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CENTRO DE INVESTIGACIÓN EN BIOINGENIERÍA UTEC

CONCYTEC

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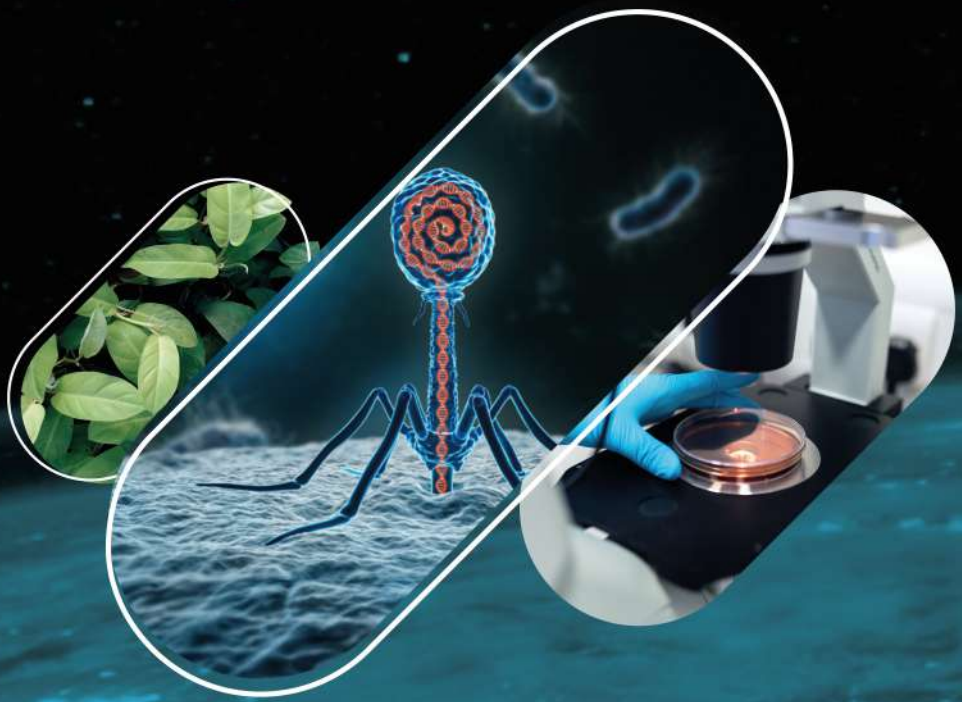
Programa Nacional de Investigación Científica y Estudios Avanzados

FIRST INTERNATIONAL CONGRESS OF

Bacteriophages and their Applications for the Environment

Real world solutions with bacteriophages technology



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First International Congress of Bacteriophages and their Applications for the Environment (FICBAE)

Jr. Medrano Silva 165, Barranco, Lima, Perú
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Contents

Welcome to FICBAE 2023

Welcome to The First International Congress of Bacteriophages and their Applications for the Environment (FICBAE). This conference aims to connect with various technology-related universities, agencies, industries, start-ups, and academia to cover topics related to national and international studies of bacteriophages, focusing on efforts for environmental solutions.

Bacteriophages are remarkable viruses that exclusively infect bacteria. These microscopic entities are natural predators of bacteria and are pivotal in shaping microbial ecosystems. The event aims to review the use of bacteriophages for environmental solutions and their impact in Peru and the South America region.

The Congress FICBAE 2023 consists of 4 workshops, 6 keynote talks, 11 specialized talks, and 2 poster sessions. They are organized into 7 topics:

- Phage–based nanomaterials
- Genetic modification of bacteriophages
- Biosecurity
- Bacteriophages in the food industry
- Phage therapy in aquaculture
- Phage therapy in agriculture
- Phage genome analysis

In addition, we have distinguished invited speakers from Peru, Mexico, USA, and Argentina for the keynote seminars. We would like to thank all the researchers, students, and individuals for their contributions. This event is organized by the Bioengineering program and the Bioengineering Research Center, CentroBio, at UTEC University, with the support of PROCIENCIA.

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Topics

Phage-based nanomaterials

Innovative biotechnological materials engineered from bacteriophages, offering versatile applications in fields such as drug delivery and tissue engineering.

Genetic modification of bacteriophages

Involves altering the genetic makeup of these viruses to enhance their effectiveness in targeted bacterial infection control, contributing to advancements in biotechnology and medicine.

Biosecurity

Encompasses strategies and measures aimed at safeguarding biological materials, research, and facilities to prevent misuse or unauthorized access to potentially harmful agents, ensuring the responsible practice of science.

Bacteriophages in the food industry

Being explored as natural antimicrobial agents to combat bacterial contamination, offering a promising solution to improve food safety and quality. Also, bacteriophages are pathogenic agents against lactic acid bacteria from dairy industry and phage monitoring and control is a common practice.

Phage therapy in aquaculture

Involves the use of bacteriophages to control bacterial infections in aquatic organisms, reducing the reliance on antibiotics and promoting sustainable practices in the aquaculture sector.

Phage therapy in agriculture

An emerging approach using bacteriophages to combat plant pathogenic bacteria, offering an environmentally friendly alternative to traditional chemical pesticides.

Phage genome analysis

The process of sequencing and studying the genetic information of bacteriophages, providing insights into their diversity, evolution, and potential applications in various fields of science and biotechnology.

Program

DAY 1

8:45 - 9:15	Words of welcome Dr. Alberto Donayre Torres | UTEC - Peru
9:15 - 10:00	Keynote Talk: "Biocontrol of plant diseases through plant phage therapy" Dr. Gabriel Rincón Enríquez | Centro de Investigación CIATEJ - Mexico
10:00 - 10:30	Phage infections in the dairy industry Dra. Mariángeles Brigglier Marcó | Universidad Nacional del Litoral - Argentina
10:30 - 11:00	**Coffee Break**
11:00 - 11:30	Environmental phages in foods Dra. Maryoris E. Soto Lopez | Universidad de Córdova - Colombia
11:30 - 12:00	Use of bacteriophages to combat bacterial canker on kiwis Dr. Roberto Bastías Romo | Pontificia Universidad Católica de Valparaíso - Chile
12:00 - 12:45	Discussion Panel: Bacteriophages in the environment and proposed solutions - What are the challenges for food and agriculture?
12:45 - 14:00	**Lunch**
14:00 - 14:15	Words of welcome Javier Bustamante | UTEC - Peru
14:15 - 14:30	Venue Dr. Julio Valdivia | UTEC - Peru
14:30 - 14:45	Congress Overview (Objectives & MISTI | UTEC Program) Dr. Alberto Donayre
14:45 - 15:30	Keynote Talk: "Bacteriophages for real world applications and environmental solutions" Dr. Angela Belcher | MIT - USA
15:30 - 16:00	Bacteriophages and heavy metals at UTEC Dr. Alberto Donayre | UTEC - Peru
16:00 - 16:30	**Coffee Break**
16:30 - 17:15	Keynote Talk: "Phages and Biomaterials for bioremediation" Dr. Nimrod Heldman | MIT - USA
17:15 - 17:45	Scale-up systems for bacteriophage production Msc. Jay Sacane | USA
17:45 - 18:15	Discussion Panel: Bacteriophages and bioremediation with biomaterials
18:15 - 18:30	**Poster hanging**
18:30 - 19:45	**Poster Session**

DAY 2

9:30 - 11:00	1st Workshop Using of Molecular Cloning Techniques for phage genome (vector M13KE) editing Dr. Nimrod Heldman | MIT - USA
11:00 - 11:30	**Coffee Break**
11:30 - 14:00	2nd Workshop Amplification of recombinant phages at high scale. Purification using centrifugation and chemical precipitation Dr. Nimrod Heldman | MIT - USA
14:00 - 15:30	**Lunch**
15:30 - 16:10	Keynote Talk: "Bacteriophages in the environment experiences in Perú" Dr. Miguel Talledo | UNMSM - Peru
16:10 - 16:40	Detection, isolation and characterization of bacteriophages against pathogens of farmed fish. Dra. Violeta Flores | IMARPE - Peru
16:40 - 17:10	Phagotherapy in Aquaculture and experiences at the Instituto del Mar del Perú. Dra. Carla Ivonne Fernandez Espinel | IMARPE - Peru
17:10 - 17:50	Keynote Talk: "The Comeback of Phage – Episode II" Dr. Pohl Milón | UPC - Peru
17:50 - 18:20	**Coffee Break**
18:20 - 19:00	Discussion Panel: Experiences with bacteriophages in Peru and regulations for their use
19:00 - 19:45	**Poster Session**

DAY 3

9:00 - 10:30	3rd Workshop Transformation of phage genome into XL1-Blue cells using chemical transformation Msc. Jay Sacane | USA
10:30 - 11:00	**Coffee Break**
11:00 - 12:30	4th Workshop Titering of M13 bacteriophages for selection of cells expressing phages Quantification of phage concentration using a spectrophotometer Msc. Jay Sacane | USA
12:30 - 14:00	**Lunch**
14:00 - 14:45	Keynote Talk: "Bacteriophages and endolysins active against Staphylococcus aureus: from the counter to the patient" Dr. Hector Morbidoni | Argentina
14:45 - 15:20	Genomic characterization and bioinformatics analysis of bacteriophages Dr. Cristian Suarez | CONICET - Argentina
15:20 - 16:00	Regulations associated with the use of bacteriophages in Peru: Biosafety and access to genetic resources. Dr. David Eduardo Castro | MINAM - Peru
16:00 - 16:30	**Coffee Break**
16:30 - 17:00	Discussion Panel: Genetic diversity of bacteriophages and genetic manipulations
17:00 - 17:30	**Poster Award**
18:00 - 19:00	**Event Closing**

Workshops description

Workshop 1: Using of Molecular Cloning Techniques for phage genome (vector M14KE) editing

Workshop 1 offers an exciting exploration of Molecular Cloning Techniques, focusing on phage genome editing using the M14KE vector. This workshop covers the innovative Gibson assembly technique, enabling seamless DNA fragment assembly with an efficient enzyme mix. Participants will gain hands-on experience with tools to unlock the potential of molecular cloning in research and biotechnology.

Workshop 2: Amplification of recombinant phages at high scale. Purification using centrifugation and chemical precipitation

