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Isolation Of Bacteriophage Infecting Haloalkaliphilic Bacteria In Lake Magadi, Kenya

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Abstract:
Samples of water were taken from Lake Magadi, a soda lake in the Rift Valley of Kenya. Bacteriophage lysate was prepared and stored in the dark at -4°C. Bacteria isolated using media for haloalkaliphiles and Aiba and Ogawa medium for marine cyanobacteria were cultured by spread plate technique to form lawns which were tested for plaque formation with phage lysate. Growth rate of the bacteria with and without phage infection was measured using optical density. Genomic DNA was extracted from the bacteria and PCR product obtained using 16S primers. The sequences obtained were aligned to sequences in the NCBI database and phylogenetic analysis done. One of the isolates had over 99% similarity to Idiomarina sp , a member of the Gammaproteobacteria while one had 99% similarity to Rhodobacter sp.of the class Alphaproteobacteria. Phylogenetic analysis showed close clustering of each isolates to members of their respective classes but separated from one another.

Key words: *Phage lysate, Plaque formation, 16S rRNA, Phylogeny*

1.Introduction

Lake Magadi is a soda lake with a salinity of up to 30% w/v (Grant, 1992) situated in the Rift Valley of Kenya. The lake harbours haloalkalophilic bacteria and archaea which act as hosts for numerous bacteriophages that inhabit the waters. Since their discovery, phages have been used for various practical applications, including in human and veterinary medicine. Therapeutic applications of bacteriophages were however largely forgotten after the widespread acceptance of antibiotics during the 1940s and 1950s.The emergence of antibiotic-resistant bacteria has rekindled interest in practical uses of phages. Bacteriophages also served as model microorganisms for some of the most significant discoveries in the field of molecular biology, including deciphering the genetic code and the discovery of the transduction phenomenon (Aze and Pasternack,2010).Work on phages inhabiting soda lakes is quite limited and much needs to be done to discover novel ones and their hosts.

2.Materials and Methods

2.1.Water Sampling Methods

Water samples were collected from the lake in the month of January. Three samples of water were collected from each site and placed in sterile and labeled bottles. These were carried in a cool box and stored at about 4^{o} C prior to analysis.

2.2.Phage Isolation

Phage growth media was prepared using the following components; 1.5 g/l KH_2PO_4,3g/l Na_2HPO_4, 1.0g/l NH_4 Cl,0.2g/l $MgSO_4.7H_2O$, 10.0g/lGlycerol,5.0g/l Acid hydrolysed casein, 0.01g/l dl-Tryptophan,0.02g/l Gelatin. The media were sterilized in the autoclave at 121 °C. To ensure an adequate supply of phages, the water sample was enriched with phage growth media for 48 hrs and incubated at 37°C. An equal amount of lysing medium was added to the growth medium during the last 6 hrs of incubation to

augment the lysing properties of phage. The lysing medium was composed of 0.98g/l NaCN (Brown, 2005).Debris and miscellaneous bacteria was removed from the enrichment culture medium by a triple centrifugation process. This was done by centrifuging at 2,500 rpm for 10min.The supernatant culture medium was decanted into another set of tubes which was centrifuged in the same manner. A third and final decantation and centrifugation was done. Filtration of the culture medium was then done using filters with a pore size of 0.22µm. The phage filtrate was then poured into sterile tubes, wrapped in foil and stored at -4°C.

2.3.Bacterial Isolation

Two types of culture media were used. These were the Aiba and Ogawa medium and the media for halophilic bacteria. Aiba and Ogawa medium for marine cyanobacteria was prepared as two separate solutions to avoid precipitation

Solution A: This consisted of 2.0g/l NaCl, 0.4g/l $MgSO_4 \cdot 7H_2O$,2.0g/l K_2SO_4, 80mg/l $CaCl_2 \cdot 2H_2O$,5g/l $NaNO_3$, 20mg/l $FeSO_4 \cdot 7H_2O$, 2.0ml/l Gaffron micronutrients

Solution B: This consisted of 1.0g/l K_2HPO_4, 8.06 g/l Na_2CO_3, 27.22 g/l $NaHCO_3$

After autoclaving, Solution A was mixed with Solution B asceptically after cooling to about 60°C giving a final pH 9.4-9.8.

