

# Bacteriophage Morphological Characterization by Using Transmission Electron Microscopy

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**Abstract:** Bacteriophages or more commonly “phages” are bacterial viruses. They are ubiquitous and good indicators of bacterial contaminations since their prevalence is high in those environments where their hosts are abundant. Phage classification is based on morphology and for this reason, even though it is considered an old technique, TEM (Transmission Electron Microscopy) still plays a key role in their characterization. In the present work, the authors focused on TEM analysis of phage  $\phi$ Apr-1 isolated against *Lactococcus lactis* (*L. lactis*), implicated in industrial fermentations and of phage  $\phi$ IZSAM-1, active against *Listeria monocytogenes* (*L. monocytogenes*), isolated from the environment. For observation with TEM (EM 900T-Zeiss), phages were harvested in liquid media and were negative stained with fosfotungstic acid 2%. An accurate viral ultrastructure analysis by using TEM is fundamental not only in the first approach of characterization of newly isolated phages but also for providing useful information to go further to the selection process as potential bio-decontaminants.

**Key words:** Bacteriophages, bacteria, bio-decontaminants, morphology, pathogens, TEM (Transmission Electron Microscopy).

## 1. Introduction

Bacteriophages are viruses that recognise bacteria as their specific hosts. Lytic bacteriophages in particular are prokaryote's natural enemies, in fact, after having infected the cell, they lyse it as final consequence of their replication.

There are an estimated  $10^{31}$  bacteriophages on the planet [1-4]. Their specificity for a particular bacterium is expressed towards the strain, the species and more rarely the genus level [3], while they are totally innocuous for eukaryotic cells, animals and humans [5, 6].

Early papers on bacteriophages are dated around the 20's. At the beginning, phages were employed as diagnostic tools in bacteria [3, 7-15]. Lately they started to be used for prophylaxis and therapy both in animals and humans in Eastern Countries. Their

natural anti-bacterial activity was scientifically and clinically confirmed and they were administered particularly in those cases where antibiotics failed.

Today bacteriophages are more and more recognised as safe, efficacious [6, 16] and innovative alternatives to the use of chemotherapies (phage-therapy) [17-19]. This would enable to prevent bacterial antibiotic resistance development. Moreover they are also identified as active substances to be used against unwanted bacteria for bio-decontamination in flocks and livestock but also in hospitals and along the chain of food productions (bio-decontaminants) [3, 20].

Another aspect to take in consideration is the undesirable implication in cheese making when specific lactic phages infect and lyse LAB (lactic acid bacteria) which are indispensable for milk curdling [21, 22].

Since they are ubiquitous and their prevalence is high in the same environment where their hosts are

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abundant, bacteriophages can be considered good indicators of the presence of bacteria [23]. For instance it is clearly demonstrated the correlation between coliphages (phages active against *Escherichia coli*) and bacteria responsible of colibacillosis in animals [24].

Bacteriophages are differentiated on the bases of their morphology and for this reason TEM is still irreplaceable [25]. They present a great shape variability and their primary classification is based on six groups established from Bradley in 1967 [26]. The groups A, B, C, D and E are distinguished according to head shape (icosahedral or elongated) and tail (presence or absence). In case of presence, the tail can be contractile or non-contractile and short or long when compared to head diameter. Some phages can also show appendices (tail-fibers). Filamentous phages, instead, belong to group F. From phage's ultrastructure it is also possible to define some genome characteristics (single/double DNA chains or single RNA chains) [26].

Another phage classification always based on morphology is used for *Campylobacter* lytic bacteriophages. In particular they are identified into three groups in relation to head diameter and genome size [27].

In the present work It focused on morphological characterization of one phage implicated in industrial fermentations ( $\phi$ Apr-1 active against *L. lactis*) and of another phage ( $\phi$ IZSAM-1) that is currently being assayed for future applications against *L. monocytogenes*.

$\phi$ Apr-1 and  $\phi$ IZSAM-1 were morphologically compared with  $\phi$ P100, a phage active against *L. monocytogenes*.

Moreover in the authors' study, they confirmed the positive correlation between phages and their hosts in the environment and we also demonstrate how a punctual TEM ultrastructure analysis of viral particles can contribute to their further selection process as bio-decontaminants.

## 2. Materials and methods

### 2.1 Bacteriophages and Hosts

The first phage to be assayed for morphological characterization was  $\phi$ Apr-1, active against *L. lactis* and implicated in cheese fermentation failures. The phage and its host were isolated from whey starter cultures used for the production of D.O.P. Italian water buffalo mozzarella cheese.

