UV IRRADIATION OF *SHIGELLA DYSENTERIAE* INDUCED THE TRANSFORMATION AND EXCISION OF A PRESUMED INTEGRATED LYSOGENIC PROPHAGE *Shd-4L10*INTO A LYTIC PHASE

F. K. Rodrigues[1], G. Armah[2], J. Fobil[3], M. Steiner-Asiedu[4], J. Efavi[5], B. Addy[1]

[1]School of Applied Sciences, Central University College, P. O. Box 2305,Tema, Ghana

[2]Department of Electron Microscopy, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, Ghana

[3]School of Public Health, College of Health Sciences, University of Ghana, Legon, Ghana

[4]Department of Nutrition and Food Science, University of Ghana, P. O. Box LG Legon, Ghana

[5]Department of Material Science, Faculty of Engineering Sciences, University of Ghana, Legon, Ghana

ABSTRACT

Repeated exposures of Shigella dysenteriae strain A to ultra-violetradiation (253.7 nm) with intervening outgrowth of survivors gave rise to clear bacteriophage plaques. Isolation, propagation and partial purification of the new Shd-4L10 phages showed that they are similar in morphology to the Myxobacteriaphage Mx-4described earlier. The new phages retained the general characteristics of S. dystenteriae phageShd-4L3,including serological properties and phage typing. It is suggested that ultra-violet irradiation may have played a role in the transformation and excision of the presumed lysogen of S. dysenteriaestrain A into a lytic phase. PhageShd-4L10was subsequently partially characterized. It has a density of 1.61, a DNA: protein ratio 0f 0.42 and thus a cryptogram of D/2:54.3/32.5:X/X: B/O. The phage was further characterised by fractionation of its protein using SDS-polyacrilamide gel electrophoresis. DNA extracted from phages was hydrolysed with restriction endonuclease R., EcoR1. The restriction fragments were catalogued and their apparent molecular weights calculated from electrophoresis gels calibrated with fragments from DNA of coliphage λ. From the total fragments obtained with nuclease R., EcoR1, the apparent minimum molecular weight of phage Shd-4L10DNA was found to be 54.3 x 10^6 Daltons. The molecular weight of the phage DNA was also calculated from measurements of contour length of purified DNA samples, using the formula MW = 1.97 x 10^{10} l/(magnification), where l is the measured length of DNA in centimetres). The very close relatedness with phage Shd-4L3 was confirmed by these techniques.

INTRODUCTION

Microorganisms have over the years been exposed to continued environmental stress. This frequently results in the selection of strains resistant to the imposed conditions (Wommack and Colwell, 2000; Park *et al*., 2000). When the stress agent is also mutagenic, as in the case of UV radiation, an increase in general mutation rates, combined with the selective process, may accelerate the emergence of more uv-resistant strains (Petruk *et al*., 2004; Goh *et al*., 2005).Several investigators have reported the development of increased uv-resistance within bacterial populations when the survivors were repeatedly grown and re-exposed to irradiation (Jordi *et al*., 1995; Dodd and Sherwin, 2005; Zahng *et al*., 2010).

Bacteriophages are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery (El-Shibiny *et al*., 2005). They are estimated to be the most widely distributed entities in the biosphere. They are ubiquitous and can be found in all reservoirs populated by bacterial hosts, such as soil or in the intestines of animals. Phages have been used for over 90 years as an alternative to antibiotics in the Soviet Union and Eastern Europe as well as in France. They were used as a possible therapy against multi drug resistant strains of many bacteria (Roney *et al*., 2001; Skurnik and Strauch, 2006).

In general there are two major types of bacteriophages; lytic and lysogenic. The lytic phages also known as virulent phages are a good choice for developing therapeutic phage preparations (Toth *et al*., 2003; Lee *et al*., 2004; McGrath and van Sinderen, 2007). They multiply inside the host cell, and release a burst of phages through the cell membrane. Some DNA phages, called temperate or lysogenic phages, only lyse a small fraction of bacterial cells; in the remaining majority of the bacteria, the phage DNA becomes integrated into the bacterial chromosome as a prophageand replicate along with it.The phage DNA remains dormant until it is induced by exposure to adverse conditions which favor the termination of the lysogenic state (McGrath and van Sinderen, 2007; St. Pierre and Endy, 2008). Such conditions include desiccation, exposure to UV radiation or ionizing radiation, and exposure to mutagenic chemicals. These adverse conditions lead to the production of proteases which destroy the repressor protein (Breibach *et al*., 2011). This in turn leads to the expression of the phage genes, reversal of the integration process and lytic multiplication.

