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A diversity of bacteriophage forms and genomes can be isolated from the surface sands of the Sahara Desert

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Abstract The surface sands of the Sahara Desert are exposed to extremes of ultraviolet light irradiation, desiccation and temperature variation. Nonetheless, the presence of bacteria has recently been demonstrated in this environment by cultivation methods and by 16S rDNA analyses from total DNA isolated from surface sands. To discern the presence of bacteriophages in this harsh environment, we searched for extracellular phages and intracellularly located phages present as prophages or within pseudolysogens. Mild sonication of the sand, in different liquid culture media, incubated with and without Mitomycin-C, was followed by differential centrifugation to enrich for dsDNA phages. The resulting preparations, examined by electron microscopy, revealed the presence of virus-like particles with a diversity of morphotypes representative of all three major double-stranded DNA bacteriophage families (*Myoviridae*, *Siphoviridae* and *Podoviridae*). Moreover, pulsed-field gel electrophoresis of DNA, extracted from the enriched bacteriophage preparations, revealed the presence of distinct bands suggesting the presence of putative dsDNA phage genomes ranging in size from 45 kb to 270 kb. Characterization of the bacteriophages present in the surface sands of the Sahara Desert extends the range of environments from which bacteriophages can be isolated, and provides an important point of departure for the study of phages in extreme terrestrial environments.

Keywords Bacteriophages · Electron microscopy · Biodiversity · Phage morphology · Sahara Desert · Sand

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Introduction

Microorganisms have been found to inhabit virtually every niche examined to date. The majority of microbes in natural environments live in biofilm communities attached to surfaces and covered with an extracellular matrix composed of microbially produced polysaccharides (Davey and O'Toole 2000; Donlon 2002). The biofilm structure protects the enclosed microorganisms from extreme conditions that can occur in the environment (e.g., radiation, desiccation, extremes of pH, etc.) (Elasri et al. 2000), and from virus infections (Davey and O'Toole 2000); although certain phages can alter the surface of specific biofilms (Dobrindt and Reidl 2000) and the proximity of the organisms within the biofilm can ease the transport of phages among the microbial population (Jiang and Paul 1998).

Bacteriophages are the most abundant biological entities in aquatic environments, with numbers ranging from 10^4 to 10^8 virus particles per milliliter (10^7–10^{11}/l) (Fuhrman 1999; Paul 2000). Phages can affect many biogeochemical and ecological processes through their influence on community composition via their involvement in bacterial mortality, genetic exchange, evolution, biodiversity and species distributions (Bergh et al. 1989; Jiang and Paul 1998; Fuhrman and Schwalbach 2003). Transducing phages are believed to play a significant role in horizontal gene transfer among bacteria, and it has been hypothesized that they may act as a gene pool for horizontal transfer among bacterial populations (Bergh et al. 1989; Jiang and Paul 1998). Bacterial

morbidity and mortality is also highly affected by phage activity (Proctor and Fuhrman 1990; Fuhrman and Noble 1995). Despite their importance and relative abundance, little is known about phage populations and diversity in natural ecosystems, and even less so in terrestrial environments. Although phages have been isolated from a variety of habitats, most studies of natural phage populations have been performed in aquatic environments, with many fewer analyses occurring in soil or other semisolid environments (Ashelford et al. 1999) where over 90% of the Earth's bacterial population is believed to be located (Curtis et al. 2002).

The use of amplified 16S rRNA genes from total DNA from natural environments has demonstrated that over 99% of bacteria, and thus their viruses, cannot be cultivated under known laboratory conditions (Staley and Konopka 1985; Rondon et al. 2000). Studies with cultivated bacteria have shown that each bacterial species can be infected by one or more different bacteriophages and can contain one or more copies of complete or cryptic prophage DNA, either as a plasmid or integrated in the host genome (Reviewed in Wommack and Colwell 2000). Bacteria can also be found to contain virus-like particles in their cytoplasm, possibly as a result of unfavorable conditions for the completion of the phage lytic cycle. These chronically infected bacteria are called "pseudolysogens" (Paul 2000). Studies of aqueous ecosystems have shown that bacteriophages can outnumber their bacterial hosts by up to 100-fold (Alonso et al. 2001; Bergh et al. 1989; Marie et al. 1999). Thus, bacteriophages most likely represent the most diverse and abundant organisms on the Earth, but also the most unknown (Pedulla et al. 2003; Weinbauer 2004). Despite their key role in microbial ecology, their abundance and their diversity, bacteriophages in their natural habitats have not been extensively studied. As phages do not have the equivalent of a highly conserved ribosomal RNA (e.g., 16S) gene, recent studies have attempted to use metagenomic approaches in order to examine phage populations from natural ecosystems via the cloning of phage genome fragments followed by DNA sequence characterization (Breitbart et al. 2004).