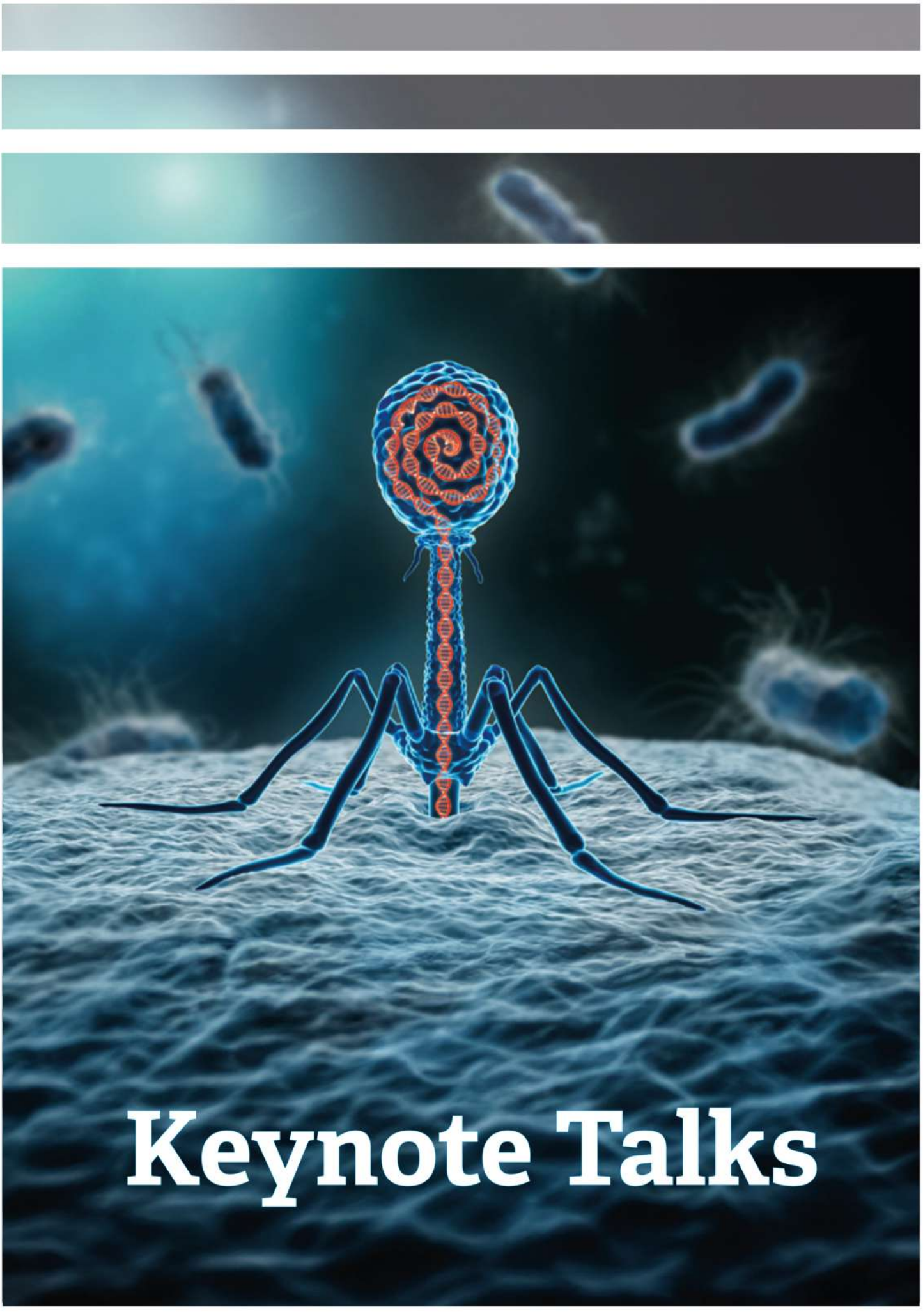
Workshop 2 immerses participants in high-scale recombinant phage amplification and purification techniques using centrifugation and chemical precipitation. This workshop encompasses key experiences for bacterial selection and gene expression studies, providing practical skills vital for molecular biology and biotechnology applications.

Workshop 3: Transformation of phage genome into XL1-Blue cells using chemical transformation

The workshop begins with an experience centered on phage titering, a technique used to assess bacteriophage concentration. This involves serial dilutions of a genetically transformed bacterial culture and plating on agar plates to visualize phage plaque formation. Participants will gain practical insights into the phage titering process and the subsequent steps for phage plating using Dilution tube 8 ($1 : 10^8$), preparing them for hands-on molecular biology applications.

Workshop 4: Titering of M13 bacteriophages for selection of cells expressing phages Quantification of phage concentration using a spectrophotometer

This workshop covers the process of amplifying and purifying bacteriophages, specifically M13KE. The key steps include creating overnight cultures, amplifying the phages, and conducting purification rounds. The final stage involves quantifying phage concentration using a NanoDrop. This workshop provides essential techniques for producing high-quality phage samples.



Keynote Talks

Biocontrol of plant diseases through plant phage therapy

Dr. Gabriel Rincón Enríquez | Centro de Investigación CIATEJ - Mexico

Video

Plant phage therapy focuses on controlling phytopathogenic microorganisms that can lead to 100% crop losses. Alternatives to chemical antibiotics and antifungals involve biological control, such as the use of bacteriophages, viruses that target bacteria, known for their specificity, abundance, and lack of contamination. Laboratory studies evaluated bacteriophages like ΦXaF18, which demonstrated resistance to UV light in specific formulations (F2 and F4). In greenhouse trials, these phages reduced diseases by up to 95%. Additionally, research into ΦXaF13 aimed to identify genes conferring UV resistance and enhance virulence. Both phages were combined to create "Fagolytic", a product competitive with commercial antibiotics and antifungals. Production scaled from 0.5L to 100L, with pricing adaptable to the agricultural market.

Figure 1. Fagolytic

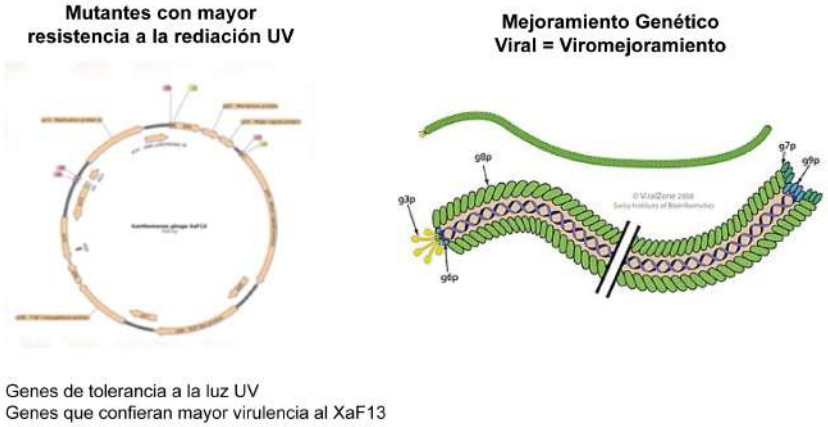


Figure 2. Viral breeding

Bacteriophages for real world applications and environmental solutions

Dr. Angela Belcher | Massachusetts Institute of Technology - USA

Video

Genetic Control of Materials explores how organisms utilize their genetic information to create protective shields and hard materials. This research delves into the potential of leveraging genetic data for the development of batteries and solar cells, with particular attention to the promising role of bacteriophages in this domain. The laboratory's diverse research interests encompass the fields of energy, healthcare, and environmental applications, all revolving around the central theme of utilizing biological processes to engineer nano-scale materials. In this context, the unique characteristics of phages come to the forefront, as their high manipulability makes them indispensable tools for gene modification. Phages are harnessed to cultivate materials for electric batteries, offering enhanced capacity and reaction rates. They find applications in energy storage, conversion, and environmental remediation, making them versatile assets. Beyond these areas, phages also show promise in cancer research, further underscoring their adaptability and potential as platforms for diverse functionalities.

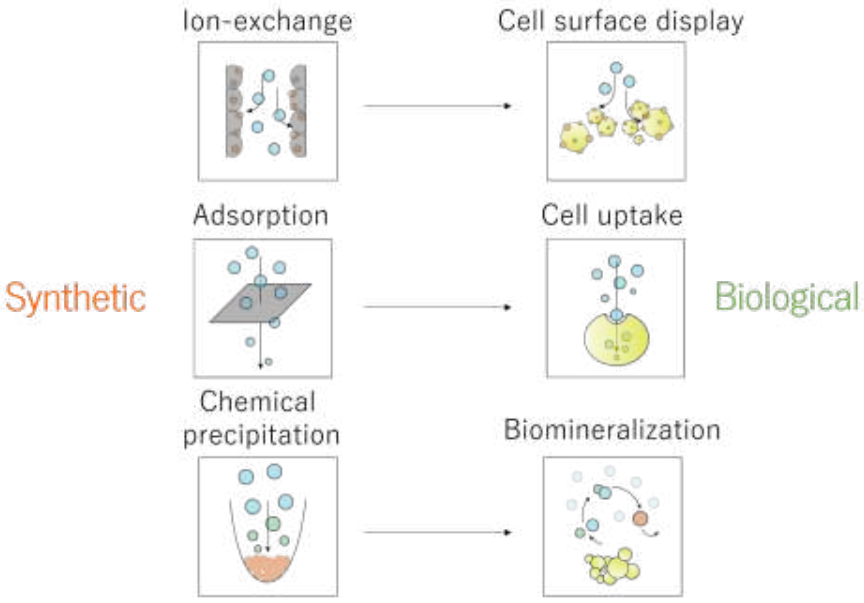


Figure 3. Synthetic vs. Biological metal remediation

Phages and Biomaterials for bioremediation

Dr. Nimrod Heldman | Massachusetts Institute of Technology - USA

Video

This study highlights the versatility of genetic manipulation, focusing on bacteriophages, to transform material characteristics for applications in medicine, energy, imaging, and environmental solutions. Emphasizing the controlled engineering of specific phage components, the research explores real-world applications, including pollution control, climate change mitigation, and viral deactivation. Of particular significance are carbon phage nanofibers that absorb and break down pollutants, providing avenues for protective clothing and virus filtration, notably in the context of the SARS-CoV-2 pandemic. Additionally, the study delves into the realm of hydrogels, using phage modification for light controlled crosslinking to create 3D objects. These hydrogels can integrate therapeutic proteins for potential medical applications, further showcasing the innovative potential of genetic control in material science.

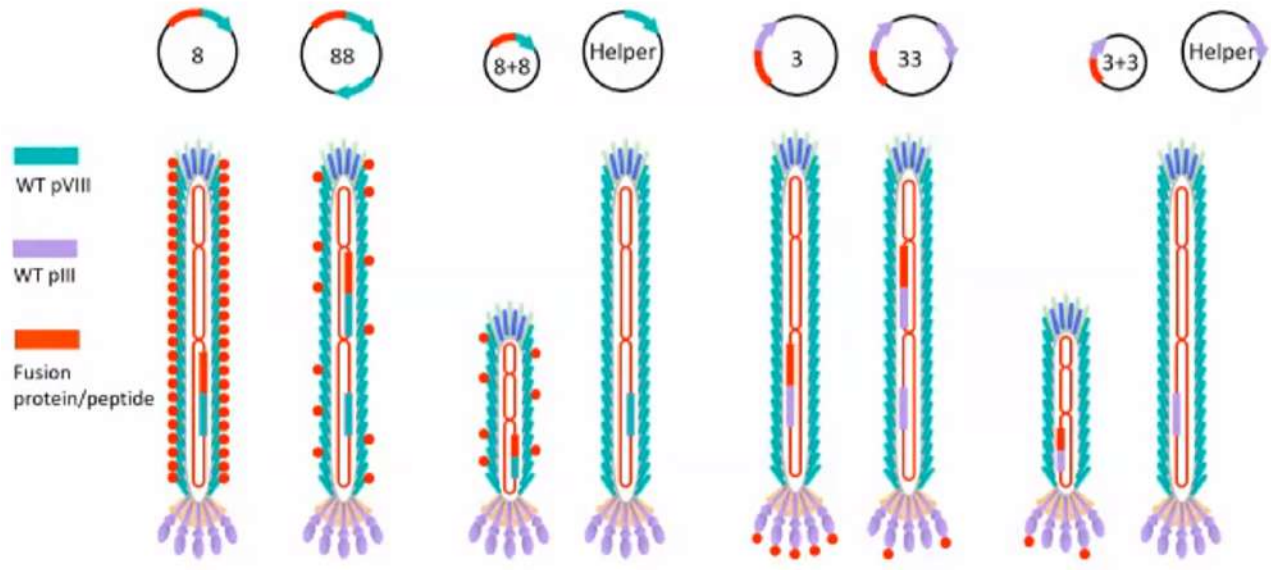


Figure 4. Synthetic vs. Biological metal remediation

Bacteriophages in the environment experiences in Peru

Dr. Miguel Talledo | Universidad Nacional Mayor de San Marcos - Peru

Video

Established at La Chira beach during a 1990s cholera epidemic in Peru, the Basic Characterization Laboratory in Biotechnology for Remediation initially focused on practical Vibrio detection using bacteriophage mixtures, with an emphasis on detection rather than comprehensive characterization. Subsequent research led to the isolation of bacteriophages like K14 *Vibrio cholerae*, which showed resistance to chloroform and low infection rates. Phages of Listeria (ASCF1) with high temperature and pH tolerance were also isolated, along with other phages like FS01 from *Salmonella typhimurium*. Emphasizing the importance of morphology in characterization, the team also explored phage adsorption on specialized substrates like degradable polyester microfibers loaded with bacteriophages, aiming to standardize substrate applications, with bacteriophages remaining a central focus in their research efforts.

