Bioinformatic studies of annotated proteins from marine phage CR1 and its evolutionary relationship with other phages

A Dissertation report for

Course code and Course Title: MBO 381 Dissertation

Credits: 08

Goa University

Submitted in partial fulfilment of degree of

Masters in Science in Marine Biotechnology

by

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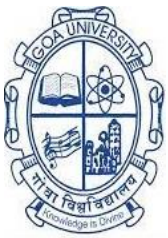

Goa University

APRIL, 2023

DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, " Bioinformatic studies of annotated proteins from marine phage CR1 and its evolutionary relationship with other phages" is based on the results of investigations carried out by me in the Marine Biotechnology discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of Prof. Sanjeev C. Ghadi and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation / internship report **Bioinformatic studies of annotated proteins from marine phage CR1 an its evolutionary relationship with other phages** is a bonafide work carried out by **Mr. Samuel Singh** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of **Masters in Science** in **Marine Biotechnology** at the School of Biological Sciences and Biotechnology, Goa University.

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ACKNOWLEDGEMENT

First off all, I am sincerely thankful to Department of Biotechnology (DBT) India for providing me this opportunity and with the financial support. Secondly, School of biological science and Biotechnology, Goa university for providing me all the facilities, instruments, and chemicals to carry out my research work.

I would like to thank my dissertation guide Prof. Sanjeev C. Ghadi, head of department, Discipline Marine Biotechnology, School of Biological Sciences and Biotechnology, for his valuable guidance, suggestions and for patiently answering all my queries regarding the project.

I owe my sincere gratitude to PhD scholar Ms. Devika Jadhav for constantly guiding and supporting me, for clearing my doubts, for helping me in my experiments and for proof reading of the draft.

I would also like to thank my teachers Prof. Savita Kerkar (Dean of School of Biological Sciences and Biotechnology), Dr. Meghnath Prabhu, Dr. Dharmendra Tiwari, Dr. Samantha Fernandez D'Mello, Prof. Usha D. Muraleedharan, Dr. Sanika Samant, Ms. Snesha Bhomkar, Ms. Dviti Mapari and all the lab staff for their constant support and guidance.

I would also like to thank the non-teaching staff, Mr. Sameer, Mr. Ashish, Mr. Serrao, Ms. Sandhya, Ms. Jaya who provided us with apparatus and materials required for the project work.

I am also thankful to my classmates for helping me in need, for making the lab a clean and healthy place to work and for their constant support and motivation.

At last, I would like to thank my parents for believing in me and for their constant love and mental, emotional, and financial support.

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1.INTRODUCTION

Viruses are the most prevalent "lifeforms" in the oceans and ubiquitous companions of cellular life forms. Most of the genetic variation within the aquatic ecosystem is stored in viruses (Curtis A. Suttle, 2007). Every cellular creature under investigation seems to have its own viruses, or at the very least, selfish genetic components resembling viruses. Recent environmental investigations have revealed that the "most abundant biological entities on the planet" are viruses, particularly bacteriophages. On Earth, there are thought to be 10^{31} viruses, the majority of which are bacteriophages (Breitbart M., Rohwer F., 2005).

Bacteriophages are nano-sized biological entities that infect bacteria for their survival and replication. The term “bacteriophage” comes from the Greek bækˈtɪərioʊ (bacterio)- bacteria and feɪdʒ (phage) —to kill or devour. They have been isolated from almost everywhere on our planet: common environments like soil and surface waters as well as extreme environments like hot springs, deep seas, etc. (Romancer et al., 2006). In nature, they keep the dominant bacterial population under control, and their infection is usually self-limiting. They are considered to play a key role in bacterial adaptive evolution by means of gene transfer (Davies, 2016).

The most frequently used classification divides tail components of bacteriophages according to their biological cycle (Fauquet and Pringle, 2000). Two groups are identified, lytic, or virulent, and lysogenic, or temperate, bacteriophages. In marine ecosystems, though they are anticipated to carry out infection mechanisms by both mechanisms, temperate phages are predicted to be more common (Tuttle M. J., Buchan A., 2020). Lytic phages, upon infection, cause the lysis of the host cell, while temperate phages insert their genome into the host genome and are then termed prophages. Bacterial hosts containing such phages are called lysogens. Prophages do not express their lytic genes until the host is under stress. This stress signals the virus to induce the expression of lytic genes, causing bacterial cell lysis. This lysis is visible in the form of plaques.

In the present study, a bacterial strain, CR1, is used and studied. Bacterial strain CR1 is a marine polysaccharide-degrading bacteria belonging to the genus *Microbulbifer (*Poduval et al., 2019). The genome studies of CR1 strain indicate the presence of lysogenic prophage.

Polysaccharides are the most frequent and complex organic substances in the ocean. Gammaproteobacteria belonging to and related to the genus *Microbulbifer* are an emerging group of complex carbohydrate-degrading marine bacteria (Nathan et al., 2005). Members of this genus can

degrade complex carbohydrates such as cellulose, alginate, and chitin. The cell walls of macroalgae, in particular, include a high diversity of sulfated polysaccharides (carrageenans, agarans, fucoidans, ulvans, etc.), many of which are highly sulfated in contrast to terrestrial polysaccharides. These sulfated polysaccharides are a crucial source of nutrition for heterotrophic organisms and are biosynthesized by macroalgal primary producers (Aquino et. al., 2005).

OBJECTIVES

- Induction of lysogenic phage in *Microbulbifer* CR1 strain.
- Time dependence study to achieve maximum phage titre.
- Evolutionary studies of prophage proteins identified from the genome of *Microbulbifer* CR1 strain.

LITERATURE REVIEW

Marine bacteriophages:

The most prevalent type of biological organism is a bacteriophage. They can be found in extreme habitats, such as those with very high or very low temperatures, soil and seas, oceanic and terrestrial surfaces, and harsh environments in general (Clokie et al., 2011). Bacteria are the most common type of living organism in the oceans and are infected by most marine viruses, commonly referred to as "bacteriophages." Marine viruses are found at concentrations up to ∼1 × 108 ml−1, resulting in an estimated ∼4 × 1030 viruses in the oceans (Suttle 2005). However, the majority of bacteriophages are found in the vast, uncultured dark matter of the microbial biosphere. Marine bacteriophages have a significant impact on the makeup of microbial communities and the biogeochemical cycling of crucial components (Perez et al., 2016). One kilogram of marine sand may contain a million different viral genotypes, and it is estimated that there are 5,000 viral genotypes in 200 litres of saltwater. On the other hand, certain molecular and cultural studies have found that viruses move between various biomes. These findings suggest that viral diversity can be high locally but low globally. Additionally, viruses can assist horizontal gene transfer by hopping between environments (Breitbart, Rohwer, 2005). Viruses reach their host by passive diffusion, and the contact is proportional to the host's size and abundance (Murray and Jackson, 1992).

The importance of lysogeny in marine environments:

When a phage infects an appropriate host, one of two main replication routes can take place. One is the lytic cycle, in which the freshly generated viral offspring are released as the host cell explodes and the phage genome replicates right away after infection. The second is the lysogenic cycle, in which the host cell passes on the phage genome from generation to generation in an infectious state until the lytic cycle is triggered by elements like UV radiation or rapid host growth. Temperate phages are those that can develop lysogeny with their hosts, whereas virulent phages cannot.

Because of their lytic powers, bacteriophages are known to be numerous and significant parts of oceanic food webs. Marine phages use a variety of infection tactics, but despite their apparent predominance, non-lytic phage-host interactions, such as lysogeny, are still poorly understood. According to estimations from sequenced marine genomes, lysogenization occurs in around half of marine bacteria. Prophages are frequently referred to as "time bombs" because of their delay before induction (Paul, 2008; Brum *et al*., 2016). Lysogeny is frequently associated with ocean conditions that are unfavorable to rapid host growth. Many of the marine phages can develop a symbiotic association with their hosts, termed as "lysogeny" (Ackermann and Dubow, 1987). A long-lasting relationship with the bacterial host is maintained by temperate phages that enter lysogeny, as opposed to lytic phages. The advantages of lysogeny from the perspective of phages are obvious, most notably the capability to survive times of low host abundance and the fact that it may provide a refuge from stressors. This situation can lead to the evolution of cooperative relationships that promote effective phage and bacterial reproduction. Large DNA insertions from temperate phages, which have the potential to interfere with gene expression and burden the cell's fitness, are integrated into the bacterial chromosome. They have also been demonstrated to help their bacterial hosts by supplying additional capabilities in a bacterium-phage symbiotic relationship known as lysogenic conversion (Feiner et. al. 2015).

Role of phages in biogeochemical cycles:

Viruses are commonly thought of as pathogens that wreak harm on plants and animals. Twort (1915) and d'Herelle (1917) were the first to describe the presence of substances that may both infect and kill bacteria. d'Herelle (1926) was one of the first researchers to look at viruses in aquatic environments was (Wilhelm SW, Subtle CA, 1999) The viral shunt pathway is a mechanism that prevents marine microbial particulate organic matter (POM) from migrating up trophic levels by recycling it into dissolved organic matter (DOM), which can be readily taken up by microorganisms. It has been demonstrated that the lysis of bacteria by viruses promotes nitrogen cycling and the growth of phytoplankton. In the water, there are roughly 1023 viral infections per second. These infections affect a variety of creatures, from prawns to whales, and are a significant cause of mortality. As a result, viruses have a significant impact on the structure of marine communities and drive several biogeochemical cycles. But in recent years, it has become more and more obvious that they are

essential to the world's oceans. The effect of viruses on the cycling of nutrients and carbon in the oceans is currently of significant interest (Steven W. Wilhelm, Curtis A. Suttle, 1999). They play a key role in the biogeochemical cycling of major elements, for example, by diverting the flow of carbon into dissolved and particulate organic matter through the lysis of their bacterial hosts, thus influencing the amount of carbon that is sequestered in the deep ocean by the biological pump.

Quantifying nutrient and energy flux requires an understanding of the mechanisms for the supply and recycling of organic carbon in aquatic systems. Because all organisms store energy in the form of chemical bonds inside complexes made of carbon, carbon can be thought of as a general tracer of energy transfer through biological systems. The majority of carbon enters the biological system through photosynthesis, where it is changed into sugars by plants and algae. Dissolved organic carbon (DOC) and particulate organic carbon (POC) are the two operating pools into which organic carbon in marine environments is typically divided. One of the main components of marine dissolved organic matter (DOM) is polysaccharides, and bacteria-derived polysaccharidases are primarily responsible for their breakdown. The identification of the mechanisms by which marine dissolved organic matter (DOM) is produced and regenerated is critical to developing a robust prediction of ocean carbon cycling (Lelchat et. al., 2019).

Numerous biomedical research has shown that in order to access their primary receptors, bacteriophages depolymerize their host capsule, slime, or biofilms via polysaccharidases (Bayer *et al*. 1979; Sutherland 1999; Sutherland *et al*. 2004). These enzymes, which are often found on phage tails, exhibit a great degree of functional and molecular diversity, and have the ability to affect viral biological characteristics, including host specificities (Sutherland 1999; Scholl, Adhya, and Merril 2005; Cornelissen *et al*. 2011, 2012; Yele *et al*. 2012). To the best of our knowledge, there is no experimental proof that marine phages have polysaccharidase activity. However, polysaccharides, also known as EPS, are actively produced by marine bacteria and either attached to the cell surface of the bacteria or discharged into the surrounding environment (Leiman *et al*. 2007).

A computational study of prophages:

Experimental approaches for the detection of lysogeny have certain limitations, such as that they require exposure to stressors or dilution of lysogen (Howard-Varona et al., 2017). They are inefficient

at detecting defective phages. With the advances in sequencing technologies, computational approaches are now being adopted for the detection of lysogeny.

Bacteriophages, especially those featuring a temperate lifestyle, are also the major driving force of horizontal gene exchange and bacterial evolution (Brussow et al., 2003; Casjens SR, 2005; Hendrix et al., 2004). Many bacterial genomes contain defective remnants of prophages that are a sign of previous infections or complete, fully functional prophages. They frequently make up a sizeable amount of the genetic material of bacteria (Klummp et al., 2013). A prophage region is described as a cluster or stretch of genes, possibly encoding proteins with bacteriophage-like core functions (i.e., terminase, portal, capsid), interspersed with genes of unknown function or or activities besides host lysis, morphogenesis, packaging, immunity, or phage replication. A real prophage is ultimately inducible, as opposed to defective prophages, which are the non-inducible by products of once-functional temperate bacteriophages. Every infection can provide fresh genetic material to a host or a progeny virus, accelerating the evolution of both the host and the viral assemblages. Exciting new information is always being discovered by probing this enormous pool of genetic and biological variation.

Any type of functional genomics study requires the full genome sequence of the bacteriophage being studied. Computational identification and examination of bacteriophage genomes, followed by phylogenetic analyses, are needed to understand the relationships between phage proteins and their roles.

Materials and Methods

4.1. Host bacteria and culture conditions:

The *Microbulbifer* strain CR1 was obtained from the culture collection of the Biotechnology- School of Biological Sciences and Biotechnology Goa University. The given bacterial strain grows only in specific media, i.e., artificial seawater (ASW). Hence, it was grown and maintained on ASW agar plates at room temperature. The agarolytic activity of the bacteria was checked by flooding the ASW + 2% agar plate with lugol's iodine after incubation at RT for 48 hrs.

4.2. Induction of phages by nutrient starvation:

- **Primary inoculation** - The Microbulbifer CR1 was inoculated and grown in ASW broth containing 0.2% agar under shaking conditions for 24 hours at room temperature.
- **Secondary inoculation** - 100µl of the primary culture broth was inoculated in ASW broth containing 0.2% glucose and was allowed to grow for 24 hours under shaking conditions at room temperature.
- **Induction** - 100µl of ASW from the secondary culture was inoculated in ASW media without any nutrient source. The flask was kept in a shaker for the induction of lysogen.

4.3. Solid propagation of phages:

The double agar overlay technique, as described by Clokie and Kropinski, 2008, was used for phage propagation. The sterile petri-plates were first poured with ASW bottom agar (ASW + 2% agar). 1 mL of lysate was mixed with 1 mL of the grown host culture. This host-phage mix was then seeded into 2 ml of sterile molten soft agar (ASW + 1%) mixed, and poured onto sterile bottom agar. The plates were incubated at 30 °C for 24 hours for the visualization of plaques.

4.4. Induction of phage at different time intervals:

The phage induction by the starvation method was carried out for various time intervals (hours, 5 hours, 7 hours, 10 hours, 24 hours, and 48 hours). After incubation, the phages were visualized using the soft agar overlay method, as described above. The phage titre was calculated in terms of phage forming units per millilitre (pfu/ml).

4.5. Phylogenetic tree analysis:

The prophage sequences predicted from the CR1 bacterial genome were used for further analysis (Poduval et al., 2018, Renuka more, 2021). Out of the three complete prophage regions that were identified, one was used for this study. The annotated proteins (excluding hypothetical and predicted sequences) were used for phylogenetic analysis. The amino acid sequences with a sequence similarity cut-off of >90% and a minimum query coverage of 45% (in some proteins >25% also taken) for each protein were retrieved from the non-redundant protein database and the UniProt/SwissProt database by blastp. The amino acid sequence was aligned using the default parameters of MUSCLE on MEGA11. The sequences were trimmed, and gaps were removed. The neighbour-joining tree and maximum-likelihood trees were then built using default parameters and a bootstrap value of 1000.

Results and Discussion

5.1. Host Bacteria:

The bacterial strain CR1 was grown on ASW agar plates at room temperature for 48 hours. The bacteria formed a clearance zone when Lugol's iodine was poured on the plate. This indicates the agar degrading activity of CR1.

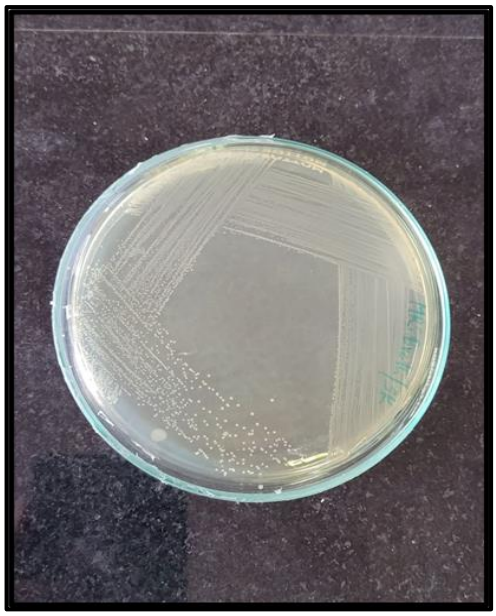

***Fig. 5.1a**. Bacterial strain CR1 on ASW agar plate.*

***Fig. 5.1b**. Agarolytic activity of strain CR1.*

5.2. Induction of Prophage:

The bacterial strain CR1 was allowed to grow in a medium without any carbon source. After incubation, the double agar overlay technique was performed by inoculating 1 ml of the culture. The plates showed the formation of clear plaques of about 1-2 mm in diameter. The formation of the plaques indicated the induction of prophage.

Nutrient starvation is a novel approach for the induction of prophages described by (Poduval et al., 2019). Limited nutrients resulted in the creation of a stressful environment for the bacteria, leading to the release of prophages from the host genome and the subsequent activation of lytic genes. Lysis was evident in the form of visible plaques.

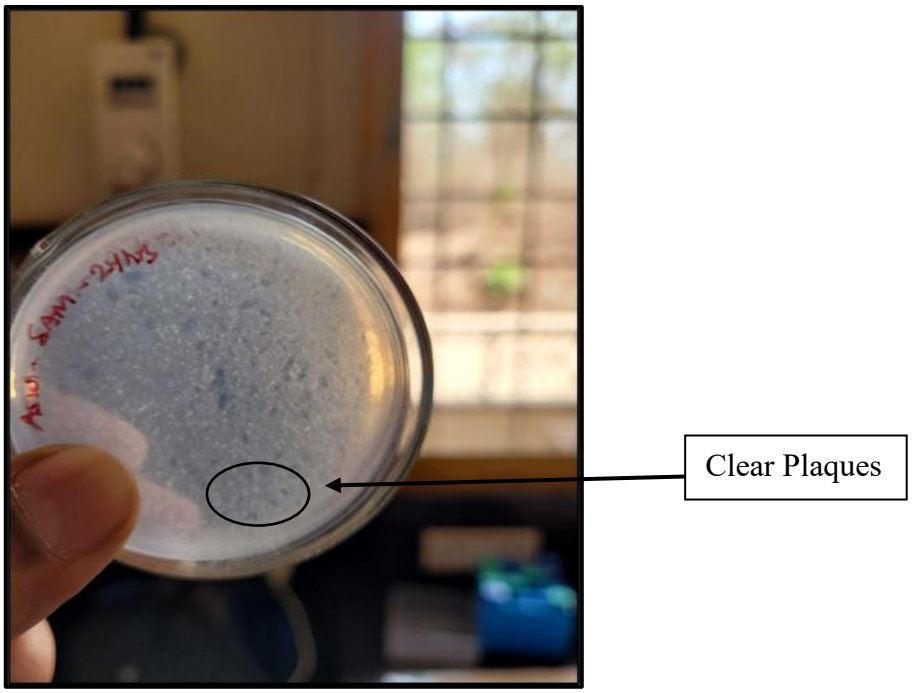

Fig. 5.2. Induction of Prophage. *Double agar overlay shows clear plaques of phage CR1*

5.3. Induction of phage at different time intervals:

1 ml of the host culture growing in the absence of a carbon source was used to perform a double agar overlay at different time intervals. Previous studies showed that complete lysis was observed after 24 hours of incubation, and thereafter, the titre gradually decreased. Hence, in order to attain a high titre of plaques, the double agar overlay was done within the 24-hour time interval. The plaques were counted for each interval, and the Plaque forming unit per ml (Pfu/ml) was calculated.

