# New virulent bacteriophages isolated from the surface of Epoisses cheese infect ripening bacteria

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## Context

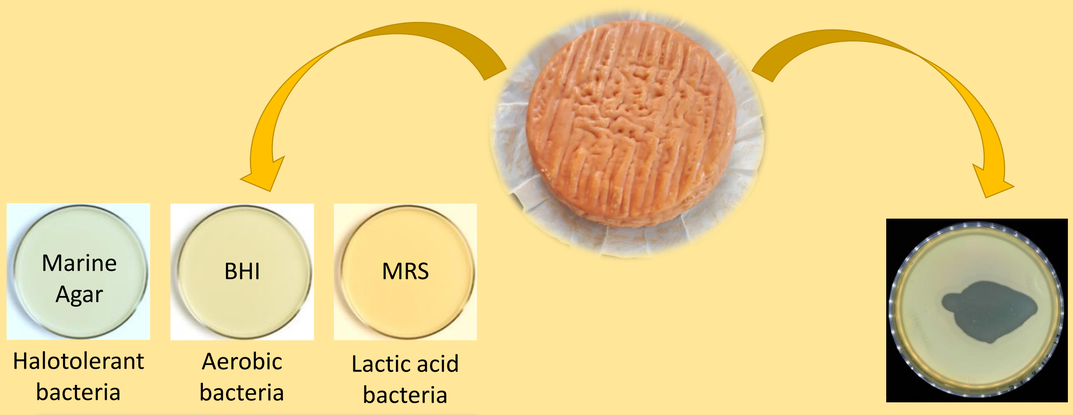
Bacteriophages are known to be major drivers of microbial communities in several ecosystems. In cheese, phages infecting lactic acid bacteria (LAB) starter cultures are well described. However, very little is known about those infecting ripening bacteria and their impact on the microbial successions observed during cheese maturation [1]. Recently, we used a viral metagenomics approach combined with transmission electron microscopy (TEM) to highlight the viral diversity present on the surface of Epoisses cheese [2]. Another group was also able to isolate and characterize several phages infecting the very common ripening culture *Brevibacterium aurantiacum* from smear and washed-rind cheeses or their production environment [3]. In order to evaluate the possible ecological role of phages present on the cheese surface, an effort was made to isolate and characterize some representative phages from Epoisses cheese.

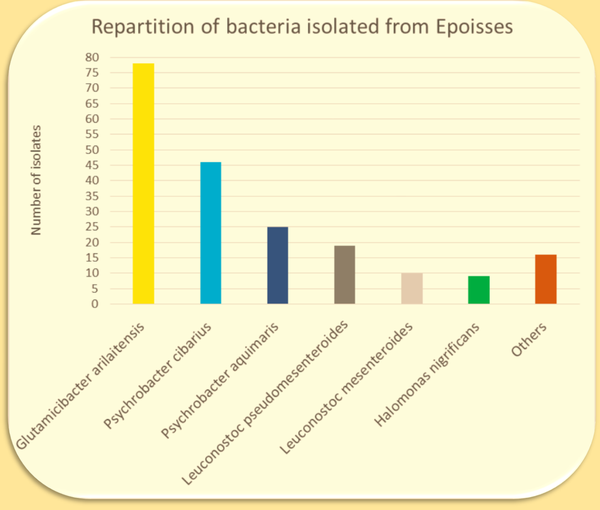
## Materials & Methods

A collection of almost 200 bacterial isolates was retrieved from the surface of three Epoisses cheese and identified by sequencing the 16S rRNA gene. In parallel, the viral fraction was also purified according to the protocol described previously [2] containing the following steps: centrifugation (5,000 × g for 45 min), filtration (0.22µm), PEG precipitation and chloroform treatment. This viral particle pool was used to infect the collection of bacterial isolates through spot assays. Phages forming clear plaques were then isolated and purified three times. After purification, phages went through different characterization steps:

- Sequencing and annotation of complete phage genomes: after DNA extraction, long-reads were produced by a MinION device (Oxford Nanopore) and short reads by the NovaSeq platform (Illumina). An assembly was obtained from long-reads with Trycycler [4] and polished with short reads using Pilon [5]. The annotation was performed using RAST [6], supplemented with additional functional predictions using CDD [7], HHpred [8] and PHROGs [9].
- Morphological analysis of the virions by TEM
- Host range evaluation by spot assays against a range of reference strains corresponding to the same species as the original host as well as other species from the same genus.