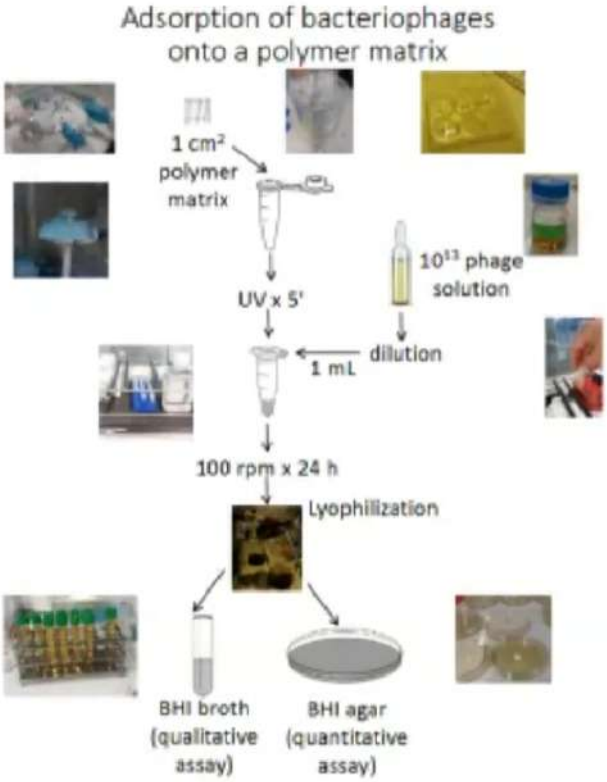


Figure 5. Adsorption of bacteriophages

The Comeback of Phage – Episode II

Dr. Pohl Milón | Universidad Peruana de Ciencias Aplicadas - Peru
Video

Phage therapy, a potential solution for antibiotic resistance, employs phages as carriers for resistance genes. Strategies include early resistance gene detection and novel antibiotic development. Certain phages, like P4, require coexisting phages for replication. Mobile genetic elements play a pivotal role in resistance gene dissemination, prompting environmental impact mitigation efforts. Pandemic-related systems detecting SARS-CoV-2 have been repurposed for resistance gene detection, with a national supply system now established. Collaborative research highlights integron significance in gene transfer. Ongoing research involves river sampling in Oxapampa using mobile detection systems. The incorporation of CRISPR-Cas technology adds specificity to the process, with research exploring its application in natural environments. Undergraduate students actively participate in sample extraction and analysis. Antibiotic development involves conformational change sensor modification and M13 phage-based phage display technology. Combinatorial nucleic acid technologies are also under exploration. Anthropogenic markers signify resistance genes evolving due to human influence, modifying mobile genetic elements.

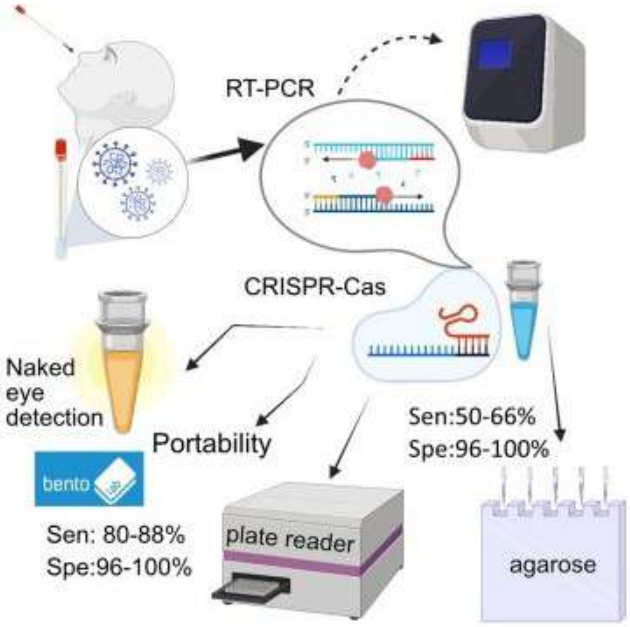


Figure 6. SARS-CoV-2 detection

Bacteriophages and endolysins active against Staphylococcus aureus: from the counter to the patient

Dr. Hector Morbidoni | Rosario National University - Argentina

Video

Bacteriophages, abundant biological entities, have been instrumental in advancing biological research and are increasingly harnessed for various applications, including capturing bacteria and biosensing. Argentina has made notable contributions to bacteriophage sequencing. In contrast, endolysins, responsible for bacterial lysis, offer versatility in targeting peptidoglycan domains, making them attractive for therapy. Phages have been isolated from environmental samples, showcasing genetic homology with known phage genes in Latin America. Their potential application in therapy, while showing some variations, is promising. Research on temperate phages facilitates comparative genomics and therapeutic development for diverse strains. Detecting proteins within phage structures is vital for tool development and modification, enhancing their role in proactive research. Organized endolysin domains hold potential for therapeutic development, offering adaptability in recognition and enzymatic activities, shaping the evolving landscape of phage-based solutions.

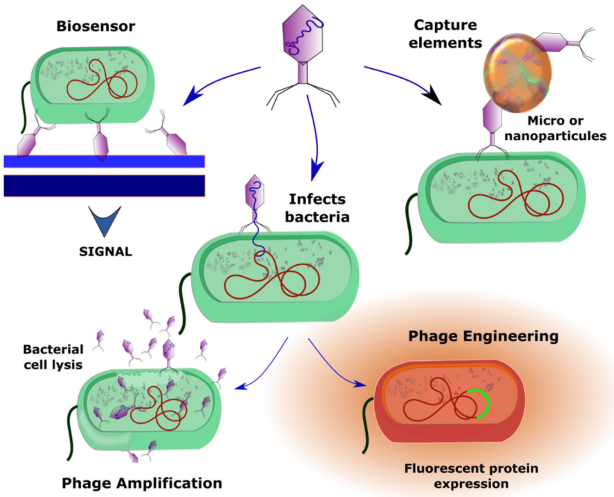
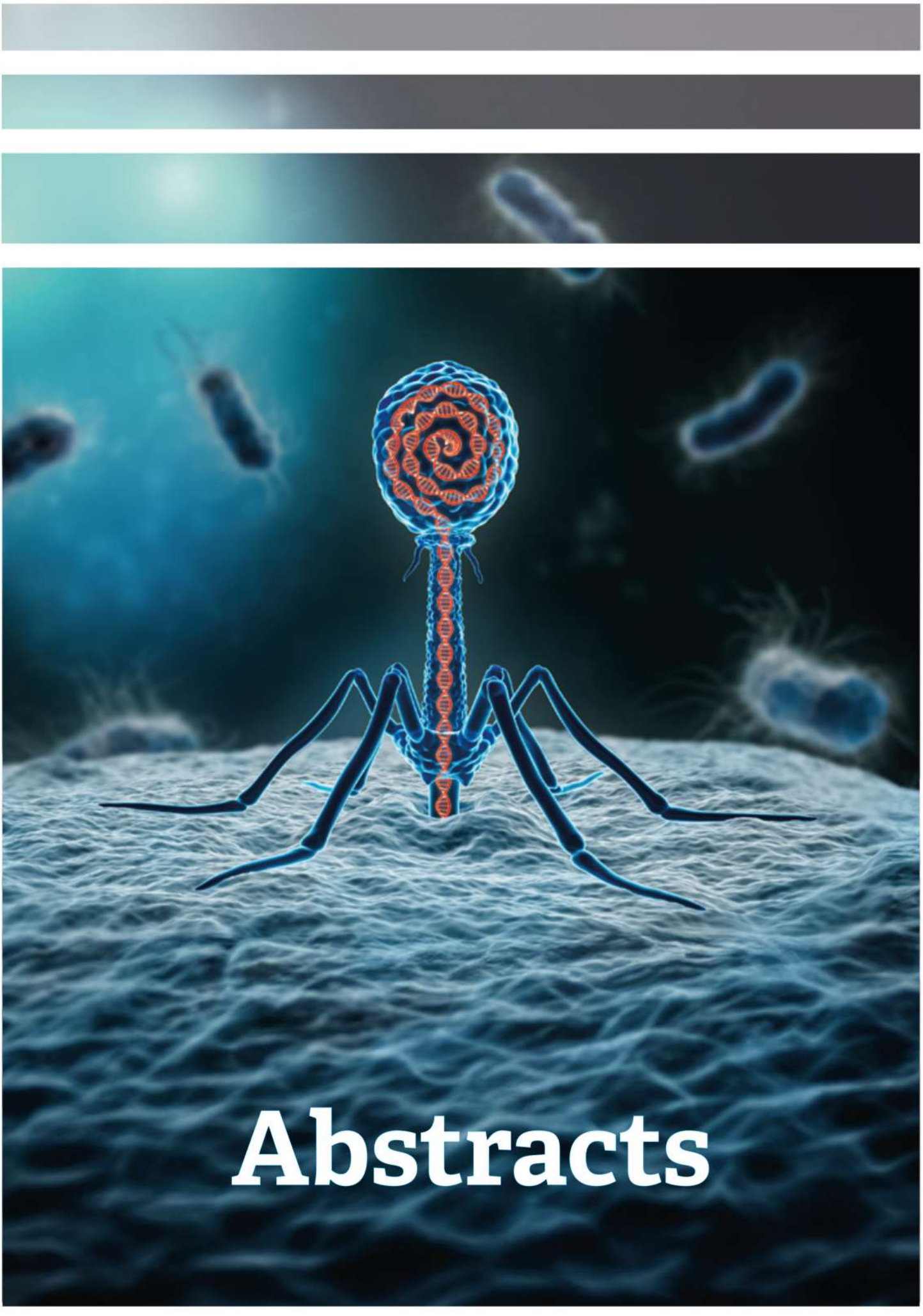


Figure 7. Bacteriophage-based methods for specific bacterial detection



Abstracts

Genetic improvement of bacteriophage φ*XaF13* for the biological control of bacterial spot in peppers

Rubén Antonio Olivares-Terrones [1]
Evangelina Esmeralda Quiñones-Aguilar [1]
Guillermo Alejandro Solís-Sánchez [1]
María Inés Siri Tomás [2]
Gabriel Rincón-Enríquez [1]

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco[1]
Universidad de la República[2]

Abstract:
Xanthomonas is a genus of Gram-negative bacilli, phytopathogenic in more than 400 species. Bacteriophages are viruses that depend on bacterial cells to propagate. These have increasingly attracted attention for their potential for biological control of diseases caused by Xanthomonas. To generate effective biological control strategies, it is necessary to have phages that meet a series of characteristics that allow them to adapt to conditions, so the variety of wild phages may not be sufficient. For this reason, a useful strategy is to improve these phages through random mutagenesis and select those with the best characteristics of resistance to a variety of conditions and biological control. Phage φ*XaF13* is a lytic life cycle inovirus with good characteristics for the biological control of bacterial spot in peppers and this work it is aimed to improve it with radiation mutagenesis.

Keywords: *Xanthomonas spp.*, phages, mutagenesis

Engineering p8 protein from *M13 bacteriophage* with high-affinity heavy metal-binding peptides

Jose M. Cisneros Mandujano [1]
Gladys M. Cavero Rozas [1]
Nimrod Heldman [2]
Jifa Qi [2]
Angela M. Belcher [2]
Alberto J. Donayre-Torres [1]

Universidad de Ingeniería y Tecnología[1]
Massachusetts Institute of Technology[2]

Abstract:
This study aims to address heavy metal contamination in water using engineered phage as adsorbent for contaminated water treatment. The objective was to engineer the M13 phage to display along phage surface binding peptides with high affinity to heavy metals. Three peptides with high affinity to Pb(II), Cr(III), and Cd(II) were inserted into the M13 Phage P8 protein gene. The successful genetic editing was confirmed through Sanger sequencing. Transmission electron microscopy (TEM) confirmed structural viability of Pb, Cr, and Cd phages remaining a stable and flexible structure, filamentous shape and a suitable length. TEM revealed phage's structural stability and resilience after Pb(II) exposure and suggested lead adsorption and mineralization along the phage surface. These findings offer promising prospects using engineered M13 phage for heavy metal adsorption from contaminated aqueous solutions. The proposed presents a viable approach for mitigating heavy metal contamination and promoting environmental sustainability.