Incubation time (In hrs)	3 hours	5 hours	7 hours	10 hours	24 hours	72 hours
Pfu/mL	84	67	56	45	30	12

Table 1. *Phage titre at different time*

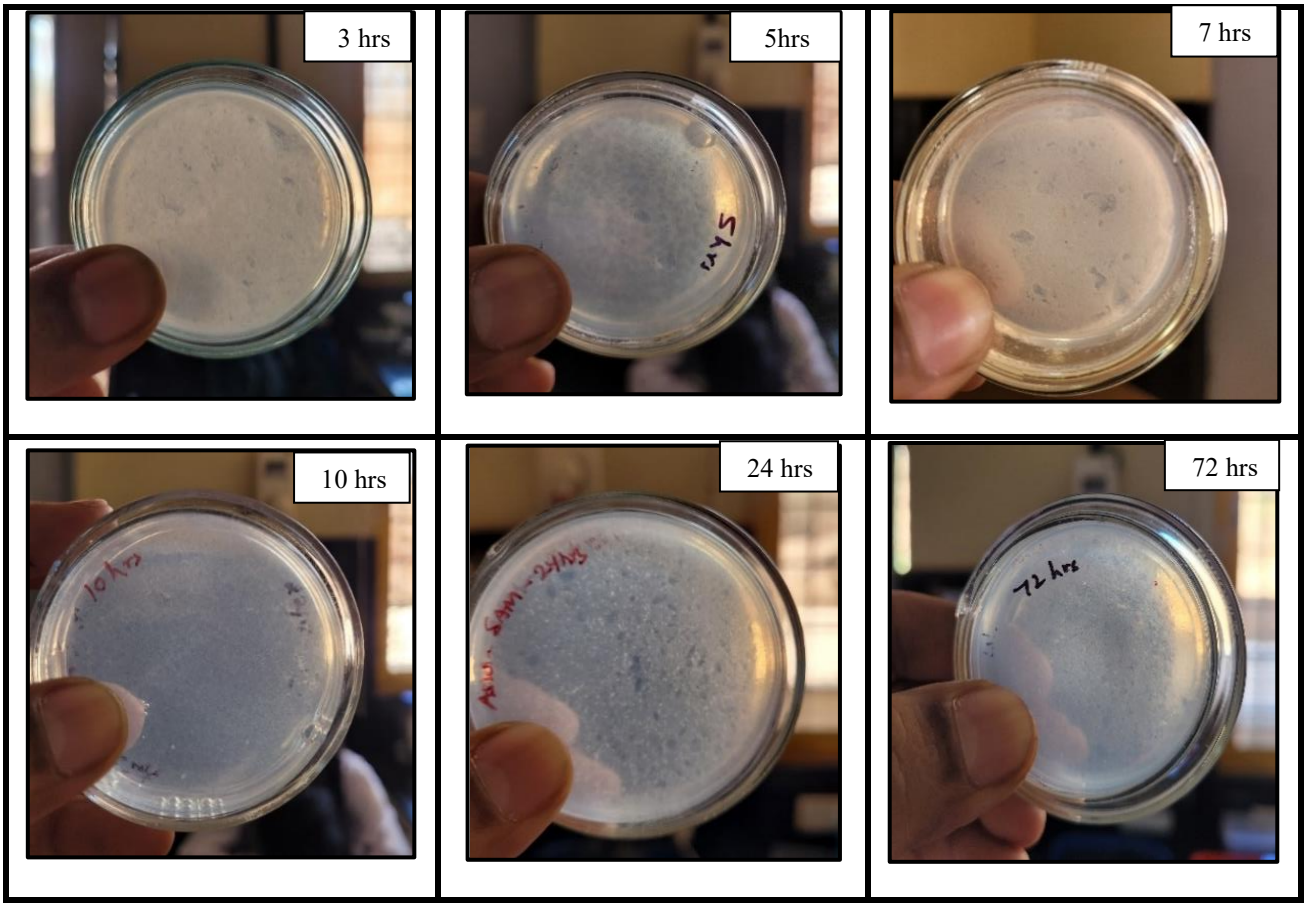

Fig 5.3. Phage titre at different time intervals

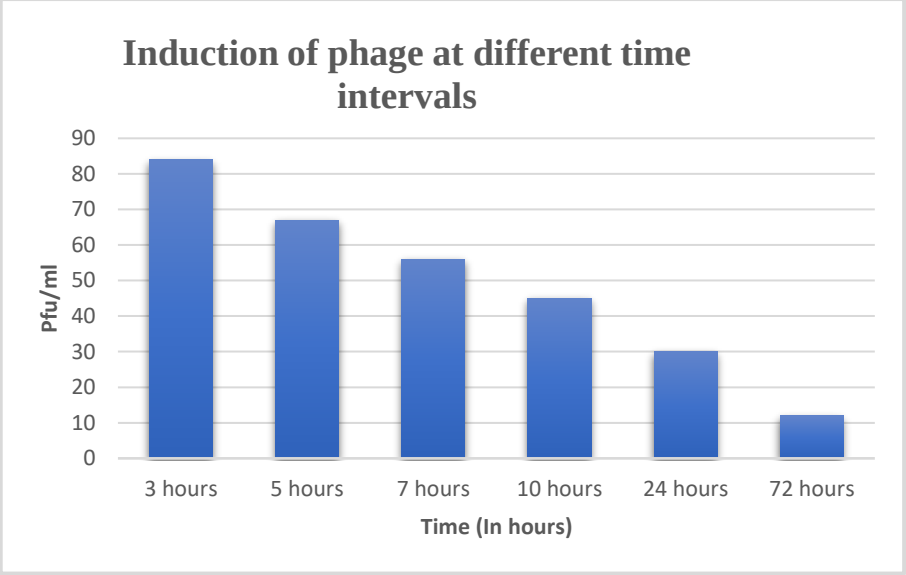

Fig 5.4. Induction of phage at different time intervals.

5.4. Sequence alignment and phylogenetic tree analysis:

Based on the previous studies (Poduval et al., 2019; More, 2021), the CR1 strain has several complete and incomplete prophages. One of the complete prophages, i.e., region 1, has been taken to study its phylogenetics. Region 1 had 33 proteins annotated, out of which 14 are hypothetical and not included in the study, while the rest 19 were studied.

Below are the annotations and position of proteins studied:

S. NO.	ANNOTATION	POSITION
HEAD PROTEINS		
1.	PHAGE head completion-stabilization protein	13427..13633
2.	PHAGE gpE+E	23265..23381
BASEPLATE PROTEIN		
3.	PHAGE putative baseplate assembly protein V	16470..17186
4.	PHAGE: gp16	21143..22309
5.	PHAGE baseplate assembly protein J	17527..18534
6.	PHAGE gp33	11104..12117
CAPSID PROTEINS		
7.	capsid scaffolding protein	10245..11066
8.	PHAGE CR1 gp36	7299..8324
9.	PHAGE gp15	22347..22856
10.	PHAGE gp23	15519..15965
TAIL PROTEINS		

11.	PHAGE tail component protein	13427..13633
12.	PHAGE tail protein	17183..17530
13.	PHAGE tail completion protein-like protein	15095..15526
14.	PHAGE tail tape measure protein	23393..25897
15.	PHAGE putative phage tail protein	25905..26333
16.	PHAGE phage tail protein D	26333..27406
17.	PHAGE gp9	29360..29674
18.	PHAGE gp13	22957..23238
19.	PHAGE tail collar domain protein	19150..21051

Table 2. PHAGE CR1 protein annotations and its position.

5.4.1. PHAGE CR1 Gp9 protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Gp9 protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Gp9 phage protein of *Pseudomonas mirosoviensis* exhibited a 65% sequence similarity with *Phage CR1* Gp9 protein, the phylogenetically closest species with standing members in the tree. Phage protein Gp9 is a component of non-contractile tail, by hydrolyzing the glycosidic connection between rhamnose and galactose in the O-antigen polysaccharide, which is a part of the phage protein Gp9, the capsid is brought close to the cell membrane and mediates initial attachment to the lipopolysaccharides (LPS) of the host cell. *Pseudomonas mirosoviensis* are mostly environmental bacteria widely distributed in soil, water, and air. Most of the host species found in top hits are present in all habitats but their common habitat is terrestrial, except *Comamonas piscis,* which is marine.

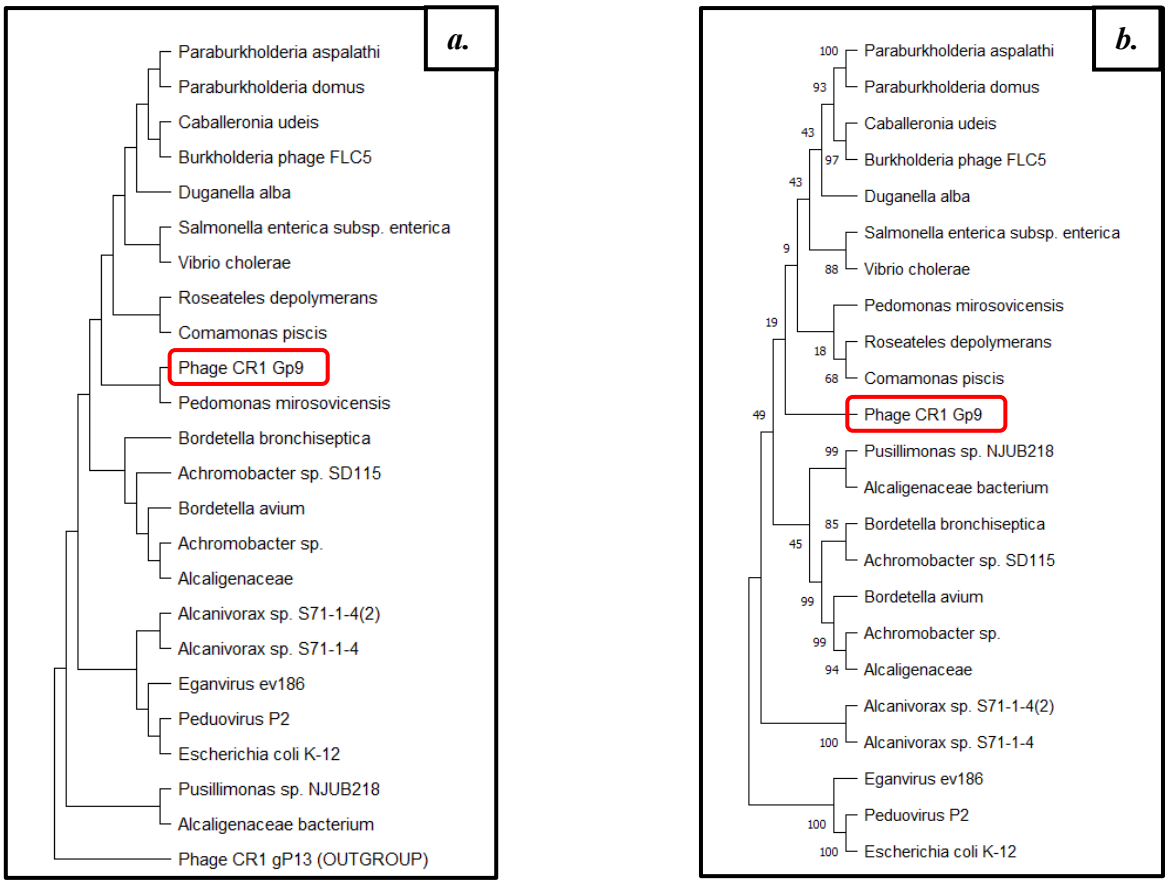

Fig 5.5. Phylogenetic analysis of Phage CR1 Gp9. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

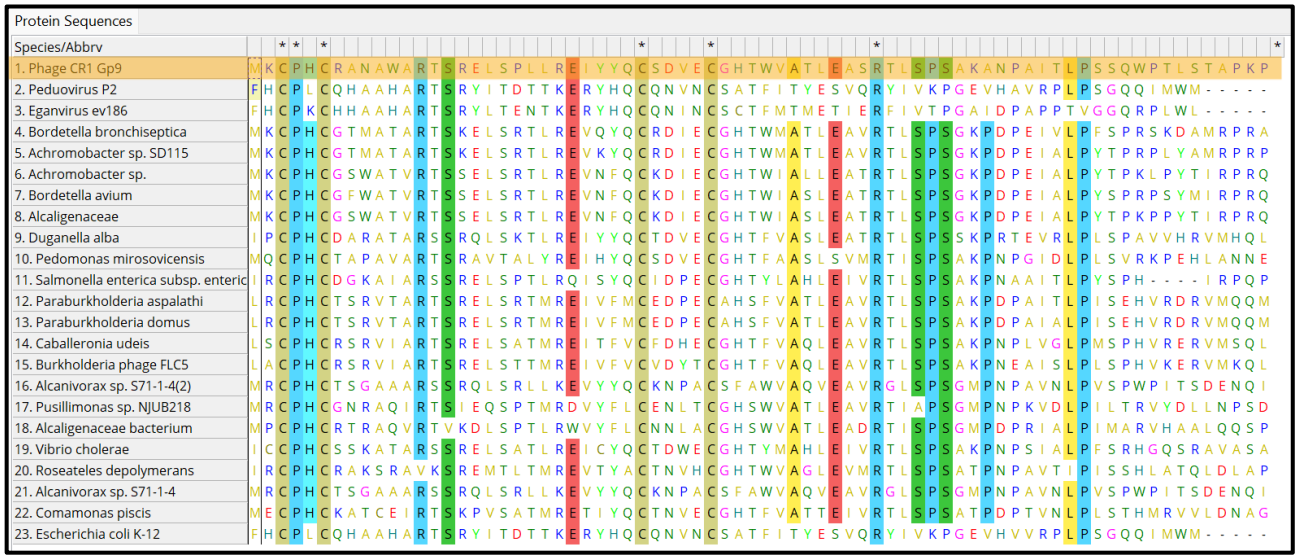

Fig. 5.6*. Multiple sequence alignment of Phage CR1 Gp9 protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Gp9 protein from bacteriophages of various hosts it was observed that cysteine, proline, histidine, cytosine, and others are highly conserved at amino acid positions 3, 4, 5, 6 and 14 other positions, respectively

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5.4.2. PHAGE CR1 Gp13 protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Gp13 protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage Gp13 protein of *Saudiphocaensis* and *Stutzerimonas kunmingensis* exhibited 89% sequence similarity with *Phage CR1* Gp13 protein, the phylogenetically closest species with standing members in the tree. To break down the peptidoglycan layer and make it easier for the viral genome to enter the host bacteria, phage protein Gp13, which is connected to tail proteins, makes contact with the host cell wall first. Essential for the tail assembly.*Marinobacter sp., Candidatus Oceanisphaera merdipullorum,* and *Halomonas gudaonesis* are marine species. *Halomonas gudaonesis* is isolated from a saline soil contaminated by crude oil and involved in its degradation (Wang et al., 2007).

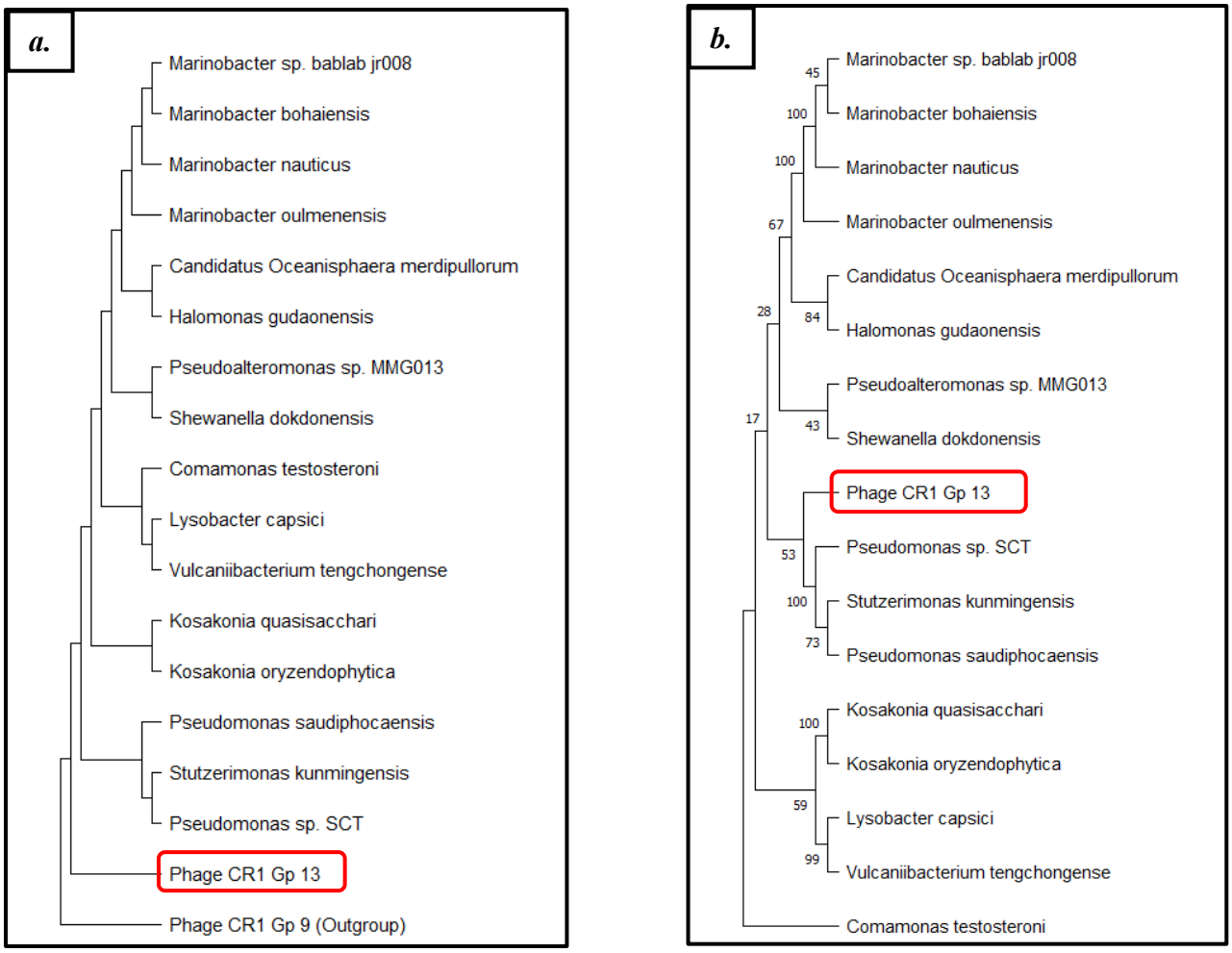


Fig. 5.7. Phylogenetic analysis of Phage CR1 Gp13. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

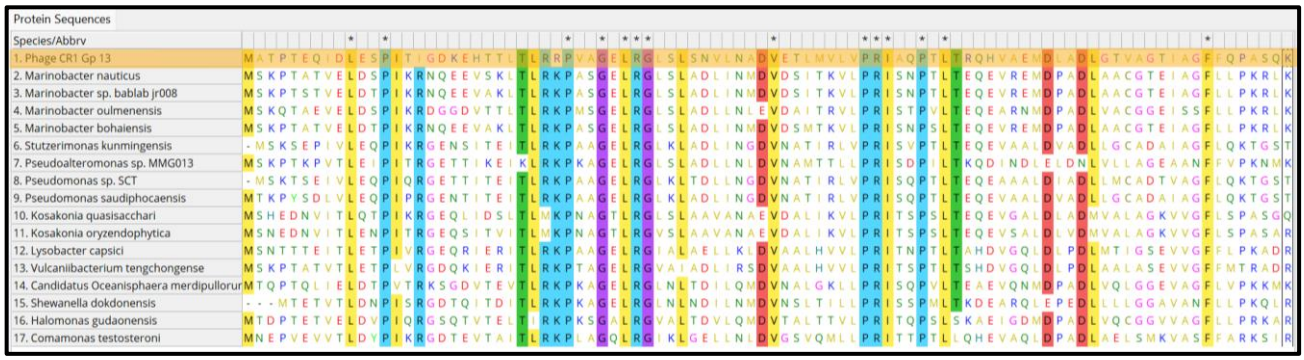


Fig. 5.8. *Multiple sequence alignment of Gp13 protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Gp15 protein from bacteriophages of various hosts it was observed that methionine, leucine, proline, and others are highly conserved at amino acid positions 1, 10, 13, and 24 other positions, respectively.

5.4.3. PHAGE CR1 Gp15 protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Gp15 protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. During the development of the capsid, the phage Gp15 protein assembles and becomes connected with the gp16. The gp15-gp16 complex attaches to the host inner membrane as well as the viral DNA, likely guiding the genome's leading end through the periplasm and regulating the amount of DNA that is translocated into the host cell. Phage Gp15 protein of *Burkholderia phage BcepMu USA/Summer/2002,* exhibited 60% sequence similarity, and *Pseudomonas psychrotolerans* exhibited 100% sequence similarity with *Phage CR1* Gp15 protein, the phylogenetically closest species with standing members in the tree.

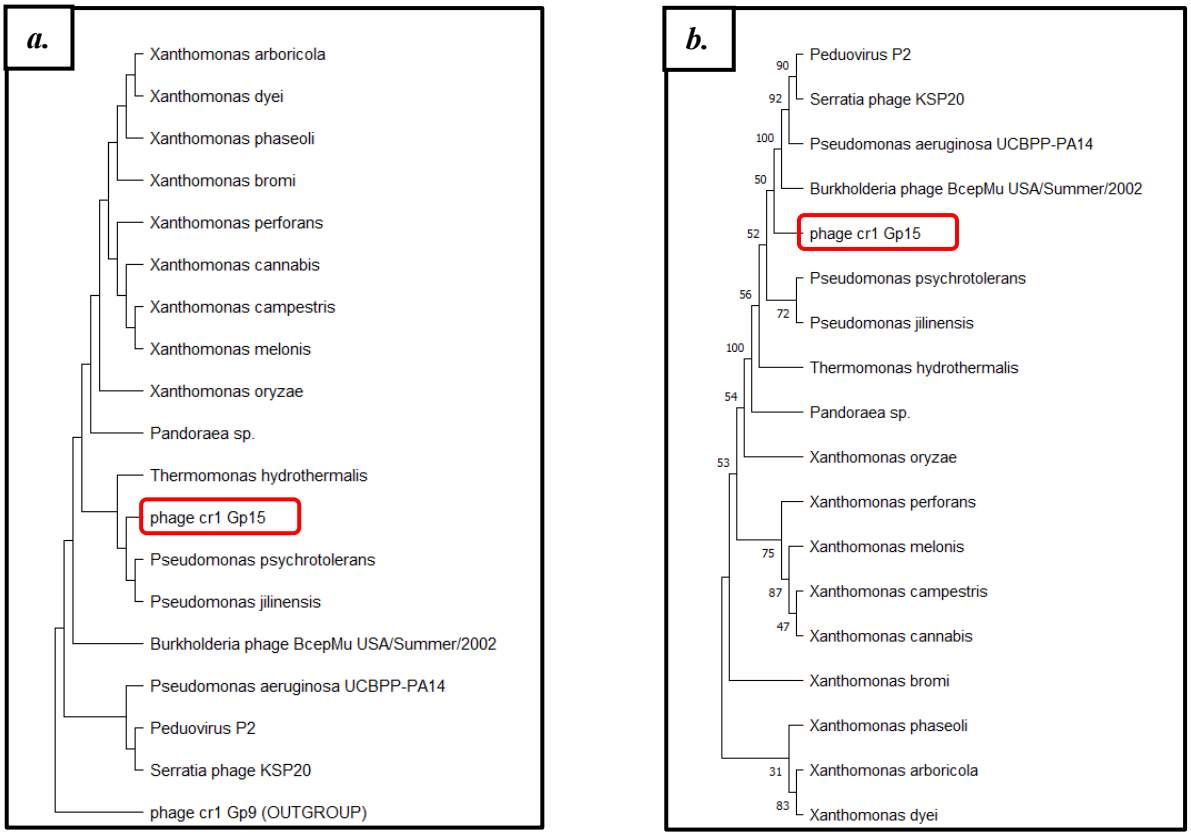
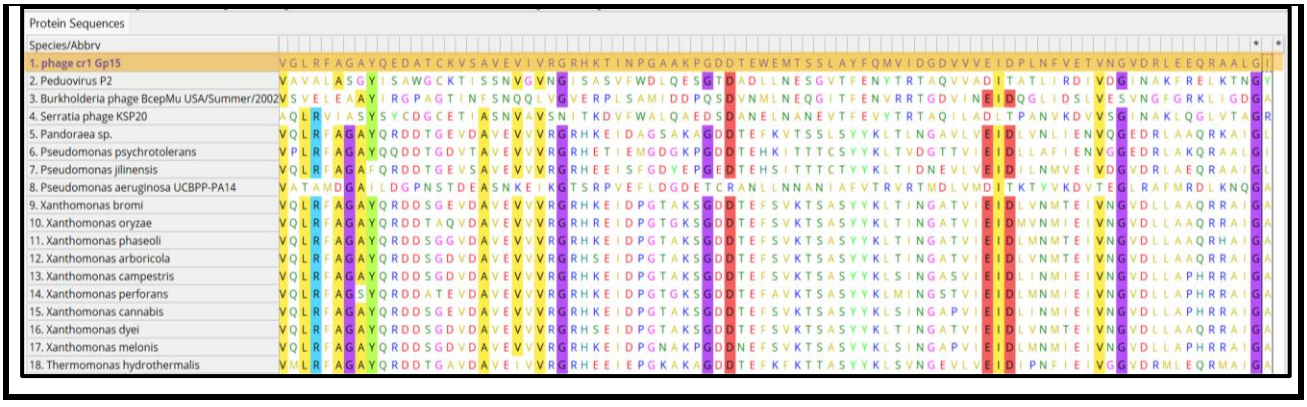


Fig. 5.9. Phylogenetic analysis of Phage CR1 Gp15. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.