## Isolation of bacteria and phages from Epoisses rind



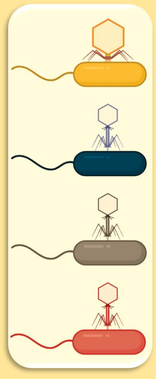




Glutamicibacter φ Voltaire
Host species: *Glutamicibacter arilaitensis*

Psychrobacter φ d'Alembert
Host species: *Psychrobacter aquimaris*

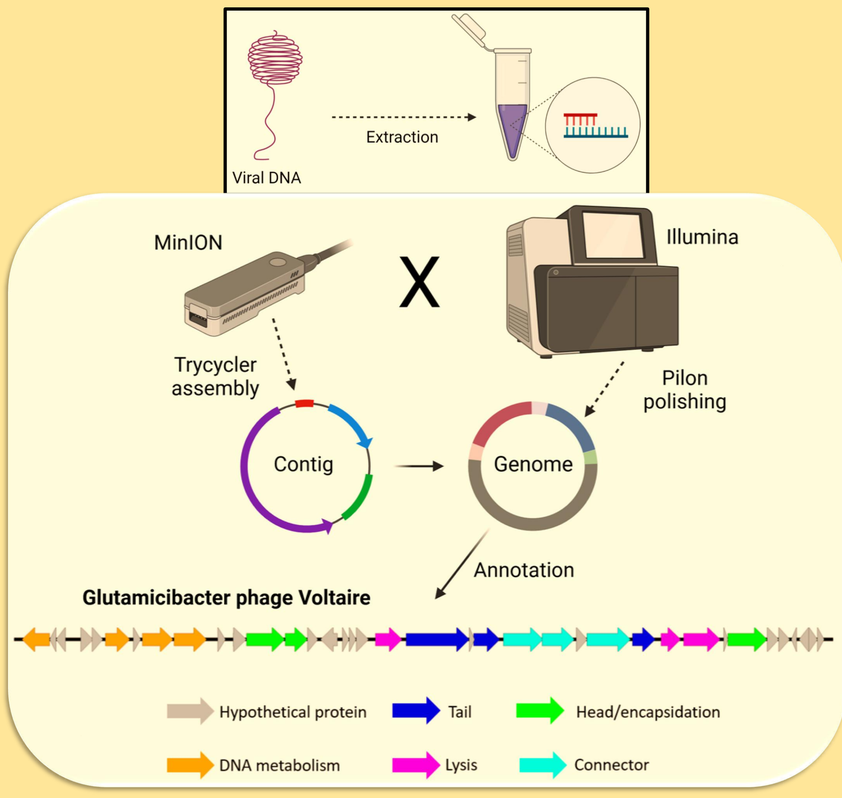
Leuconostoc φ Diderot
Host species: *Leuconostoc pseudomesenteroides*

Brevibacterium φ Rousseau
Host species: *Brevibacterium aurantiacum*



Four virulent phages were isolated from the cheese rind. They infect four different bacterial species: *G. arilaitensis* and *B. aurantiacum* which are often used as ripening culture in the manufacture of washed-rind cheeses, *L. pseudomesenteroides* which is a non-starter lactic acid bacteria (NSLAB) and *P. aquimaris* which is frequently detected as a dominant species in washed-rind cheeses and is generally considered as an endogenous species.

