and was allowed to solidify. Thereafter, the petri dishes were incubated at 37^0c for 48 hours.

Colony purification

After 48hours of isolation, colonies with discrete growth were picked and subcultured in a fresh nutrient agar medium to obtain a pure culture (that is, 24 hours culture).

Table 1: Biochemical test for *Pseudomonas aeruginosa*

Test	Gram Stain	Catalase	Citrate	Nitrate	Oxidase	Coagulase
Results	–	+	+	+	+	+

(+) = Positive

(–) = Negative

Table 1.1: Biochemical Test and Identification of *Pseudomonas aeruginosa*

Characteristics	Shape (Cocci/Diplococci/Rods)	Motility (Motile / Non-Motile)	Capsule (Capsulated/Non-Capsulated)	Spore (Sporing/Non-Sporing)	Flagella (Flagellated/Non-Flagellated)
Pseudomonas aeruginosa	Rods	Motile (Unipolar)	Non-Capsulated	Non-Sporing	Single Flagella

Table 1.2: Sugar Fermentation of *Pseudomonas aeruginosa*

Fermentation	Fructose	Glucose	Lactose	Maltose	Sucrose
P.aeruginosa	–	–	–	–	–

(–) = Negative

Table 1.3: Gram-Staining isolate under light microscopic view

Isolate	Shape	Margin	Elevation	Size	Colour	Opacity	Consistency	Surface	Texture	Gream reaction
S^{-1}	Circular	Entire	Flat	Small	Creamy	Opaque	Brittle	Dull	Rough	+ cocci
S^{-2}	Irregular	Undulate	Raised	Moderate	Creamy	Opaque	Sticky	Shiny	Rough	- cocci
S^{-3}	Irregular	Undulate	Flat	Moderate	Creamy	Opaque	Sticky	Dull	Rough	+ cocci
S^{-4}	Circular	Entire	Flat	Moderate	Creamy	Opaque	Brittle	Shiny	Smooth	-
S^{-5}	Irregular	Undulate	Flat	Moderate	Creamy	Opaque	Sticky	Shiny	Rough	+ cocci
S^{-6}	Circular	Entire	Flat	Small	Creamy	Opaque	Sticky	Shiny	Smooth	+ cocci
S^{-7}	Irregular	Undulate	Flat	Small	Creamy	Opaque	Sticky	Dull	Rough	+ cocci
S^{-8*}	Circular	Undulate	Flat	Moderate	Creamy	Opaque	Sticky	Shiny	Smooth	+ cocci
S^{-9*}	Circular	Entire	Flat	Moderate	Creamy	Opaque	Sticky	Shiny	Smooth	- Rod
S^{-10*}	Circular	Entire	Flat	Small	Creamy	Opaque	Sticky	Shiny	Smooth	+ cocci
S^{-11}	Irregular	Undulate	Flat	Small	Creamy	Opaque	Sticky	Dull	Rough	+ cocci
S^{-12}	Irregular	Undulate	Raised	Small	Creamy	Opaque	Sticky	Shiny	Rough	+ cocci
S^{-13}	Irregular	Undulate	Raised	Moderate	Creamy	Opaque	Brittle	Dull	Smooth	+ cocci
S^{-14}	Circular	Entire	Raised	Moderate	Creamy	Opaque	Brittle	Dull	Smooth	- cocci

Isolate code	Gene size	Taxonomic affinity	Accession number	Similarity (%)