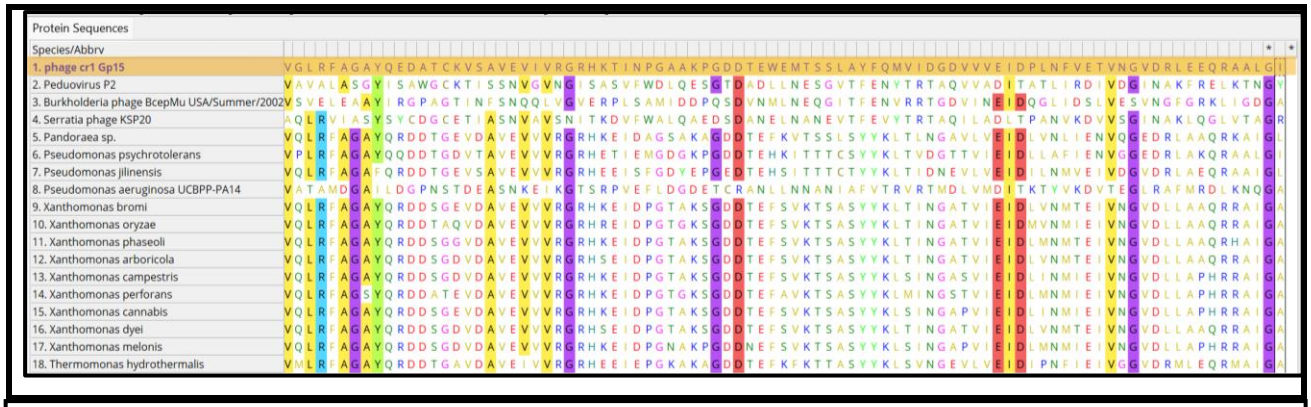


Fig. 5.10. *Multiple sequence alignment of Gp15 protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Gp15 protein from bacteriophages of various hosts it was observed that methionine, asparagine, glycine, and others are highly conserved at amino acid positions 1, 11, 13, 15 and 37 other positions, respectively.

5.4.4. PHAGE CR1 Gp16 protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Gp16 protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. At the baseplate's bottom, phage Gp16 creates a dome that closes the central channel. Gp16 protein opens a channel at the baseplate's bottom for DNA ejection when calcium activates it. Phage Gp16 protein of *Alcanivorax sp. S71-1-4,* exhibited 99% sequence similarity with *Phage CR1* Gp16 protein, the phylogenetically closest species with standing members in the tree.

The genus *Alcanivorax* is one of the most abundant and well-studied organisms for oil degradation. Due to its affinity for metabolising hydrocarbons and derivatives of crude oil, the ubiquitous marine bacterial genus *Alcanivorax* is categorised as an obligatory hydrocarbon clastic bacterium (OHCB).

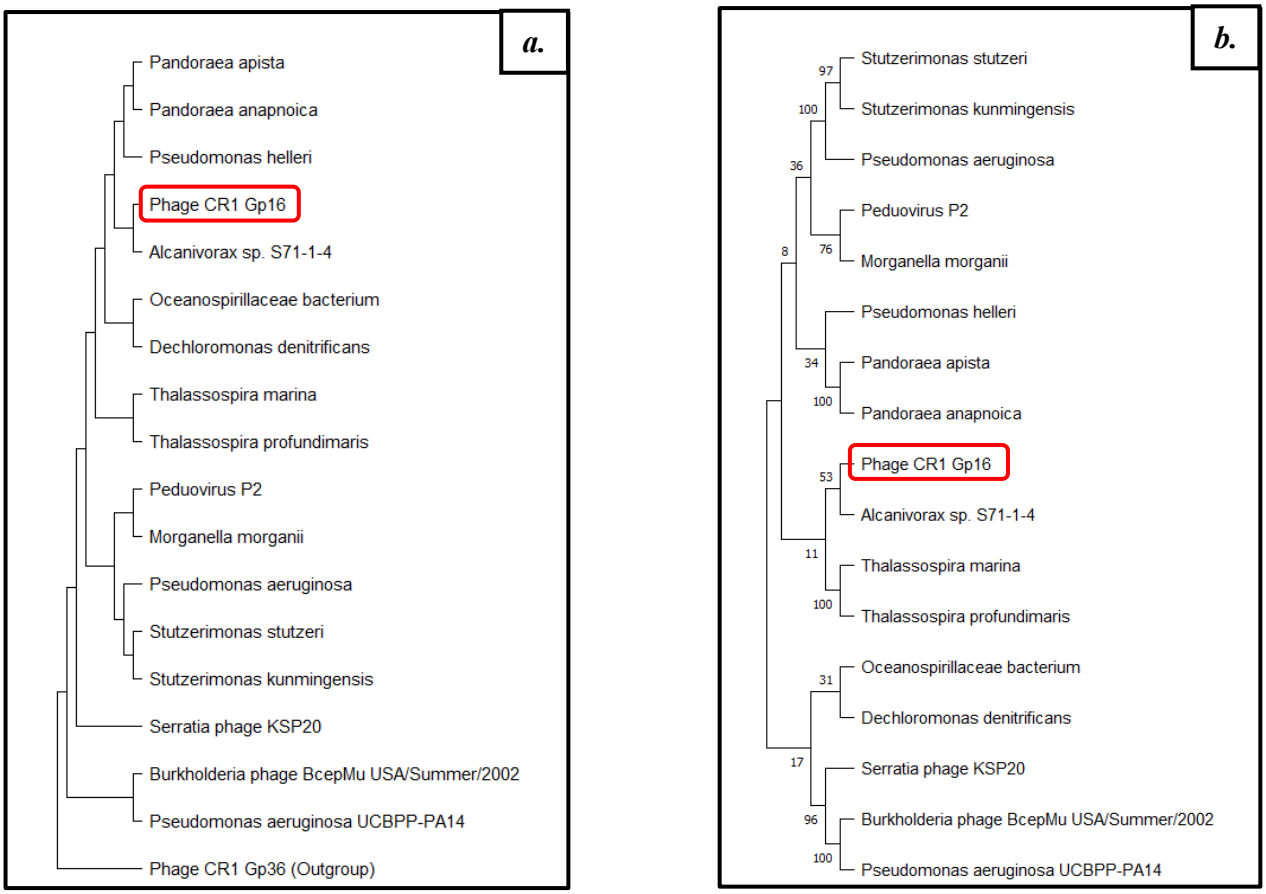
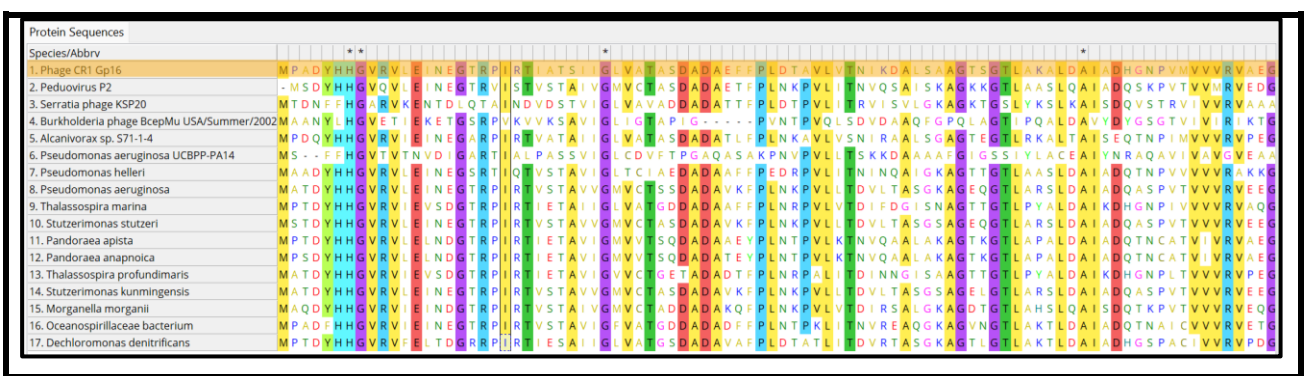


Fig. 5.11. Phylogenetic analysis of Phage CR1 Gp16. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.



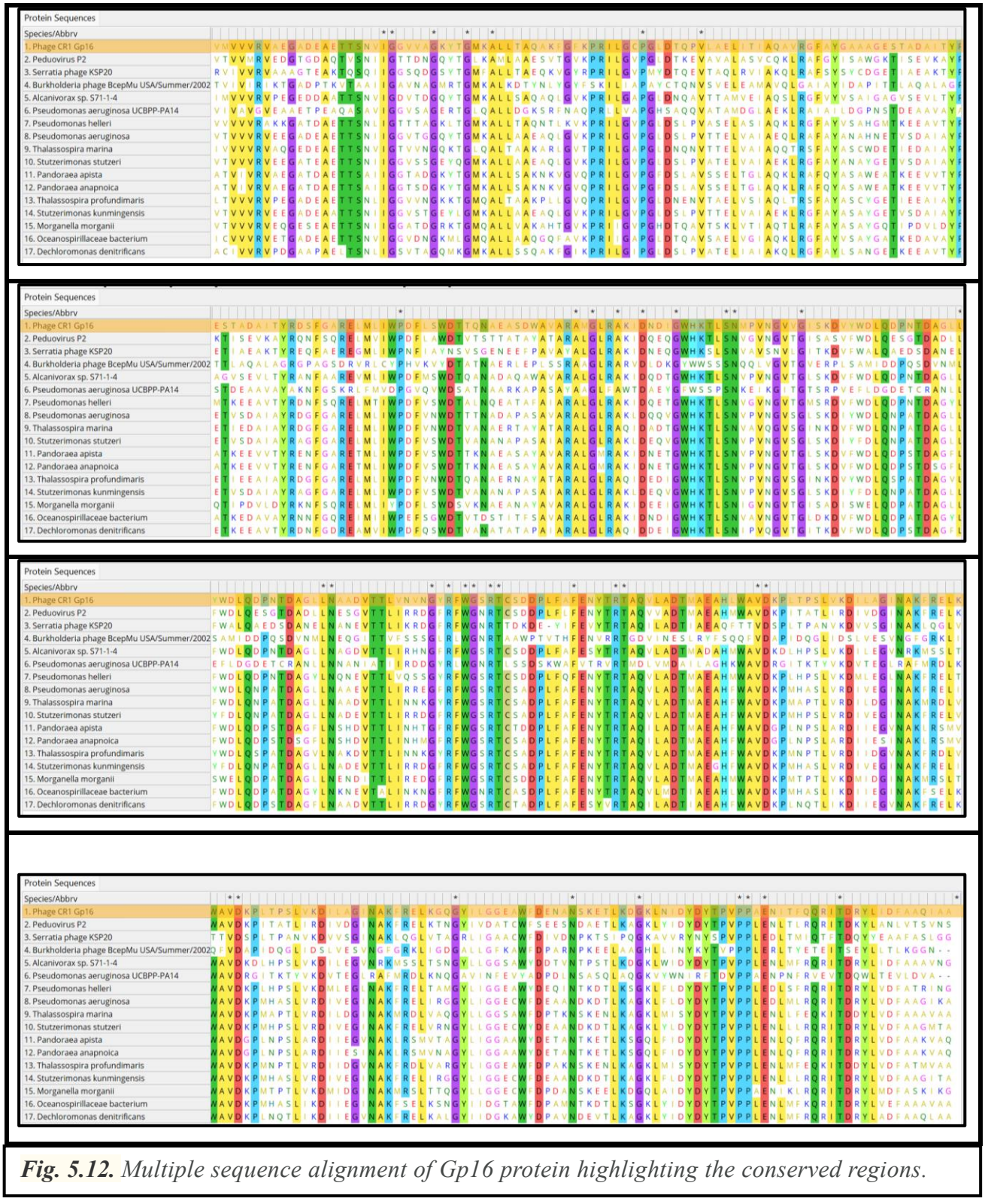


Fig. 5.12. *Multiple sequence alignment of Gp16 protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Gp16 protein from bacteriophages of various hosts it was observed that methionine, tyrosine, histidine, and others are highly conserved at amino acid positions 1, 5, 6, 7 and 144 other positions, respectively

5.4.5. PHAGE CR1 Gp23 protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Gp23 protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. The majority of the capsid is generated by the hexamerization of the phage Gp23 protein. Two chaperones are necessary to aid in the folding of the main capsid protein; these are the host chaperone GroL and the phage-encoded gp23-specific chaperone, gp31. Phage Gp23 protein of *Marinobacterium sedimentorum* exhibited 97% sequence similarity with *Phage CR1* Gp23 protein, the phylogenetically closest species with standing members in the tree. *Marinobacterium sedimentorum* is present in deep sea sediments.

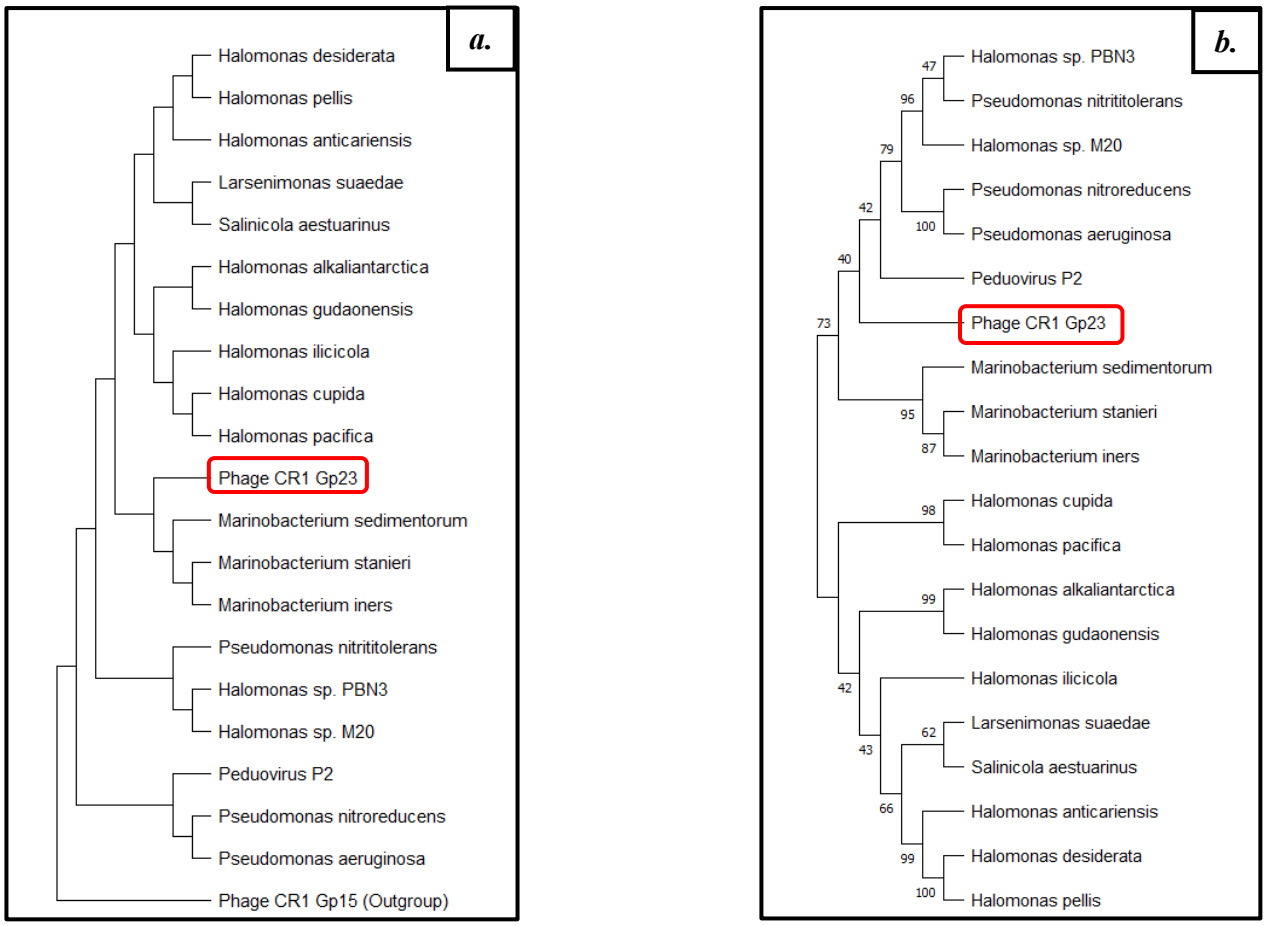
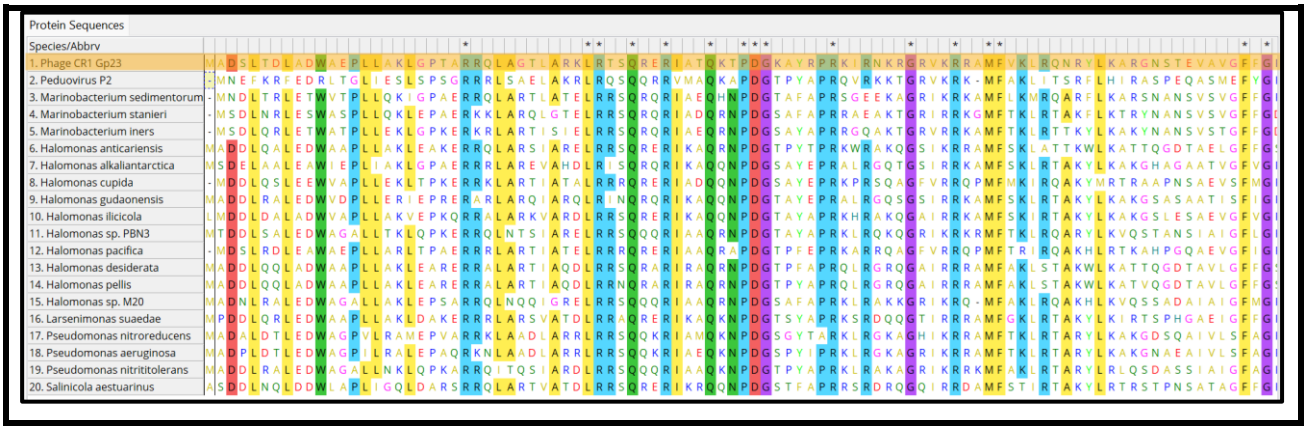


Fig. 5.13. Phylogenetic analysis of Phage CR1 Gp23. (a.) Maximum likelihood tree (b.) Neighbourhood Joining tree.



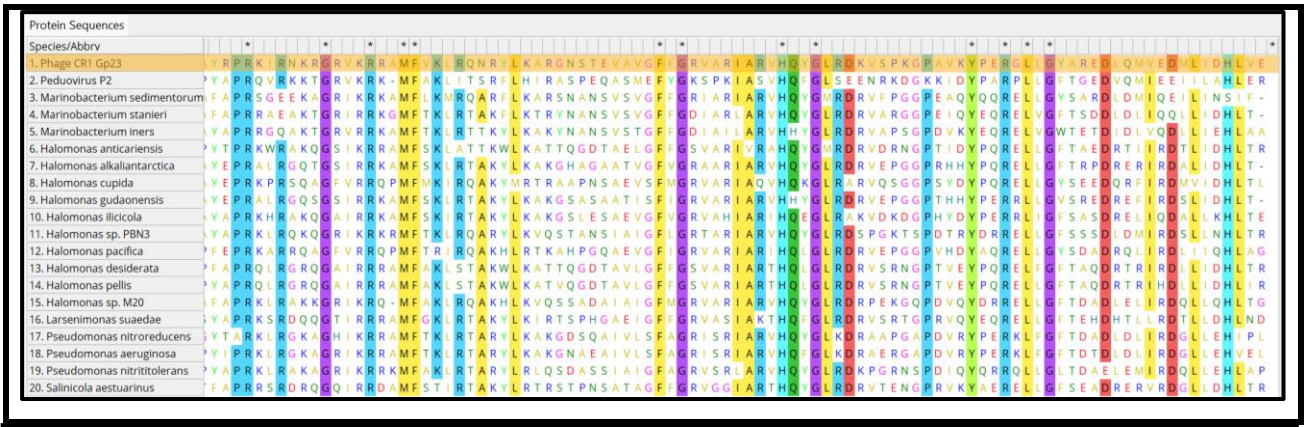


Fig. 5.14. *Multiple sequence alignment of Gp23 protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Gp23 protein from bacteriophages of various hosts it was observed that aspartic acid, leucine, tryptophan, and others are highly conserved at amino acid positions 3, 5, 8, 11 and 57 other positions, respectively.

5.4.6. PHAGE CR1 Gp33 protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Gp16 protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. The function of Gp33 protein is when the sliding clamp is present, it activates transcription at late promoters. binds to the upstream dsDNA as well as the host RNA polymerase (RNAP). Phage Gp33 protein of *Alcanivorax sp. S71-1-4,* exhibited 99% sequence similarity with *Phage CR1* Gp33 protein, the phylogenetically closest species with standing members in the tree.