The extremely dry surface sand of a desert presents a unique challenge for life, in that it is highly deficient in humidity, highly exposed to UV radiation, and undergoes large shifts in temperature (Heulin et al. 2003). Recent studies have revealed the presence of bacteria living on the surface sands of the Sahara Desert at concentrations estimated on the order of 10^3– 10^4 bacteria/g of sand (Heulin et al. 2003). In order to determine if phages are also present in this extreme environment, we utilized a combination of mechanical and chemical extraction of bacteria and phages from the sand, followed by differential centrifugation, in order to enrich for phages with dsDNA genomes, as these are more easily separated from fragments of chromosomal DNA and ribosomes than ssDNA and ssRNA phages. Our approach proved sufficiently effective to allow the recovery of a diversity of bacteriophage morphologies, as revealed by electron microscopy, and genome sizes characterized after nucleic acid separation by pulsed field gel electrophoresis.

Materials and methods

Samples

Samples of surface sands were collected from 13 different locations in April 2002, in the Sahara Desert in Morocco and Tunisia (Fig. 1). The surface sands were scooped into sterile, 50 ml conical plastic centrifuge tubes, and then mixed in equal proportions (by volume) under sterile conditions, as one sample (a kind gift from T. Heulin, CEA Cadarache, France; Heulin et al. 2003). Samples were stored in the dark at room temperature for several months until use.

Bacteriophage extractions

Direct extraction of the sand

Nine grams of sand were resuspended in 9 ml calcium-free Mu buffer (20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 20 mM $MgSO_4$, 0.1% (w/v) gelatin) and sonicated on ice for 17.5 s at 33% duty cycle and maximum output (4) using a microprobe on a Branson model 450 Sonifier. Free DNA was degraded by the addition of 50 U/ml of S7 micrococcal nuclease (Roche) in 1 ml of reaction buffer (100 mM Na-glycine, pH 8.6, 10 mM $CaCl_2$), plus 0.1 mg/ml (final concentration) of nuclease-free bovine serum albumin (BSA - New England BioLabs) followed by incubation for 30 min at 37°C. The S7 micrococcal nuclease was inactivated by chelating free calcium ions via the addition of 50 µl of 0.25 M ethylene glycolbis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; pH 7.8). The sample was then filtered through a 0.45 µm pore-size filter (Nalgene), the filtrate was collected and bacteriophage particles larger than 100S were collected by ultracentrifugation at 100,000 *g* for 2.5 h at 4°C (Beckman TL-100 ultracentrifuge, TLS 100.4 rotor). The supernatant fluid was discarded and the pellet overlaid with 50 µl cold (4°C) Mu buffer (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 20 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1% (w/v) gelatin) at 4°C overnight. The pellet was then gently resuspended and stored at 4°C.

Extraction with incubation

Five grams of sand were resuspended in 10 ml 2x TGY media (1% Tryptone, 0.2% Glucose, 0.6% Yeast extract) or 10 ml 0.25x TS media (Tryptic Soy—Difco). Samples were sonicated on ice for 17.5 s at 33% duty cycle and maximum output (4) using a microprobe on a Branson model 450 Sonifier, and then incubated at 30°C for 3 h and 18 h with gentle shaking (150 rpm).

Supernatant fluids were collected after gravity sedimentation of the sand particles, and completed to 10 ml with the same media. Half of the sample was then incubated, after addition of Mitomycin-C to a final concentration of 1 μg/ml, for 30 min at 30°C with gentle shaking. The cells were washed twice by centrifugation at 6,000 *g*, 10 min, 4°C and then resuspended in 10 ml of culture media to remove the Mitomycin-C from the media, and incubated for 3 h at 30°C with gentle shaking. The supernatant fluids were collected after centrifugation (as above) and filtered through a 0.45 μm pore-size filter. The bacteriophages were collected by ultracentrifugation and resuspended in Mu buffer as described in the previous section.

Electron microscopy

Formvar, carbon-coated copper electron microscopy grids (400 mesh) were overlaid with 10 μl of enriched bacteriophage suspensions for 5 min, and the attached phage particles negatively stained with 1% (w/v) phospho-tungstenic acid for 1 min. Excess stain was removed and the grids allowed to air-dry prior to examination using an EM 205 Philips electron microscope (microscope accelerating voltage 80 kV). Examples of phage particle morphologies observed at least four times on different grids were photographed for further examination.