(+) = Positive

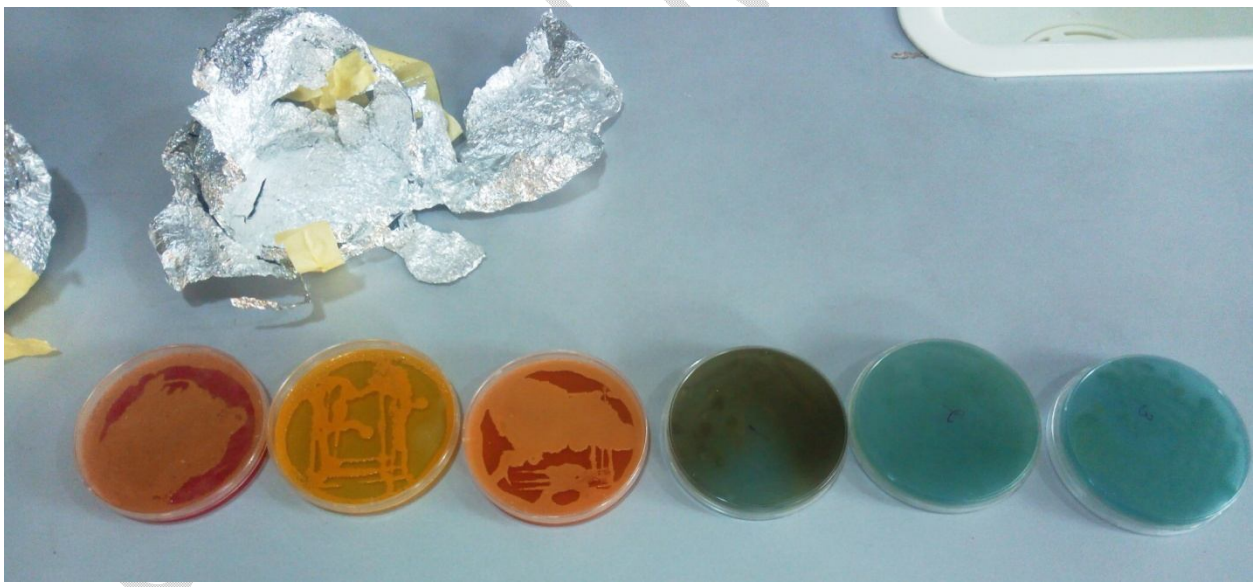
(–) = Negative

Table 1.4: Result showing molecular identification of *Pseudomonas aeruginosa*

S 1	1537bp	*Pseudomonas aeruginosa* strain NBAII AFP-7	HM439964.1	100%
S 2	1316bp	*Pseudomonas aeruginosa* strain WES2	MN960116.1	100%

Double Agar Overlay Plaque Assay

All the plates show positive for the isolation of Bacteriophages. Round, clear and transparent plagues were observed in all plates, indicating that the sample collected at works department Akwa Ibom State University contained bacteriophages that were infectious against *Pseudomonas aeruginosa*. The phages produce clear, medium sized (1.0 – 3.1mm in diameter) plagues with well-defined edges in bacterial lawn, showing that the isolated phages have lytic effect against *Pseudomonas aeruginosa.*



A B C D E G

Fig 1: Plates showing Bacteriophages from cultured plates of nutrient Agar and CLED agar.

Discussion, Conclusion:

Results of this study indicated that both lytic phage isolates could be used as a biological control agents against urinary track infection.

Bacteriophage ineffective to *Pseudomonas aeruginosa* can be isolated from raw sewage. The most probable reason is using the cultured enrichment for phage isolation. In conclusion, the bacteriophages isolated from raw sewage were able to infect *Pseudomonas aeruginosa*. All the 14 isolated phages are virulent phages which produced round, clear and medium sized (1.0 – 3.0mm in diameter) plaques. Sampling site provided favorable condition for the growth of phages. Based on the number of phages isolated, higher number of host bacteria population was present in C,D, E than A and B.