Lysogenic phages sometimes express gene products that have subtle effects on the phenotype of the host cell. They, for example, carry genes that can enhance the virulence of the bacterial host in a process called lysogenic conversion. Lysogenic phages have been shown to carry genes that can modify the *Salmonella* O antigen, which is one of the major antigens to which the immune response is directed (Wood and Revel, 1976; Vogel and Schmeiger, 1986; Sternberg and Coulby, 1987). Toxin production by *Corynebacteriumdiphtheriae* is mediated by a gene carried by a phage (Martin, 1988; Shi *et al*., 2011; Goodsell, 2001).

The present work describes the application of UV radiation to a defined organism, *S. dysenteriae* strain A and it slysogenic derivative containing a prophage, with the objective of isolation, propagation, purifying and partially characterizing the associated phage. It is thought that such a system would provide a useful model for subsequent study of the genetics and mechanisms of resistance in microorganisms.

MATERIALS AND METHODS

Bacteria, phage strains and chemicals

Shigelladysenteriae strain A was obtained from the Bacteriology Unit of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. Several unidentified strains of *S.dysenteriae* were obtained from the Microbiology Department of the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong, Akwapim. Mutants of *S. Dysenteriae* strain Aresistant to UV irradiation were earlier isolated following mutagenesis (Rodrigues *et al*., 2006). Nutrient Broth, Nutrient Agar, and other chemicals were obtained from Fluka Chemie AG, CH, Switzerland. The phage λ used as a source of DNA in this work was λcI857S_7.

Growth conditions of bacteria and phage isolations

S. dysenteriae strain A was cultured in Nutrient Broth. All incubations were at 37°C. New phages isolated from uv-irradiation of *S. dysenteriae,* were harvested by first suspending in Nutrient Broth (1% w/v) medium and filtered through Whatman number one filter paper to remove debris. Sufficient (5ml) chloroform ($CHCl_3$) was added and the mixture agitated by vortex swirling. They were partially clarified by low speed centrifugation. The supernatants were passed through a bacteriological filter

(0.2mm) and the filtrates tested for the presence of phage by plating a portion (0.1ml) with parent *S. Dysenteriae* strain A (Fiegna and Velicer, 2003; Poza *et al*., 2003) as indicators. Nutrient Broth Agar (1.5% w/v) and (0.8%w/v) was used as underlay and overlay respectively. Any plaques observed were removed with sterile wooden cocktail stick and an attempt was made to propagate and purify the phage.

Plaques of phages *Shd-4L10* were discovered in plates of *S. dysenteriae* strain A. The phages were grown by confluent lysis using Nutrient Broth Agar plates and *S. dysenteriae* strain A as indicator. The lyzates were stored over chloroform 4°C. For electron microscopy, the phages were purified by centrifugation through sucrose gradient column as described for phage Mx-1 (Matsuo and Kamiya, 2005; Stine *et al*., 2005).

Serological methods

Antiserum against phage *Shd-4L10* was raised in New Zealand white rabbit by intramuscular injection of approximately 10^{13} plaque forming unit (pfu) in an emulsion with complete Freund's adjuvant and then at 4 day intervals with approximately 10^{12},2 x 10^{12}, 5 x 10^{12}, and 10^{13} pfu with $Al(OH)_3$ (aluminum precipitate) as adjuvant. The serum was titred by phage inactivation (Queralt *et al*., 2003; Burnet, 2005; Mattseliukh and Burova, 2004) to establish the relatedness of the isolated *Shd-4L* group of phages (*Shd-4L3 and Shd-4L10)*.

Electron microscopy

Negatively stained preparations of phage *Shd-4L10*were made (Rodrigues *et al.,* 1981; Norcum *et al*., 2005; Vegge *et al*., 2005) and observed with Hitachi H600 transmission electron microscope at an accelerated voltage of 75KV (Norcum *et al*., 2005; Ackermann, 2011). The images were photographed at a magnification of between 80,000 and 160,000.