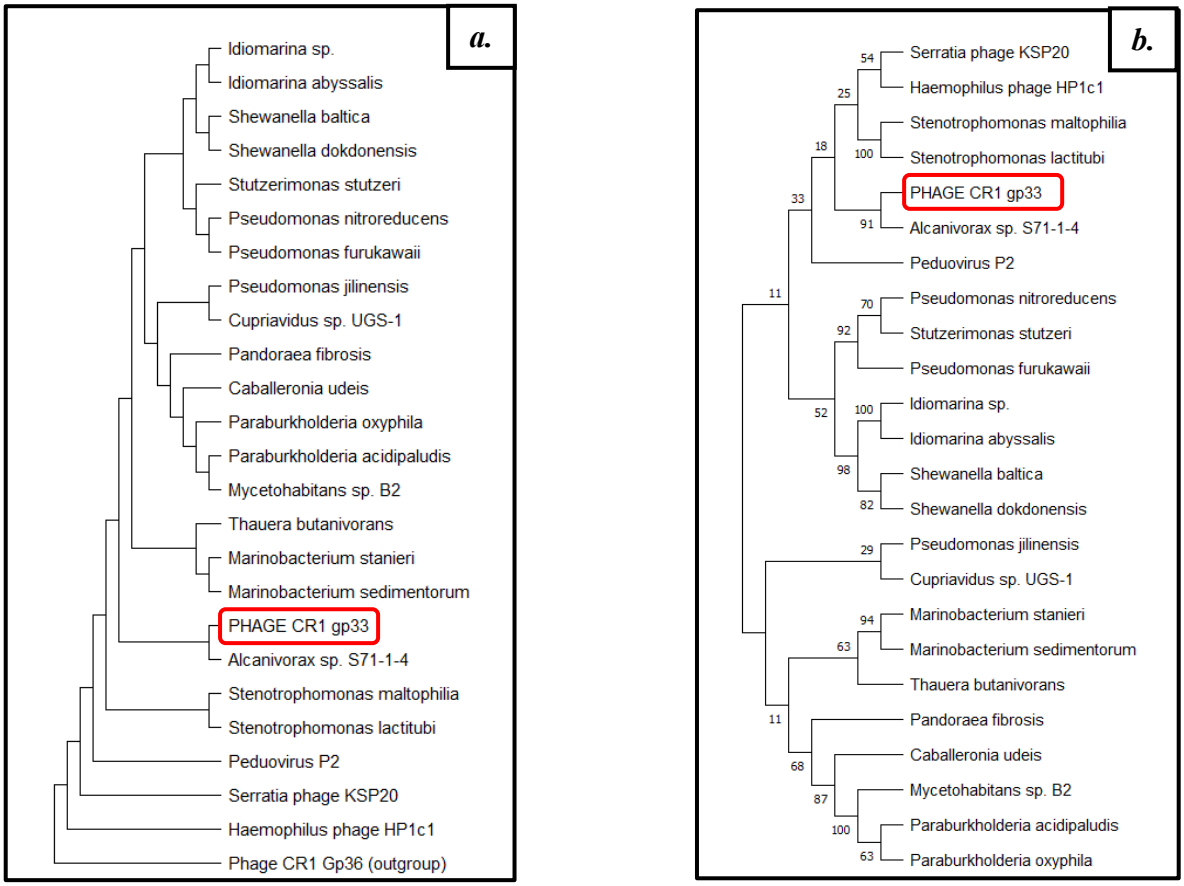


Fig. 5.15. Phylogenetic analysis of Phage CR1 Gp33. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

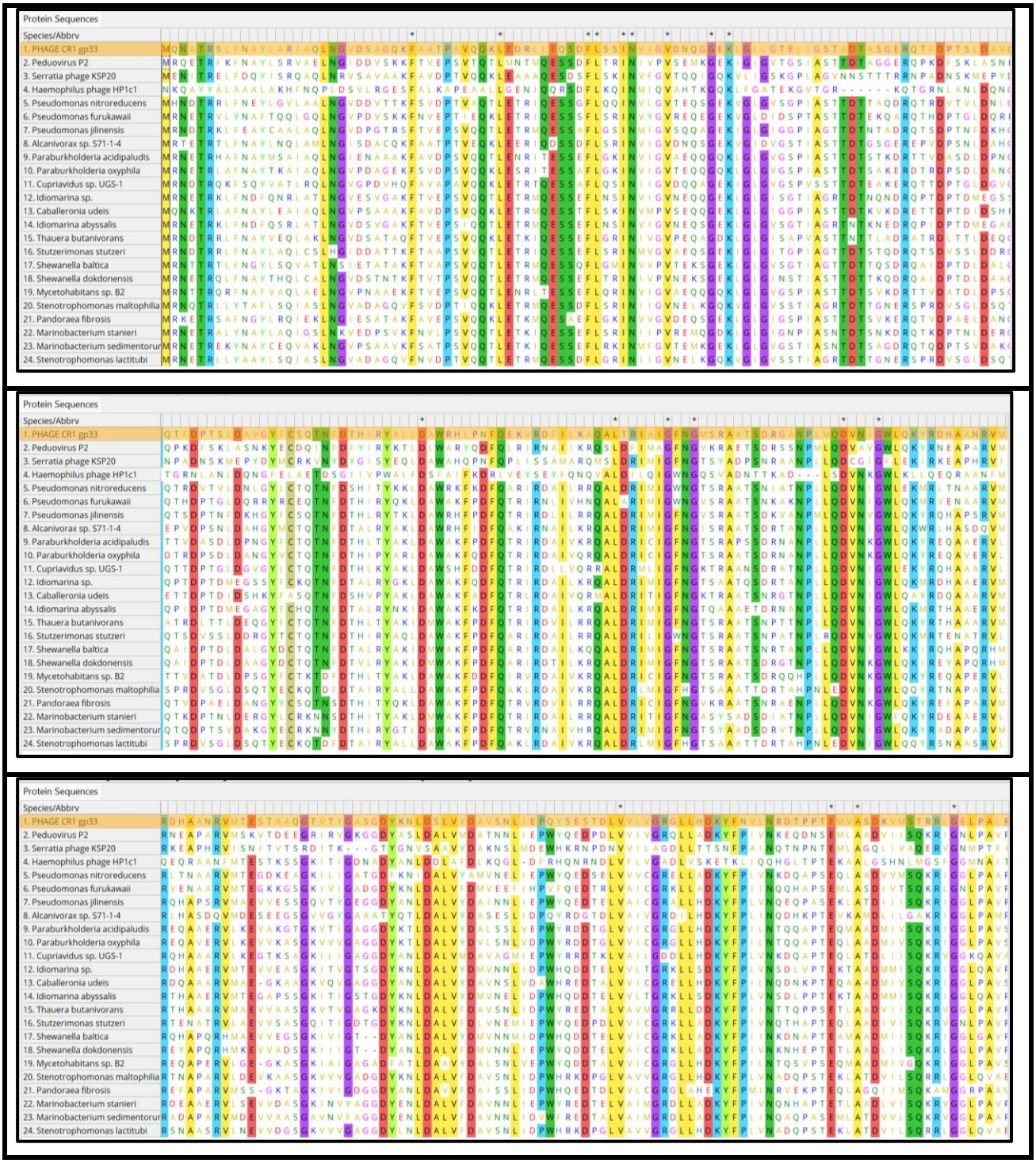

Protein Sequences
Species/Abbrv
1. PHAGE CR1 gp33
2. Peduovirus P2
3. Serratia phage KSP20
4. Haemophilus phage HP1c1
5. Pseudomonas nitroreducens
6. Pseudomonas furukawaii
7. Pseudomonas jilinensis
8. Alcanivorax sp. S71-1-4
9. Paraburkholderia acidipaludis
10. Paraburkholderia oxyphila
11. Cupriavidus sp. UGS-1
12. Idiomarina sp.
13. Caballeronia udeis
14. Idiomarina abyssalis
15. Thauera butanivorans
16. Stutzerimonas stutzeri
17. Shewanella baltica
18. Shewanella dokdonensis
19. Mycetohabitans sp. B2
20. Stenotrophomonas maltophilia
21. Pandoraea fibrosis
22. Marinobacterium stanieri
23. Marinobacterium sedimentorur
24. Stenotrophomonas lactitubi

Fig. 5.16. *Multiple sequence alignment of Gp33 protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Gp33 protein from bacteriophages of various hosts it was observed that methionine, asparagine, threonine, and others are highly conserved at amino acid positions 1, 3, 5, and 61 other positions, respectively.

5.4.7. PHAGE CR1 Gp36 protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Gp36 protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. When a procapsid is being formed, the phage Gp36 protein assembles on the inner side of the capsid and aids in the ejection of bacteriophage DNA into the host cellcatalyzes the cleavage of the host pep tidoglycans while acting as an exolysin. Phage Gp36 of *Alcanivorax sp. S71-1-4*, exhibited 99% sequence similarity with *Phage CR1* Gp36 protein, the phylogenetically closest species with standing members in the tree.

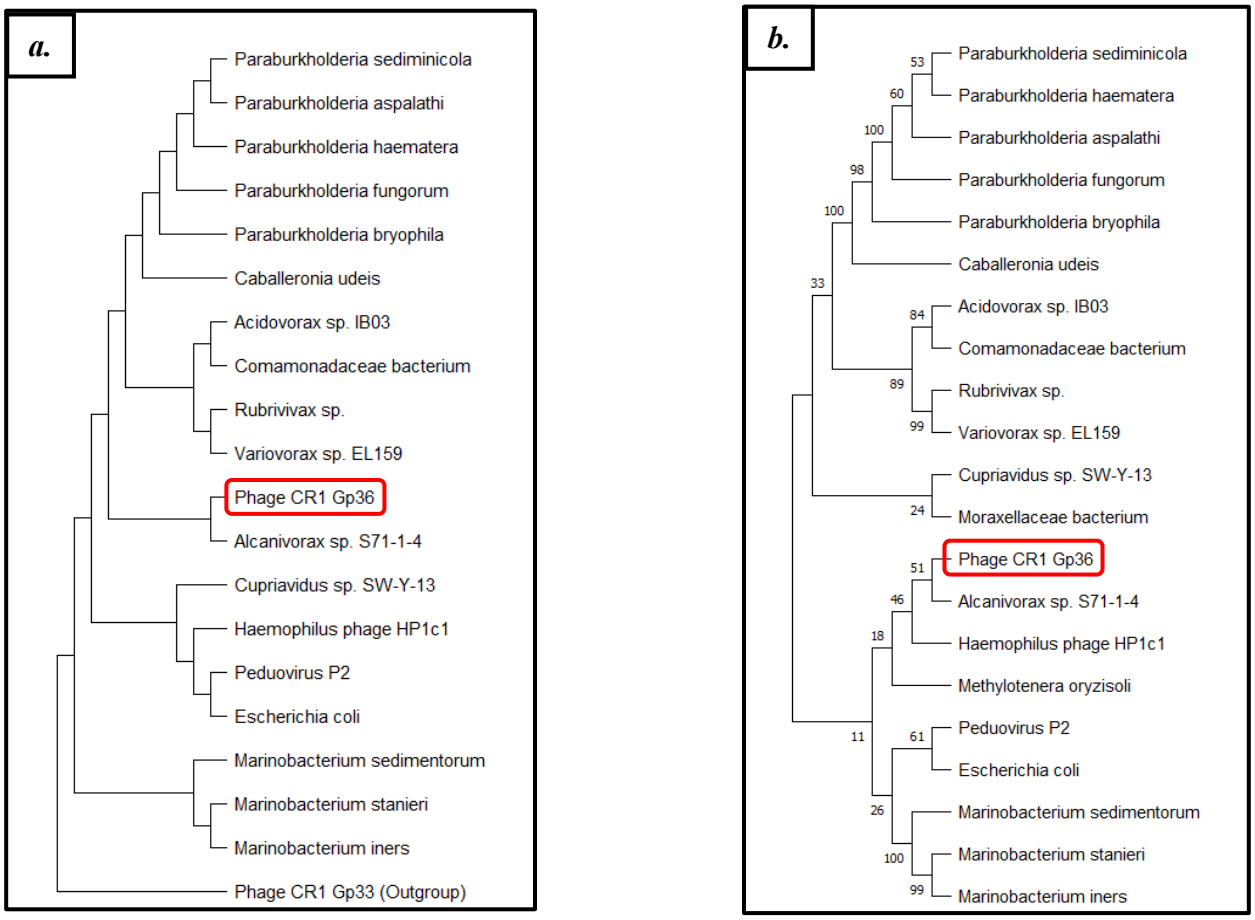


Fig. 5.17. Phylogenetic analysis of Phage CR1 Gp36. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

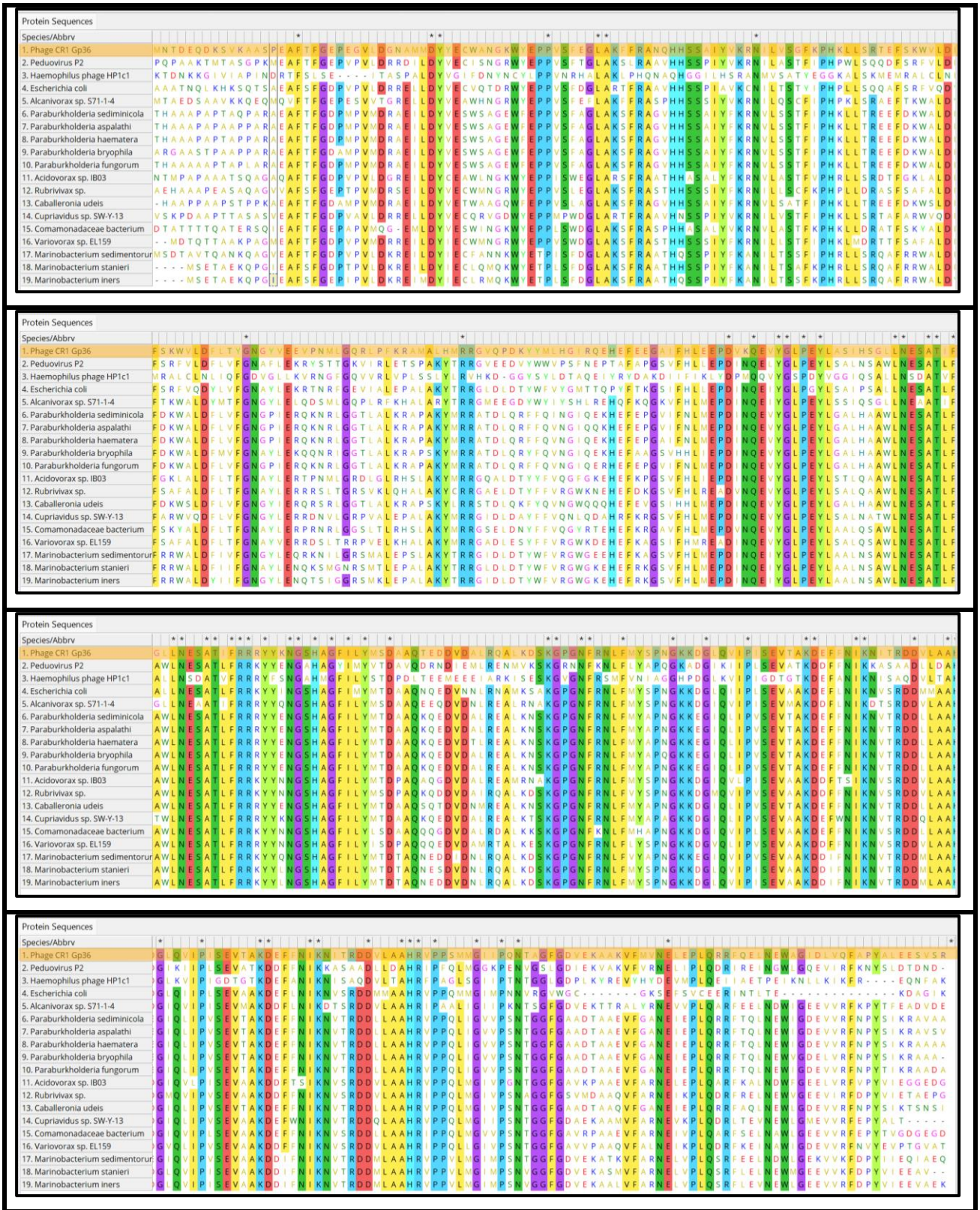


Fig. 5.18. *Multiple sequence alignment of Gp36 protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Gp36 protein from bacteriophages of various hosts it was observed that aspartic acid, leucine, tryptophan, and others are highly conserved at amino acid positions 3, 5, 8, 11 and 57 other positions, respectively.

5.4.8. PHAGE CR1 GpE+E protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 GpE+E protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. The host translocase MraY activity, which catalyses the production of lipid I, an essential step for the host cell wall biosynthesis, is inhibited by the phage GpE+E protein, resulting in host cell lysis. Phage GpE+E protein of *Halomonas spp*, exhibited 92% sequence similarity with *Phage CR1* GpE+E protein, the phylogenetically closest species with standing members in the tree. *Halomonas spp.* are Gram-negative bacteria belonging to the family of Halophiles that prefer to grow in saline environments (commonly referred to NaCl or KCl), such as salt lakes and marshes, oceans, or other saline areas on earth.

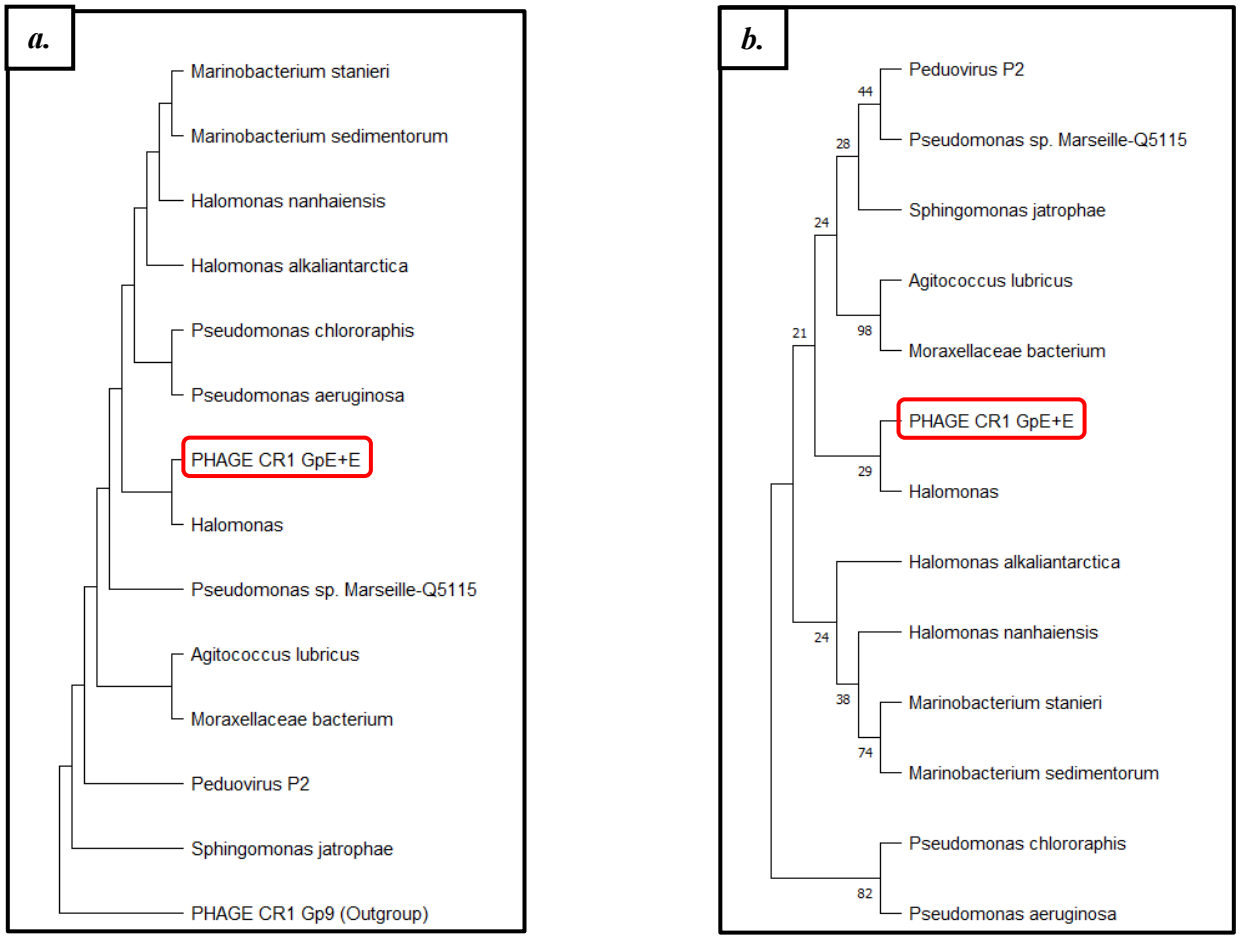


Fig. 5.19. Phylogenetic analysis of Phage CR1 GpE+E. **(a.) *Maximum likelihood tree* (b.) *Neighbourhood Joining tree***.

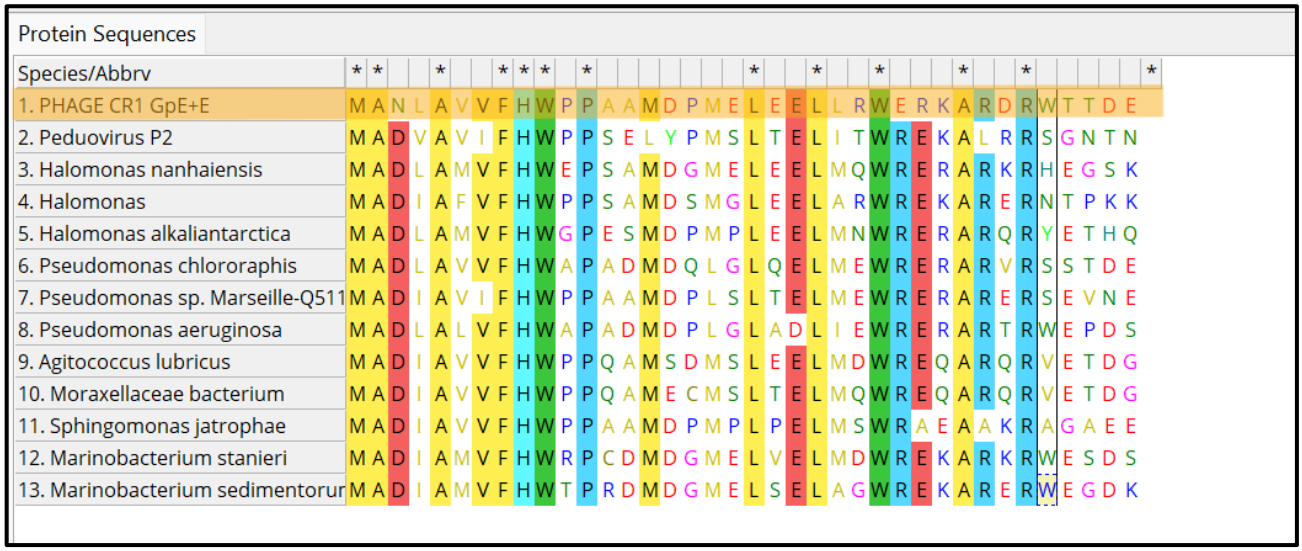


Fig. 5.20. *Multiple sequence alignment of GpE+E protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* GpE+E protein from bacteriophages of various hosts it was observed that methionine, alanine, aspartic acid, and others are highly conserved at amino acid positions 1, 2, 3, and 16 other positions, respectively.