References

Ackermann, H.W. (2006). Classification of bacteriophages. In The Bacteriophages, Ed. Calendar R, Oxford University Press, ISBN 0-19-514850-9, New York, USA, pp. 8–16

Ackermann, H.W. (2007). 5500 Phages examined in the electron microscope. Archives of Virology Vol.152, No.2, pp. 227-243. PMID 17051420

Ackermann, H.W. (2004).Bacteriophage classification. In Bacteriophages. Biology and Applications. Eds Kutter E, Sulakvelidze A, CRC Press ISBN 978-0-8493-1336-3, Boca Raton, USA, pp. 67–89Adams, M.B.; Hayden, M. & Casjens, S. (1983). On the sequential packaging of bacteriophage P22 DNA. The Journal of Virology,Vol.46, No.2, pp. 673-677.

Aksyuk, A.A.; Leiman, P.G.; Kurochkina, L.P.; Shneider, M.M.; Kostyuchenko, V.A.; Mesyanzhinov, V.V. & Rossmann, M.G. (2009). The tail sheath structure of bacteriophage T4: a molecular machine for infecting bacteria.The EMBO Journal, Vol.28, pp 821–829

Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K. & Watson, J. D. (1989). Molecular Biology of the Cell,2nd ed. New York: Garland Publishing,. ISBN 0824036956

Anderson, D.L.; Hickman, D.D. & Reilly, B.E. (1966). Structure of Bacillus subtilis bacteriophage phi 29 and the length of phi 29 deoxyribonucleic acid. The Journal of Bacteriology,Vol.91, No.5, pp. 2081-9.

Arnold, H.P.; Ziese, U. & Zillig, W. (2000). SNDV, a novel virus of the extremely thermophilic and acidophilic archaeon Sulfolobus. Virology , Jul 5; Vol.272, No.2, pp. 409-416.

Azam, A. H., and Tanji, Y. (2019). Bacteriophage-host arm race: an update on the mechanism of phage resistance in bacteria and revenge of the phage with the perspective for phage therapy. *Appl. Microbiol. Biotechnol.* 103, 2121–2131. doi: 10.1007/s00253-019-09629-x

Barrangou, R. (2013). CRISPR-Cas systems and RNA-guided interference. *Wiley Interdiscip. Rev. RNA* 4, 267–278. doi: 10.1002/wrna.1159

Batinovic, S., Wassef, F., Knowler, S. A., Rice, D. T. F., Stanton, C. R., Rose, J., et al. (2019). Bacteriophages in natural and artificial environments. *Pathogens* 8:100. doi: 10.3390/pathogens8030100

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res.* 27, 573–580. doi: 10.1093/nar/27.2.573

Bertelli, C., Laird, M. R., Williams, K. P., Lau, B. Y., Hoad, G., Winsor, G. L., et al. (2017). IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res.* 45, W30–W35. doi: 10.1093/nar/gkx343

BIG Data Center Members (2019). Database resources of the BIG data center in 2019. *Nucleic Acids Res.* 47, D8–D14. doi: 10.1093/nar/gky993

Bishop-Lilly, K. A., Plaut, R. D., Chen, P. E., Akmal, A., Willner, K. M., Butani, A., et al. (2012). Whole genome sequencing of phage resistant *Bacillus anthracis* mutants reveals an essential role for cell surface anchoring protein CsaB in phage AP50c adsorption. *Virol. J.* 9:246. doi: 10.1186/1743-422X-9-246

Biswas, A., Raymond, H. J. S., Morales, S. E., Fineran, P. C., and Brown, C. M. (2016). CRISPRDetect: a flexible algorithm to define CRISPR arrays. *BMC Genomics* 17:356. doi: 10.1186/s12864-016-2627-0

Bland, C., Ramsey, T. L., Sabree, F., Lowe, M., Brown, K., Kyrpides, N. C., et al. (2007). CRISPR recognition tool (CRT): a tool for automatic detection of clustered regularly interspaced palindromic repeats. *BMC Bioinformatics* 8:209. doi: 10.1186/1471-2105-8-209

Blasco, L., Ambroa, A., Lopez, M., Fernandez-Garcia, L., Bleriot, I., Trastoy, R., et al. (2019). Combined use of the Ab105-2phiDeltaCI lytic mutant phage and different antibiotics in clinical isolates of multi-resistant *Acinetobacter baumannii*. *Microorganisms* 7:556. doi: 10.3390/microorganisms7110556