Buoyant density centrifugation

For the buoyant density centrifugation of phage *Shd-4L10* clarified lysate (15ml) was layered over three layers of Caesium Chloride (CsCl) in Tris EDTA (TES) of densities 1.30, 1.45 and 1.70 (2ml per layer) in tubes for the SW25 rotor of a Beckman model 2-65B centrifuge and was centrifuged for 3h at 22000 rev. min^{-1} at 4°C. The tubes were removed and the turbid band of phage between the layers of density 1.45 and 1.70 was removed with a hypodermic syringe. The phages were diluted to 4.5ml and CsCl added to adjust the density to 1.45. The samples were centrifuged for 48 hours at 40,000 rev. min^{-1} and 4°C in an SW50.1 rotor of a Beckman model L2-65B centrifuge and fractionated. The density of CsCl in each fraction was determined from the refractive index measurements. The fractions were diluted, the absorbance at 260nm (A_{260}) was measured and the phage titre determined.

RESULTS

Isolation of a new phage

Repeated one minute exposure of *S. Dysenteriae* strain A to short wave (germicidal) ultraviolet radiation produced clear plaques of same morphology as those obtained following treatment of phage *Shd-4L3* with uv light or with chemical mutagens (Fig.1). Purification and examination of the phage in the plaque revealed that the new phages(*Shd-4L10)*had a morphology similar to phage *Shd-4L3* described earlier (Rodrigues *et al*., 2006) and different from the known turbid *Shd-4L* type plaques (Kuo *et al*., 2000) (Fig. 2). They are however not plaque morphology mutants of *Shd-4L3*. The nature of

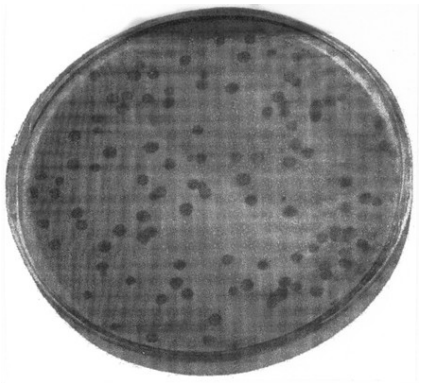

Fig. 1: Clear plaques of phage *Shd-4L10*on *Shigella dysenteriae* strain as host

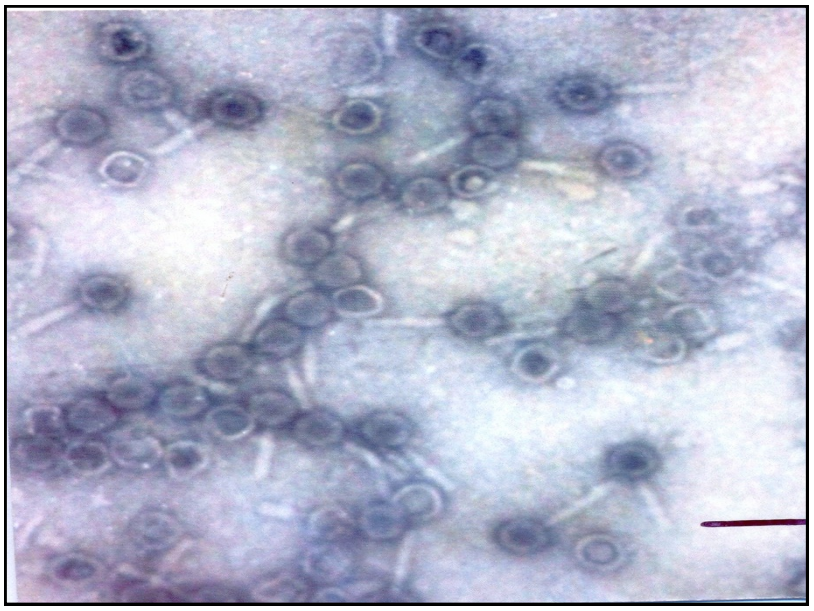

Fig. 2: Negatively stained images of bacteriophage (*Shd-4L10*) for *Shigella dysenteriae* Bar marker is 0.1 µm

the molecular events that gives rise to *Shd-4L10* during the irradiation process is not known.