Keywords: M13 bacteriophage; water remediation; heavy metal ions

Isolation of lytic *Raoultella* bacteriophage

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Mónica Velarde [1]
Ana-Isabel-Flor Gutierrez-Román[1]

Universidad Nacional Federico Villarreal[1]

Abstract:
Certain species within *Raoultella* and *Klebsiella* genera have become increasingly important due they have been identified as pathogens able to infect plants, some mammals, and humans. As an alternative to antibiotics for bacterial control, several studies have reported the potential use of bacteriophages. The main of this study is to report the isolation of a lytic bacteriophage (vB_Rte) and its bacterial host BP3, which was identified as a Raoultella terrigena strain based on 16S rRNA sequencing. The bacterial host was isolated from a potato with soft rot and the bacteriophage from an agricultural sample using double-layer agar and spot assays. The vB_Rte was able to inhibit bacterial growth of *Raoultella terrigena* up to MOI 10^{-6} but was unable to infect other bacteria causing soft rot such as *Klebsiella variicola* and *Klebsiella oxytoca* both isolates from banana wet rot disease and *Pectobacterium coratovorum* isolated from potato with soft rot.

Keywords: Bacteriophage; *Raoultella terrigena*; Bacteria

Isolation and characterization of a lytic bacteriophage isolate from chicken intestine and specific for Multidrug-resistant *Salmonella infantis*

Arlene Cuyutupa-Guillen [1]
Renzo Punil-Luciano [1]
Egma Mayta-Huatuco [1]
Deysy Ramirez-Soto[1]
Enrique Mamani-Zapana [1]

National University of San Marcos[1]

Abstract:
Background: The aims were to isolate and characterize a lytic bacteriophage from markets in Lima, Peru, specific for multidrug-resistant *Salmonella infantis*; **Methods:** Phage 52 was isolated by enrichment, processed by spot test and was purified by the double layer method. It was evaluated under different conditions of pH, temperature and exposure to chloroform for 1 hour. It was stored at 25, 4 and -20 °C for 4 months. The optimal multiplicity of infection (MOI) was determined and the one-step growth curve was performed; **Results:** Bacteriophage 52 is specific for *S. infantis MDR*, has an optimal MOI of 0.001, is stable at temperatures from 40 to 60 °C and at pH values from 3 to 10. It showed tolerance to chloroform and has storage stability at temperatures of 4 and -20 °C. Phage 52 has a latency period of 10 minutes and a burst size of 32,93 PFU/cell; **Conclusions:** Phage 52 has great potential to be applied as a control agent for strains for multiresistant *Salmonella infantis* strains.
Keywords: Bacteriophage; Characterization; *Salmonella infantis MDR*

Morphological and molecular characterization of a lytic bacteriophage isolated from soils from bean crops

Erika Camacho Beltran [1,2]
Gabriel Rincón Enríquez [1]
Melina López Meyer [2]
Evangelina Esmeralda Quiñones Aguilar [1]

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco[1]
Instituto Politécnico Nacional[2]

Abstract:
Given the need to address the biological control of bacterial phytopathogens with a sustainable approach that reduces the use of copper salts and antibiotics, the use of bacteriophages has been considered a strategy in the area of agriculture. Said development depends on the molecular and biological characteristics, therefore, a study of the genome and biology of lytic phages is essential for the development of bacteriophages as biological control agents. The objective was to characterize morphologically and molecularly lytic bacteriophages of bean crops. A bacteriophage of bean bacterial strains from Sinaloa was isolated. Genetic characterization by PCR-RFLPs and morphological characterization by TEM was performed. The lytic bacteriophage φ25-4 measured 37.25 nm from vertex to vertex of the capsid and its genome was single-stranded, circular DNA. This bacteriophage could be used for the biological control of phytopathogenic bacteria that worsen the health of beans in Sinaloa, Mexico.

Keywords: Biological control, phage, *Phaseolus vulgaris*

Isolation of lytic bacteriophages against strains of *Klebsiella pneumoniae* and *Salmonella spp.* multidrug-resistant

Elsa Gladys Aguilar-Ancori [1,2]
Duly Nuñez-Carazas[1]
Enrique Mamani-Zapana [3]
Laura Campo-Pfuyo [1]
Marishani Marin-Carrasco [2]

National University of San Antonio Abad[1]
University Institute of Tropical Diseases and Biomedicine[2]
National University of San Marcos[3]

Abstract:
Background: This study aims to isolate lytic phages against *Klebsiella pneumoniae* and *Salmonella spp.* multidrug-resistant; **Method:** 50 strains of *K. pneumoniae* and 4 multidrug-resistant *Salmonella spp.* strains were characterized as ESBL and carbapenemases, coming from patients of the different hospitals of Cusco. The search for bacteriophages was carried out in the Huatanay River and processed by spot test and double layer. Results: We isolated 14 phages against *K. pneumoniae* and 13 for *Salmonella spp.* obtaining concentrations 3x109 PFU/mL. Likewise, 4 bacteriophages with greater lytic capacity were selected (36% on hospital strains of *K. pneumoniae*) and 03 bacteriophages (80% on Salmonella spp strains). Currently, these data are being processed by transmission electron microscopy for phages, whole genome sequencing using NGS for multi-resistant strains and phages. Conclusion: The liytic phages isolated have potential application in MDR infections against *Klebsiella pneumoniae* and *Salmonella spp.*.

Keywords: Bacteriophage1; *Klebsiella pneumoniae* MDR 2; *Salmonella spp.*MDR 3

Scaling of formulated bacteriophages for biological control of bacterial spot (*Xanthomonas euvesicatoria*) in pepper crops

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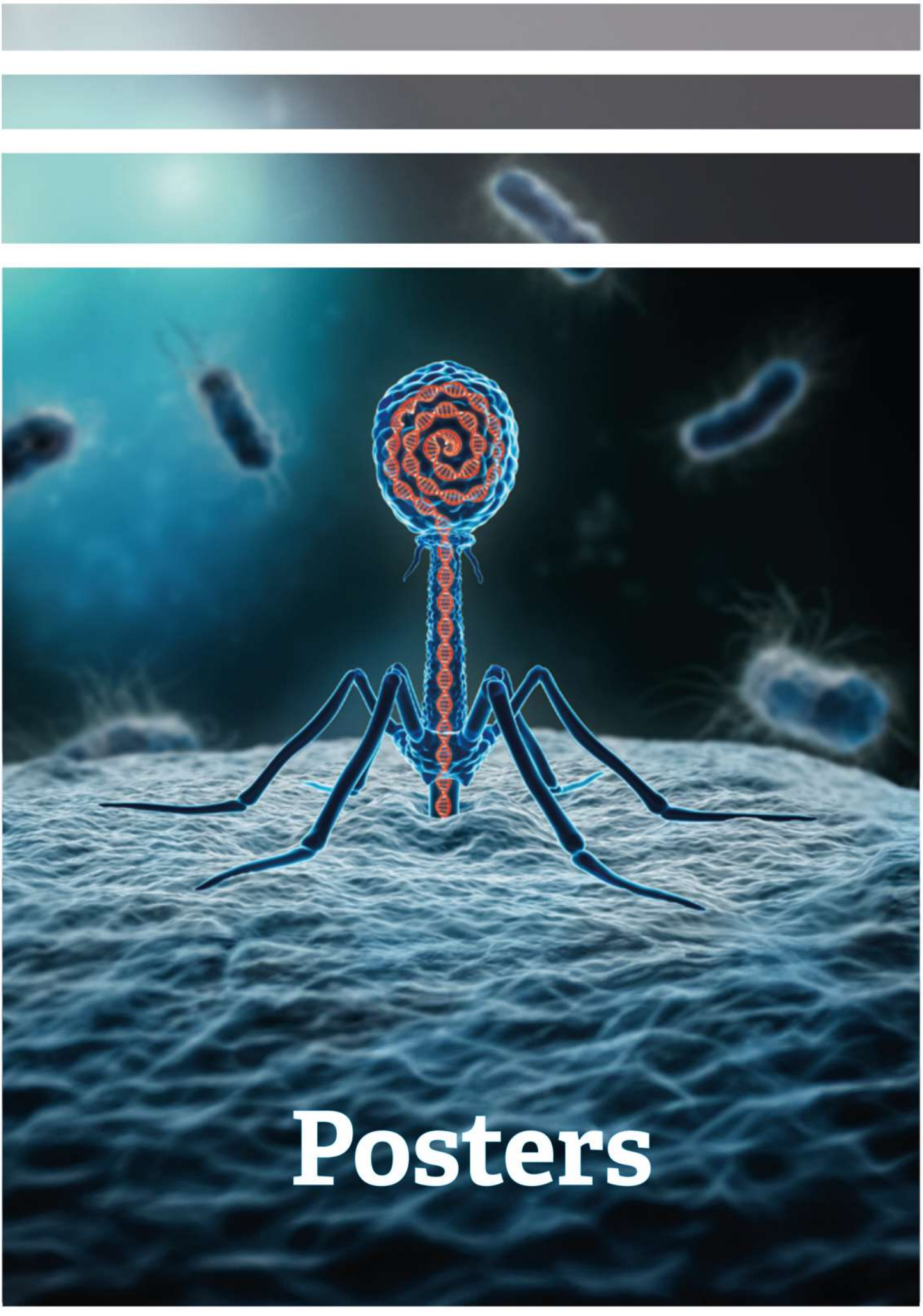
Evangelina Esmeralda Quiñones Aguilar [1]
Guillermo Alejandro Solís Sánchez [1]
Melchor Arellano Plaza [1]
Gabriel Rincón Enríquez [1]

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco[1]

Abstract:
The scaling of the photoprotective formulation called Fagolytic based on zinc nanoparticles was evaluated by measuring the growth of its bacteriophages (φXaF13 and φXaF18) in a pilot plant of 20 L and its biological effectivity for *Xanthomonas euvesicatoria* control in pepper crops at greenhouse conditions. The bacteriophages produced in 20 L bioreactors presented concentrations of $1x10^8$ UFP/mL y $1.8x10^8$ UFP/mL respectively. The statistical analysis was made using the methods of Tukey Pairwise Comparisons and Dunnet Multiple Comparisons with a Control. Both analyses showed that different symptoms behavior were statistically equal in the healthy plants $(control)$ and the plants treated with Fagolytic, such as chlorotic spots, necrotic spots, severity scale, and damaged leaf area $(p \leq 0.05)$. Given the statistical equality between the Fagolytic treatment, the present commercial preventive treatments and the control in different variables, the formulated bacteriophages show a promising alternative for bacterial spot control in pepper crops.

Keywords: Fagolytic; pepper crops, pilot plant



Posters

Genetic improvement of bacteriophage ϕXaF13 for the biological control of bacterial spot in peppers.