Bondy-Denomy, J., Pawluk, A., Maxwell, K. L., and Davidson, A. R. (2013). Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature* 493, 429–432. doi: 10.1038/nature11723

Boral, B., Unaldi, Ö, Ergin, A., Durmaz, R., Eser, Ö, and Acinetobacter Study Group (2019). A prospective multicenter study on the evaluation of antimicrobial resistance and molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* infections in intensive care units with clinical and environmental features. *Ann. Clin. Microbiol. Antimicrob.* 18:19. doi: 10.1186/s12941-019-0319-8

Broniewski, J. M., Meaden, S., Paterson, S., Buckling, A., and Westra, E. R. (2020). The effect of phage genetic diversity on bacterial resistance evolution. *ISME J.* 14, 828–836. doi: 10.1038/s41396-019-0577-7

Chai, G., Yu, M., Jiang, L., Duan, Y., and Huang, J. (2019). HMMCAS: a web tool for the identification and domain annotations of CAS proteins. *IEEE/ACM Trans. Comput. Biol. Bioinform.* 16, 1313–1315. doi: 10.1109/TCBB.2017.2665542

Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (2021) NCEZID, Division of Healthcare Quality Promotion (DHQP)

Chapman, B., and Chang, J. (2000). Biopython: python tools for computational biology. *SIGBIO Newsl.* 20, 15–19. doi: 10.1145/360262.360268

Chen, N. (2004). Using RepeatMasker to identify repetitive elements in genomic sequences. *Curr. Protoc. Bioinformatics* Chapter 4:Unit4.10. doi: 10.1002/0471250953.bi0410s25

Cock, P. J., Antao, T., Chang, J. T., Chapman, B. A., Cox, C. J., Dalke, A., et al. (2009). Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* 25, 1422–1423. doi: 10.1093/bioinformatics/btp163

Cook, R., Brown, N., Redgwell, T., Rihtman, B., Barnes, M., Clokie, M., et al. (2021). INfrastructure for a PHAge REference database: identification of large-scale biases in the current collection of phage genomes. *bioRxiv* [preprint]. doi: 10.1101/2021.05.01.442102

Couvin, D., Bernheim, A., Toffano-Nioche, C., Touchon, M., Michalik, J., Néron, B., et al. (2018). CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Res.* 46, W246–W251. doi: 10.1093/nar/gky425

Doron, S., Melamed, S., Ofir, G., Leavitt, A., Lopatina, A., Keren, M., et al. (2018). Systematic discovery of antiphage defence systems in the microbial pangenome. *Science* 359:eaar4120. doi: 10.1126/science.aar4120

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

Fedrigo, N. H., Xavier, D. E., Cerdeira, L., Fuga, B., Marini, P. V. B., Shinohara, D. R., et al. (2021). Genomic insights of *Acinetobacter baumannii* ST374 reveal wide and increasing resistome and virulome. *Infect. Genet. Evol.* 97:105148. doi: 10.1016/j.meegid.2021.105148

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., et al. (2014). Pfam: the protein families database. *Nucleic Acids Res.* 42, D222–D230. doi: 10.1093/nar/gkt1223

Finn, R. D., Clements, J., and Eddy, S. R. (2011). HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res.* 39, W29–W37. doi: 10.1093/nar/gkr367

Gordillo Altamirano, F., Forsyth, J. H., Patwa, R., Kostoulias, X., Trim, M., Subedi, D., et al. (2021). Bacteriophage-resistant *Acinetobacter baumannii* are resensitized to antimicrobials. *Nat. Microbiol.* 6, 157–161. doi: 10.1038/s41564-020-00830-7