Partial characterisation of phage Shd-4L10

Digestion of phage *Shd-4L10*DNA with Restriction endonuclease Eco RI generated a series of linear duplex DNA fragments which were fractionated according to size on agarose gel. No change in the pattern was observed as a result of sequential addition of more enzymes or prolonged incubation time. The pattern obtained thus represents the limit product of digestion. No attempt was made at eluting the fragments and measuring their size by electron microscopy.

However as the distance migrated by each fragment on the gel is inversely related to the molecular weight for each of the fragments and since the λ DNA digested fragment are expected to be in stoichiometric amounts, the approximate size of the native phage DNA molecule could be estimated from the sum of the MW of each species of fragments. The restriction fragments were catalogued and their apparent molecular weights estimated from electrophoresis gels. From the total fragments obtained with nuclease R., EcoR1, the apparent minimum molecular weight of phage *Shd-4L10*DNA was found to be 54.3 x 10^6 Daltons. Thus the phage has a genomic molecular weight similar to those of *Myxococcus xanthus Mx-4* type phages (Rodrigues *et al*., 2006).The digestion profile of phage *Shd-4L10* and *Shd-4L*3 are very similar and showed no obvious differences from each other, although point differences, small deletions and additions in DNA sequence between sites could not be detected by this test (Fig. 3).

The molecular weight of the phage DNA was also determined from measurements of contour length of purified DNA samples, using the formula MW = 1.97 x 10^{10} l/(magnification), where l is the measured length of DNA in centimetres) to be 50.8 x 10^6 Daltons (Fig. 4). Such a range in size would be expected to be accom-

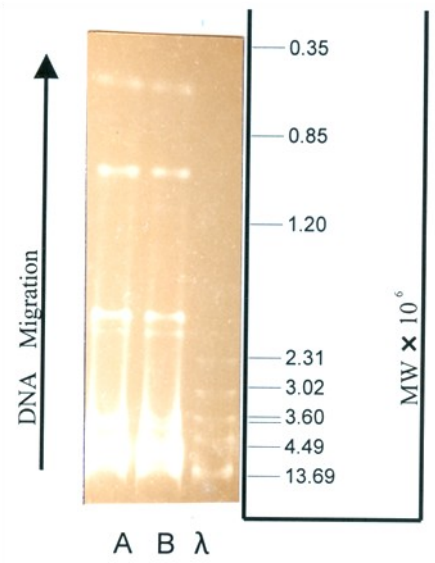

Fig. 3: Bacteriophages *Shd-4L10* and *Shd-4L3* DNA fragments from digests with restriction endonuclease R., EcoR1. fractionated by agarose gel electrophoresis. Digested λ DNA was fractionated as a marker. DNA bands were visualised under uv light by soaking the gel in ethidium bromide solution (0.4µg/ml) for 30 minutes
A *Shd-4L10*DNA
***B* *Shd-4L3*DNA**
λ Marker DNA

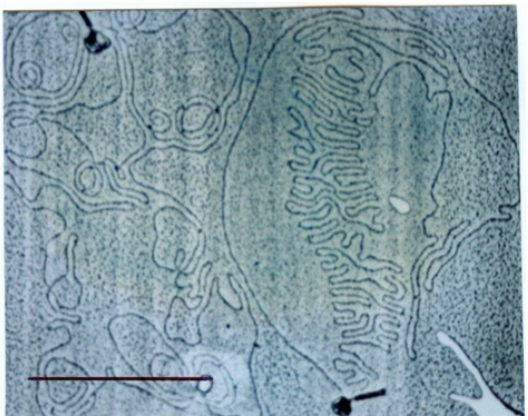

Fig.4: Electron micrograph of bacteriophage *Shd-4L10* DNA showing released DNA molecules. Bar marker is 0.1µm

modated in a head of fixed geometry. For example, it is a size for which deletion and insertion mutants of phage Mx-4 (MW 49.8 x 10^6 Daltons) can form viable particles. A variation in genome size in a set of phages that are morphologically similar and demonstrate also a weak serological relationship would normally suggest that the phages may mature their genomes by a sequence specific mechanism rather than by packaging a "head-full" of DNA.