5.4.9. PHAGE CR1 Baseplate assembly protein J:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Baseplate assembly protein J** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage baseplate assembly protein J of *Pseudomonas sp. TCU-HL1* exhibited 97% sequence similarity with *Phage CR1* Baseplate assembly protein J protein, the phylogenetically closest species with standing members in the tree. *Pseudomonas sp. TCU-HL1* degrading plant-produced monoterpenes, specially Borneol, a plant terpene that is widely used in traditional Chinese medicine (Tsang et al., 2016).

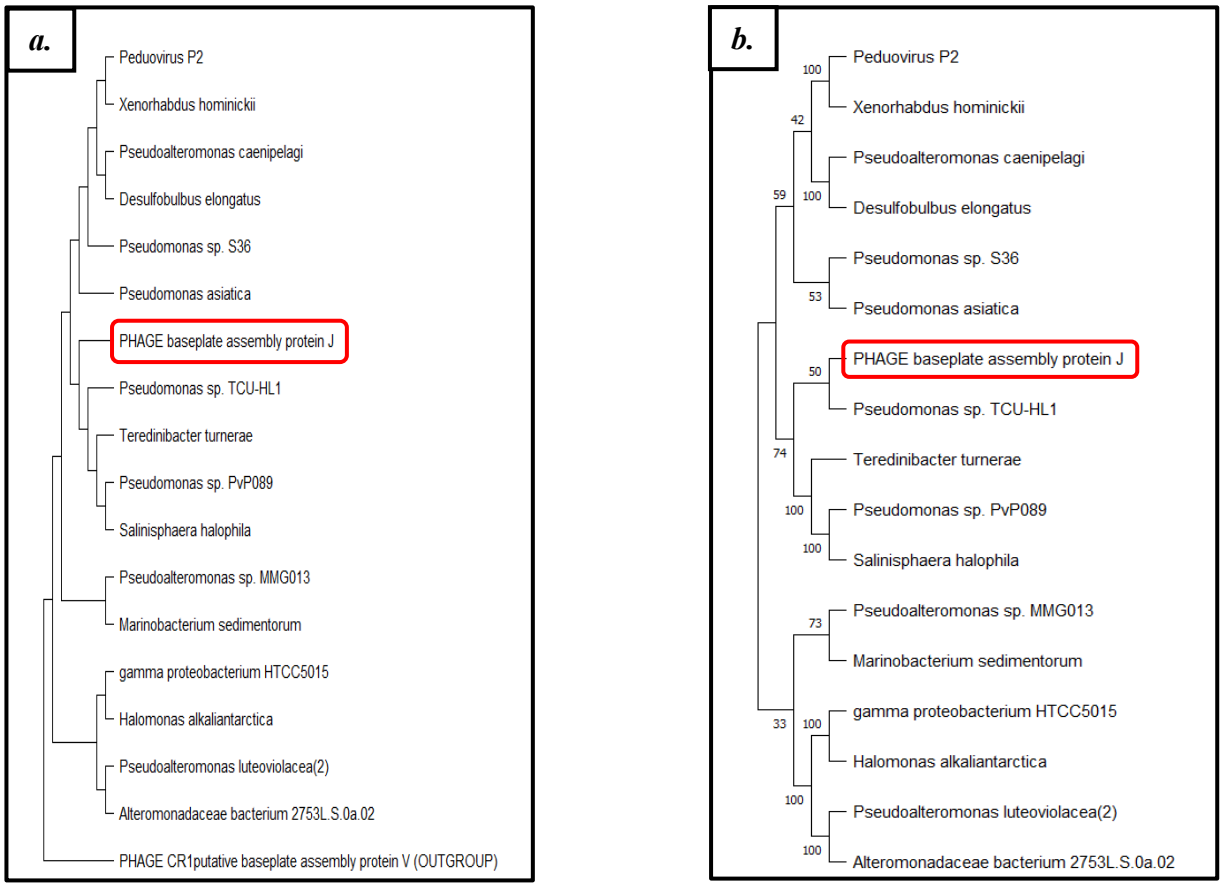


***Fig. 5.21. Phylogenetic analysis of Phage CR1* baseplate assembly protein *J.* (a.) *Maximum likelihood tree* (b.) *Neighbourhood Joining tree*.**

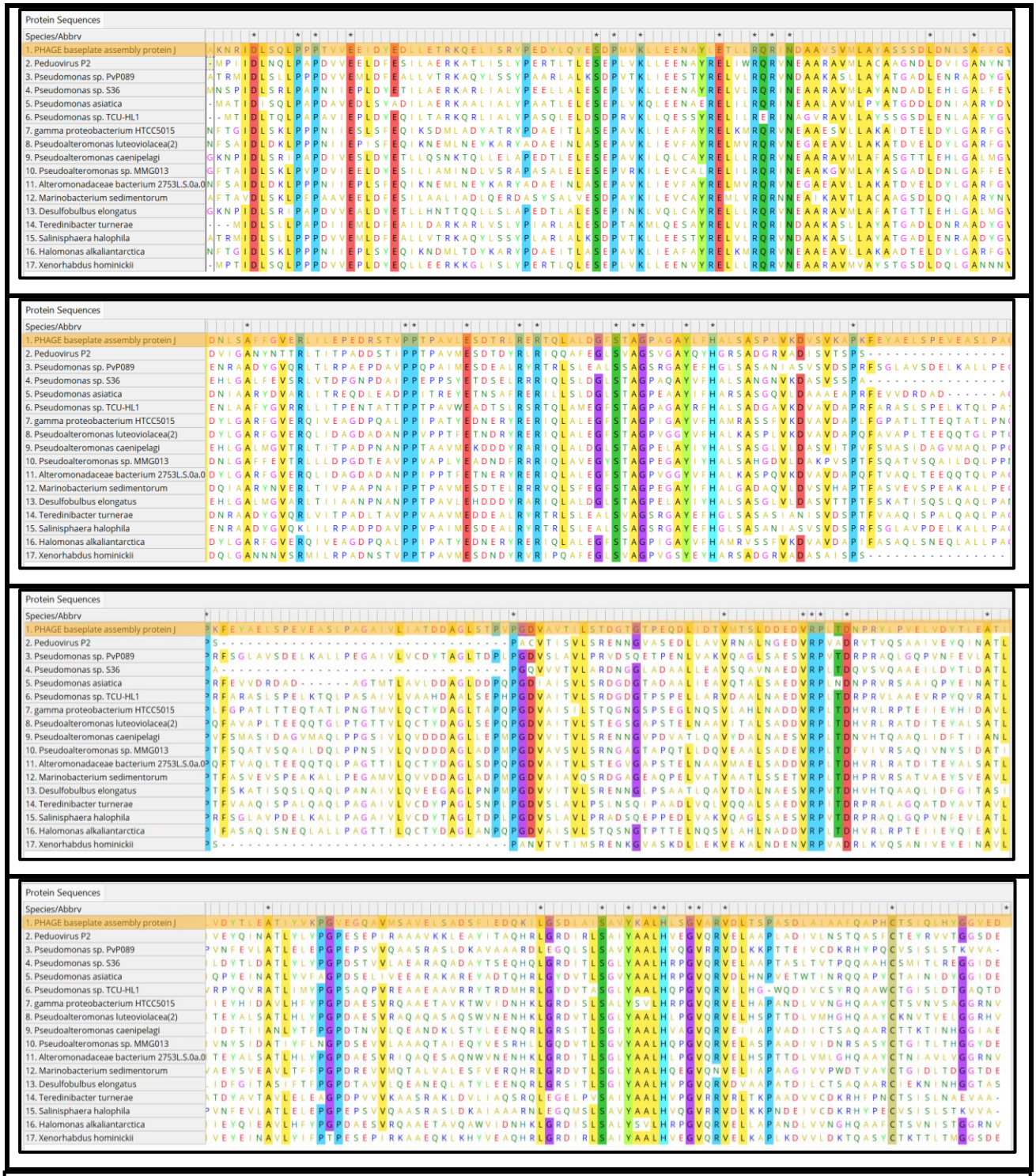


Fig. 5.22. *Multiple sequence alignment of Phage CR1* **Baseplate assembly protein J** *highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Baseplate assembly protein J protein from bacteriophages of various hosts it was observed that isoleucine, aspartic acid, leucine, and others are highly conserved at amino acid positions 5, 6, 7, and other positions, respectively.

5.4.10. PHAGE CR1 Capsid scaffolding protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Capsid scaffolding protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage capsid scaffolding proteim of *Alcanivorax sp. S71-1-4,* exhibited 99% sequence similarity with *Phage CR1* Capsid scaffolding protein, the phylogenetically closest species with standing members in the tree.

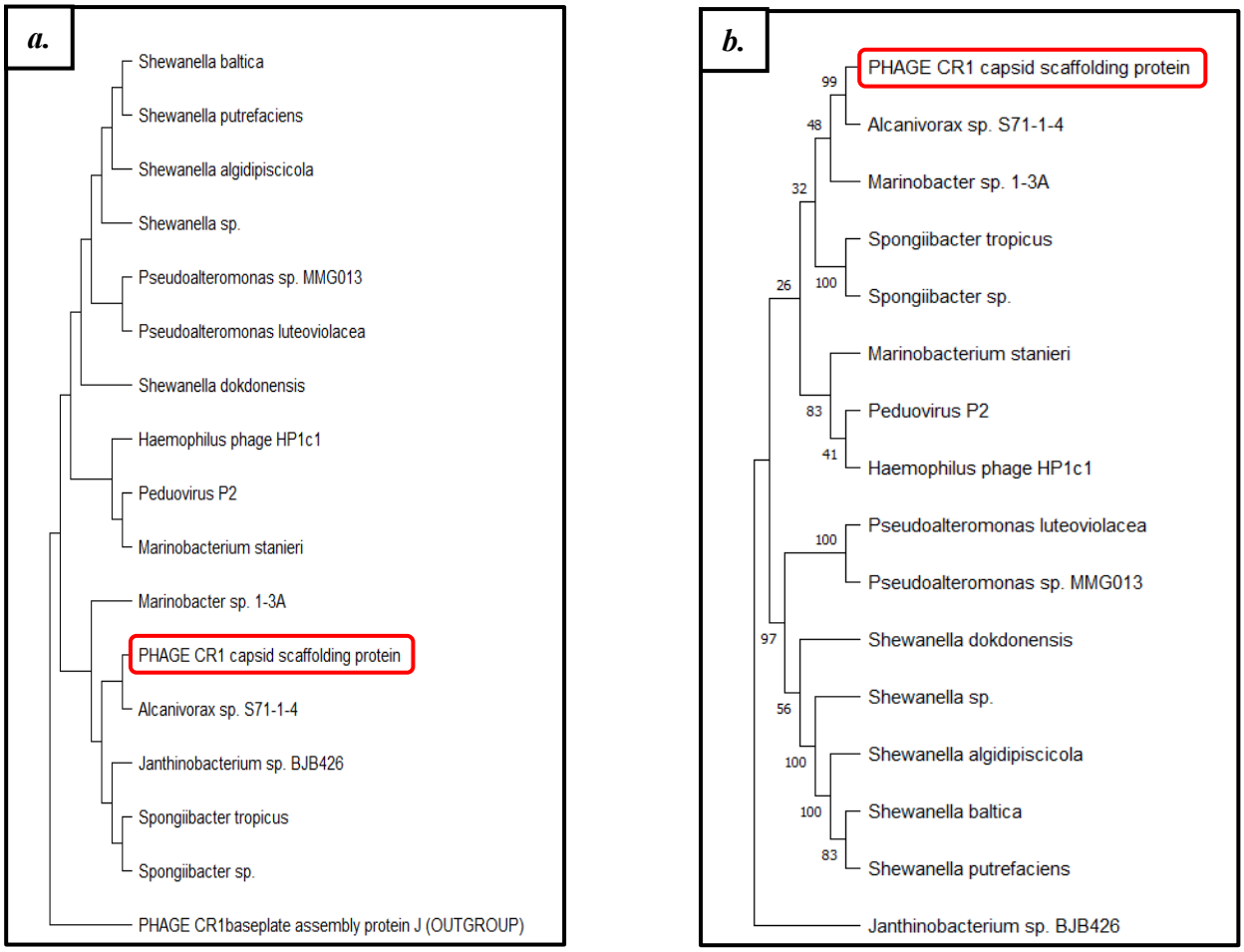


Fig. 5.23. Phylogenetic analysis of Phage CR1 Capsid scaffolding protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

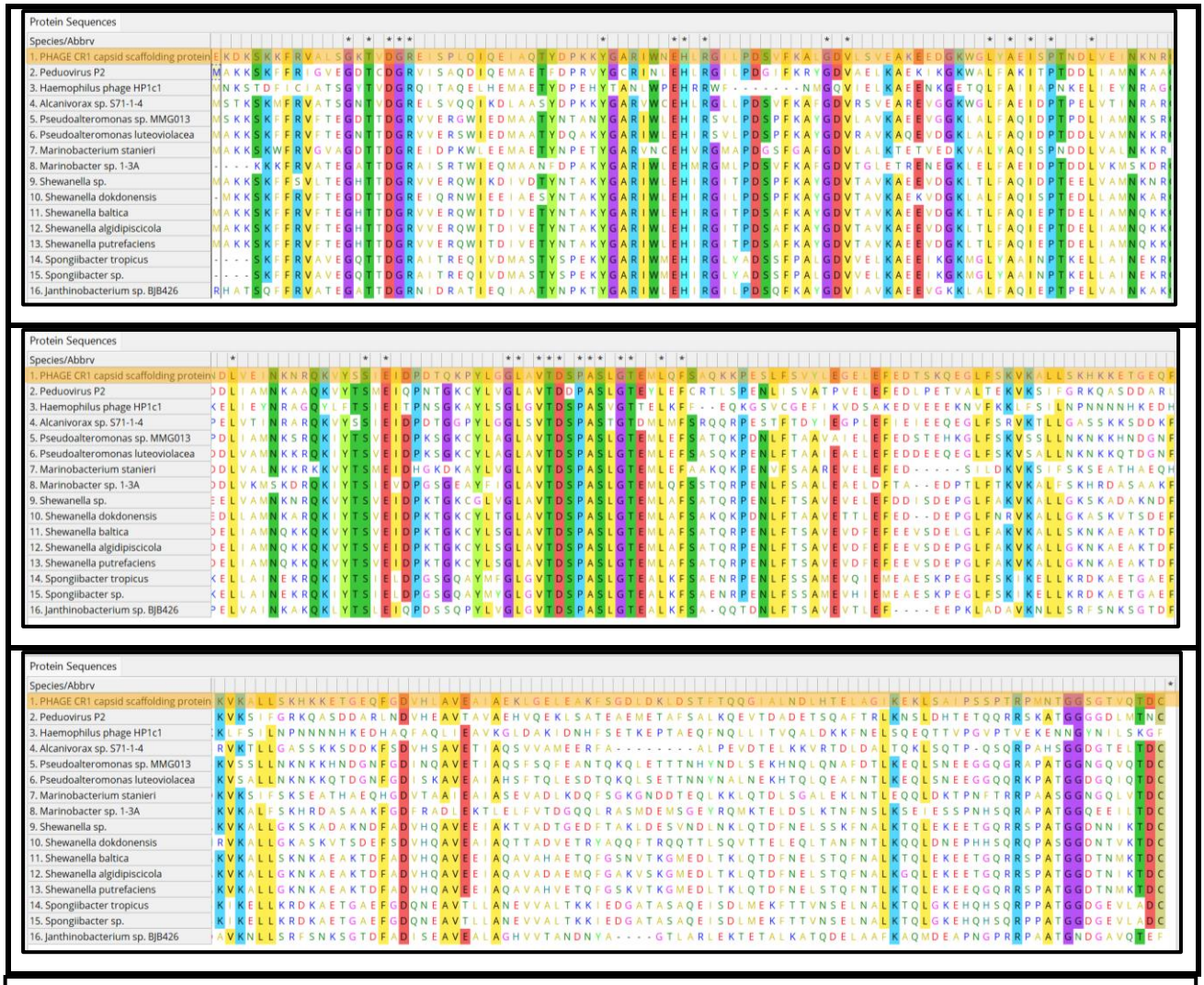


Fig. 5.24. *Multiple sequence alignment of* ***Capsid scaffolding protein*** *highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Capsid scaffolding protein from bacteriophages of various hosts it was observed that serine, lysine, phenylalanine, and others are highly conserved at amino acid positions 5, 6, 8, and 88 other positions, respectively.

5.4.11. PHAGE CR1 head completion-stabilization protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 head completion-stabilization protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage head completion-stabilization protein *Chitinolyticbacter meiyuanensis* and *Chromobacterium alkanivorans*, exhibited 100% sequence similarity with *Phage CR1* head completion-stabilization protein, the phylogenetically closest species with standing members in the tree. *Chitinolyticbacter meiyuanensis* capable of hydrolysing chitin and shrimp shell to *N*-acetyl glucosamine (GlcNAc). Chitin the most abundant amino-polysaccharide polymer occurring in marine environment, and *C. meiyuanensis* is capable of hydrolysing by producing chitinase enzyme (Zhang et al, 2020).

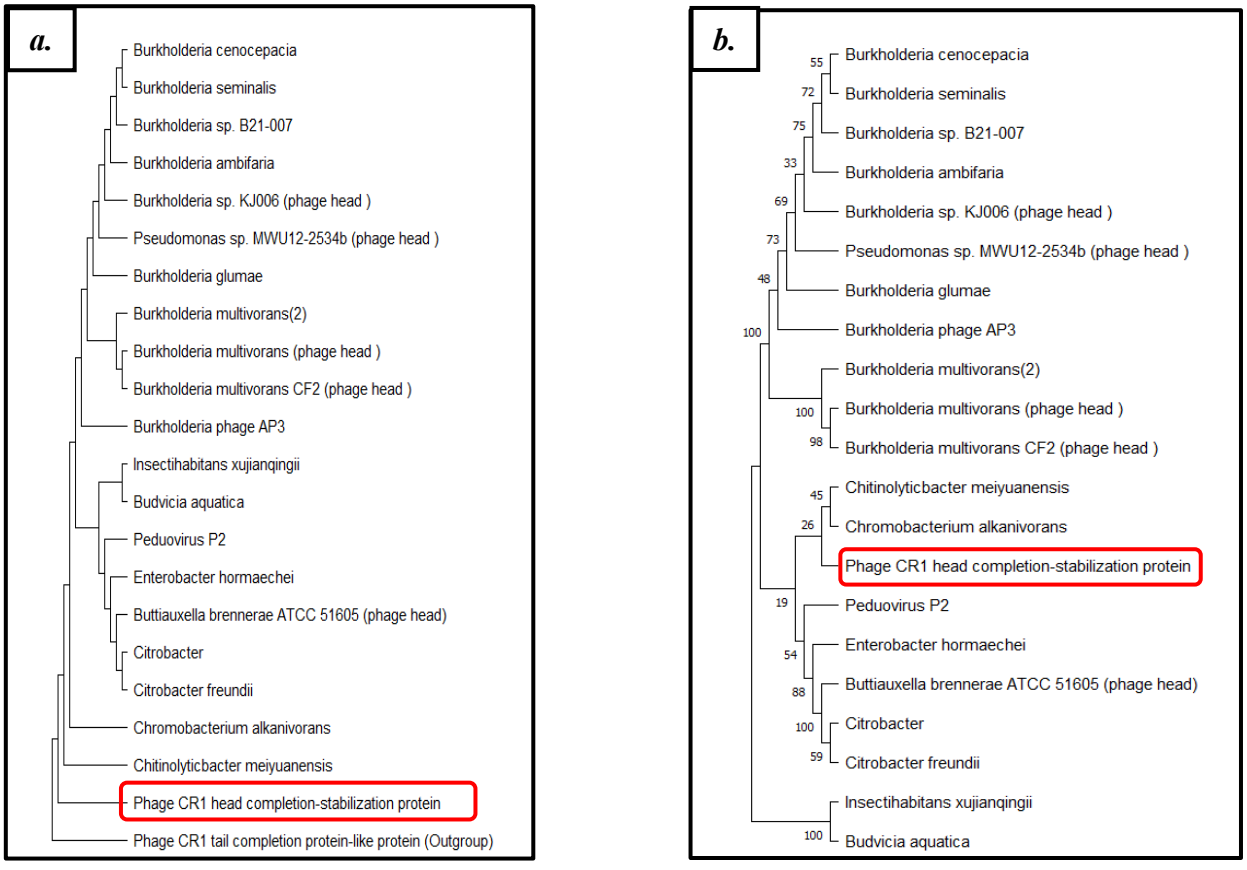


Fig. 5.25. Phylogenetic analysis of Phage Capsid scaffolding protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

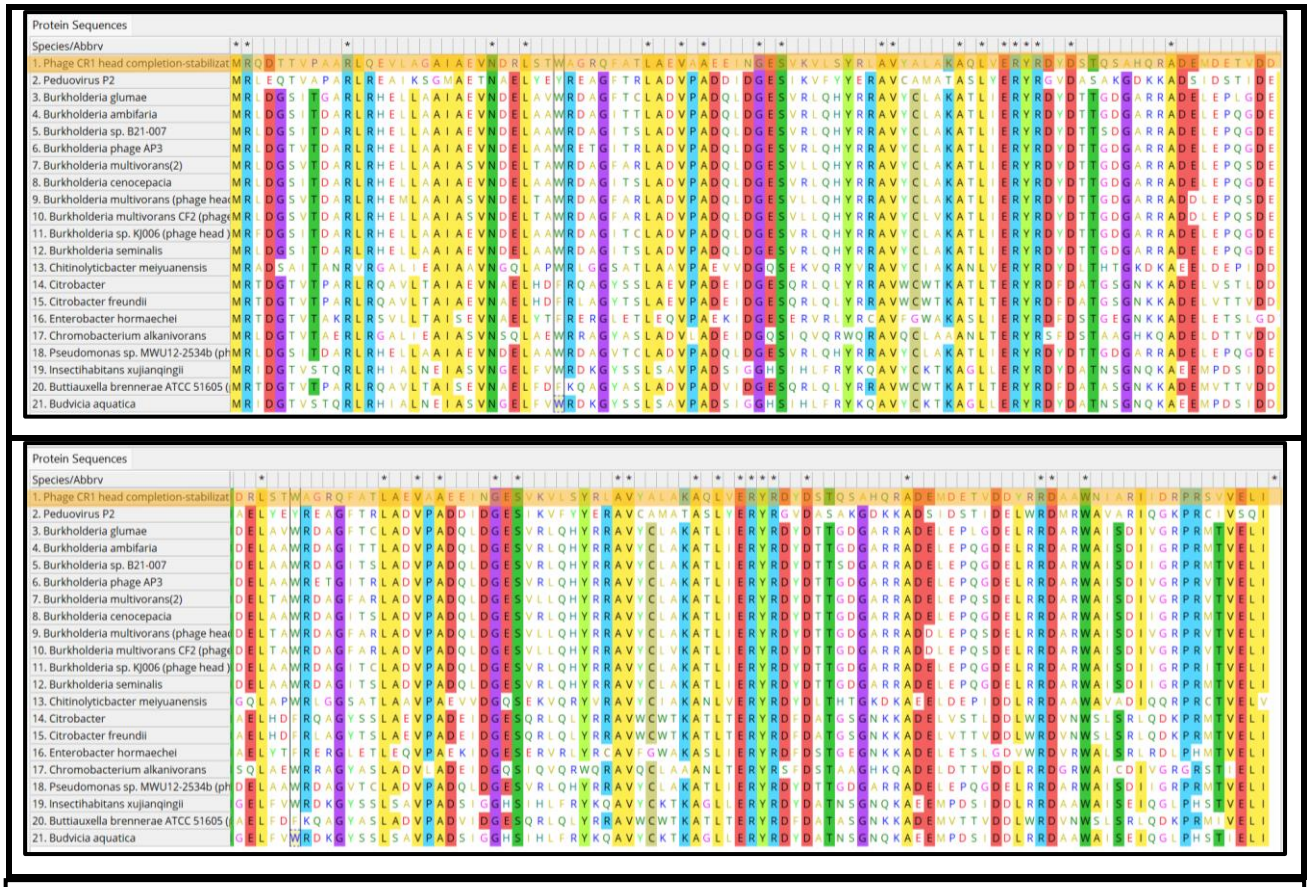


Fig. 5.26. *Multiple sequence alignment of* **Capsid scaffolding protein** *highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* Capsid scaffolding protein from bacteriophages of various hosts it was observed that methionine, arginine, aspartic acid, and others are highly conserved at amino acid positions 1, 2, 4, and 56 other positions, respectively.