The second phage,  $\phi$ IZSAM-1, active against *L. monocytogenes*, was isolated from waste waters of a cheese plant that was monitored for *L. monocytogenes* contamination and where this pathogen was constantly detected. The host used for phage harvesting was *L. monocytogenes* ATCC 7644, serotype 1/2 c.

The third phage,  $\phi$ P100, is also an anti-*Listeria* phage and it is commonly used in U.S.A. to prevent *L. monocytogenes* contamination in Ready To Eat food [28-32] as principal component of a product called Listex™ P100 (Micros, Wageningen, Holland).

All bacteriophages were cultured in liquid media [33] for 24 h and filtered with 0.45  $\mu$ m filters (lysates).

### 2.2 TEM Analysis

For each phage a 200 mesh copper grid coated with carbon-stabilizer formvar was inserted into a tube for airfuge (Beckman), filled with 120  $\mu$ L of each lysate, centrifuged at 20 psi for 15 min and negative stained with 2% phosphotungstic acid. Each sample was then observed with TEM EM 900 T (Zeiss) between 12000x and 80000x magnification.

## 3. Results

The ultrastructure analysis of the three bacteriophages delivered the following results:

$\phi$ Apr-1: icosahedral-isometric head of about 50 nm diameter. Thin, long, non-contractile, flexible tail of about 110 nm in length. Total phage length is about 160 nm. From the analysis of these data phage  $\phi$ Apr-1 was located in the *Caudovirales* order, *Siphoviridae*

family [34], Group B, Morphotype B1 [35]. It is a double stranded DNA virus (Fig. 1).

$\phi$ IZSAM-1: icosahedral-isometric head of about 60 nm in diameter. Long, non-contractile and flexible tail of about 170 nm in length. Total phage length is about 230 nm. Also this bacteriophage is related to *Caudovirales* order, *Siphoviridae* family [34], Group B, Morphotype B1 [35] and it is therefore a double stranded DNA virus (Fig. 2).

$\phi$ P100: icosahedral-isometric head of about 80 nm in diameter. Neck of about 20 nm in length connected to a long, rigid and contractile tail of about 90 nm lengths. The tail is constituted of an internal tube and of a clearly visible external contractile sheath. The total phage length is about 190 nm. Also base-plate and the tail-tube protruding from the contracted tail were clearly identified. The different pattern of this phage's tail located it in the *Myoviridae* family of the Order *Caudovirales* [34], Group A, Morphotype A1 [35]. This is also a double stranded DNA virus (Fig. 3).

#### 4. Discussion

TEM contributes to a high level of phage classification. Nature and organization of phage genetic material is directly deduced from viral morphology.

When compared with genome analysis, negative staining is much faster to perform and data deriving from virus shape observation provide useful information in short time. In addition to classification, morphological findings are useful also for comparing and selecting bacteriophages to employ in prophylaxis (phage therapy) or in bio-decontamination.

Phages with contractile tail like  $\phi$ P100 present a higher genetic complexity and different mechanisms of DNA injection during infection when compared with phages having non-contractile tails (e.g.  $\phi$ Apr-1 and  $\phi$ IZSAM-1) [36].

Important differences involving assembly pathways can be derived from tail lengths. Bacteriophages with

long tails (longer than head diameter) like the three phages speculated in this work, assemble heads and tails separately and then add them together. Instead short-tailed phages (tail shorter than head diameter) add the tail sequentially onto completed heads [36].

Tail lengths give information also about phage stability and resistance in the environment. In fact short and not-tailed phages are generally more resistant while long tails tend to be damaged easier, resulting in loss of infectious activity.

Tailed phages are constituted of double stranded DNA while not-tailed phages have completely different genomic pattern, with single-stranded DNA or RNA [26].

Moreover TEM enables to distinguish between “full” infective virus particles and empty “ghost” particles ((Fig. 4). “Ghost” particles, in particular, are represented by viruses after loss of their genetic material as consequence of stress factors (e.g. heat, UV radiations, high pressures) [6]. Empty phages cannot replicate but their lytic activity is still preserved because of the presence of cell wall degrading enzymes (lysis from without) [6, 37].

Some other useful information could arise from the observation of “phage agglomerates”. These spatial

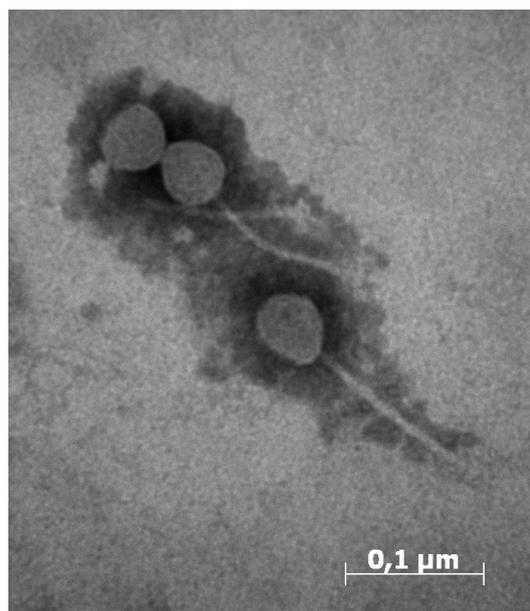


Fig. 1 Phage  $\phi$ Apr-1.