Phage density and proteins

Phage density was determined from the evaluation of plaque forming units, absorbance at 260nm and density of caesium chloride fractions (Fig. 5). The density of phage *Shd-4L10* was found to be 1.61. DNA to protein ratio was 0.32 thus its cryptogram (Rodrigues *et al*., 1981; Ackermann, 2007) is: D/2:54.3/32.5:X/X: B/O. From measurements of negatively stained images the phage head has a diameter of 62nm and the tail is 98nm on the average. Analysis of proteins specified by phages*Shd-4L10* and *Shd-4L3* by polyacrylamide gel elec-

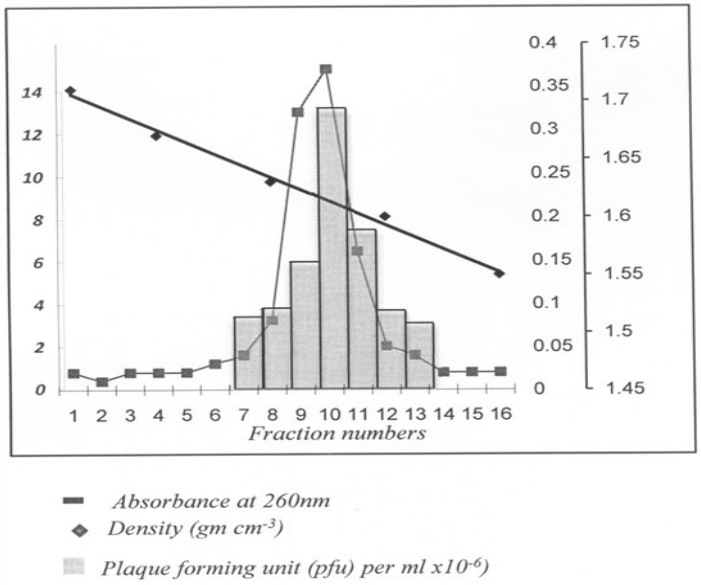

Fig.5: Fractionation of bacteriophage by caesium chloride buoyant density centrifugation, measurement of absorbance of fraction at 260nm and determination of plaque forming units per fraction

trophoresis was carried out in a discontinuous system. Anti *Shd-4L10* serum was raised in rabbits by intramuscular injection of phage particles emulsified with complete Freund's adjuvant and used to test the relatedness of phages *Shd-4L10* and *Shd-4L3*. The results show that although there is some detectable cross reactivity they are only closely related and not the same.

DISCUSSION

This work has shown that the *Shd-4* group of phages are commonly associated with a wide variety of bacteria species including *S. dysenteriae*, that they can readily be isolated and that some of them are potentially useful generalized lysogens to the host strain. We contend that the fact that these phages are lysogenic to their host strains make them suitable for further genetic analyses of *Shigella* strains without defects in their developmental cycles. It is however important to be prepared to modify a phage stock by passing through a new host with initially a very high multiplicity of infection in order to overcome the apparent restriction system that might develop in the CSRPM strains. The nature of the processes that gave rise to *Shd-4L10* is unknown, but it is clear that *S. dysenteriae* strain A contains corresponding prophages, which can generate plaque forming particles following some kind of recombinational event enhanced by irradiation of germicidal ultra violet light presumably implicating the presence in the cell of damaged *Shd-4L10* linked DNA. Super infection by a phage with damaged DNA may be a technique of potentially general application for isolation of new phages from *Shigella* and possibly other groups of bacteria. It must be recognised that some bacteria are themselves an initial source of phage which they seem to carry in some intracellular form. Cultures of such bacteria always contained free phage particles although the bacteria seemed otherwise normal.

CONCLUSION

We have demonstrated that phages of the Mx-4 and Shd-4 group are commonly associated with a wide variety of bacterial species including *S. dysenteriae* strain A and that such phages can readily be isolated following repeated exposure to germicidal range of uv light. We also contend that the phages can be very useful potential generalized transducers of genetic markers, being originally prophages of their host bacterial strains. We suggest further that super infection by a phage with damaged DNA may be a technique for isolating new phages from *Shigella* strains and possibly from other groups of bacteria. Normally cells have mechanisms to repair this damage, but if the duration of exposure to uv light is sufficient, the repair mechanisms are unable to keep up with the rate of DNA modification, the degree of inactivation by uv radiation being directly related to the uv dose applied.

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