Bacteriophage genome diversity and size estimation

The nucleic acids of the bacteriophage suspensions (approximately 100 μl) were extracted from the virions with buffer-saturated phenol (pH 8), followed by ether extraction and ethanol precipitation. Approximately 25% of the purified phage nucleic acids (roughly equivalent to 1 g of sand) were then loaded onto a 1% agarose gel and subjected to electrophoresis in 0.5x Tris-acetate-EDTA (TAE) buffer (20 mM Tris-acetate pH 8.0, 5 mM EDTA) for 15 h at 14°C using a Bio-Rad CHEF Mapper pulsed field gel electrophoresis apparatus at 6 V/cm, 120°, ramping: 0.2–22 s linear slope. DNA was visualized under UV illumination after ethidium bromide staining.

Results

Bacteriophage extractions

We established the optimum amount of sonication to apply to the sand sample in liquid media required to dislodge attached bacteria via a determination of the parameters required to release the greatest amount and colony diversity of cultivatable microorganisms from the sand biofilm (data not shown). We then used the direct extraction protocol (see Materials and methods) to estimate the capacity to recover extracellular bacteriophages from the sand of the Sahara Desert. Any free DNA released from bacterial cells should be degraded by nuclease treatment, and the remaining bacterial cells and debris were removed by filtration. However, this direct extraction protocol did not allow us to recover virus-like particles in sufficient quantities to be detected using electron microscopy, nor distinct phage-sized genomes after pulsed field gel electrophoresis (data not shown).

However, in addition to free particles (or virions), phages can exist as prophages (present as extrachromosomal plasmids or integrated within the genome of their hosts), or as virus particles in the cytoplasm of "pseudolysogenic" (also called "carrier state") bacteria. Thus, we introduced incubation steps in culture media, in the absence and presence of Mitomycin-C, to allow for phages within pseudolysogenic bacteria, and SOS-inducible temperate bacteriophages, to be released from bacteria in the sand samples (Williamson et al. 2001). Using this extraction protocol, we recovered and observed a variety of virus-like particles from the sand.

Virus-like particle morphologies

Based on structural criteria (Ackermann and DuBow 1987), the virus-like particles observed by electron microscopy revealed at least 12 seemingly different phage-like morphologies and sizes (Figs. 2, 3). The virus-like particles depicted here likely represent the most abundant phages present in the samples, and each virus-like particle represents an example of a phage morphotype observed independently at least four times on a grid (Table 1). The sizes of the similar virus-like particle types were found to vary by 5% or less. Six virus-like particle types, having potentially contractile tails, were discerned and categorized as *Myoviridae*-type bacteriophages (Fig. 2). Two short-tailed virus-like particles with long hexagonal capsids were classified as *Podoviridae* (Fig. 3a, b), and four virus-like particle types with long noncontractile tails were assigned to the *Siphoviridae* family (Fig. 3c–f).

The *Myoviridae*-type bacteriophages were composed of a nonenveloped capsid, separated by a neck-like structure, and a complex tail with a central tube and contractile sheath, a base plate and terminal fibers. The capsids exhibit icosahedral symmetry and appear hexagonal in outline. Particle sizes range from head dimensions between 82 nm and 185 nm in diameter, and tails of 129–385 nm in length and 25–55 nm in diameter.

The largest phage particle type we observed (Fig. 2a) has an apparent capsid diameter of 185 nm with a 55 nm wide and 385 nm-long tail, terminating with 4–6 short fibers arranged in a rake-like structure. A second particle type observed by electron microscopy had a capsid of 130 nm in diameter and a tail 180 nm long and 30 nm in diameter, with a single sword-like terminal fiber as long as the tail itself (Fig. 2b). Two particle types with a capsids

