ENVIRONMENTAL BIOLOGY
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Bacterial resistance as a key factor for a successful phage therapy

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Abstract

The practice of **phage therapy** for the control of bacterial diseases rose in popularity due to the emergence of multidrug-resistant bacteria, commonly known as "superbugs",. In that context, the use of phages for the management of many important bacterial diseases in the aquaculture environment is auspicious. ***Vibrio harveyi***, a well-known and serious bacterial pathogen is responsible for many disease outbreaks in aquaculture resulting in huge economic and production losses. To this direction, we isolated a novel bacteriophage, designated as **Vibrio phage Virtus**. Phage profiling was followed by the development of **phage-resistant bacterial strains** and the determination of their phenotype. A **trade-off** between resistance and fitness was revealed. The majority of the phage-resistant strains appeared to have lower growth rates, were less virulent and more susceptible to antibiotics. We then employed these mutants for enrichment cultures of marine environmental samples in order to isolate and characterize novel bacteriophages capable to infect them. Ultimately, we aim at using this strategy as a preliminary step towards the design and optimization of an efficient "phage cocktail" that can overcome the limitations of phage therapy and possibly treat vibriosis outbreaks.

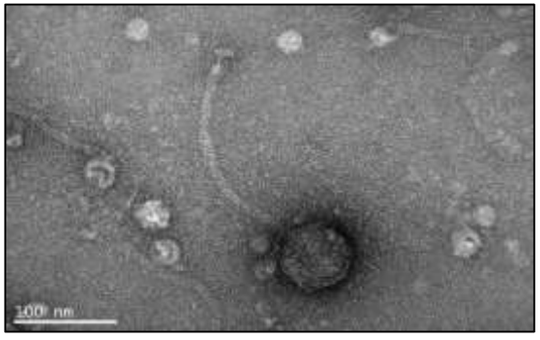


Figure 1. Transmission electron microscopy picture of Vibrio phage Virtus showing a typical morphology of siphoviruses.

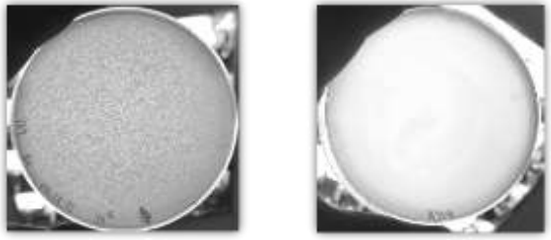
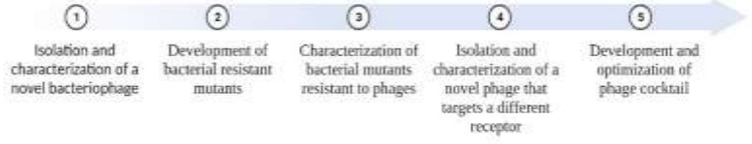


Figure 2. Direct plating of *Vibrio harveyi* VH2 infected with Vibrio phage Virtus (A) and a phage resistant mutant R9D4 infected with Vibrio phage Virtus (B). Plaque formation is observed in the susceptible strain VH2 while no plaques are observed in the resistant strain.

Experimental Design



Material and Methods

The workflow for the biological profiling of Vibrio phage Virtus included TEM sample preparation, host range determination, one step growth, *in vitro* bacterial lysis and thermal and pH stability assays. For genomic characterization we employed several bioinformatic tools and databases. We then developed phage-resistant host mutants following the method described in Thomas Denes et al. (ref). Antibiotic susceptibility tests and *in vitro* assays were conducted to further characterize the phenotype of the mutants. Phage therapy trials and challenges in gilthead seabream were conducted in order to assess bacteria virulence and phage efficacy.