The Modified medium for haloalkaliphilic bacteria consisted of 53.0g/l Na_2CO_3, 42.0g/l $NaHCO_3$, 29.5 NaCl (sea salt) ,1.5g/l TSB, 15.0 g/l Agar

Salts were sterilized in an autoclave at 121 ^oC for 20 min separately then mixed with the rest of the sterilized ingredients at 60 ^oC just before pouring onto plates.

Bacteria were cultured using the enrichment culture method by mixing water samples with each liquid media in1:1 ratio. This was followed by the spread plate technique on agar plates of each media. One set of each media culture was incubated at room temperature with plenty of sunlight, while the other was incubated at 37°C. Discrete colonies that developed were streaked on fresh plates to obtain pure isolates. The pure cultures obtained were sub cultured in tubes containing each liquid media. Isolates were characterized by Gram stain and catalase test. These were kept at 4°C.

2.4.Determination Of Cyanophage Host Range

About 0.1 ml of phage filtrate, of serial dilution1:100, and 2 ml of each bacterial liquid culture was added to tubes of 2ml soft nutrient agar(consisting of 7g/l agar and 8g/l nutrient broth)which was kept from solidifying by keeping the tubes in a water bath at 50°C. The soft agar was layered over lawns of bacterial isolates on hard nutrient agar and incubated at 37°C.Observation was done after 3 hrs. Visible plaques were counted and the plaque forming units per ml (PFU/ml) recorded after 48 hrs.

2.5.Isolation Of Phage From Plaques

Phage infecting bacterial isolate MB5 was isolated by cutting out agar sections containing the plaques using a sterile blade. These were placed in 8% NaCl sterile solution and incubated at 37°C for 12 hrs. The solution was then centrifuged and filtered through a 0.22µm filter to remove any remaining bacteria. The filtrate was stored in tubes wrapped in foil at -4°C.

2.6.Measurement Of Growth Rate

80ml of each bacterial isolate broth culture had growth rate monitored by measurement of optical density using a spectrophotometer (Beckmann Coulter Du 530 model) at wavelength of 680 nm (Brown, 2005). Measurement was done after every one hour after an initial incubation period of 12 hrs at 37°C.On the fifth hour each bacterial culture was divided into two portions of 40 ml. Two ml phage lysate was added to one of the portions while a similar amount of culture media was added to the other portion to equqlise the amounts. The portions were then incubated at 37°C and optical density measurement resumed after every hour.

2.7.Nucleic Acid Analysis (16S Rrna Gene Sequencing)Of Host Bacteria

2.7.1.DNA Isolation

Bacterial cells were harvested in the early log phase of growth in liquid broth (about 24 hr after innoculation).Total DNA of the microbial isolates was isolated using a DNEasy Blood and Tissue kit, from Qiagen, according to the manufacturer's instructions(http//www.qiagen.com). The genomic DNA obtained was stored at -25 ^oC.

2.7.2.Polymerase Chain Reaction (PCR)

Base sequences of the primers used for amplification were:

16S F 5'- AGA GTT TGA TCH TGG CTY AG -3'

16S R 5'- ACG GNT ACC TTGTTACGACTT – 3'

The reaction mixture contained the following components; 38.25µl PCR water; 4µl DNA template diluted 1: 10 in PCR water; 5µl thermopol – 10 x buffers; 1.25µl dNTP mix; 0.5µl each of forward and reverse Universal Archaea or 16S primer diluted 1: 10 with PCR water; 0.5µl Taq polymerase, totalling 50µl. The thermocycler program used was 95 ^oC for 3 min, 95 ^oC for 30 sec, 55 ^oC for 30 sec, 72 ^oC for 1 min for extension of the PCR product, 30 cycles were performed with a final extension of 3 min at 72 ^oC. The reaction was held at 4 ^oC and the product removed and stored at -25 ^oC.To purify the PCR products a QIAquick Purification KIT from Qiagen was used according to instructions(http//www. qiagen.com).About 50µl of eluate was collected and stored at -25 ^oC.

Agarose gel electrophoresis was carried out and band images taken. The PCR product obtained was sequenced and aligned and phylogenetic tree constructed using MEGA 5 program (Http;//www.megasoftware.net).

3. Results

3.1. Bacterial Isolates

Two Gram negative, rod shaped isolates that were catalase positive were obtained. They were named isolate MB3 and MB5.