5.4.12. PHAGE CR1 putative baseplate assembly protein V:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 putative baseplate assembly protein V** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage putative baseplate protein V of *Alcanivorax sp. S71-1-4,* exhibited 99% sequence similarity with *Phage CR1* putative baseplate protein, the phylogenetically closest species with standing members in the tree.

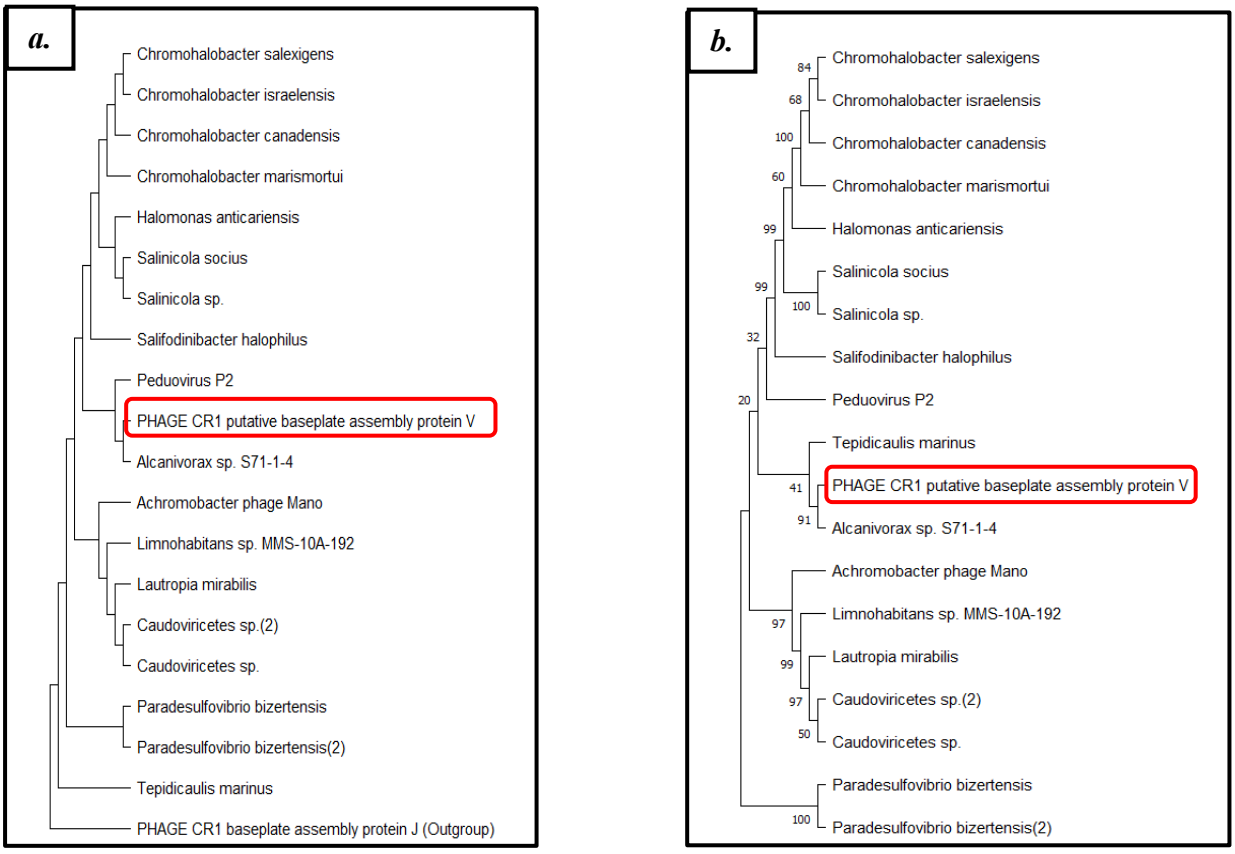


Fig. 5.27. Phylogenetic analysis of Phage CR1 putative baseplate assembly protein V. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

Fig. 5.28. *Multiple sequence alignment* ***putative baseplate assembly protein V*** *highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* putative baseplate assembly protein V from bacteriophages of various hosts it was observed that glutamic acid, arginine, asparagine, and others are highly conserved at amino acid positions 6, 9, 813 and 44 other positions, respectively.

5.4.13. PHAGE CR1 tail component protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 tail component protein** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits.Phage tail protein of *Marinobacter sp. X15-166B,* exhibited 100% sequence similarity with *Phage CR1* tail component protein, the phylogenetically closest species with standing members in the tree. The *Marinobacter sp. X15-166B* cultivated from marine sediments near the harbours.

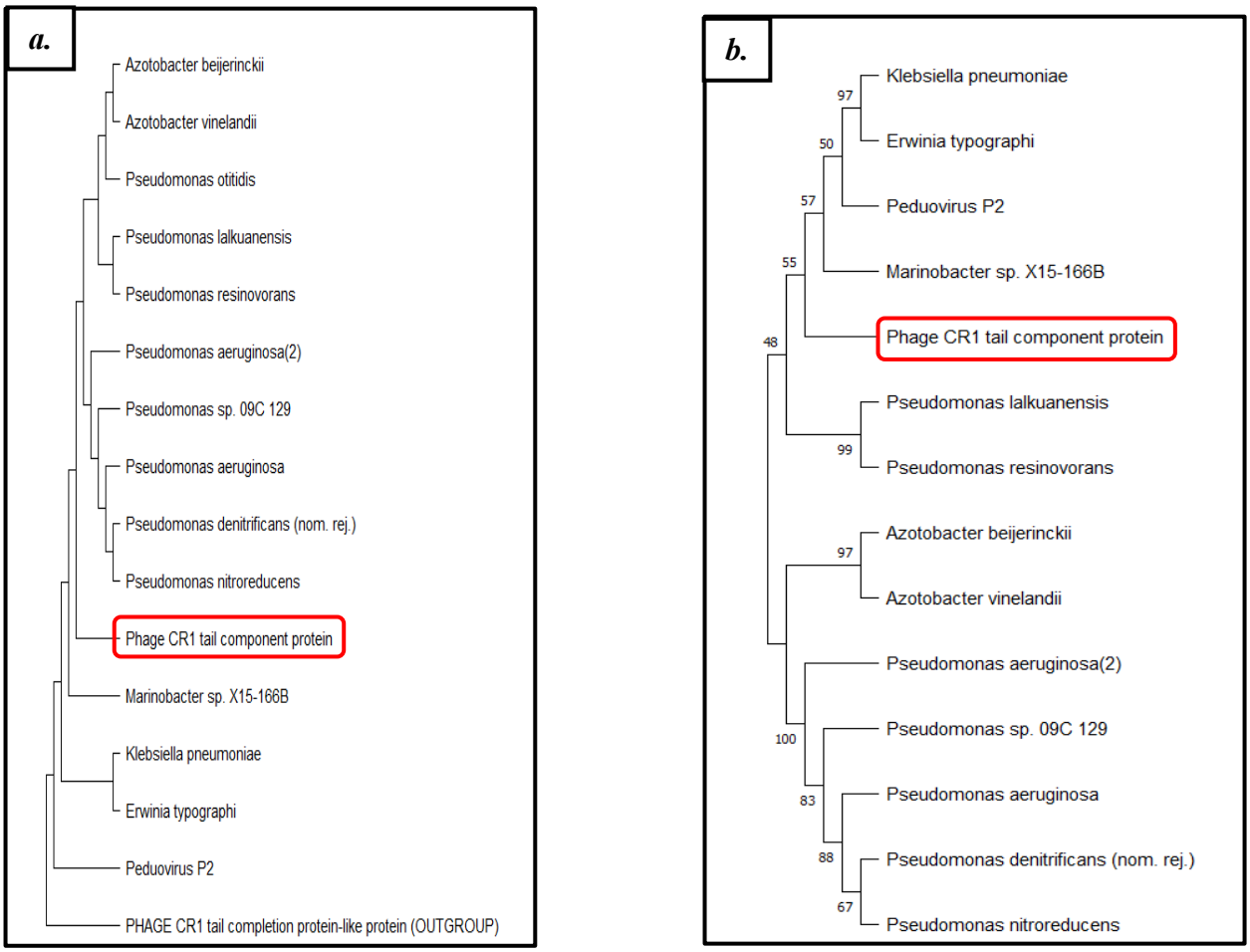


Fig. 5.29. Phylogenetic analysis of Phage CR1 tail component protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

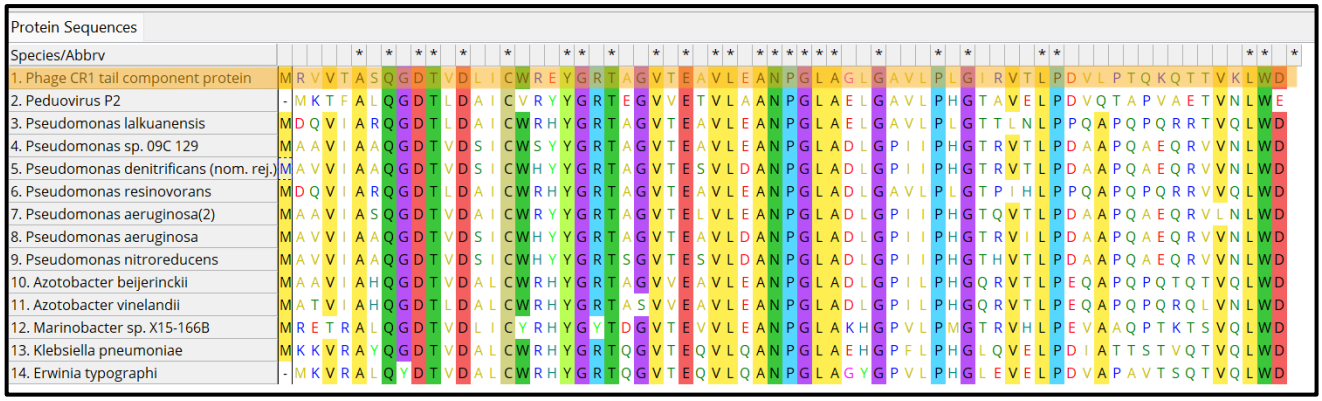


Fig. 5.30. *Multiple sequence alignment of* ***tail component protein*** *highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* tail component protein from bacteriophages of various hosts it was observed that methionine, valine, alanine, and others are highly conserved at amino acid positions 1, 4, 6, and 33 other positions, respectively.

5.4.14. PHAGE CR1 tail completion protein-like protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 tail completion protein-like protein,** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage tail protein of *Marinobacter sp. X15-166B* and *Alcanivorax sp. S71-1-4,* exhibited 100% sequence similarity with *Phage CR1* tail completion protein-like protein, the phylogenetically closest species with standing members in the tree.

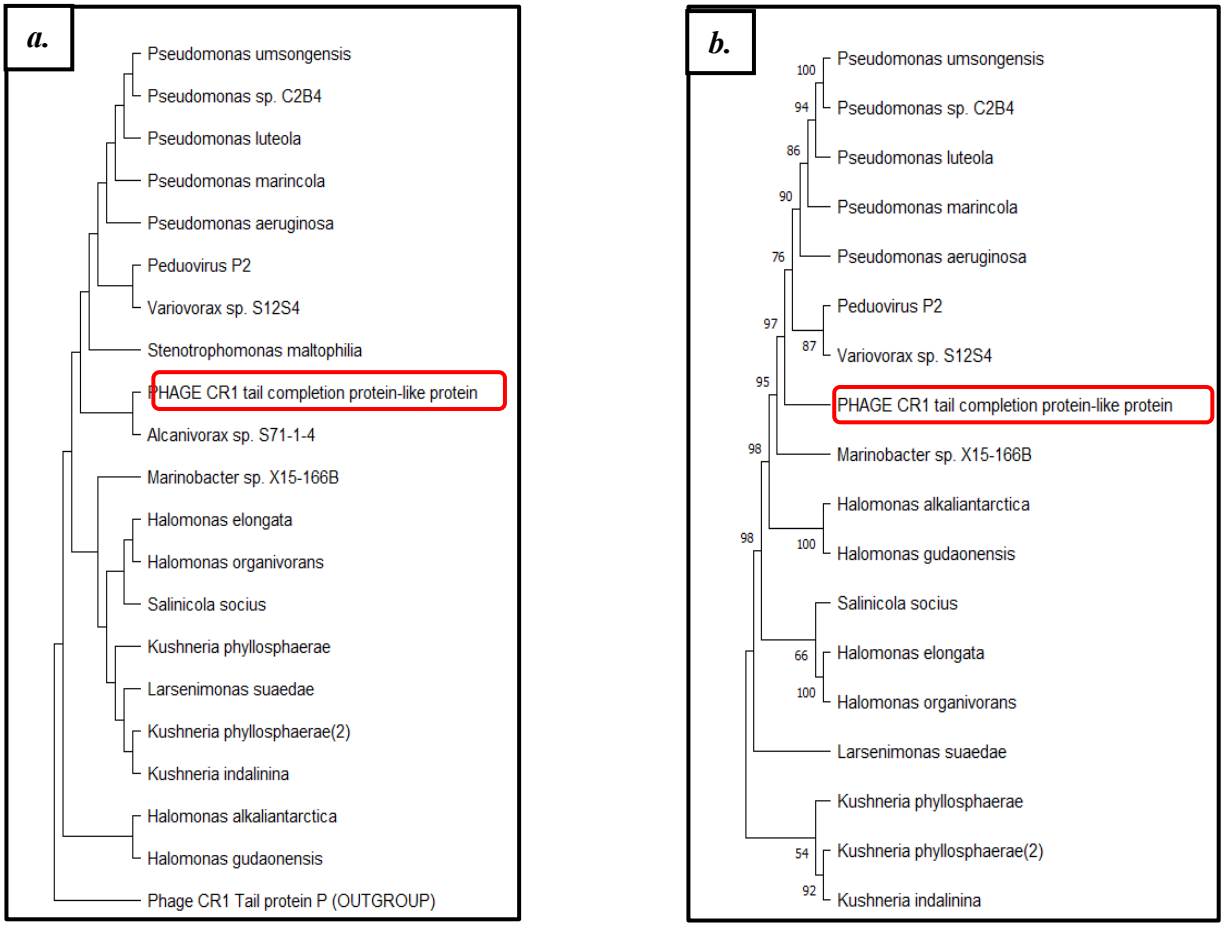


Fig. 5.31. Phylogenetic analysis of Phage CR1 tail completion protein-like protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

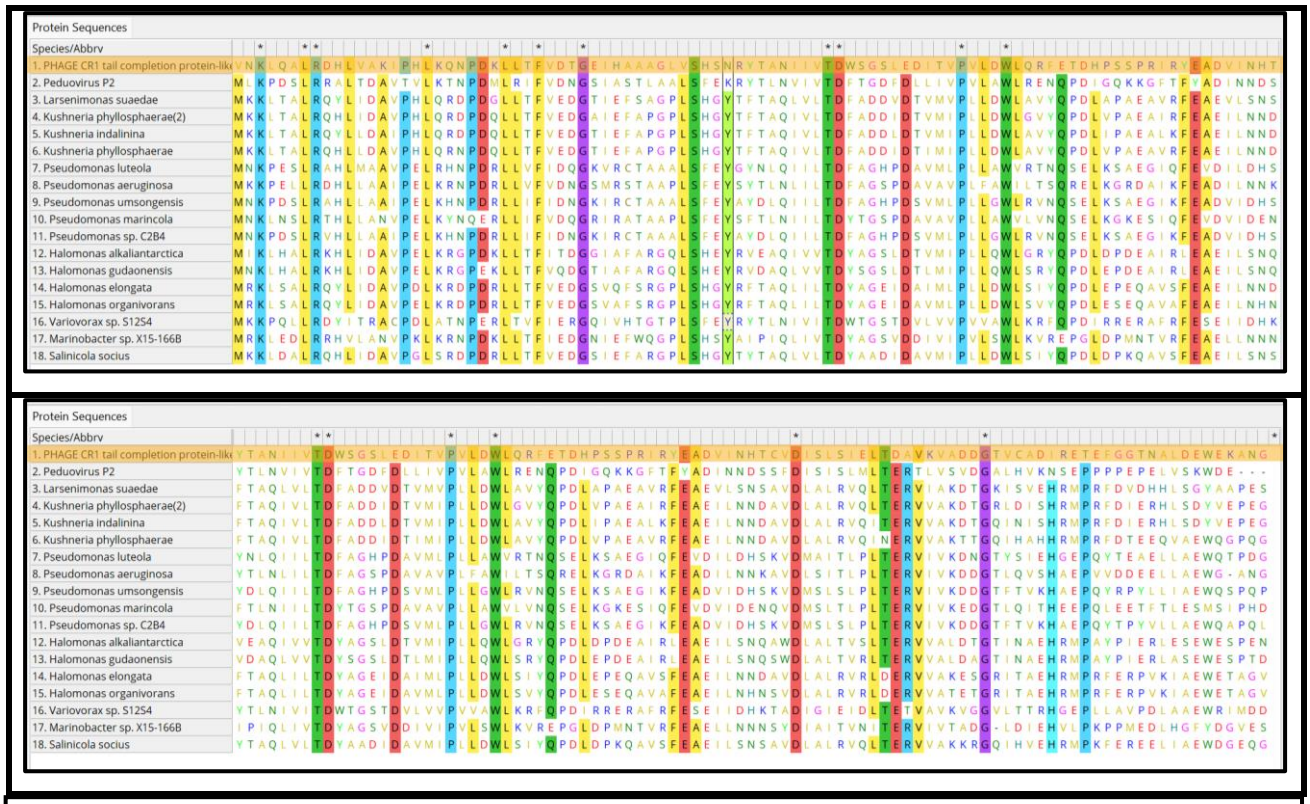


Fig. 5.32. *Multiple sequence alignment of* tail completion protein-like *protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* tail completion protein-like protein from bacteriophages of various hosts it was observed that methionine, lysine, leucine, and others are highly conserved at amino acid positions 1, 3, 7, and 35 other positions, respectively.

5.5.15. PHAGE CR1 tail protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 tail protein,** has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage tail protein of *Marinobacter sp. X15-166B,* exhibited 100% sequence similarity with *Phage CR1* tail protein, the phylogenetically closest species with standing members in the tree.