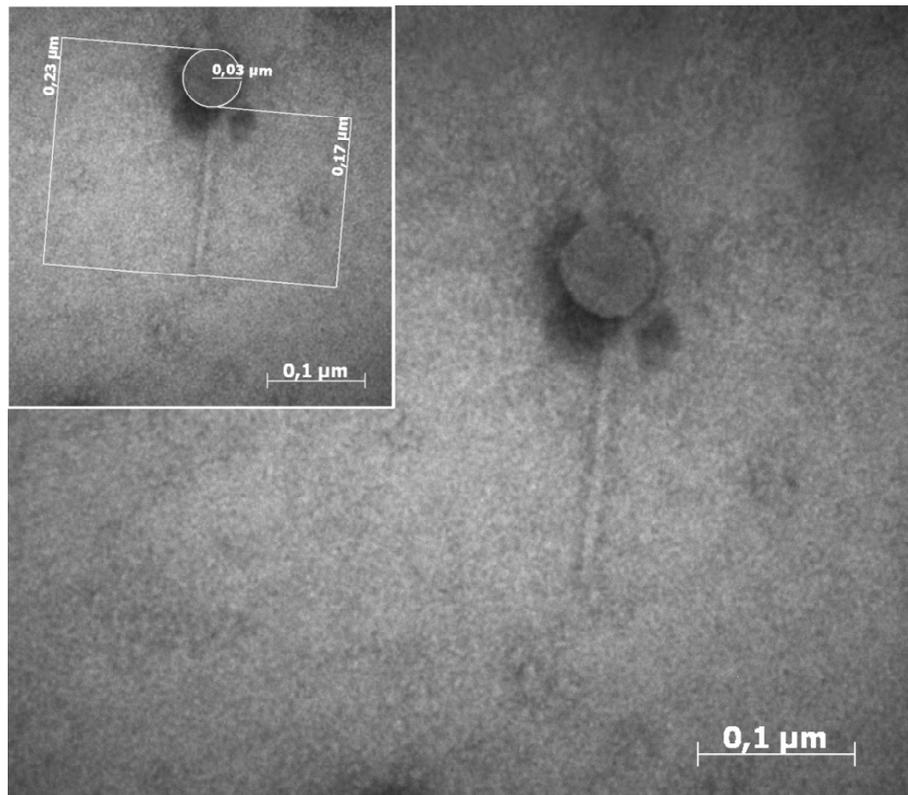


Fig. 2 Phage  $\phi$ IZSAM-1.

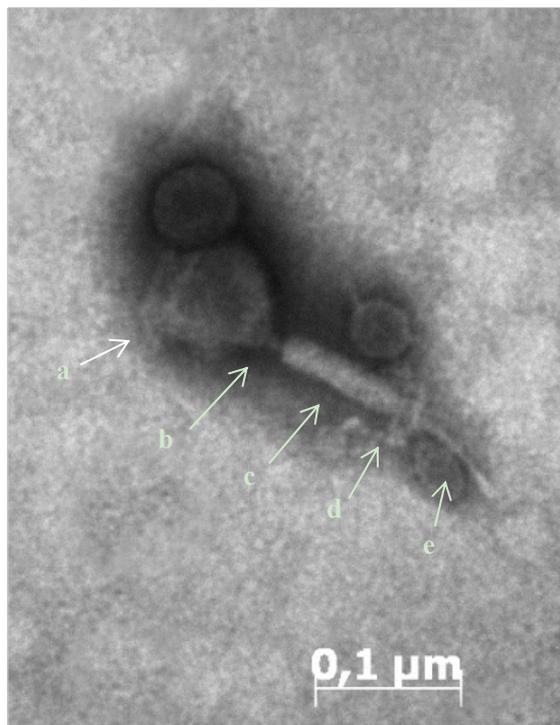


Fig. 3 Phage  $\phi$ P100 with identification of body elements (a—head, b—neck, c—contracted sheath, d—baseplate, e—tail tube).