3.2. Plaque Formation

When isolates MB3 and MB4 were inoculated with phage lysate there was development of plaques with isolate MB-5. The plaques were of about 1mm to 2 mm in diameter. The bacterial concentration was about $1.4x10^4$CFU/ml while the phage concentration was about$1.2x10^4$ PFU/ml.

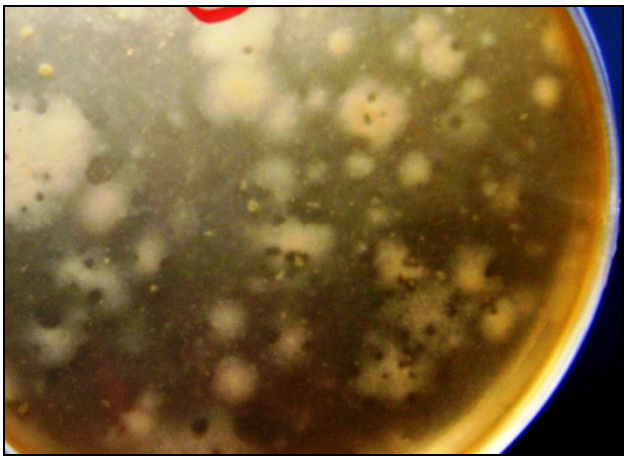

Figure 1: Image Of Plaques That Developed On Isolate MB5

3.3. Growth Rates

Growth was monitored by measurement of optical density of the culture. Measurement was done after every one hour after an initial incubation period of 12 hrs at 37°C.On the fifth hour the culture was divided into two portions. Phage lysate was added to one of the portions while a similar amount of culture media was added to the other portion. The two portions were then incubated at 37°C and optical density measurement resumed after every hour.

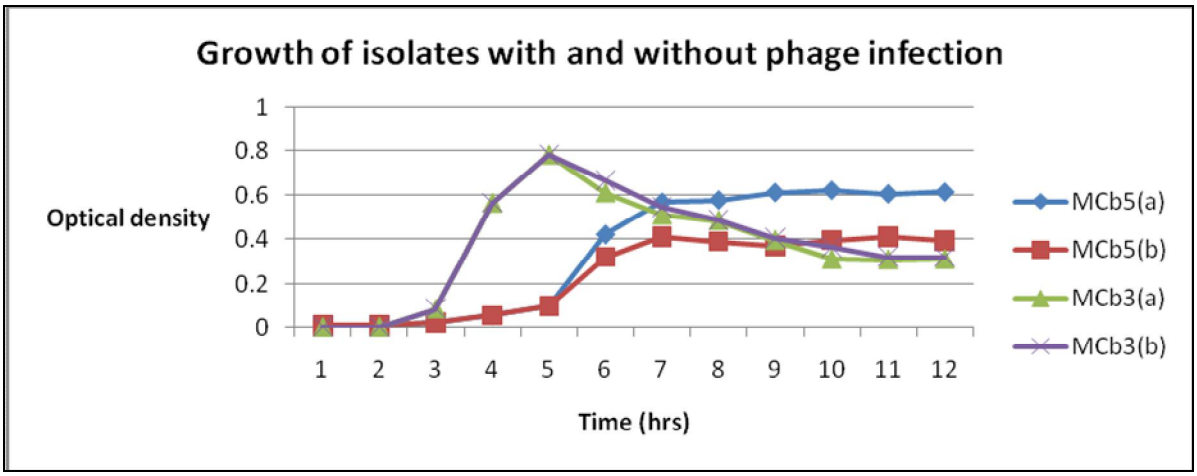

Figure 2: Graph Of Optical Density Against Time For Infected And Non-Infected Bacterial Isolates

The growth curves labeled (a) had no phage lysate while those labeled (b) had phage lysate added. The curve obtained with the infected isolate MB5 show reduction in concentration which can be attributed to cell lysis by the phage.

3.4. Nucleic Acid Analysis

Genomic DNA extracted from the isolates was cloned and the PCR product sequenced. Gel electrophoresis was carried out and band images taken.

