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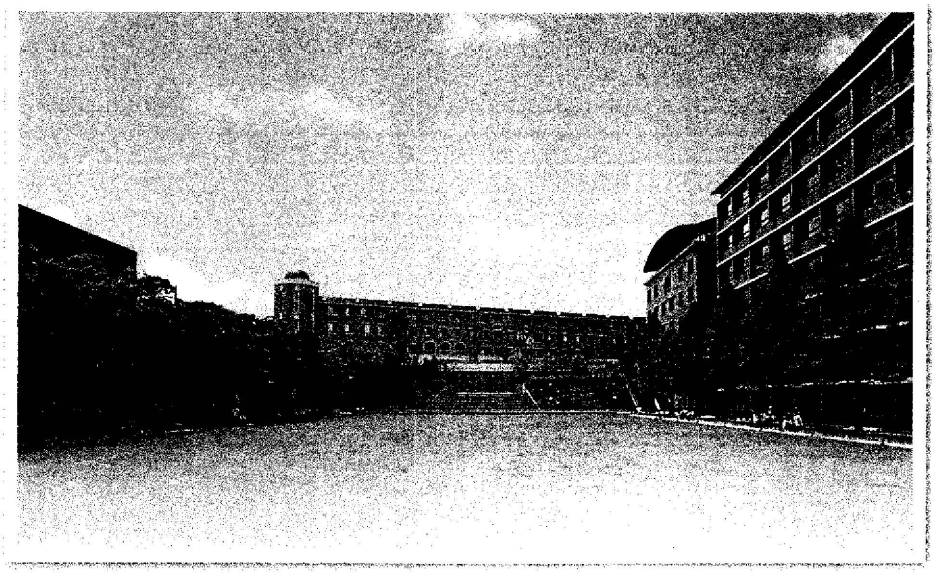

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Novel Bacteriophage Capable of Inducing Biosurfactant Rhamnolipid Production in *Pseudomonas aeruginosa* USM AR2

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Abstract

A bacteriophage infecting Pseudomonas aeruginosa USM AR2 was successfully isolated from hydrocarbon-contaminated muddy soil. Morphological study revealed a phage with icosahedral capsid head and long non-contractile tail. The genome of the phage was determined as double stranded DNA. Thus, the phage could be a member of the Siphoviridae family. Genome profiling using different restriction enzymes showed different digestion patterns when compared to the most common Siphoviridae phage lambda and other Siphoviridae phages infect P. aeruginosa. Hence, the isolated phage could potentially be a new member of the Siphoviridae family. A very peculiar observation was noticed regarding the plaques formation when the phage infected P. aeruginosa USM AR2. The plaques formed were different from the usual where slimy substance was observed in addition to clear zones produced. When P. aeruginosa USM AR2 and the isolated phage were cultured together in broth, the broth became slimy as well. Orcinal assay was carried out on the broth and confirmed the presence of biosurfactant rhamnolipid with production could reach more than one hundred percent of the cell biomass in a shake flask. An extensive literature search using various databases, as well as, different websites of search engines could not find anything on phages that are capable of inducing rhamnolipid production either in Pseudomonas or in any other biosurfactant producing bacteria. Perhaps, this is the first ever phage capable of inducing rhamnolipids production and potentially new and novel phage.

1. INTRODUCTION

One of the members in microbial communities is bacteriophage or phage for short refer to viruses that infect bacteria. Phages are among the most common biological entities on earth and are found in all habitats in the world where bacteria and archaea proliferate (Clokie *et. al.* 2011). Recent estimates suggest that there exist globally ~100 million phage species; however, only a small fraction of phages have so far been characterized (Rohwer, 2003 & McNair *et. al.* 2012). Out of the estimation of more than 10^{31} phages, only approximately 5100 have been identified and reported towards the end of last century (Ackermann, 2001 & Parisien *et. al.* 2008). On a global scale, it was estimated that ~10^{25} phages initiate an infection every second (Hendrix *et. al.* 1999). Therefore, it is not surprising that phages play major roles in the ecological balance of microbial life and indirectly influence the world ecology as well.

Biosufactants, produced by varieties of microorganisms, are well known and well documented for their role in enhancement of the emulsification of hydrocarbons, potentially solubilizing the hydrocarbon contaminants and increasing their availability for microbial degradation (Salwa, *et. al.*, 2009; Sekhon *et. al.*, 2012). Biosurfactants are structurally diverse and can have various chemical compositions mainly consisting of fatty acids, glycolipids, lipopeptides, lipopolysaccharides and lipoproteins depending on the producing microorganisms, raw matter and process conditions (Makkar *et. al.*, 2011 and Sekhon *et. al.*, 2012). Biosurfactants have the potential to be applied in various fields such as cleaning industry, pharmaceuticals, cosmetics, food industry and medical (Banat *et. al.*, 2000; Kitamoto *et. al.*, 2002; Zeraik & Nitschke, 2010). To date, biosurfactants play an important application in petroleum-related industries included enhanced oil recovery, cleaning oil spills, and bioremediation (Bertrand *et. al.*, 1994 & Daziel, *et. al.*, 1996).