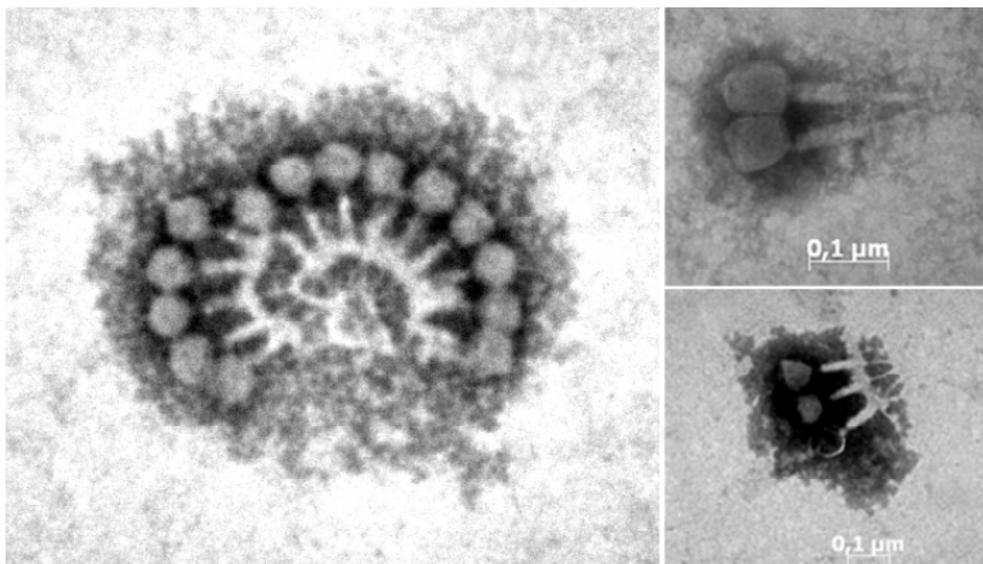
dispositions are consequence of high titre suspensions of large bacteriophages [38], with phageheads adhering together and tails left free towards the inner side of “micelles”. These phage suspensions generally present a very low infectivity grade (Fig. 5).

Full/ghost particles and phage agglomerates in particular are useful targets for scientists to screen and evaluate the “quality” of phage lysates.

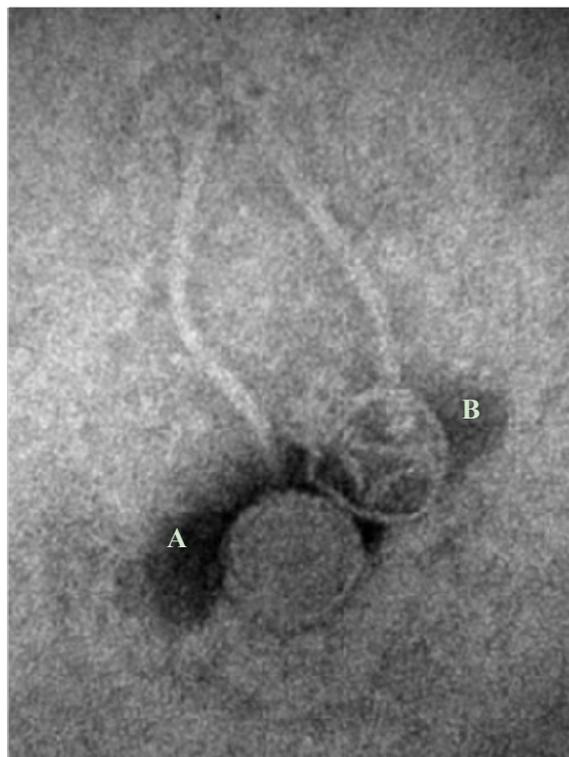
## 5. Conclusions

Since the discovery of Transmission Electron Microscopy about 70 years ago, bacterial viruses and TEM are deeply linked. Microscopy demonstrated that bacteriophages are viruses with complex sizes and shapes, with intracellular obligate development and unique assembly activities.

TEM provided from the beginning the elements for establishing bacteriophage orders and families and its role is still actively recognised. In fact with the development of new scenarios that locate



**Fig. 4** Bacteriophage  $\phi$ P100 “agglomerates”.



**Fig. 5** Phage  $\phi$ IZSAM-1: “full” (A) and “ghost” (B) particles.

bacteriophages in a different framework of applications, shifting from fields of diagnosis to phage-therapy and bio-decontamination, Electron Microscope plays a key role in classifying “novel” phages that are actively being isolated into families.

The features that derive from a punctual viral

morphological analysis are useful also for comparing phages and completing their selection process.

Moreover the results confirm bibliographical data about correlation between phages and host present in the same environments, identifying these viruses as good indicators of bacterial contaminations.

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