Guan, L., Han, Y., Zhu, S., and Lin, J. (2016). Application of CRISPR-Cas system in gene therapy: pre-clinical progress in animal model. *DNA Repair (Amst.)* 46, 1–8. doi: 10.3389/fonc.2020.01387

Haft, D. H., Selengut, J. D., and White, O. (2003). The TIGRFAMs database of protein families. *Nucleic Acids Res.* 31, 371–373. doi: 10.1093/nar/gkg128

Hampton, H. G., Watson, B. N. J., and Fineran, P. C. (2020). The arms race between bacteria and their phage foes. *Nature* 577, 327–336. doi: 10.1038/s41586-019-1894-8

Harms, A., Brodersen, D. E., Mitarai, N., and Gerdes, K. (2018). Toxins, targets, and triggers: an overview of toxin-antitoxin biology. *Mol. Cell* 70, 768–784. doi: 1016/j.molcel.2018.01.003

Hesse, S., Rajaure, M., Wall, E., Johnson, J., Bliskovsky, V., Gottesman, S., et al. (2020). Phage resistance in multidrug-resistant *Klebsiella pneumoniae* ST258 evolves via diverse mutations that culminate in impaired adsorption. *mBio* 11:e02530-19. doi: 10.1128/mBio.02530-19

Hoikkala, V., Ravantti, J., Díez-Villaseñor, C., Tiirola, M., Conrad, R. A., Mcbride, M. J., et al. (2021). Cooperation between different CRISPR-cas types enables adaptation in an RNA-targeting system. *mBio* 12:e03338-20. doi: 10.1128/mBio.03338-20

Kamruzzaman, M., and Iredell, J. R. (2019). CRISPR-Cas system in antibiotic resistance plasmids in *Klebsiella pneumoniae*. *Front. Microbiol.* 10:2934. doi: 10.3389/fmicb.2019.02934

Karah, N., Samuelsen, Ø, Zarrilli, R., Sahl, J. W., Wai, S. N., and Uhlin, B. E. (2015). CRISPR-cas subtype I-Fb in *Acinetobacter baumannii*: evolution and utilization for strain subtyping. *PLoS One* 10:e0118205. doi: 10.1371/journal.pone.0118205

Koonin, E. V., Makarova, K. S., and Wolf, Y. I. (2017). Evolutionary genomics of defence systems in archaea and bacteria. *Annu. Rev. Microbiol.* 71, 233–261. doi: 10.1146/annurev-micro-090816-093830

Krupovic, M., Makarova, K. S., Forterre, P., Prangishvili, D., and Koonin, E. V. (2014). Casposons: a new superfamily of self-synthesizing DNA transposons at the origin of prokaryotic CRISPR-Cas immunity. *BMC Biol.* 12:36. doi: 10.1186/1741-7007-12-36

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

*Kutter, Elizabeth; De Vos, Daniel; Gvasalia, Guram; Alavidze, Zemphira; Gogokhia, Lasha; Kuhl, Sarah; Abedon, Stephen (1 January 2010). "Phage Therapy in Clinical Practice: Treatment of Human Infections". Current Pharmaceutical Biotechnology. **11** (1): 69–86. doi:10.2174/138920110790725401. PMID 20214609. S2CID 31626252*

Kumar, S., Tamura, K., and Nei, M. (1994). MEGA: molecular evolutionary genetics analysis software for microcomputers. *Comput. Appl. Biosci.* 10, 189–191. doi: 10.1093/bioinformatics/10.2.189

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327. doi: 10.1038/nrmicro2315

Liao, W., Liu, Y., Chen, C., Li, J., Du, F., Long, D., et al. (2020). Distribution of CRISPR-Cas systems in clinical carbapenem-resistant *Klebsiella pneumoniae* strrains in a Chinese Tertiary hospital and its potential relationship with virulence. *Microb. Drug Resist.* 26, 630–636. doi: 10.1089/mdr.2019.0276

Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., et al. (2019). ICEberg 2.0: an updated database of bacterial integrative and conjugative elements. *Nucleic Acids Res.* 47, D660–D665. doi: 10.1093/nar/gky1123

Lopatina, A., Tal, N., and Sorek, R. (2020). Abortive infection: bacterial suicide as an antiviral immune strategy. *Annu. Rev. Virol.* 7, 371–384. doi: 10.1146/annurev-virology-011620-040628

López, M., Mayer, C., Fernández-García, L., Blasco, L., Muras, A., Ruiz, F. M., et al. (2017). Quorum sensing network in clinical strains of A. *baumannii*: AidA is a new quorum quenching enzyme. *PLoS One* 12:e0174454. doi: 10.1371/journal.pone.0174454

López, M., Rueda, A., Florido, J. P., Blasco, L., Fernández-García, L., Trastoy, R., et al. (2018). Evolution of the Quorum network and the mobilome (plasmids and bacteriophages) in clinical strains of *Acinetobacter baumannii* during a decade. *Sci. Rep.* 8:2523. doi: 10.1038/s41598-018-20847-7

López, M., Rueda, A., Florido, J. P., Blasco, L., Gato, E., Fernández-García, L., et al. (2016). Genomic evolution of two *Acinetobacter baumannii* clinical strains from ST-2 clones isolated in 2000 and 2010 (ST-2_clon_2000 and ST-2_clon_2010). *Genome Announc.* 4:e01182-16. doi: 10.1128/genomeA.01182-16

Lu, S., Wang, J., Chitsaz, F., Derbyshire, M. K., Geer, R. C., Gonzales, N. R., et al. (2020). CDD/SPARCLE: the conserved domain database in 2020. *Nucleic Acids Res.* 48, D265–D268. doi: 10.1093/nar/gkz991

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., et al. (2011). Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol.* 9, 467–477. doi: 10.1038/nrmicro2577

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., et al. (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nat. Rev. Microbiol.* 13, 722–736. doi: 10.1038/nrmicro3569

Makarova, K. S., Wolf, Y. I., Iranzo, J., Shmakov, S. A., Alkhnbashi, O. S., Brouns, S. J. J., et al. (2020). Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. *Nat. Rev. Microbiol.* 18, 67–83. doi: 10.1038/s41579-019-0299-x

Mangas, E. L., Rubio, A., Álvarez-Marín, R., Labrador-Herrera, G., Pachón, J., Pachón-Ibáñez, M. E., et al. (2019). Pangenome of *Acinetobacter baumannii* uncovers two groups of genomes, one of them with genes involved in CRISPR/Cas defence systems associated with the absence of plasmids and exclusive genes for biofilm formation. *Microb. Genom.* 5:e000309. doi: 10.1099/mgen.0.000309

Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., et al. (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Res.* 43, D222–D226. doi: 10.1093/nar/gku1221

McGrath S and van Sinderen D (editors). (2007). Bacteriophage: Genetics and Molecular Biology *(1st ed.). Caister Academic Press.* ISBN 978-1-904455-14-1.

Mintzer, V., Moran-Gilad, J., and Simon-Tuval, T. (2019). Operational models and criteria for incorporating microbial whole genome sequencing in hospital microbiology – A systematic literature review. *Clin. Microbiol. Infect.* 25, 1086–1095. doi: 10.1016/j.cmi.2019.04.019

Mzia Kutateladze and Revaz Adamia (2010). Bacteriophages as potential newtherapeutics to replace or supplement antibiotics. Trends in Biotechnology, 28-12.

Mzia, Kutateladze and Revaz, Adamia (2019). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in Biotechnology*, 28-12.