Rubén Antonio Olivares-Terrones[1], Evangelina Esmeralda Quiñones-Aguilar[1], Guillermo Alejandro Solís-Sánchez[1], María Inés Siri Tomás[2] and Gabriel Rincón-Enríquez[1]*

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GRAPHICAL ABSTRACT

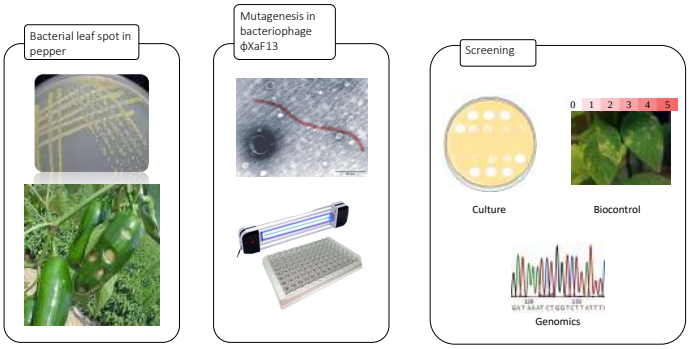

Figure 1: Graphical abstract

INTRODUCTION

Xanthomonas is a genus of Gram-negative bacilli, phytopathogenic in more than 400 species, including pepper (*Capsicum annuum*). Bacterial spot caused by *X. euvesicatoria* pv. *euvesicatoria*, *X. euvesicatoria* pv. *perforans, X. hortorum* pv. *gardneri* and *X. vesicatoria* is the bacterial disease that causes the greatest economic damage in pepper crops.

Control of these disease is currently managed in Mexico by cultural and chemical methods, like use of certified seed and cupper salts, but there is not commercially available Phage-based product.

Bacteriophages are viruses that depend on bacterial cells to propagate. These have increasingly attracted attention for their potential for biological control of diseases caused by *Xanthomonas spp.* To generate effective biological control strategies, it is necessary to have phages that meet a series of characteristics that allow them to adapt to in field conditions (Ibarra-Rivera, 2019).

Phage ϕXaF13

Phage ϕXaF13 is a filamentous bacteriophage of the *inoviridae* family with lytic life cycle, infecting to the phytopathogenic bacteria *X. euvesicatoria.* This phage is highly sensitive to chloroform and have a moderate resistance to UV radiation. Its genome consist of a 7,045-b scDNA encoding 12 ORFs in the positive stand and 2 in the negative strand (Juarez-García, 2022; Solis-Sanchez *et al.*, 2020).

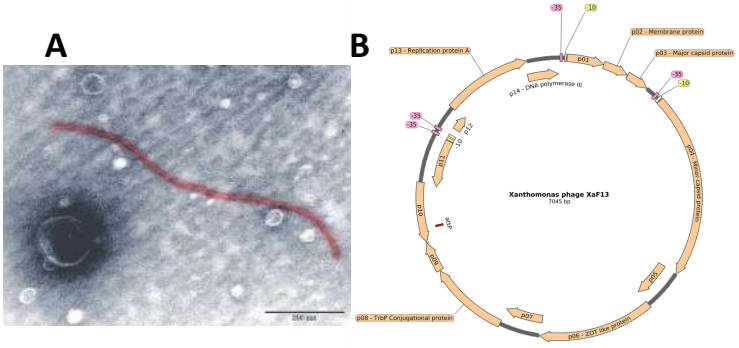

Figure 2. (a) TEM micrography of the phage ϕXaF13 (b) ϕXaF13 genoma organization (obtained from Solis *et al.*, 2020)

METHODS

In order to improve the characteristics of phage ϕXaF13 it will be irradiated with UV-c light to generate random mutations. The stability of the mutant phages will be screened at the in vitro culture level after exposure to various condition to find those more promising.

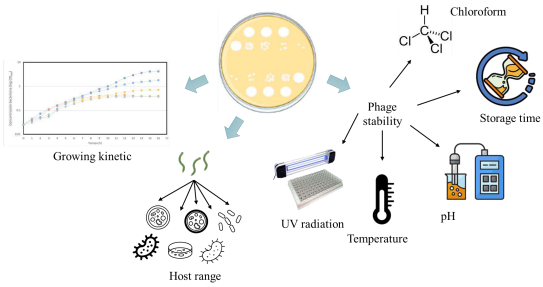

Figure 3. Culture characteristics that will be screened.

In addition to the microbiological culture characteristics, the genomes of the mutant phages will be sequenced to observe the exact sites where gene mutations occur and whether patterns are detected between the appearance of mutations in certain genes and changes in the characteristics of the bacteriophages.

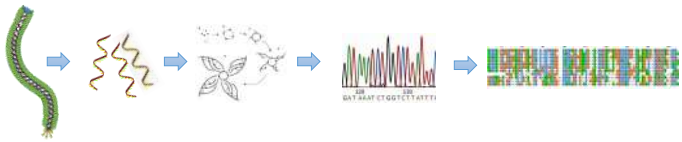

Figure 4. Schematics representation of the genomics analysis.

The ability of mutant phages to control bacterial leaf spot in pepper under greenhouse conditions will also be examined, it will be evaluated according to a severity index, as well as the development of the plant.

Figure 5. Schematics representation of the greenhose tests.

PERSPECTIVES

The results of this work will help to develop more effective phage-based formulations for the control of bacterial spot in pepper and will also help to understand the mechanism of infection of the ϕXaF13 phage and of the phages of the *inoviridae* family.

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Solís-Sánchez, Guillermo Alejandro, Evangelina Esmeralda Quiñones-Aguilar, Saul Fraire-Velázquez, Julio Vega-Arreguín, y Gabriel Rincón-Enríquez. 2020. "Complete Genome Sequence of XaF13, a Novel Bacteriophage of Xanthomonas vesicatoria from Mexico". Microbiology Resource Announcements 9(5). doi: 10.1128/MRA.01371-19.

Engineering p8 protein from M13 bacteriophage with high-affinity heavy metal-binding peptides

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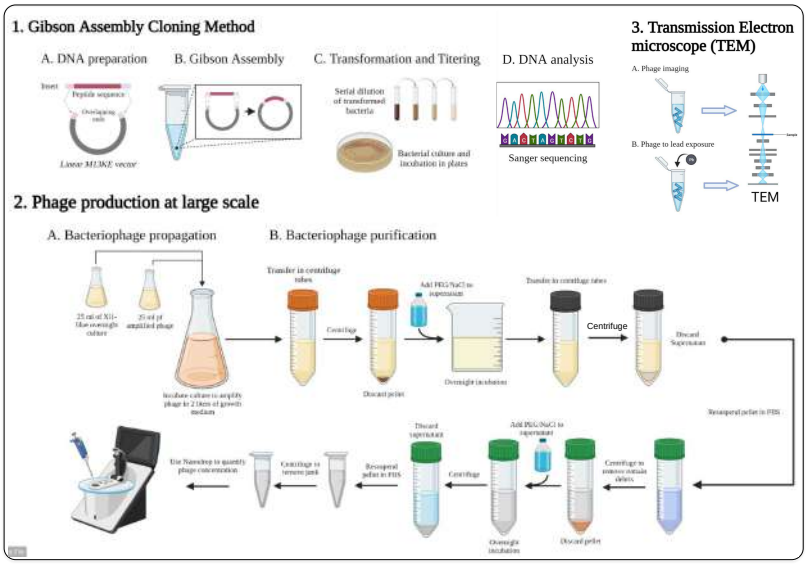
GRAPHICAL ABSTRACT



INTRODUCTION

Heavy metals, originating from industrial activities and natural sources, pose significant risks to human health and ecosystems when present in water, ground and air. Rural communities are particularly vulnerable to substantial exposure due to a high incidence of illegal mining activities [1]. According to the Peruvian Health Ministry (MINSA), it is estimated that one-third of the Peruvian population resides or works in proximity to sources of toxic compounds, including heavy metals in drinking water, which represents a critical exposure [1]. Even trace amounts of heavy metal intoxication in humans can lead to severe damage to important organs such as the brain, lungs and liver [2]. Current methods for removing heavy metals from wastewater include mostly ion-exchange, adsorption and membrane filtration; however, they are expensive and require complex equipment and infrastructure, or are inefficient to remove low concentrations of metal ions [3]. Developing efficient systems for heavy metal detection and removal holds immense potential to significantly improve the lives of millions of people affected by these pollutants.

The M13 bacteriophage is a filamentous virus that can be modified to create novel nanomaterials with different and useful properties to biomedicine, biosensing and energy applications. This versatility is possible through a combination of genetic editing and chemical modification on its surface proteins, particularly p8, which covers most of the phage structure [4]. Previous studies have successfully fused binding peptides to the p8 protein, allowing the mineralization of metals on the M13 bacteriophage surface [5]. By fusing gold and cobalt binding peptides to the p8 protein, Au and Co particles were able to bind to the M13 bacteriophage, in order to design nanowires for lithium flexible batteries [5]. In another study, rare earth metals like neodymium (Nb) were recovered from aqueous solution using a M13 phage decorated on its surface with Nb binding peptides linked to the p8 protein [6]. The adsorption of heavy metal binding peptides onto the M13 p8 protein presents a promising approach to use it as adsorbent for the removal of metal ions from contaminated aqueous solutions. Moreover, the genetic editing and large scale production of M13 bacteriophage are affordable and straightforward processes, enabling the scalable synthesis of millions of viruses. This holds significant potential for applications in heavy metal remediation.

In this study, we genetically engineered three different M13 bacteriophage with heavy metal-binding peptides expressed as fusions to the p8 protein with the aim of using them as absorbents for metal ions from aqueous solutions. Using the Gibson assembly technique, we successfully inserted peptides with high affinity for Pb(II), Cr(III), and Cd(II) ions into the N-terminal region of the M13 bacteriophage p8 protein. Large-scale phage amplification by bacterial infection was performed to obtain highly concentrated bacteriophage stocks. The structural viability of the recombinant phages was visualized by transmission electron microscopy (TEM). In addition, we evaluated the adsorptive viability of recombinant phages to lead exposure using TEM. These innovative nanostructured materials show promise in mitigating heavy metal contamination in waterways and promoting environmental sustainability.

METHODS

Phage engineering using Gibson Assembly:
Nine peptides with affinity for heavy metal **(Table 1)** were expressed as a fusion to the p8 protein of the M13 bacteriophage. Peptide sequences were inserted by substituting "EGD" amino acids at the amino terminus of the p8 protein. Gibson assembly was used to introduce the peptide sequences to the M13 bacteriophage genome (M13KE plasmid).

1. **Insert Preparation:** DNA sequences codifying for peptides (Table 1), were designed with adjacent identical regions from the M13KE vector. Complementary corresponding combo primers were designed for each peptide sequence. Annealing reactions were performed to create each DNA fragments.

1. **Linearized Recipient vector:** The M13KE vector was linearized by PCR amplification using divergent primers.

A Gibson Assembly Master Mix contains three enzymes:

- First enzyme, a T5 exonuclease, generates single strand DNA 3' overhangs in insert oligo and linear vector. Complementary segments between insert and linear vector anneal.
- A second enzyme, a DNA polymerase, performs an extension to fill the gaps in the annealing.
- Finally, a DNA ligase covalently links the DNA fragments.

The resulting plasmid from the Gibson assembly reaction is used to transform bacteria. Colonies are then picked for miniprep, followed by Sanger sequencing to determine if the genetic editing was successful. The supernatant from the miniprep is stored for use in large-scale amplification in the event of a positive colony.