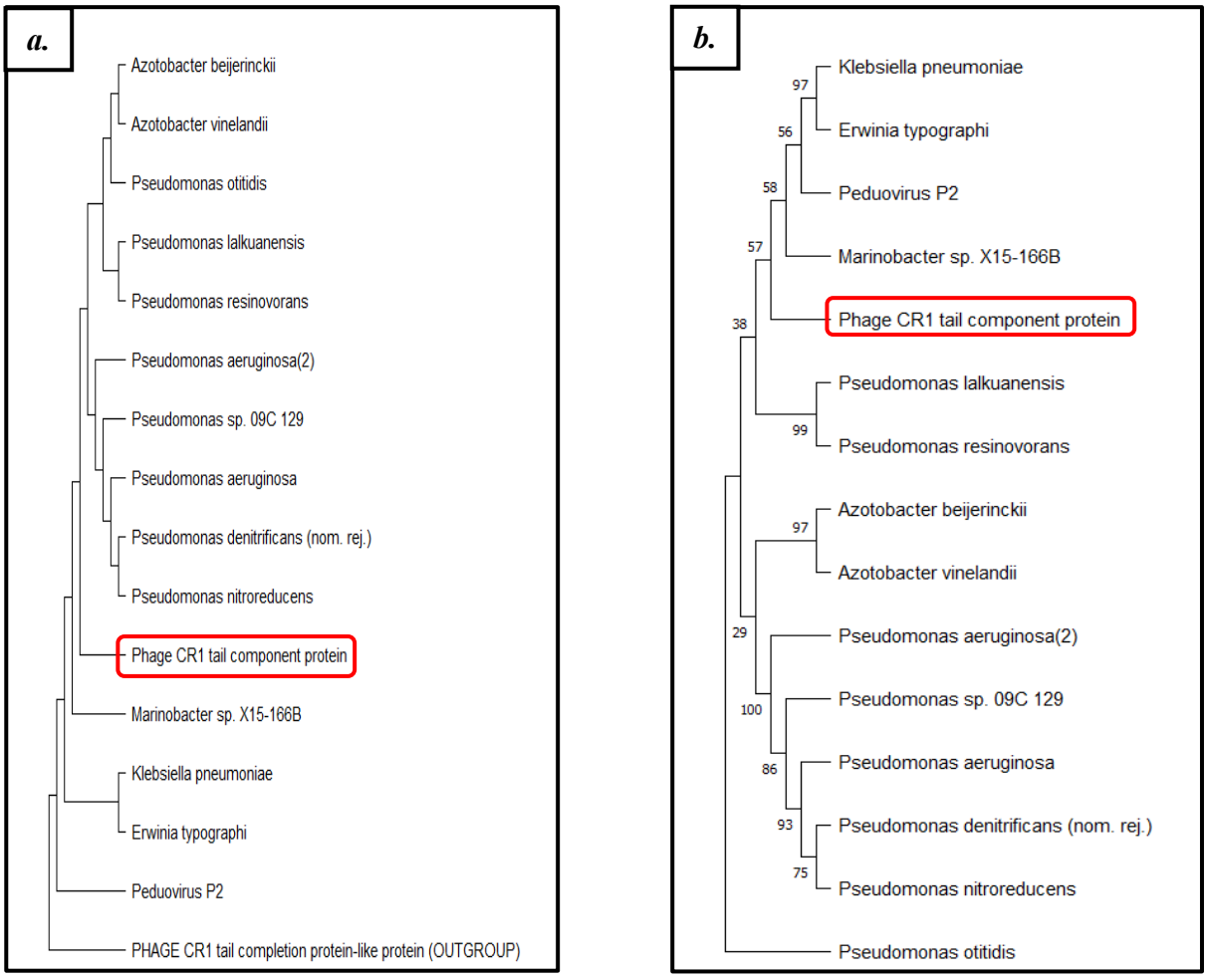


Fig. 5.33. Phylogenetic analysis of Phage CR1 tail protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

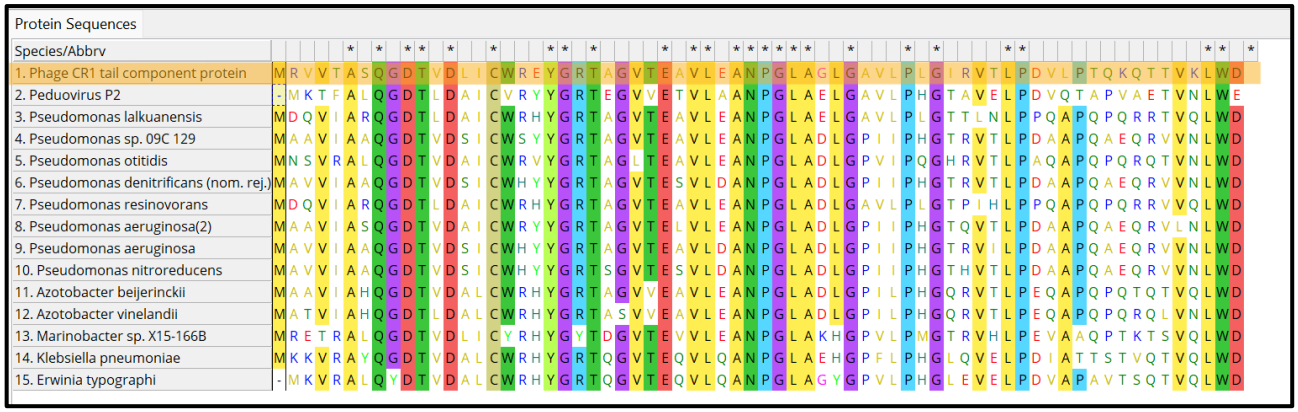


Fig. 5.34. *Multiple sequence alignment of tail protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* tail protein from bacteriophages of various hosts it was observed that methionine, valine, alanine, and others are highly conserved at amino acid positions 1, 4, 6, and 33 other positions, respectively.

5.5.16. PHAGE CR1 tail collar domain protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 tail collar domain protein**, has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage tail protein of *Desulfoluna butyratoxydans,* exhibited 59% sequence similarity with *Phage CR1* tail collar domain protein, the phylogenetically closest species with standing members in the tree. *Desulfoluna butyratoxydans,* an anaerobe isolated from estuarine sediments.

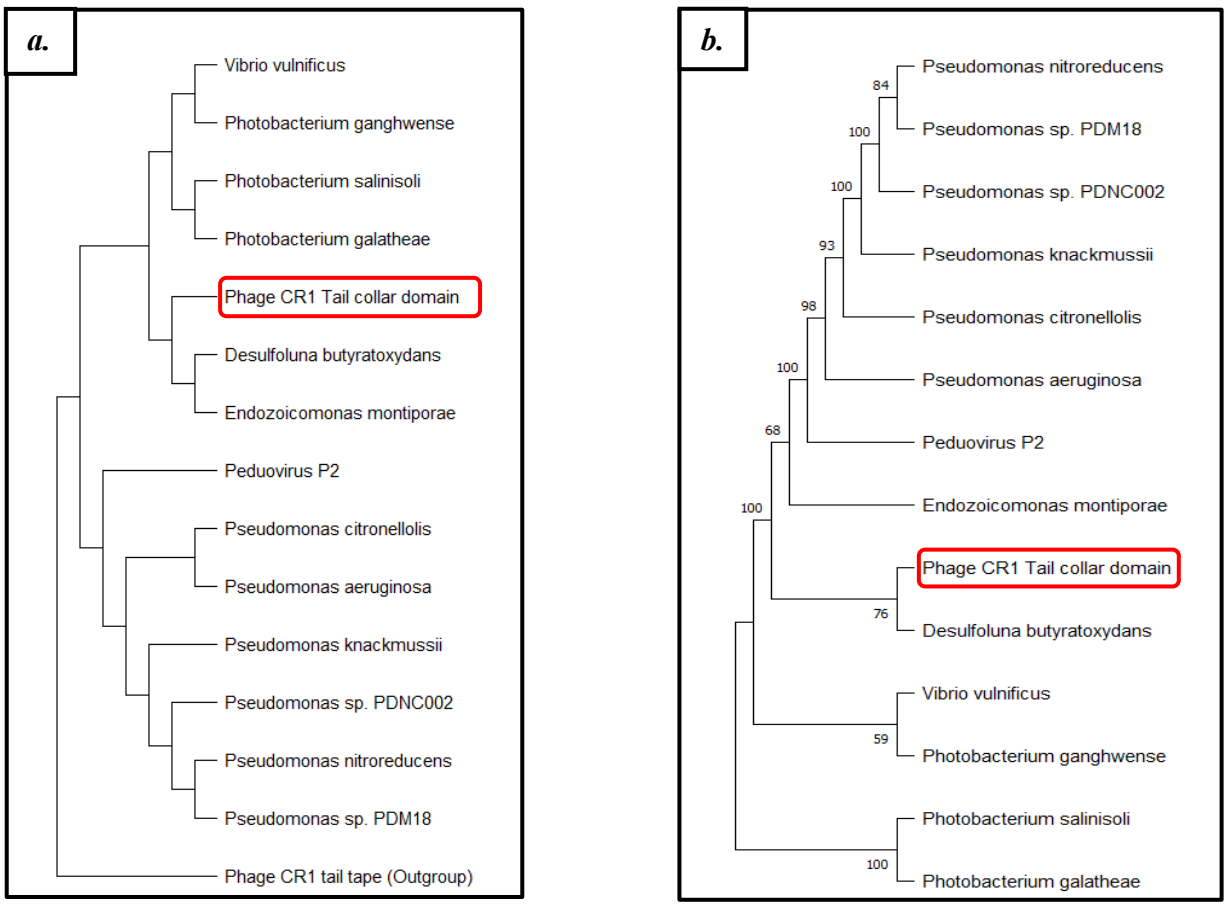


Fig. 5.35. Phylogenetic analysis of Phage CR1 tail collar domain protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

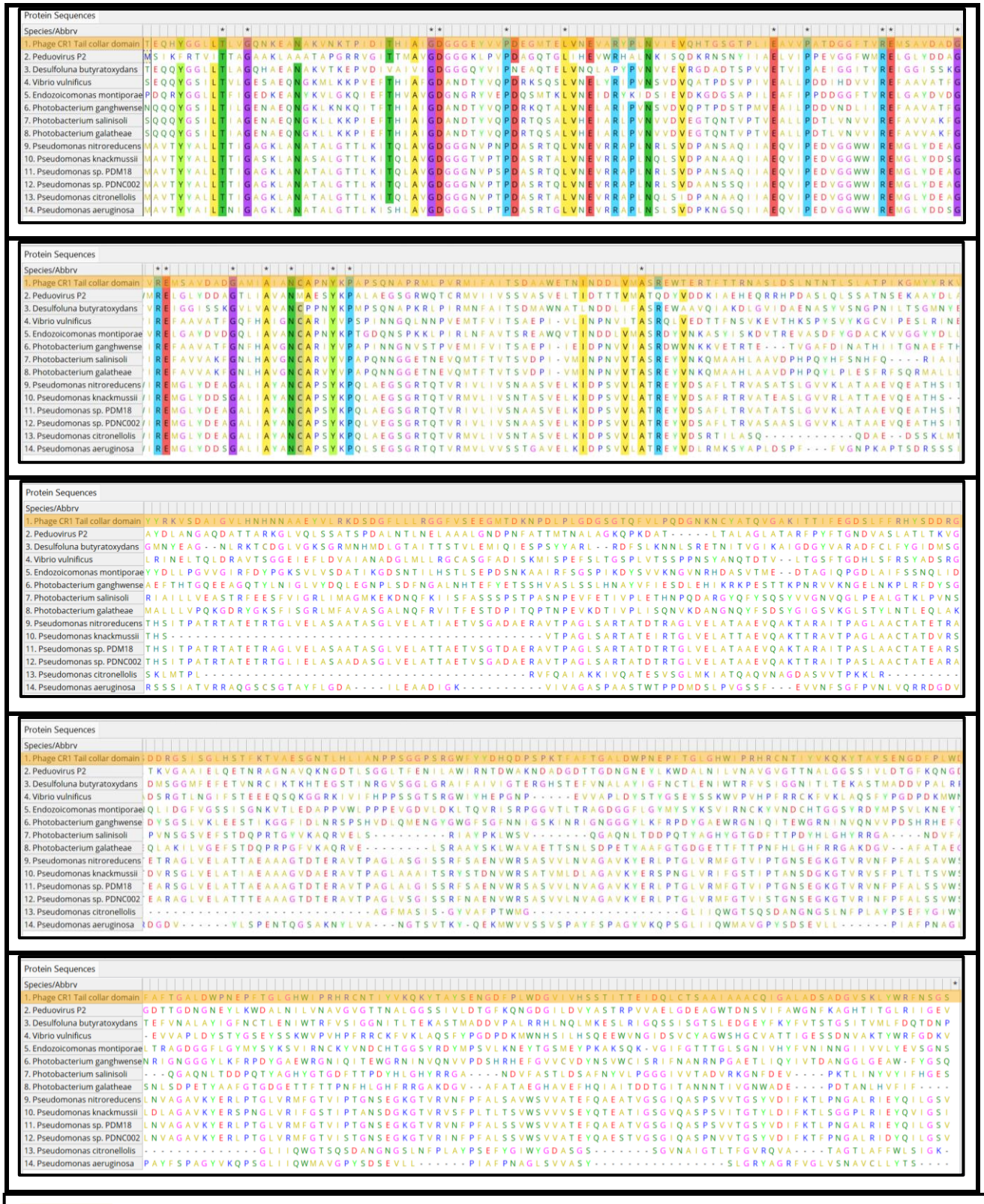


Fig. 5.36. *Multiple sequence alignment of tail collar domain protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* tail collar domain protein from bacteriophages of various hosts it was observed that tyrosine, leucine, threonine, and others are highly conserved at amino acid positions 5, 9, 10, and 36 other positions, respectively.

5.5.17. PHAGE CR1 tail tape measure protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 tail tape measure protein**, has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage tail tape measure protein *Peduovirus P2,* exhibited 74% sequence similarity with *Phage CR1* tail tape measure protein, the phylogenetically closest species with standing members in the tree. *Peduovirus P2* is an *E. coli* phage.

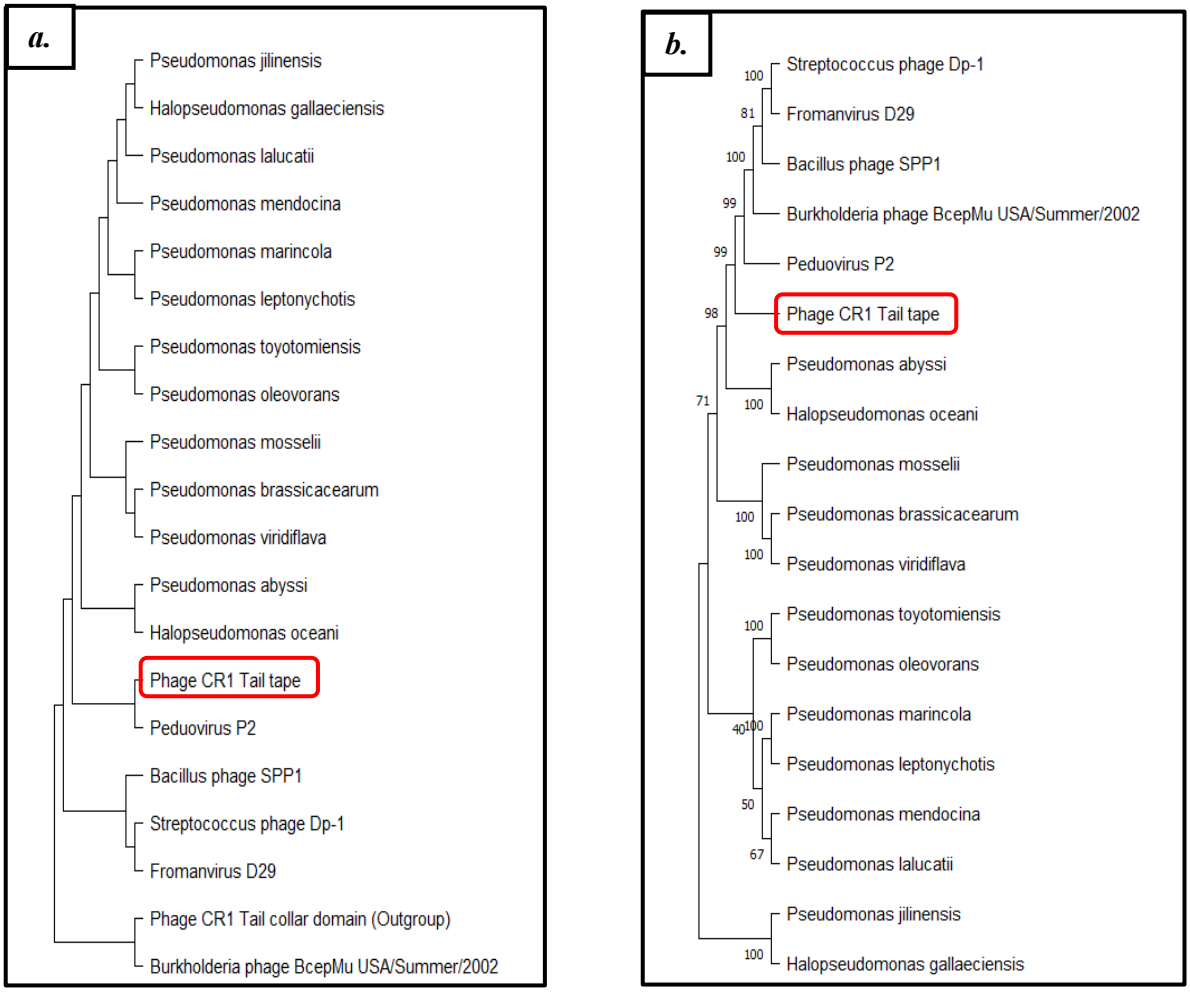
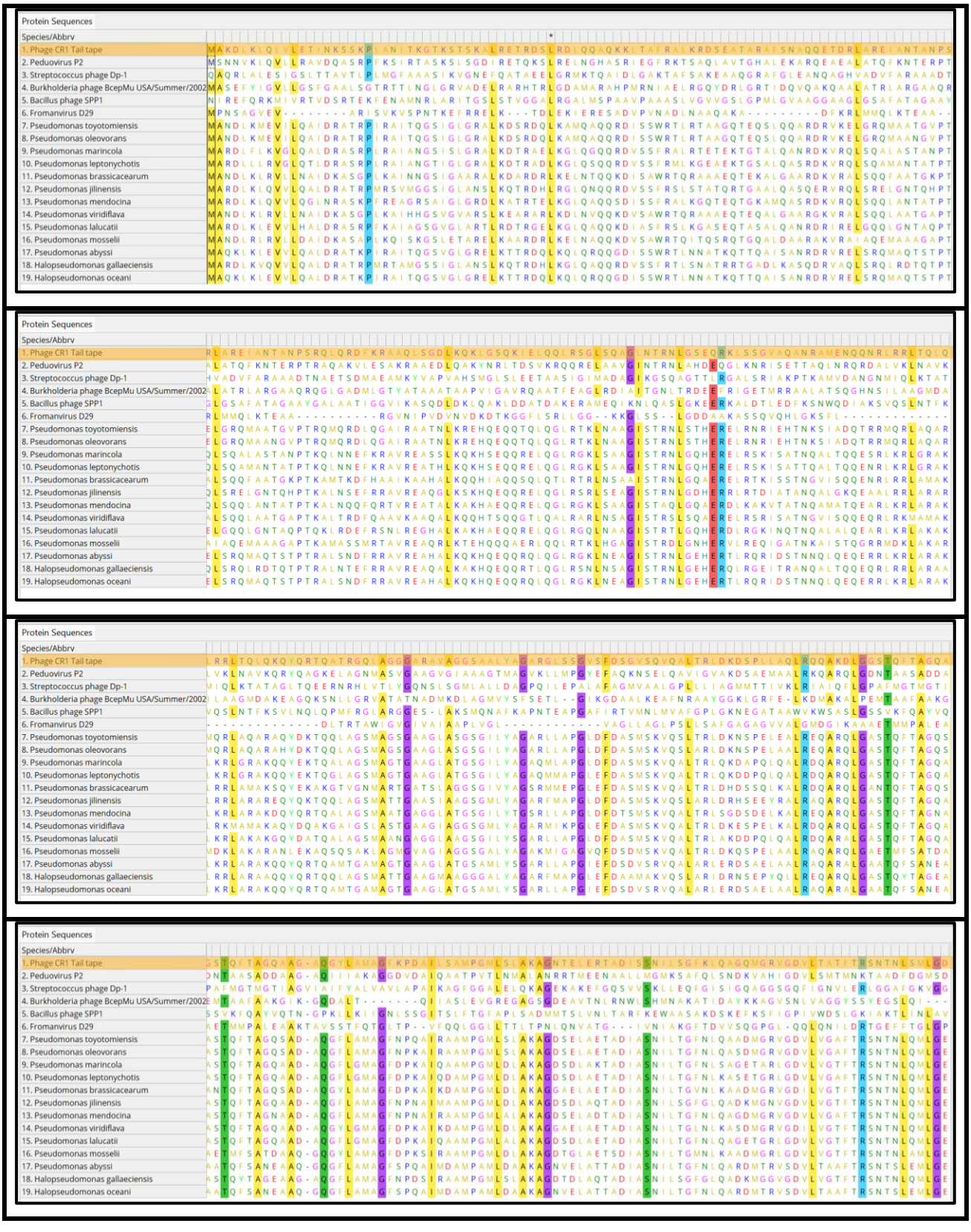
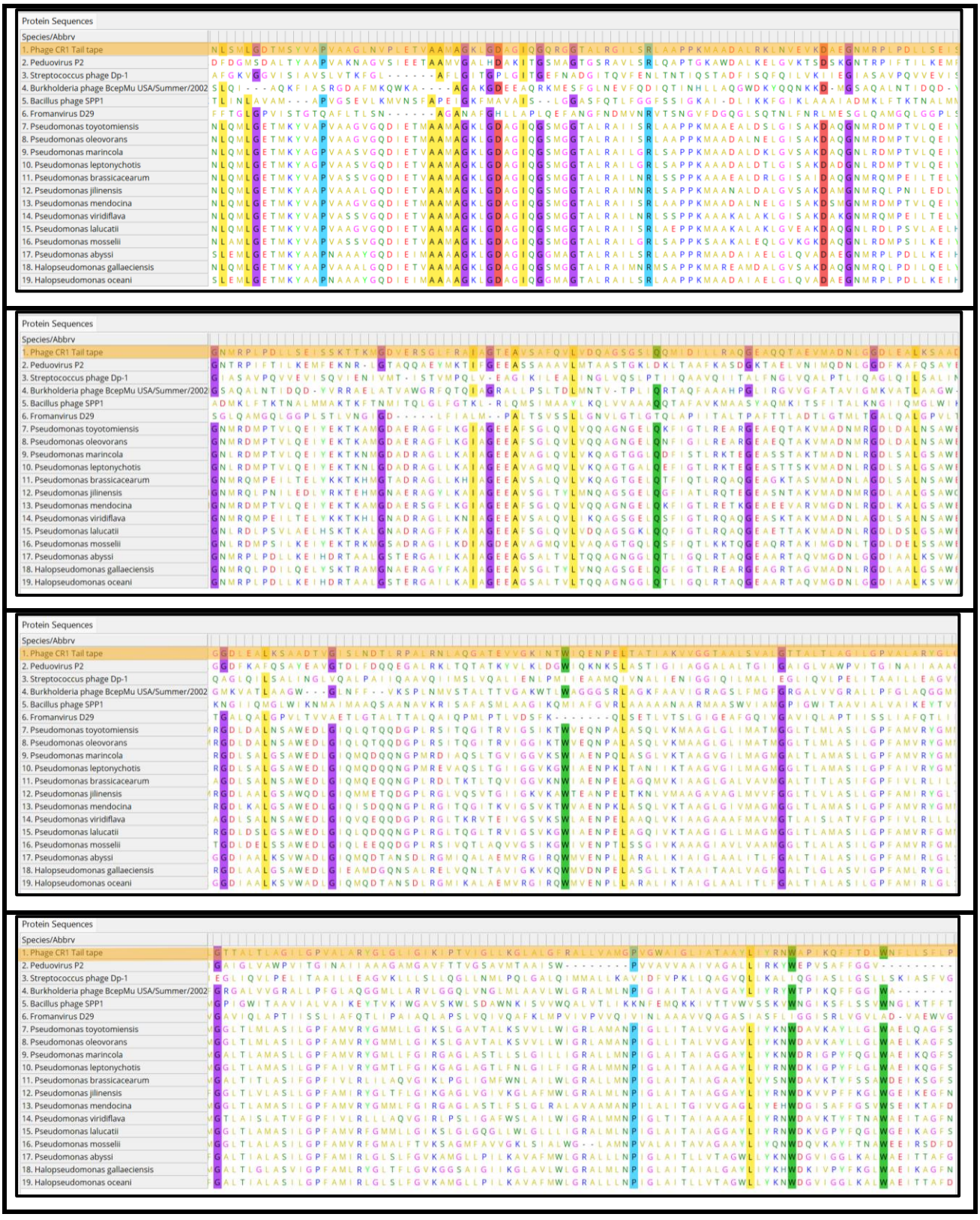


Fig. 5.37. Phylogenetic analysis of Phage CR1 tail tape measure protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.