Mojica, F. J., and Rodriguez-Valera, F. (2016). The discovery of CRISPR in archaea and bacteria. *FEBS J.* 283, 3162–3169. doi: 10.1111/febs.13766

"Novel Phage Therapy Saves Patient with Multidrug-Resistant Bacterial Infection". UC Health – UC San Diego. Retrieved 13 May 2018

Oechslin, F. (2018). Resistance development to bacteriophages occurring during bacteriophage therapy. *Viruses* 10:351. doi: 10.3390/v10070351

Oliveira, P. H., Touchon, M., and Rocha, E. P. (2014). The interplay of restriction-modification systems with mobile genetic elements and their prokaryotic hosts. *Nucleic Acids Res.* 42, 10618–10631. doi: 10.1093/nar/gku734

Otsuka, Y., and Yonesaki, T. (2012). Dmd of bacteriophage T4 functions as an antitoxin against *Escherichia coli* LsoA and RnlA toxins. *Mol. Microbiol.* 83, 669–681. doi: 10.1111/j.1365-2958.2012.07975.x

Prescott, L. (1993). Microbiology, Wm. C. Brown Publishers, ISBN 0-697-01372-3

Ptashne M. Lambda's switch: lessons from a module swap. Curr Biol. 2006 Jun 20;16(12):R459-62.

Pinilla-Redondo, R., Mayo-Muñoz, D., Russel, J., Garrett, R. A., Randau, L., Sørensen, S. J., *et al.* (2020). Type IV CRISPR-Cas systems are highly diverse and involved in competition between plasmids. *Nucleic Acids Res.* 48, 2000–2012. doi: 10.1093/nar/gkz1197

Rando, O. J., and Verstrepen, K. J. (2007). Timescales of genetic and epigenetic inheritance. *Cell* 128, 655–668. doi: 10.1016/j.cell.2007.01.023

Rhoads, DD; Wolcott, RD; Kuskowski, MA; Wolcott, BM; Ward, LS; Sulakvelidze, A (June 2009). "Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial". Journal of Wound Care. ***18*** *(6): 237–8, 240–3. doi:10.12968/jowc.2009.18.6.42801. PMID 19661847.*

Reimann, V., Ziemann, M., Li, H., Zhu, T., Behler, J., Lu, X., et al. (2020). Specificities and functional coordination between the two Cas6 maturation endonucleases in Anabaena sp. PCC 7120 assign orphan CRISPR arrays to three groups. *RNA Biol.* 17, 1442–1453. doi: 10.1080/15476286.2020.1774197

Rho, M., Wu, Y. W., Tang, H., Doak, T. G., and Ye, Y. (2012). Diverse CRISPRs evolving in human microbiomes. *PLoS Genet.* 8:e1002441. doi: 10.1371/journal.pgen.1002441

Simmonds P, Aiewsakun P. Virus classification - where do you draw the line? Arch Virol. 2018 Aug;163(8):2037-2046.

Schooley, R. T., Biswas, B., Gill, J. J., Hernandez-Morales, A., Lancaster, J., Lessor, L., et al. (2017). Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrob. Agents Chemother.* 61:e00954-17. doi: 10.1128/AAC.00954-17

Shen, J., Lv, L., Wang, X., Xiu, Z., and Chen, G. (2017). Comparative analysis of CRISPR-Cas systems in Klebsiella genomes. *J. Basic Microbiol.* 57, 325–336. doi: 10.1002/jobm.201600589

Shmakov, S. A., Utkina, I., Wolf, Y. I., Makarova, K. S., Severinov, K. V., and Koonin, E. V. (2020). CRISPR arrays away from cas genes. *CRISPR J.* 3, 535–549. doi: 10.1089/crispr.2020.0062

Sievers, F., and Higgins, D. G. (2014). Clustal Omega, accurate alignment of very large numbers of sequences. *Methods Mol. Biol.* 1079, 105–116. doi: 10.1007/978-1-62703-646-7_6

Song, S., and Wood, T. K. (2020). A primary physiological role of toxin/antitoxin systems is phage inhibition. *Front. Microbiol.* 11:1895. doi: 10.3389/fmicb.2020.01895