Table 1. Peptides sequences

Heavy metal affinity	Peptide sequence	Reference
Pb(II)	TNTLSNN	[7]
Cr(III)	YKASLIT ANLWPDG NNNWPWT	[8]
Cd(II)	HSQKVF HDGRSS YLPKNG AEHSQKVF AEYLPKNG	[9]

Large scale amplification of M13 phage
A small amount of virus is cultured with E. coli cells, allowing the virus to infect bacteria that produce viral proteins and lead to phage assembly. The positive colony and XL1-blue E. coli cells are amplified independently in 25 ml LB medium. Amplified phage and an E. coli culture are incubated with magnesium chloride and tetracycline in two liters of LB medium. After centrifugation of the incubated culture media, the entire supernatant is incubated with 400 ml of PEG solution to promote precipitation of the phage, while the pellets are discarded. This solution is incubated overnight at 4°C. The next day, the solution is centrifuged. The pellet (bacteriophage) is diluted with 20 mL phosphate buffer solution (PBS) and purified by centrifugation to remove dead biomass. 4 ml of PEG is then added. This solution is incubated overnight at 4°C. The next day, the solution is centrifuged and the pellet is resuspended with 2 mL of PBS. Debris is removed by centrifugation. The final step is quantification of M13 bacteriophage using a nanodrop. By measuring the absorbance at 320 nm and 269 nm, the phage concentration can be determined using the following formula:

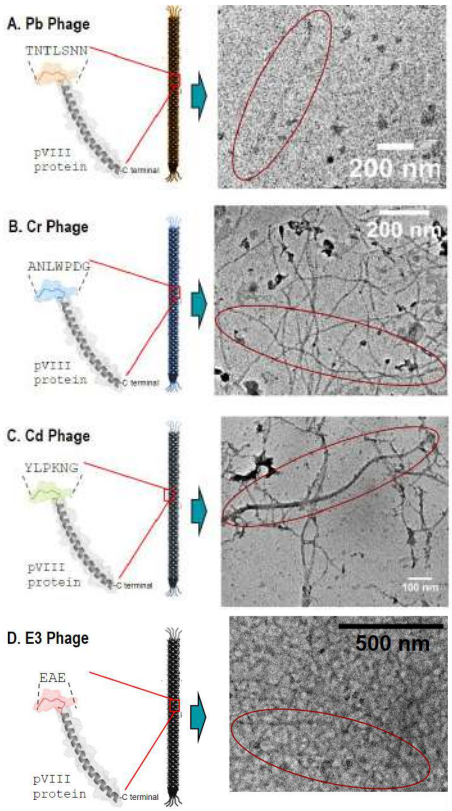
$$[\mathrm{M13}] = \frac{A_{269} - A_{320}}{7225} \cdot 6 \cdot 10^{17} [\mathrm{virions/mL}]$$

Transmission electron microscopy (TEM)
Bacteriophage imaging
Wild type, lead, chromium, and cadmium bacteriophage were prepared for TEM analysis. Additional purification was performed on the phage stock using a dialysis membrane to remove impurities. Phage solutions were first diluted to a concentration of 10e12 virions/mL. Carbon film grids were used to immobilize the phage. Bacteriophage samples were loaded onto the grids. Uranyl acetate was applied to the grids to facilitate contrast enhancement. The grids were then washed three times with water to remove excess staining. Finally, the grids were allowed to air dry and were then ready for TEM imaging.

Bacteriophage to lead exposure
Pb, E3 and wild-type bacteriophage from large scale amplification were dissolved in PBS. Pb(NO3)2 is insoluble in PBS; therefore, to prevent the precipitation of Pb(NO3)2 with phage dissolved in PBS during the lead exposure assay, dialysis was used to change solvent from PBS to water. The three phages were diluted to 10e12 virions/mL. 18 ul of phage were exposed to 2 µL 10 mM Pb(NO3)2 and incubated for 2 hours. Then, 10 µL of each incubated sample was applied to carbon film grids for TEM analysis. The grids were washed three times to remove unbound materials, and bacteriophage morphologies were imaged by TEM.

RESULTS AND DISCUSSION

Figure 1. TEM images of synthetic M13 phages

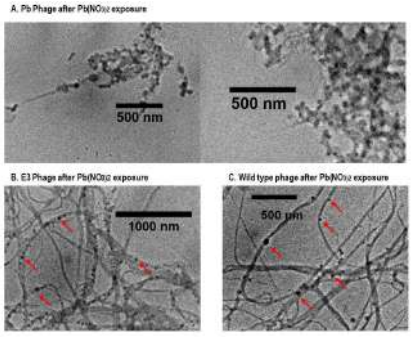


Production of synthetic phages
We obtained three M13 engineered bacteriophage, displaying peptides with affinity to lead, chromium and cadmium were found in the N-terminus of the p8 protein gene **(Figure 1)**. The lead, chromium and cadmium recombinant phages exhibit peptides fused to the p8 protein as expected (Table 1). Genome modifications were confirmed by Sanger sequencing. The addition of AE amino acids at the beginning of YLPKNG favored the insertion of this peptide into the bacteriophage protein coat. We hypothesized that negative amino acid charges favor viable bacteriophage assembly. These three bacteriophage, wild-type and E3 bacteriophage were produced on a large scale. **Table 2** shows the obtained bacteriophage volume and concentration.

Table 2. Phage amplification yield

Phage name	Volumen	Concentration (virions/ml)
Lead phage (TNTLSNN)	225 ul	6.1e13
Cr phage (ANLWPDG)	2 mL	1.6e13
Cd phage (AEYLPKNG)	2 mL	4.5e13
E3 Phage (EAE)	2 mL	3.4e14
Wild type phage	2 mL	8.3e14

Figure 2. TEM images of phage after lead exposure



TEM analysis of Bacteriophage
TEM analysis revealed a stable, flexible and ~800 nm filamentous structure for Pb bacteriophage, Cd bacteriophage, Cr bacteriophage and E3 bacteriophage **(Figure 1A-1D)**. Pb bacteriophage and E3 bacteriophage showed weak contrast under TEM, while wild type showed a destabilized structure. With the exception of the wild-type bacteriophage, the other bacteriophage show peptides with affinity to heavy metals. A more purified phage would give a clear-cut figure.

Bacteriophage to lead exposure
TEM analysis of bacteriophage exposed to lead revealed bacteriophage stability and integrity **(Figure 2)**. Pb phage showed high bacteriophage agglomeration **(Figure 2A)**. TEM suggested tightly bound and inhomogeneously distributed lead deposition along the E3 phage and wild-type phage. These results suggested lead adsorption and mineralization for E3 and wild-type phages **(Figure 2B and 2C)**.

CONCLUSIONS

- Three recombinant M13 phages displaying binding peptides with affinity for Pb(II), Cr(III) and Cd(II) in p8 were successfully synthesized to allow selective adsorption of these heavy metals. Phages were genetically modified using Gibson assembly technique and successful assemblies were validated by Sanger sequencing.
- The structural viability of the recombinant phages was validated by transmission electron microscopy (TEM) analysis, which revealed structural stability, filamentous shape, and appropriate length close to 800 nm, features of wild-type phages.
- Under lead exposure, the phages retained their characteristic morphology and showed resilience to heavy metal exposure. TEM revealed agglomeration and possible mineralization of lead ions along the M13 phage surface, suggesting adsorption of lead by M13 phages.
- Future investigation would consist of evaluating Cd phage and Cr phage exposure to cadmium and chromium ions, respectively, to determine phage adsorption capacity. We consider evaluating performance of the recombinant M13 phages using contaminated samples collected from Peruvian polluted water bodies. These real-world samples are likely to contain a complex mixture of contaminants and impurities that will likely challenge the binding efficiency.

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FICBAE

Isolation of lytic Raoultella bacteriophage

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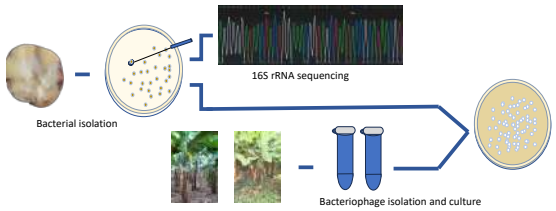
GRAPHICAL ABSTRACT



Isolation of lytic Raoultella bacteriophage

INTRODUCTION

Raoultella terrigena is a gram-negative, oxidase negative, re-classified in the Raoultella genus and separated from Klebsiella in 2001 based on 16S rRNA and the rpoβ gene sequences analysis. Microbiological identification of *R. terrigena* is difficult and isolates are frequently misidentified as Klebsiella species [1].

The Raoultella genus are ubiquitous in natural environments (water, soil, plants); R. terrigena was isolated from soft rot of chile plants [2]; and among the bacterial consortium for the wheat straw degradation [3]. Likewise, they were reported as opportunistic pathogens in human and animal infections [4,5]. The bacterial role in these biological processes is not clear and efforts should be made to identify and verify its role in vegetable waste degradation and infectious diseases.

Over the last few years, some Raoultella bacteriophages have been isolated and reported with the capability to control and balance the bacterial population [6,7]. This short report describes in Peru the isolation of a new phage vB_Rte infecting *Raoultella terrigena.*

METHODS

Bacteria host isolation and identification: The bacteria host was isolated from a potato with soft rot symptoms and subcultured on Tryptic Soy Agar (TSA) plate. The principal bacteria component of the potato with soft rot was identified using the 16S rRNA sequencing.

Lytic bacteriophage isolates: A total of thirty different agricultural samples were assayed for the presence of phages (15 banana pseudo-stem, 1 crop water, and 14 direct soil). The filtrate of each sample was assessed for bacteriophages using double agar overlay and spot test assay.

Host specificity test: The bacterial strains for the specificity test were provided by the "Laboratorio de Investigación de Bioquímica y Biología Molecular - UNFV" (LIBBM-SL10LA105). The bacteriophage infection specificity was tested against *Klebsiella variicola, Klebsiella oxytoca, and Pectobacterium coratovorum,* all of them related to soft rot. The host specificity test was assessed using double agar overlay and spot test assay.

The Minimum inhibitory multiplicity of infection (miMOI): The miMOI was assessed using fresh culture broth of bacteria host. The bacteriophages were mixed with bacterial culture at different MOIs an incubated at 30°C for 4.5 hours.

RESULTS Y DISCUSSION

The principal bacteria component obtained from potato with soft rot symptoms (BP3) was characterized by microbiology and classified as rods gram-negative and oxidase-negative. Finally, the molecular characterization identified BP3 as *Raoultella terrigena*; the sequence of 1437 nt. of the 16S rRNA gene obtained of BP3 was submitted to the GenBank (Accession number OQ804622).

One of thirty different agricultural samples (banana pseudo-stem) displayed a lytic zone. The bacteriophage was amplified and purified by the selection of single clear plaque-forming units (See Figure 1) and was named vB_Rte, using the prefix vB (bacterial virus) and the three-letter host abbreviation Rte (*Raoultella terrigena*).

The vB_Rte displayed specificity to R. terrigena and was unable to infect phylogenetically close bacteria such as *Klebsiella variicola* and *Klebsiella oxytoca* both isolates from banana pseudostem with symptoms of soft rot. Also was unable to infect *Pectobacterium coratovorum* isolate from potato with soft rot.

The stability of vB_Rte was assessed after different storage temperatures and the infectivity remained intact at 12°C storage temperature, while a 10-fold decrease in the number of plaque-forming units was shown after one hour of storage at 55°C or when storage at -20 °C. Null bacteriophage activity was shown after one hour at 65°C. The lytic activity was the same between room temperature to 45°C (See Figure 2).

The minimal MOI displayed was 10^{-6}, with higher dilution non-inhibition to the bacterial growth was shown. The absorbance of the culture broth at different MOIs increased gradually during the first minutes and then decreased according to the decrease in the MOIs value. See Figure 3

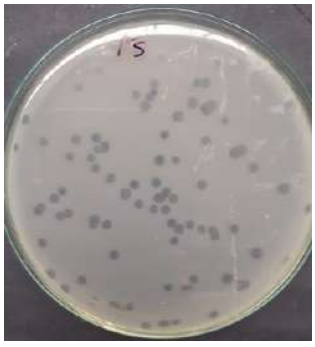


Figure 1.Plaques produced by bacteriophage vB_Rte on double-layer solid medium .

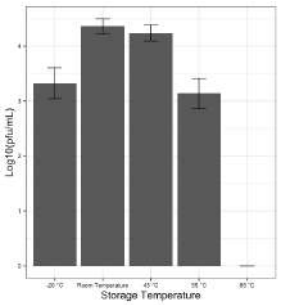


Figure 2. The influence of storage temperature on bacteriophage vB_Rte

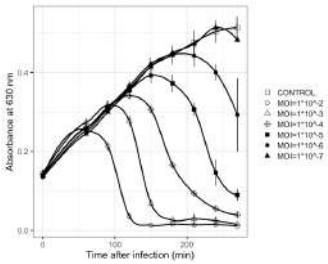


Figure 3. Bacteriophage vB_Rte lysis profile

CONCLUSIONS

This study isolated a lytic bacteriophage specific to *R. terrigena*. The lytic properties showed its potential as an innovative biocontrol agent and specific detection tool for *R. terrigena*. The results of this study need to be expanded for biocontrol as a suitable and realistic antibacterial agent to include in management strategies to the control of degradative bacteria.