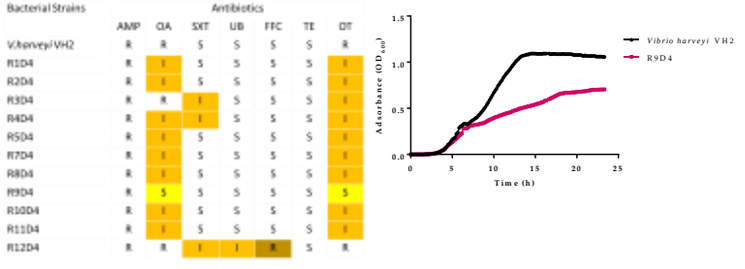


Figure 2. Antibiotic susceptibility assay in phage resistant mutants and *V.harveyi* VH2 (Left). *In vitro* growth curve of phage resistant mutant R9D4 compared to wild type *V.harveyi* VH2.

Results

- Vibrio phage Virtus is a novel cosmopolitan siphophage that can infect a wide spectrum of Vibrio spp., including strains of *V. harveyi, V. owensii, V. campbellii, V. parahaemolyticus, and V. mediterranei*. It has a latent period of 40 min with an unusually high burst size of 3200 PFU cell^{-1}. Vibrio phage Virtus has a double-stranded DNA of 82,960 base pairs with 127 predicted open reading frames (ORFs). No virulence, antibiotic resistance, or integrase-encoding genes were detected. In vivo phage therapy trials in gilthead seabream, *Sparus aurata*, larvae demonstrated that Vibrio phage Virtus was able to significantly improve the survival of larvae for five days at a multiplicity of infection (MOI) of 10, which suggests that it can be an excellent candidate for phage therapy
- Mutant bacterial strains were more sensitive to antibiotics
- Some of the mutant bacterial strains showed a significantly reduced fitness compared to the control
- The survival of the larvae tested with the R9D4 strain was significantly greater than that of the larvae tested with V. harveyi, thus showing that R9D4 was less virulent than V. harveyi VH2.

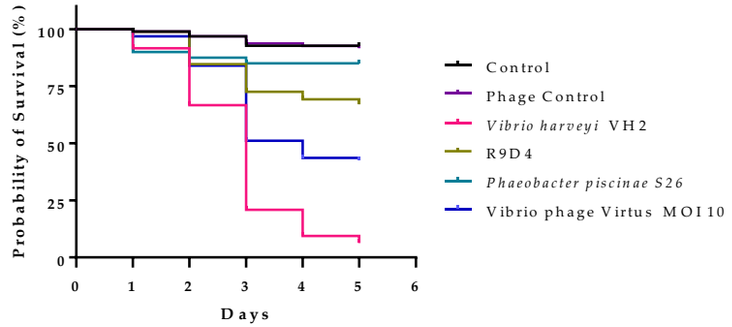


Figure 4. Survival of gilthead seabream larvae infected with *V. harveyi* VH2 in an experimental phage therapy trial during a period of 5 days. Gilthead seabream larvae that were infected with VH2 were inoculated with Vibrio phage Virtus with different multiplicities of infection (MOI) at 2 h post-infection. Gilthead seabream were also challenged with R9D4 in order to assess the virulence of the mutant compared to VH2 wild type. *Phaeobacter piscinae* S26 were used to evaluate the effect of non-pathogenic bacteria at the same concentration to fish larvae.

Conclusion

In summary, the result of the present study was the isolation and thorough genomic and biological characterization of a new bacteriophage, Vibrio phage Virtus. Its biological and genomic profile make it an interesting model for studying the microbial ecology of vibrios, but also an ideal therapeutic agent against *V. harveyi* infections. In combination with mutated bacterial strains resistant to the bacteriophage, they are an ideal model for studying bacterial resistance against phages by enriching existing knowledge and taking another step on the road to successful phagotherapy.

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References

1.Denes T, den Bakker HC, Tokman JI, Guldimann C, Wiedmann M. Selection and Characterization of Phage-Resistant Mutant Strains of Listeria monocytogenes Reveal Host Genes Linked to Phage Adsorption. Appl Environ Microbiol. 2015 Jul;81(13):4295-305. doi: 10.1128/AEM.00087-15. Epub 2015 Apr 17. PMID: 25888172; PMCID: PMC4475870.

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