Stern, A., and Sorek, R. (2011). The phage-host arms race: shaping the evolution of microbes. *Bioessays* 33, 43–51. doi: 10.1002/bies.201000071

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Twort, F. (1930). "Filter-Passing Transmissible Bacteriolytic Agents (Bacteriophage)". The Lancet. ***216*** *(5594): 1064–1067. doi:10.1016/S0140-6736(00)86452-1*

Van Houte, S., Buckling, A., and Westra, E. R. (2016). Evolutionary ecology of prokaryotic immune mechanisms. *Microbiol. Mol. Biol. Rev.* 80, 745–763. doi: 10.1128/MMBR.00011-16

Vasu, K., and Nagaraja, V. (2013). Diverse functions of restriction-modification systems in addition to cellular defence. *Microbiol. Mol. Biol. Rev.* 77, 53–72.

Wommack, K. E.; Colwell, R. R. (2000). "Virioplankton: Viruses in Aquatic Ecosystems". Microbiology and Molecular Biology Reviews. ***64*** *(1): 69–114. doi:10.1128/MMBR.64.1.69-114.2000. PMC 98987. PMID 10704475.*

Wright, A.; Hawkins, C.H.; Änggård, E.E.; Harper, D.R. (August 2009). "A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy". Clinical Otolaryngology. ***34*** *(4): 349–357.* doi:10.1111/j.1749-4486.2009.01973.x. PMID 19673983

Wang, X., Loh, B., Gordillo Altamirano, F., Yu, Y., Hua, X., and Leptihn, S. (2021). Colistin-phage combinations decrease antibiotic resistance in *Acinetobacter baumannii* via changes in envelope architecture. *Emerg. Microbes Infect.* 10, 2205–2219.

Wei, Y., Gao, Z., Zhang, H., and Dong, Y. (2016). Structural characterizations of phage antitoxin Dmd and its interactions with bacterial toxin RnlA. *Biochem. Biophys. Res. Commun.* 472, 592–597. doi: 10.1016/j.bbrc.2016.03.025

Wright, A. V., Wang, J. Y., Burstein, D., Harrington, L. B., Paez-Espino, D., Kyrpides, N. C., *et al*. (2019). A functional mini-integrase in a two-protein-type V-C CRISPR system. *Mol. Cell* 73, 727–737.e723. doi: 10.1016/j.molcel.2018.12.015

Yang, Y., Shen, W., Zhong, Q., Chen, Q., He, X., Baker, J. L., et al. (2020). Development of a bacteriophage cocktail to constrain the emergence of phage-resistant *Pseudomonas aeruginosa*. *Front. Microbiol.* 11:327. doi: 10.3389/fmicb.2020.00327

Zhang, Q., and Ye, Y. (2017). Not all predicted CRISPR-Cas systems are equal: isolated cas genes and classes of CRISPR like elements. *BMC Bioinformatics* 18:92. doi: 10.1186/s12859-017-1512-4

Zhang, Q., Doak, T. G., and Ye, Y. (2014). Expanding the catalog of cas genes with metagenomes. *Nucleic Acids Res.* 42, 2448–2459. doi: 10.1093/nar/gkt1262

Zhang, R., Ou, H. Y., Gao, F., and Luo, H. (2014). Identification of horizontally-transferred genomic islands and genome segmentation points by using the GC profile method. *Curr. Genomics* 15, 113–121. doi: 10.2174/1389202915999140328163125

Zhang, Y., Zhang, Z., Zhang, H., Zhao, Y., and Xiao, J. (2020). PADS Arsenal: a database of prokaryotic defence systems related genes. *Nucleic Acids Res.* 48, D590–D598. doi: 10.1093/nar/gkz916

Zhou, K., Aertsen, A., and Michiels, C. W. (2014). The role of variable DNA tandem repeats in bacterial adaptation. *FEMS Microbiol. Rev.* 38, 119–141. doi: 10.1111/1574-6976.12036