Protein Sequences
Species/Abbrv
1. Phage CR1 Tail tape
2. Peduovirus P2
3. Streptococcus phage Dp-1
4. Burkholderia phage BcepMu USA/Summer/2002
5. Bacillus phage SPP1
6. Fromanvirus D29
7. Pseudomonas toyotomiensis
8. Pseudomonas oleovorans
9. Pseudomonas marincola
10. Pseudomonas leptonychotis
11. Pseudomonas brassicacearum
12. Pseudomonas jilinensis
13. Pseudomonas mendocina
14. Pseudomonas viridiflava
15. Pseudomonas lalucatii
16. Pseudomonas mosselii
17. Pseudomonas abyssi
18. Halopseudomonas gallaeciensis
19. Halopseudomonas oceani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11. Pseudomonas brassicacearum
12. Pseudomonas jilinensis
13. Pseudomonas mendocina
14. Pseudomonas viridiflava
15. Pseudomonas lalucatii
16. Pseudomonas mosselii
17. Pseudomonas abyssi
18. Halopseudomonas gallaeciensis
19. Halopseudomonas oceani

Fig. 5.38. *Multiple sequence alignment of tail tape measure protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* tail tape measure protein from bacteriophages of various hosts it was observed that methionine, alanine, valine, and others are highly conserved at amino acid positions 1, 2, 9, and 96 other positions, respectively.

5.5.18. PHAGE CR1 tail protein D:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 tail protein D**, has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Baseplate hub protein of *Eganvirus ev186* and *Peduovirus P2,* exhibited 91% and 95% sequence similarity with *Phage CR1* tail protein D, the phylogenetically closest species with standing members in the tree. *Eganvirus ev186* and *Peduovirus P2* are *E. coli* phage (Seed KM., Dennis JJ., 2005, Viral host database).

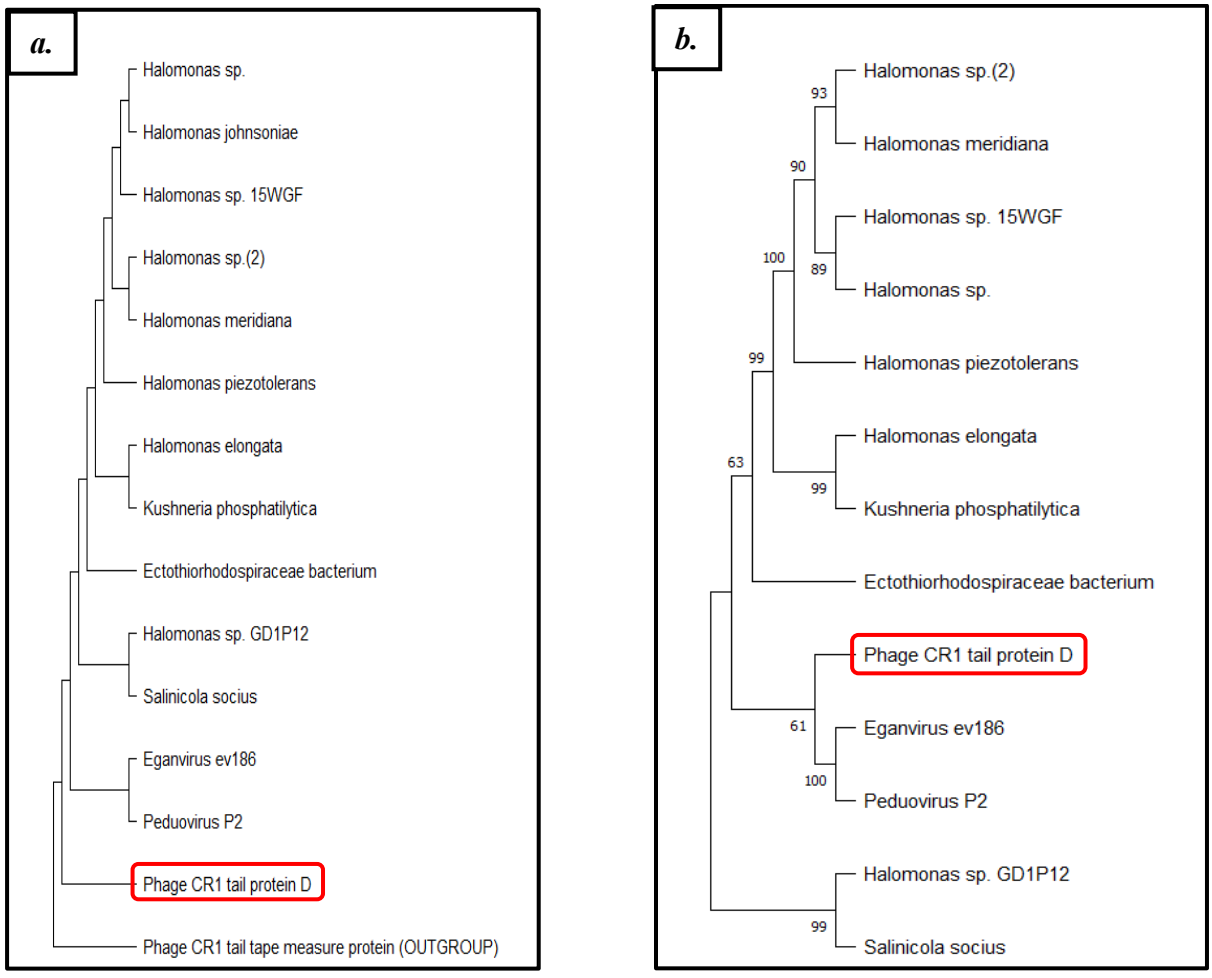

Fig. 5.39. Phylogenetic analysis of Phage CR1 tail protein D. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

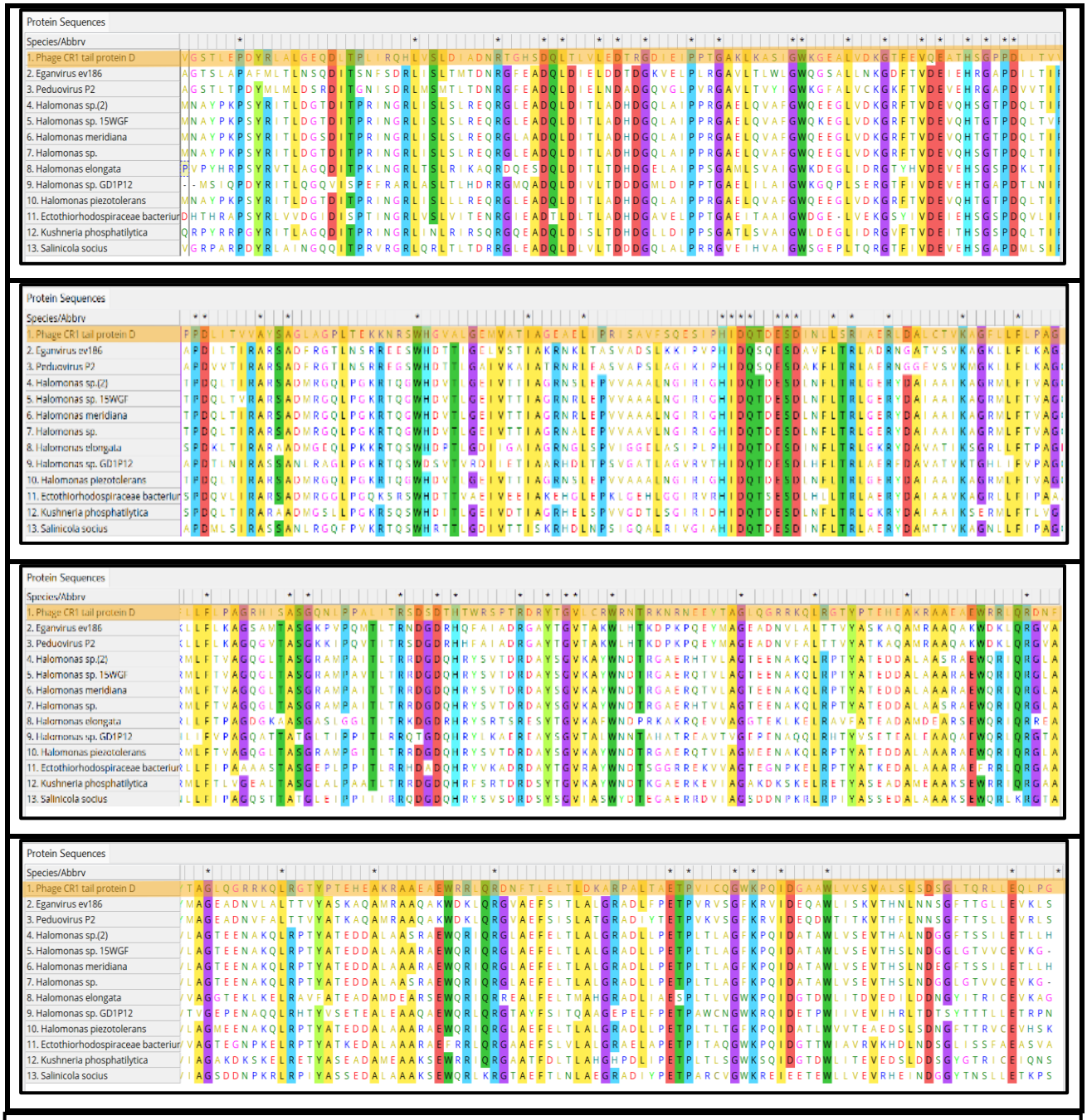

Fig. 5.40. *Multiple sequence alignment of Phage CR1 tail protein D highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* tail protein D from bacteriophages of various hosts it was observed that proline, tyrosine, arginine, and others are highly conserved at amino acid positions 7, 9, 10, and 130 other positions, respectively.

5.5.19. PHAGE CR1 Putative phage tail protein:

The BLASTp using non-redundant protein database and UniProt/SwissProt database of the protein sequence of **PHAGE CR1 Putative phage tail protein**, has been done and sequence alignment followed by phylogenetic tree analysis were performed using the top few hits. Phage tail protein of *Pseudomonas jilinensis*, exhibited 94% sequence similarity with *Phage CR1* Putative phage tail protein, the phylogenetically closest species with standing members in the tree. *Pseudomonas jilinensis* was isolated from oil production water.

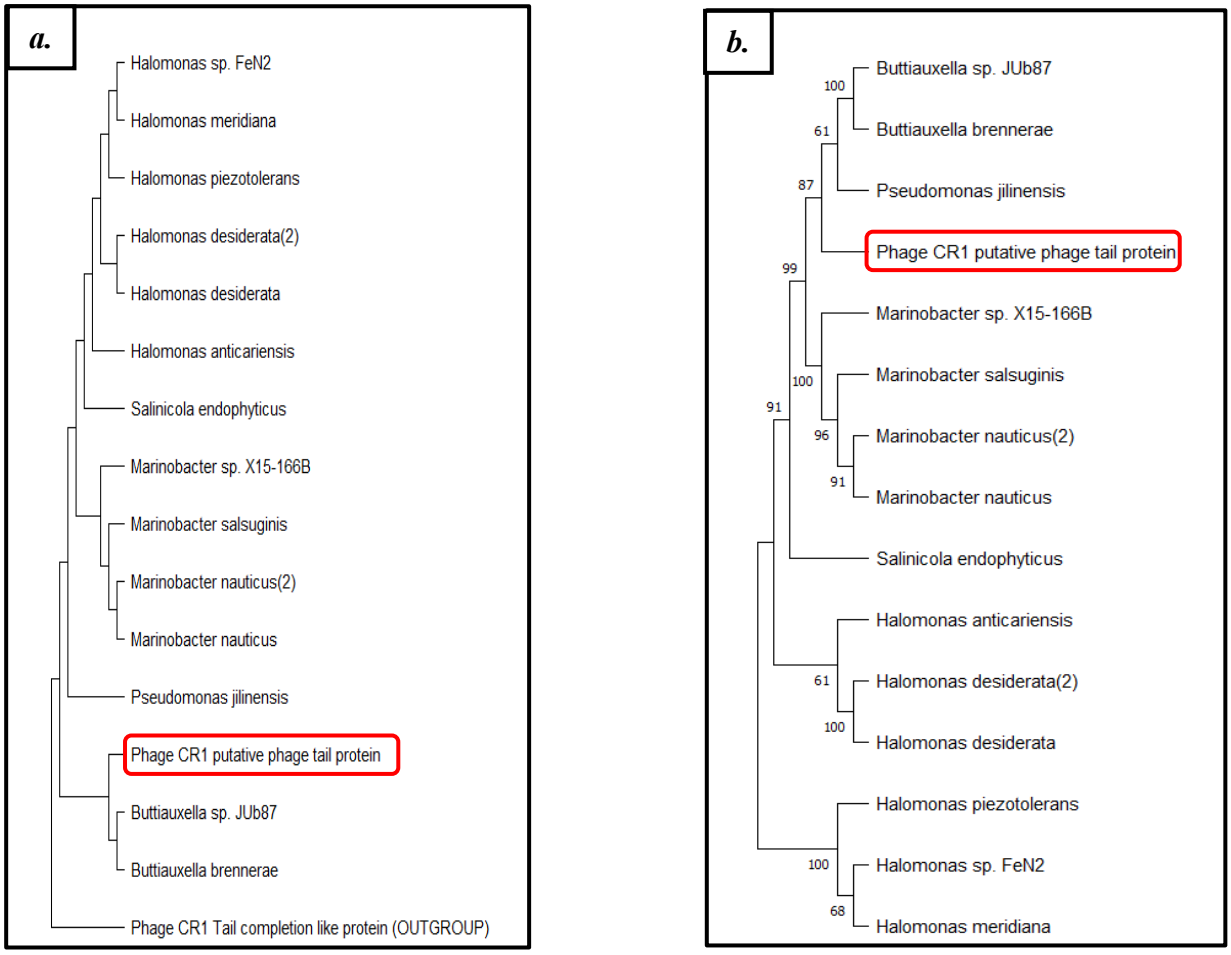

Fig. 5.41. Phylogenetic analysis of Phage CR1 putative phage tail protein. **(a.)** ***Maximum likelihood tree*** **(b.)** ***Neighbourhood Joining tree***.

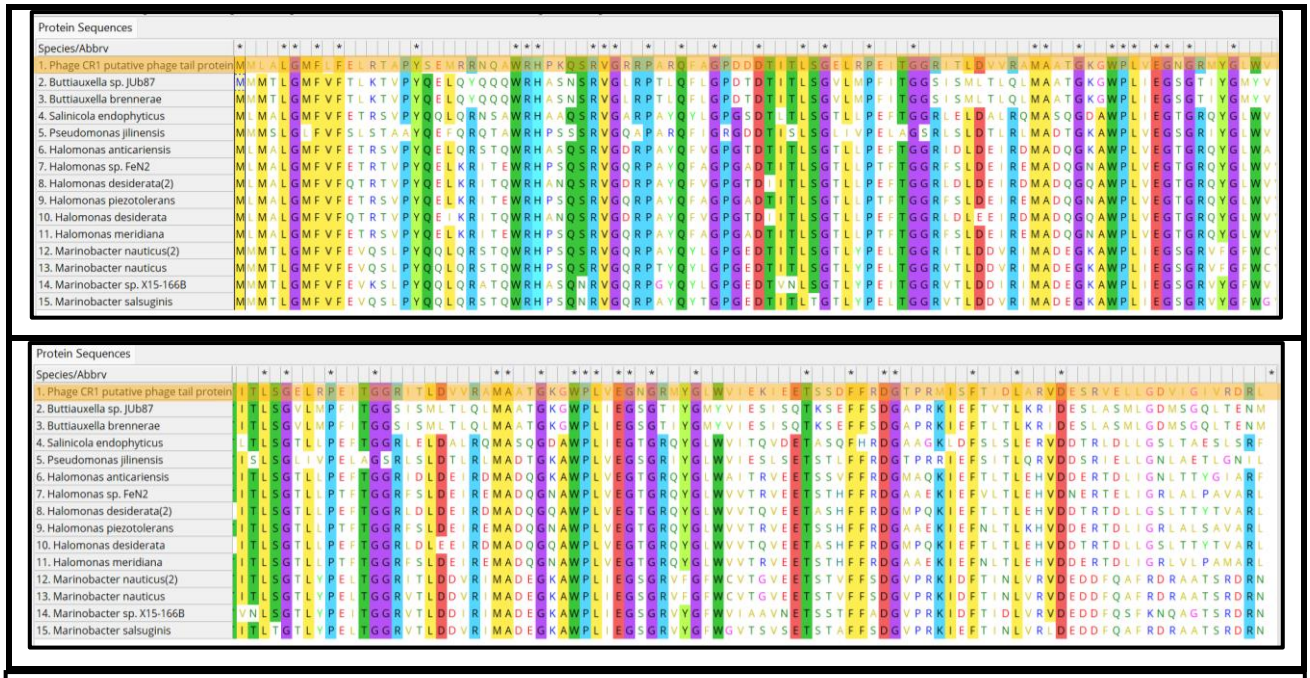

Fig. 5.42. *Multiple sequence alignment of putative phage tail protein highlighting the conserved regions.*

Based on the multiple sequence alignment of *Phage CR1* putative phage tail protein from bacteriophages of various hosts it was observed that methionine, leucine, glycine, and others are highly conserved at amino acid positions 1, 3, 5, 6 and 66 other positions, respectively.

CONCLUSION

The host of the lysogenic bacteriophage CR1 is *Microbulbifer* strain *CR1*, which is a marine polysaccharide degrading bacteria. *Microbulbifer* strain CR1 is a lysogen, whose specific phage CR1 was induced by nutrient starvation. Induction at different time intervals results in different phage titres. The titre is maximum at 3 hrs of induction and then gradually decreases as time of induction and incubation in nutrient deficient environment increases

The prophage CR1 consists of 33 proteins, out of which 14 are hypothetical proteins, 2 are head proteins, 4 proteins are involved baseplate assembly, 4 proteins are in capsid formation, and the rest 9 proteins involved with tail proteins to perform their function. The present study indicates that the proteins of phage *CR1 are* closely related to the proteins of bacteriophages associated with the host bacteria from different marine environments, viz., *Alcanivorax sp. S71-1-4, Pseudomonas jilinensis, Marinobacter sp. X15-166B, Desulfoluna butyratoxydans, Chitinolyticbacter meiyuanensis, Marinobacterium sedimentorum,* and *Halomonas spp*.

A few terrestrial species are also obtained, showing relationships in some of the trees, like *Pseudomonas saudiphocaensis, Stutzerimonas kunmingensi, Chromobacterium alkanivorans, Pseudomonas mirosoviensis*, and *Pseudomonas sp. TCU-HL1* is one of the few bacteriophages hosts whose phage proteins show close relatedness to those of the CR1 phage. Along with this, *Eganvirus ev186, Peduovirus P2,* and *Burkholderia phage BcepMu USA/Summer/2002* were among the top few blast hits. These phages have been reported to infect *E. coli.* (Seed KM., Dennis JJ., 2005, Viral host database).

Proteins from *Alcanivorax sp. S71-1-4* phage show maximum relations with *PHAGE CR1* proteins. Though the host of the bacteriophage used in the present study is a polysaccharide degrading bacterial strain, none of the closely related bacteriophages or the host bacteria have reported the presence of complex polysaccharide degrading enzymes expect *Chitinolyticbacter meiyuanensis.* The Phage CR1 head completion-stabilization protein from *Chitinolyticbacter meiyuanensis* shows close evolutionary relation to the head completion-stabilization protein from CR1 phage *(*Zhang et al., 2020). The phylogenetic analysis shows a close association with crude oil degrading bacteria like *Alcanivorax sp. S71-1-4 (*obligatory hydrocarbon clastic bacterium*),* and *Pseudomonas jilinensis*. Previous studies predicted that phage CR1 belongs to the order *Caudovirales*, and the phylogenetic tree analysis show maximum similarity with the species belong to the same order.

6. FUTURE PRSOPECTS

- Concentration and purification of phage.
- DNA isolation of the phage.
- Viral genome sequencing analysis.
- Atomic force microscopy can be explored more in order to study the lytic cycle of the phage and the morphological changes that the phage causes in host bacteria.

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A.1. Artificial seawater agar:		
1.	Tris base	6.05 g/L
2.	$MgSO_4$	12.32 g/L
3.	KCl	0.74 g/L
4.	$(NH_4)_2HPO_4$	0.13 g/L
5.	NaCl	17.52 g/L
6.	$CaCl_2$	0.14 g/L
Dissolve in distilled water and adjust the pH to 7 with concentrated HCl. Make up the volume to 1000 mL with distilled water.		
Agar: 2%		

Table 3. *Composition of ASW agar*

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Analyzed document	INTRODUCTION (1).docx (D166250463)
Submitted	2023-05-08 15:23:00
Submitted by	Veda Manerikar
Submitter email	biotech.veda@unigoa.ac.in
Similarity	0%
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1. INTRODUCTION

Viruses are the most prevalent "lifeforms" in the oceans and ubiquitous companions of cellular life forms. Most of the genetic variation within the aquatic ecosystem is stored in viruses (Curtis A. Suttle, 2007). Every cellular creature under investigation seems to have its own viruses, or at the very least, selfish genetic components resembling viruses. Recent environmental investigations have revealed that the "most abundant biological entities on the planet" are viruses