## Sequencing, assembly and annotation

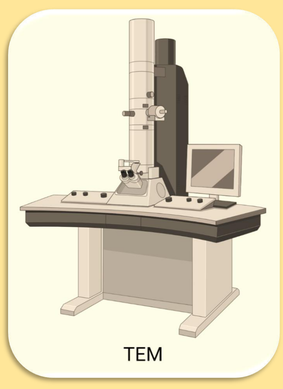


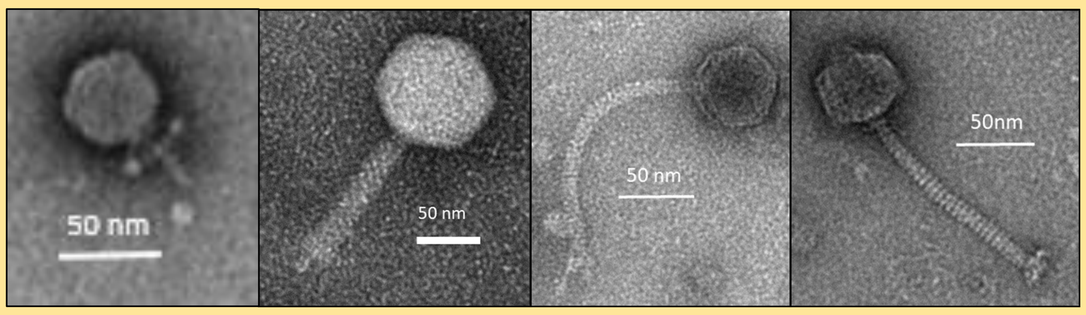

**Tab1. Genomic information about the four isolated phages.**

| Phage | Genome size (kb) | ORFs | Terminal Repeat (bp) | Best Blast hit (NCBI_viruses) |
|---|---|---|---|---|
| Glutamicibacter φ Voltaire | 18 | 28 | 175 | Brevibacterium phage Cantare 83,33% id **1% cov** |
| Psychrobacter φ d'Alembert | 92 | 158 | 5207 | Vibrio phage vB_VhaM_VH-8 83,95% id **34% cov** |
| Leuconostoc φ Diderot | 27 | 43 | / | Leuconostoc phage PhiLN03 98,20% id **98% cov** |
| Brevibacterium φ Rousseau | 40 | 71 | / | Siphoviridae sp. Isolate ctmmc7 75,54% id **0% cov** |

Following the latest proposed taxonomic criteria [10], where viruses with >70% nucleotide identity over the full genome belong to the same genus and viruses with >95% nucleotide identity belong to the same species, isolated phages may represent new genera except for Leuconostoc phage Diderot that belongs to the same species than PhiLN03.

## Morphology (TEM)



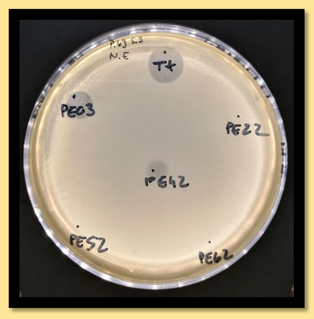

**Voltaire** **D'Alembert** **Rousseau** **Diderot**

**Tab2. Dimensions and morphologies of isolated phages**

| Phage | Capsid (nm) | Tail (nm) | Morphotype |
|---|---|---|---|
| Glutamicibacter φ Voltaire | 47 | 30 | Podophage |
| Psychrobacter φ d'Alembert | 88 | 113 | Myophage |
| Leuconostoc φ Diderot | 57 | 141 | Siphophage |
| Brevibacterium φ Rousseau | 62 | 177 | Siphophage |

Average sizes were obtained through the measure of 5 virions. They all belong to the *Caudoviricetes* class.

## Host range evaluation (Spot assay)




**Tab3. Host range of isolated phages**

| Phage | Sensitive species/Tested species (same genus as the host) | Sensitive isolates/Tested isolates (same species as the host) | |
|---|---|---|---|
| | | Isolated from Epoisses | From other sources |
| Glutamicibacter φ Voltaire | 1/4 | 9/13 | 0/4 |
| Psychrobacter φ d'Alembert | 1/5 | 3/10 | 0/5 |
| Leuconostoc φ Diderot | 2/4 | 7/9 | 0/1 |
| Brevibacterium φ Rousseau | 1/6 | 1/2 | 0/16 |

Between 17 and 25 strains were tested in triplicate for each phage.

## Conclusions

New virulent phages infecting ripening bacteria were isolated from Epoisses cheese rind, and characterized. These steps allowed to confirm the originality of their genomes, as three of them may represent new viral genera. Their very narrow host range give first clues of their ecological impact in cheese ecosystem.
New metaviromics analyses should allow to answer the question of the persistence of phages in Epoisses cheese.

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