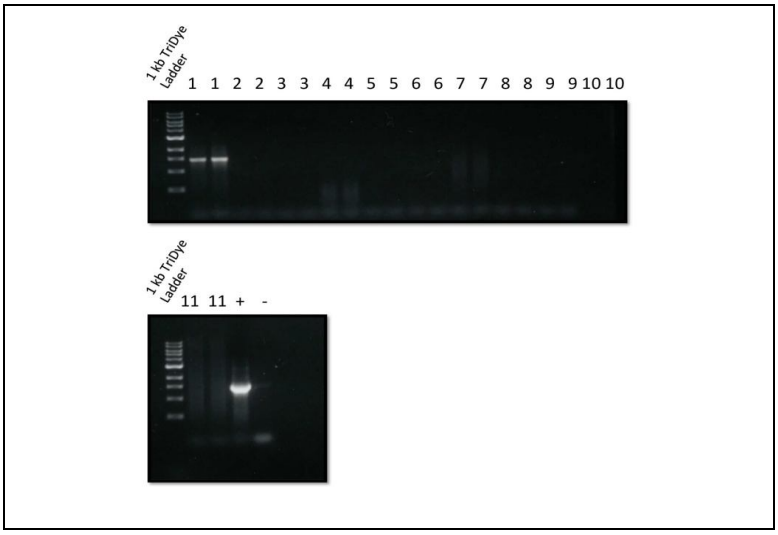

Figure 3: Image Of Ethidium Bromide-Stained Electrophoresis Gel Of PCR Product On 1% Agarose

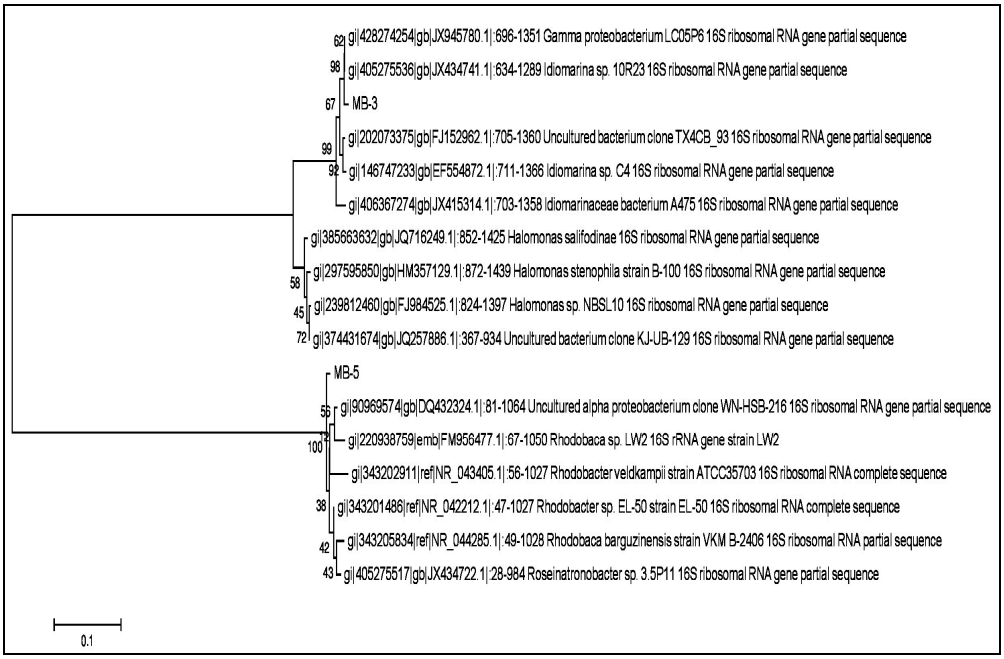

Figure 4: Phylogenetic Tree To Show Relationship Between 16S Rrna Sequences Of Isolates And Their Comparisons From NCBI Genbank

The sequence obatained was compared to other sequences in the GenBank data base. Isolate MB-3 was found to be 99% similar to Idiomarina while Isoalte MB-5 was 99% similar to Rhodobacter sp. The comparisons were used to generate a phylogenetic tree using MEGA