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FICBAE

Scaling of formulated bacteriophages for biological control of bacterial spot (*Xanthomonas euvesicatoria*) in pepper crops.

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GRAPHICAL ABSTRACT

Figure 1: graphical abstract

INTRODUCTION

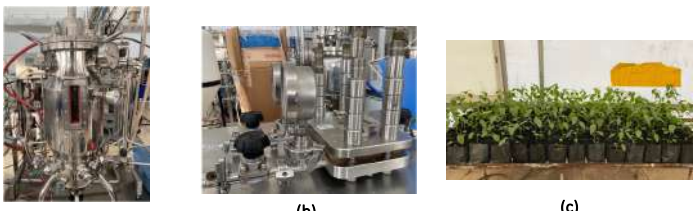
The current methods in Mexico to control the *Xanthomonas euvesicatoria* in pepper crops are primarily based on prevention, as chemical control proves intricate due to issues such as the bacterium's rapid resistance development against active compounds like copper-based compounds and antibiotics (Ibarra, 2019). Consequently, these classical pest control methods lead to long-term repercussions on pepper cultivation, elevating production costs, diminishing effectiveness, and disrupting the balance of the natural ecosystem.

Given the problems arising from ineffective strategies in controlling *Xanthomonas* spp. pests, interest has surged in the utilization of biocontrol through viruses known as bacteriophages, which have coexisted with bacteria and crops in natural conditions (Nakayinga *et al.,* 2021). When applying a bacteriophage in natural or greenhouse conditions to combat the *X. euvesicatoria* in *Capsicum annuum* agricultural production, one must consider challenges that may arise and prove challenging to manage, such as UV radiation, high temperatures, and the bacterium's potential immunity generation. UV radiation stands as a primary limitation to bacteriophage effectiveness in pest control, central to the Fagolytic product/invention.

In this study, an upscaling of Fagolytic was pursued, composed of zinc nanoparticles and the lytic phages ΦXaF13 and ΦXaF18, to a pilot plant scale of 20 L. Its biological effectiveness in controlling *X. euvesicatoria* in pepper crops under greenhouse conditions was evaluated.

METHODS

Bacteriophages were produced at the pilot plant level in a 20 L Applikon bioreactor. Centrifugation and filtration of the product was carried out using a Thermo Scientific Centrifuge Multifuge 3XR and a Millipore Tangential Filter. Finally, the biological effectiveness of Fagolytic was evaluated in pepper crops established in a plastic greenhouse using the methods of Tukey Pairwise Comparisons and Dunnet Multiple Comparisons ($p \leq 0.05$) with a control employing chlorotic spots, necrotic spots, number of leaves with symptoms, defoliation, severity scale and damaged leaf area per leaf as variables.



(a) (b) (c)

Figure 2. (a) Bioreactor (b) Tangential Filter (c) Pepper plants in greenhouse

RESULTS Y DISCUSSION

The results obtained from the titrations of batches 1 and 2 of bacteriophages produced in the 20 L bioreactors (Table 1) showed suitable concentrations for the biological effectiveness test in pepper crops under greenhouse conditions that was carried out at the end of the formulation of Fagolytic products. The concentration dimensions of the phages are close to those obtained by Payan (2022).

The statistical analysis made by the methods of Tukey Pairwise Comparisons and Dunnet Multiple Comparisons ($p \leq 0.05$) with a control showed that in many of the symptoms provoked by the *X. euvesicatoria* bacterial spot the disease, such as chlorotic spots, necrotic spots, severity scale, and damaged leaf area has statistically equal behavior when is treated with Fagolytic as when is with the present commercial preventive treatments, and in a few cases with the control treatment (healthy).

Table 1. Bacteriophage batches

Batch (date of production)	BACTERIOPHAGE	CONCENTRATION (UFP/mL)
1 (2nd of march)	ΦXaF13	$1x10^8$
2 (13th de march)	ΦXaF18	$1.8x10^8$

Chlorotic spot (Day 21)
Tukey Pairwise Comparisons ($p \leq 0.05$)

Factor	N	Mean	Grouping	
Serenade	19	31.58	A	
Formulated phages	19	28.58	A	
Non-formulated phages	19	26.68	A	
Sick plant	19	26.05	A	
Kasumin	19	23.84	A	
Fagolytic	19	18.68	A	B
Healthy plant	19	1.211		B

Figure 3. Pairwise comparisons of chlorotic spots provoked by bacterial spot disease.

Necrotic spot (Day 21)
Tukey Pairwise Comparisons ($p \leq 0.05$)

Factor	N	Mean	Grouping	
Serenade	37	18.95	A	
Formulated phages	37	18.11	A	
Non-formulated phages	37	17.8	A	
Sick plant	37	16.92	A	
Kasumin	37	15.24	A	
Fagolytic	37	11.54	A	B
Healthy plant	37	1.216		B

Figure 4. Pairwise comparisons of necrotic spots provoked by bacterial spot disease.

Given the present results, the Fagotytic treatment in pepper crops at greenhouse conditions shows statistically the same behavior in symptoms caused by the bacterial spot disease of *X. euvesicatoria* when the culture is healthy as when is treated previous the infection.

CONCLUSIONS

Given the statistical equality between the Fagolytic treatment, the present commercial preventive treatments and the control in different variables, the formulated bacteriophages show a promising alternative for bacterial spot control in pepper crops.

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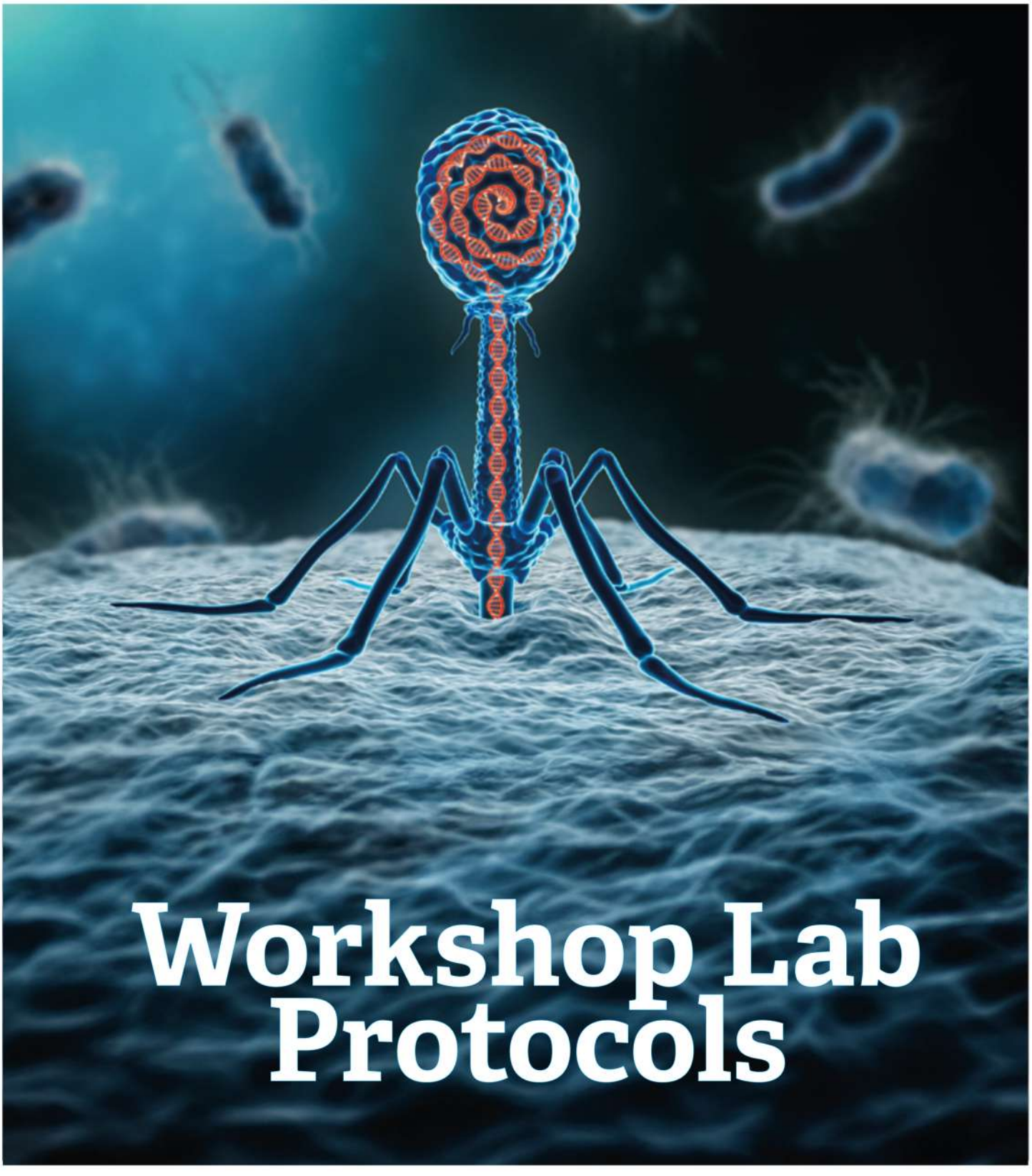

Workshop Lab Protocols

Workshop 1

Using of Molecular Cloning Techniques for phage genome (vector M14KE) editing

Video

Experience 1

Gibson assembly and primer design

In 2009, Dr. Daniel Gibson and his colleagues first described the Gibson assembly technique. Gibson assembly is a single reaction technique for the assembly of multiple overlapping DNA fragments. It enables the insertion of an insert into the desired vector.
The process begins with DNA fragments, both insert and open vector, possessing regions of homology at their ends, typically generated through PCR. DNA fragments are then incubated together with an enzyme master mix comprising three different enzymes:

1. An exonuclease, which trims back the 5' ends of the fragments, creating long overhangs that allow the single-stranded regions with homology to anneal.
2. A polymerase, which fills in the gaps.
3. A DNA ligase, which seals the nicks of the annealed and filled-in gaps.

One of the remarkable aspects of this enzyme mix is that they all function at the same temperature. Consequently, the entire reaction can be completed in about an hour at 50°C or lower. After this short incubation period, the sample is ready for immediate transformation into competent cells.

Materials and equipment:

- Snapgene

Sanger sequencing analysis

Automated DNA Sequencers produce a chromatogram with four colors, representing the results of the sequencing process, along with a text file containing the sequence data as interpreted by a computer program. The characteristics of Sequencing Chromatograms are as follows.

Workshop 2

Amplification of recombinant phages at high scale. Purification using centrifugation and chemical precipitation

Video

Experience 1: Plasmid DNA quantification in Nanodrop

Reagents:

- 1.5 mL Eppendorf Tubes with plasmid
- 1.5 mL Eppendorf Tubes with blank (water or buffer)

Materials and equipment:

- UV/Vis Spectrophotometer
- Tips (10 ul)

Protocol: Plasmid DNA quantification in Nanodrop

1. Add 2uL of Millipore deionized water to initialize the instrument.
2. Once completed, remove the fluid with Kimwipe and add 2uL of the blank solution and press ”Blank”. This will use the absorbance spectra of the solution as a reference for the sample measurement.
3. Place 2uL of the plasmid DNA solution on the Nanodrop pedestal and run the machine.
4. Record the absorbance.

Experience 2: Bacterial transformation

Description:

This manual provides a step-by-step protocol for the transformation of XL1-Blue competent cells using the M13KE gIII Cloning Vector. The transformation process involves the introduction of the M13KE vector into the bacterial cells, allowing for the expression of desired genes or DNA sequences.