2. EXPERIMENTAL PROCEDURE

2.1. Isolation and Purification of Bacteriophage

Bacteria host *P. aeruginosa* USM AR2 was cultured in LB broth and incubated overnight at 37°C with shaking at 180 rpm. Bacteria culture was mixed together with 5 g of hydrocarbon-contaminated muddy soil sample and allowed for incubation overnight at 37°C with shaking at 180 rpm. The next morning, the incubated culture was filtered with filter paper, centrifuged at 10,000 rpm and followed by filtration using bacteria filter with pore size of 0.45 µm. Then, the filtrate was serially diluted in LB broth. 100 µL of the dilutions was mixed with 100 µL of *P. aeruginosa* USM AR2 culture and plated onto LB agar. 3 mL LB top agar was overlay onto the plates and allowed to harden. The plates were incubated at 37°C overnight.

The next morning, formations of clear zones or plaques were observed indicating the infection of *P. aeruginosa* USM AR2 by phages presence in the filtrate. For purification purposes, well isolated plaques were transferred onto a new LB plate that was previously spread with *P. aeruginosa* USM AR2 and overlaid with LB top agar. The transfer was carried out using sterile toothpicks by poking through the LB top agar. The plate was incubated at 37°C overnight for plaques formation.

The LB top agar was scrapped off the LB plate and eluted in TMS buffer overnight at 4°C. The next day, 0.2 mL chloroform was added and shakes gently. The elution was centrifuged and the supernatant was filtered through 0.45 µM bacteria filter. A small amount of chloroform (not more than 5% of the filtrate volume) was added into the filtrate for storage purpose at 4°C as stock. Plaque assay was performed to determine the phage titer (pfu/mL) of the filtrate.

For bacteriophage enrichment, the stock was allowed to infect *P. aeruginosa* USM AR2 overnight. The infected *P. aeruginosa* USM AR2 culture was centrifuged, filtered, stored in chloroform as enriched bacteriophage stock.

2.2 Transmission Electron Microscopy (TEM)

A drop of phage with titer of 10^{10} pfu/mL was applied onto the surface of a carbon-coated grid (400 mesh copper grid). The sample was negatively stained with 2% methyltungstate. The morphology of the phage was observed using transmission electron microscope (Philips CM12 equipped with analysis system, Philips Electron Optics).

2.3 Genomic Profiling

Prior to genome extraction, the phage stock with was treated with DNase and RNase. The phage genome was extracted using phenol/chloroform and ethanol salt precipitation method (Sambrook and Russel, 2001). The extracted genome was digested with either DNase or RNase. Restriction enzyme digestion of the extracted genome was carried out using *Eco*RI, *Eco*RV and *Nco*I. Virtual restriction enzyme digestion patterns were performed using online programme, NEBcutter V2.0, accessible through website http://tools.neb.com/NEBcutter2/ (Vincze *et. al.*, 2003). Phages D3112 and MP29, *Siphoviridae* phages infecting *P. aeruginosa*, were used as comparison against restriction enzyme digestion patterns of the isolated phage.

2.3 Orcinol Assay

A culture of *P. aeruginosa* USM AR2 was mixed with the isolated phage to allow infection took place. The mixture was sampled at 12 hours and 24 hours post infection and centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and appropriate dilutions were prepared for Orcinal assay. The diluted supernatants were mixed with Orcinal reagent and incubated at 70°C for 40 minutes. After incubation, the mixtures were allowed to cool down to room temperature and spectrophotometer readings at OD_{421} were taken for each dilutions.

Table 1: Orcinol assay for rhamnolipid detection. Copious amount of rhamnolipid was produced within 48 hours post infection.

Sample		Average OD_{421} reading (15X dilution)	Biomass (g/L)	Amount of rhamnolipid produced (g/L)	% rhamnolipid production
24 hours	Non-infected	0.854	0.77	0.51	66.2
	Infected	1.423	1.28		
48 hours	Non-infected	0.736	0.66	0.69	104.5
	Infected	1.496	1.35		

For the isolated phage to act as an inducer for rhamnolipid production in *P. aeruginosa* USM AR2 was unheard before. Supported by an extensive literature search using various databases, as well as, different websites of search engines could not find anything on phages that are capable of inducing rhamnolipid production either in *Pseudomonas* or in other biosurfactant producing bacteria.

4. CONCLUSIONS

Based on these results, the isolated phage may be a potentially new and novel phage infecting *P. aeruginosa* USM AR2 and capable to act as an inducer for rhamnolipid production.

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