4.Discussion

Two strains of halophilic bacteria were isolated from the lake water samples and coded as isolate MB3 and MB5.The bacteria were Gram negative rods that were aerobic producing bubbles of oxygen with hydrogen peroxide in the catalase test. Phylogenetic studies based on 16SrDNA sequence analysis showed Isolate MB-3 clustered closely with Gamma proteobacterium of accession number J945780.1 and Idiomarina sp of accession numberJX434741.1.Isolate MB-5 clustered closely with Rhodobacter veldkampii strain ATCC3503 of accession numberNR_043405.1. and Rhodobacter sp of accession numberNR_042212.1. Idiomarina belonging to class Gammaproteobacteria is a heterotrophic bacteria that is able to hydrolyse food material by production of hydrolytic enzymes. Rhodobacter sp belonging to class Alphaproteobacteria is a kind of purple bacteria that is able to carry out photosynthesis. The phage isolated was host specific to Idiomarina failing to cross infect the Rhodobacter sp. The growth rate of isolate MB3 is affected by the

presence of phage showing a considerable difference in the optical density and hence cell concentration reduction. Five *Rhodobacter sphaeroides* virulent phages have been isolated from water reservoirs in Puerto Rico. This is the first report of bacteriophages that infect photosynthetic bacteria from the Caribbean, specifically purple non-sulfur anoxyphototrophs. Bacteriophages previously described in literature were isolated from Europe (Duchrow et. al., 1988; Garí et. al., 1992) and North America (Abeliovich and Kaplan, 1974; Mural and Friedman, 1974). In order to see a plaque during the initial detection and isolation steps, it was necessary to perform two host enrichment phases in the water sampled. This suggested a low concentration of viral particles and the need for their amplification in order to detect them. The bacteriophages were able to form plaques only during aerobic conditions.Transmission Electron Microscopy analysis revealed that all phages had polyhedral heads and a long tail. The TEM analysis of the isolates was similar to the bacteriophages previously described as belonging to Myoviridae and Siphoviridae family. The plaques and plaque forming units per plaque (pfu/p) produced by the isolated phages in the *R. sphaeroides* lawn had plaque zones that ranged in size from 0.9 mm to 1.2mm and concentration of 3.0 x 10^{9}pfu/p) to 1.2 x 10^{11}pfu/p(Rojas-Dulan et al,2009).

5.Conclusion

The results suggest that viruses may influence host microbe concentrations. They may also influence non host microbes through a "chain reaction" mechanism. By lysing specific hosts that are lethal to other microorganisms, viruses can influence microbial population dynamics indirectly. Such a chain reaction caused by phage or viruses has not been tested in the natural environment. (Cai, et al, 2011).Further research needs to be done on phages inhabiting soda lakes and hence affecting microbe populations some of which like Rhodobacter contribute to primary production.

6.Acknowledgements

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7.References

1. Abeliovich, A., and S. Kaplan. 1974. Bacteriophages of Rhodopseudomonas sphaeroides: isolation and charac-terization of a Rhodopseudomona sphaeroidesbacte-riophage. J. Virol.13(6):1392-1399.
2. Aze A, Pasternack, G.(2010)Industrial and regulatoryissuesin bacteriohage applications in food production and rocessing,In:Sabour P.M.,Griffiths M.W,Editors.Bacteriophages in control of food and pathogens.Washinton DC:ASM Press; pp297-326
3. Brown, A.E., 2005.Benson's Microbiological applications: Laboratory manual in General Microbiology, ninth edition. McGraw Hill Companies ptr, New York.
4. Cai W., H. Wang, Y. Tian, F. Chen and T. Zheng (2011) Influence of a bacteriophage on the population dynamics of toxic Dinoflagellates by Lysis of Algicidal bacteria Appl. Environ. Microbiol.7837: 77(21)
5. Duchrow, M., S. Heitefuss, and J. Kalkus. 1988. Isolation and characterization of a virulent phage for Rhodobacter sphaeroides. Arch. Microbiol.14
6. Garí, E., M. Ollé, and I. Gilbert. 1992. Isolation and characterization of a recombinationdefective dependent bacteriophage Rhodobacter sphaeoridesCurr. Microbiol.24:151-157 .
7. Grant, W.D., 1992. Alkaline environments, In; J.Lederberg (Ed). Encyclopedia of Microbiology, Academic press, San Diego. 73 - 80.
8. Http; //www.qiagen. com; Http;//www.megasoftware.net
9. Mural, R. J., and D. I. Friedman. 1974. Isolation and characterization of a temperate bacteriophages specific for Rhodopseudomonas sphaeroides. J. Virol.14(5):1288-1292 .
10. Rojas-Dulan M.F. R. Vasquez, C.R. Velasquez,(2009)Molecular and morphological characterization of bacteriophages on Rhodobacter spiroides from Puerto Rico; Carribean Journal of Science Vol 45 No 1,118-124