Reagents:

- SOC medium
- XL1-Blue Competent Cells Catalog #200249
- M13KE gIII Cloning Vector (10 ng/ul)

Materials and equipment:

- 1.5 mL Eppendorf Tubes
- 15 mL tubes
- Tips (10, 200 and 1000 ul)
- Water bath at 42 °C
- Ice bucket filled with ice
- Incubator at 37 °C

Transformation Protocol

1. Thaw one vial of chemically competent cells on ice and transfer 100 ul from this tube to a 1.5 ml eppendorf tube. Work in ice.
2. Add 5 ul of M13KE vector (10 ng/ul) to the tube to the competent cells (You are adding 50 ng of vector). Mix gently by pipetting up and down or by flicking the tube 4–5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for 30 seconds. Do not mix.
5. Transfer tube to ice for 2 minutes.
6. Add 900 µl of room-temperature SOC media to the tube.
7. Incubate the bacteria transformation reaction at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.

Experience 3-a: Tetracycline Preparation

Reagents:

- Tetracycline hydrochloride
- 70% ethanol

Materials and equipment:

- One tube 15 ml
- Ice bucket filled with ice
- Laminar Flow hood

Protocol Blue LB agar

1. To prepare 10 ml of 20 mg/ml tetracycline stock solution, weigh out 200 mg of tetracycline. Transfer it to a 15 ml screw-capped tube. Add 9 ml of 70% ethanol. Mix until all tetracycline dissolves completely. Once the content is mixed, make up the volume to 10 ml.
2. Store at -20°C for a long time. Protect the solution from light, 0.22 µm syringe filter.

Experience 3-b: Blue agar preparation

Reagents:

- LB medium (Dehydrated powder)
- Bacto Agar in powder
- X-Gal solution (50 mg/ml) (Promega)
- Tet 1000X
- IPTG 100mM (100X)
- Milli-Q Water

Materials and equipment:

- ~20 units of Petri Dish Boxes
- Tips (1000 ul)

Protocol:

1. To prepare 500 ml of LB-agar, in 500 ml of sterile water add:
    - 5 g/L Bacto-Tryptone
    - 2.5 g/L yeast extract
    - 2.5 g NaCl
    - 7.5 g/L Agar.
2. Autoclave and cool.
3. Then add IPTG, Xgal, and Tet solutions.
    - Add 0.4 ml of X-Gal solution $(50mg/ml)$ $(Promega)$.
    - Add 5000 ul of IPTG $(100mM)$.
    - Add 0.5 mL Tet 1000X.
4. Mix before using. Pour into Petri dishes ($\sim$25 mL per plate).

Workshop 3

Transformation of phage genome into XL1-Blue cells using chemical transformation

Video

Experience 1: Phage Plating and aliquoting with Top agar

Description:
This manual provides a detailed protocol for conducting phage titering, a technique used to determine the concentration or titer of bacteriophages present in a sample. The titering process involves serial dilutions of a genetically transformed bacterial culture and subsequent plating on agar plates to visualize the formation of phage plaques. **Reagents:**

- LB Agar Plaques with IPTG/XGal/Tet
- Top Agar
- LB Medium
- 4 mL Overnight Culture of XL1-Blue Competent Cells
- Solution with Phage

Materials and equipment:

- 1.5 mL Eppendorf Tubes
- 15 mL tubes
- 10 mL Pipette
- Micropipette Tips
- Incubator at 37 °C
- Microwave

Protocol

1. Microwave Agarose Top and store at 50-60°C. High temperature can kill samples.
2. Place 4 plates in the incubator at 37 °C upside down.

3. Do 10 serial dilutions ($1:10$ to $1:10^{10}$) with the phage solution tube. In ten 1.5 mL eppendorf tubes, add 90 ul LB medium.
   (a) **Dilution tube 1 (1:10):** Take 10uL of phage solution tube and resuspend it in the 90uL dilution media. Mix by pipetting.
   (b) **Dilution tube 2 ($1:10^2$):** Add 10uL of Dilution tube 1 to the tube with 90uL LB medium.
   (c) **Dilution tube 3 ($1:10^3$):** Add 10uL of Dilution tube 2 to the tube with 90uL LB medium.
   (d) Repeat the process until you reach Dilution tube 10 ($1:10^{10}$)).
4. Aliquot 100uL Overnight Culture of XL1-Blue Competent Cells in eleven Eppendorf tubes and label them as Tube 0, Tube 1, Tube 2, Tube 3 until Tube 10.
5. Add 10uL of the phage solution tube to Tube 0,pipetting to mix it. Add 10uL of each dilution tube to its respective tube that has 100 ul E. coli overnight culture, pipetting to mix it.
6. Retrieve plates and Agar Top. Label the back of the plates as Plate 0, Plate 1, Plate 2, and Plate 3.
7. Aliquot 3mL Agar Top into 15 mL tubes.
8. Pipette the 110uL Phage/E. coli solution (from Tube 0, Tube 1, Tube 2, Tube -3, and so on) into the 3mL Agar top and mix. You can use a vortex on low or swirl it by hand.
9. Pour the newly mixed solution onto a correspondingly labeled petri dish, swirling it to evenly cover the full area.
10. Repeat this process for each of the dilutions and Agar top samples.
11. Once plates have cooled and solidified, place them back in the incubator at 37 °C upside down overnight.

Note: The Dilution tube 8 ($1:10^8$) showed a good distribution of colonies infected with phage. For the workshop, the Phage Plating will be using Dilution tube 8 ($1:10^8$). We will start from the 4th step of this protocol.

Workshop 4

Titering of M13 bacteriophages for selection of cells expressing phages Quantification of phage concentration using a spectrophotometer

Video

Experience 1: Large scale amplification of bacteriophage

Description: This manual provides a detailed protocol about the amplification process of M13KE bacteriophages. This step involves infecting a bacterial culture with a small amount of bacteriophage so that it can replicate a larger population of new phages. Amplification can be performed in multiple rounds to increase the quantity of bacteriophages.

Reagents:

- Millipore DI water
- LB (Luria-Bertani) media
- MgCl2·6H2O
- Tetracycline (TET)
- E. coli colony (XL-1 Blue)
- Stock solution of roughly 1e13 virions/mL phage
- 10% bleach solution

Materials and equipment:

- Erlenmeyer Flasks (500mL)
- Autoclaved 250 mL Erlenmeyer flask
- 2L flask
- Polycarbonate centrifuge tubes
- Centrifuge with JLA 81,000 rotor
- 4°C refrigerator

- NanoDrop UV/Vis spectrophotometer
- Pipettes and tips
- Vortex mixer
- Microcentrifuge
- 40mL centrifuge tube

Amplification and purification Protocol

Day 0: Materials preparation and 4mL E. coli OC

1. Combine 500 mL Millipore-DI water with 10.5 g LB (in powder) in a large 1L flask and 0.5 g of MgCl2·6H2O. Prepare 2 Erlenmeyer Flasks. Autoclave the flasks with the solution inside. Cool the LB solution below 45°C and add 0.5mL of Tetracycline solution to each flask.
2. Combine 25 mL Millipore-DI water with 0.515 g LB (in powder) in a small 100 mL flask. Prepare 2 Erlenmeyer Flasks. Autoclave the flasks with the solution inside. Cool the LB solution below 45°C and add 25 ul of Tetracycline solution to each flask.
3. In a 15 mL tube, add 4mL LB, 4 ul of Tet 1000X, and a sample of E. coli Xl1-Blue. Make sure there is 167mL of PEG/NaCl for every liter of LB being amplified. If not, add 150g of PEG-8000 and 110g NaCl to 750mL of Millipore DI H2O.

Day 1: Prepare Overnight Culture (OC)

1. Small flask with E.coli: Add 250 ul of E. coli overnight culture with 25uL of TET to the flask with 25 mL of LB.
2. Small flask with Phage: Add phage stock, 250 ul of E. coli overnight culture with 25uL of TET to the flask with 25 mL of LB. Incubate overnight in a 37°C incubation shaker.

Day 2: 24 Hour Virus Amplification

1. Add 10 mL of Small flask with E.coli to 1L flask (prepared in Step 1).
2. Measure optical density at 600 nm.
3. When optical density is 0.4, add 10 mL of Small flask with Phage to 1L flask.
4. Incubate for 24 hours by shaking at 37°C and 225-250 rpm (shake for at least 6-7 hours).

Experience 2: Purification using centrifugation and protein precipitation

Reagents:

- Millipore DI water
- PEG-8000
- NaCl
- 1x PBS solution

Materials and equipment:

- Autoclaved 250 mL Erlenmeyer flask
- 15 mL centrifuge tubes
- 50 mL centrifuge tubes
- Centrifuge for 15 mL tubes
- Centrifuge for 50 mL tubes
- 4°C refrigerator
- NanoDrop UV/Vis spectrophotometer
- Pipettes and tips
- Vortex mixer

Day 3: Phage Separation and Purification Round 1

1. Extract the flask from the shaker and transfer the contents of each flask into separate polycarbonate centrifuge tubes.
2. Perform centrifugation on the overnight culture using the largest rotor available at a speed of 8,000 rpm for a duration of 30 minutes. This procedure should lead to the formation of a bacterial pellet at the bottom of the centrifuge tube. Meanwhile, the virus, due to its significantly smaller mass compared to bacterial cells, will remain suspended in the supernatant.
3. Once the centrifugation cycle is completed, decant the supernatant into a flask with a capacity of 1L. Add PEG/NaCl to each in a volume that corresponds to 1/5th of the volume of the virus solution.

4. Store the flask in a refrigerator set at a temperature of 4°C overnight. The addition of PEG and salt serves to draw water molecules away from the virus, inducing its separation from the fluid. Over the span of several hours, the virus will initiate a process of aggregation and precipitation, causing it to settle out of the solution.

5. Utilize a disposable spatula to scrape off a segment of the bacterial pellet and preserve it in a 1.5mL round bottom centrifuge tube. This sample will be reserved for DNA extraction purposes. Store this tube in the refrigerator set at a temperature of 4°C.

Day 4: Phage Purification Round 2

1. Transfer solution from Step 16 into centrifuge tubes. Spin at a speed of 8,000 rpm for 60 minutes. Make sure not to shake the solution too much.

2. Once the spinning is done, you should see a white pellet at the bottom of the tube. Quickly pour out and get rid of the liquid part into a big bottle with around 20mL of a liquid that's 10

3. Dissolve the pellet by putting 10mL of PBS (1X) in each tube. To do this, gently shake the bottles and move a pipette up and down close to the pellet. Collect the phage solution, which is the liquid part, in a 50 mL centrifuge tube. Centrifuge these tubes at 14000 rpm for 30 minutes. Transfer supernatant into clean 50 centrifuge tubes. Discard pellets (residual impurities).

4. Add 2mL of PEG/NaCl solution into each of the tubes. Put the tubes from Step 22 into a refrigerator around 4°C. Leave them in there overnight.

Day 5: Final Phage Purification and Collection

1. Spin the phage solution in the centrifuge at a speed of 14,000 rpm for 45 minutes.

2. Completely mix the solid part (pellet) in each tube with 2mL of PBS (1x).

3. Move the solution into 1.5 Eppendorf tubes. Spin them at a speed of 14000 rpm for 10 minutes.

4. Move the supernatant to new clean 1.5mL eppendorf tubes.

5. This is now your final phage solution.

Day 5: Quantification with the NanoDrop

1. Choose "UV/Vis" on the main menu.

2. Put 2uL of Millipore DI water to start the device.
3. When that's done, clean away any liquid with a Kimwipe cloth, and then add 2uL of PBS (1x) solution.
4. Push the "Blank" button. Put 2uL of phage solution from Step 27 onto the platform of the Nanodrop.
5. Write down the absorbance numbers at 269 and 320 nm.

Use the following formula to find out the final phage concentration:

$$[M13] = \frac{(A_{269} - A_{320}) \cdot 6 \times 10^{17}}{7225}$$


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