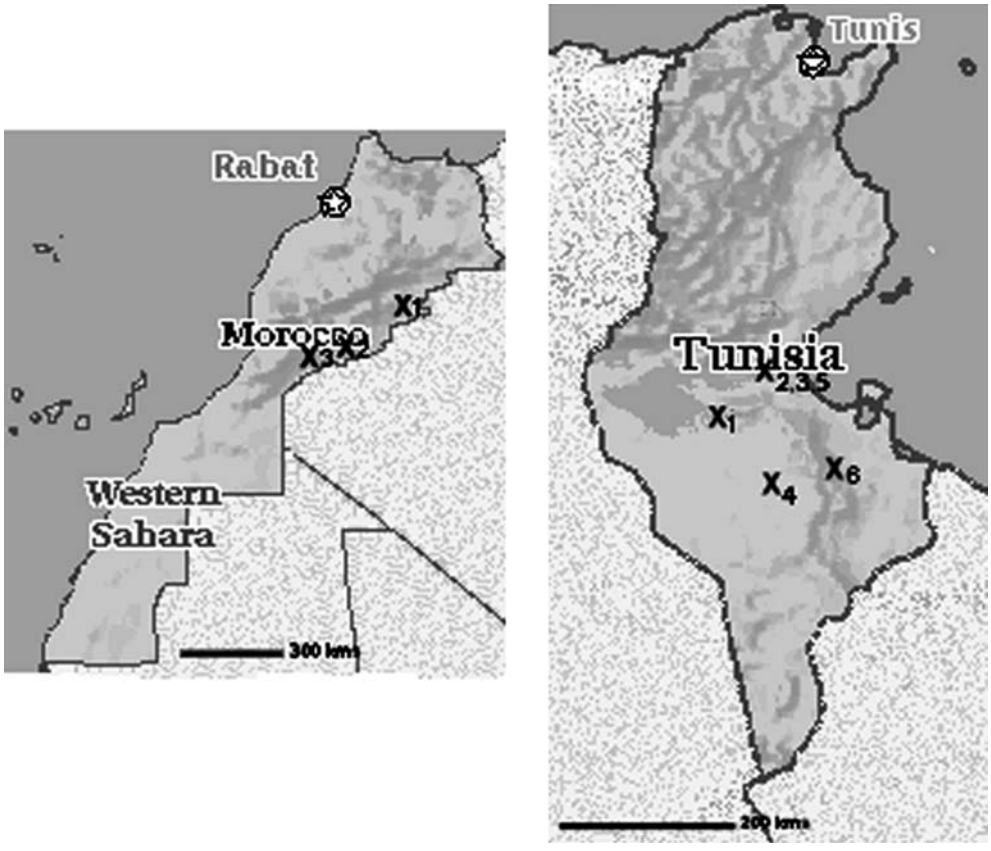

Fig. 1 The location of the sampling sites from the Sahara Desert in Morocco and Tunisia. The X's represent the location of sites where surface sand was sampled. *Scale bar* is 300 km for Morocco and 200 km for Tunisia

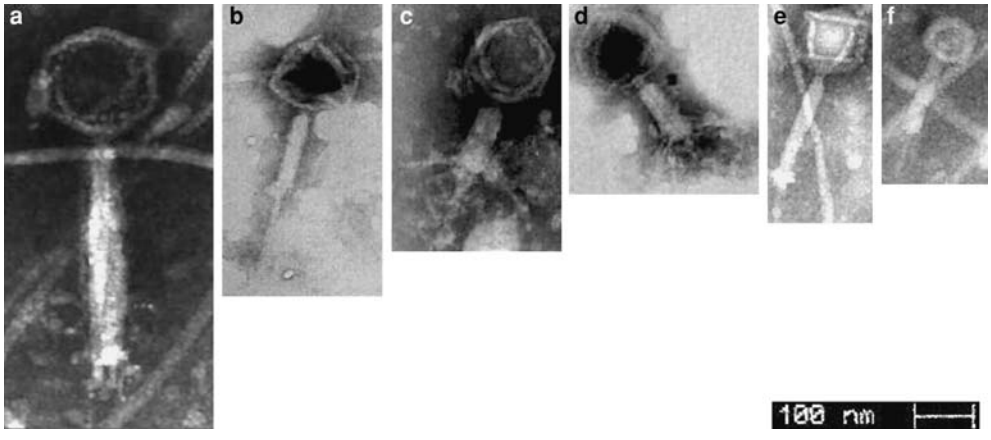

Fig. 2 Examples of phage particles of the *Myoviridae* family isolated from the Sahara Desert sample. Phage particles were isolated as described in the Materials and methods section and examined with a Philips 205 electron microscope on Formvar carbon-coated copper grids after negative staining with phosphotungstate. *Scale bar* is 100 nm

of 140 nm and 120 nm in diameter, having tails of 130 nm and 135 nm long and 40 nm and 35 nm in diameter, respectively, were observed, with terminal fibers consisting of straight fibers angled at 45° from the axis of the tail, and longer curly fibers in the axis of the tail (Fig. 2c, d). For both particle types, the axial canal of the tail is distinct, in contrast with the structure of the sheath, suggesting that the tails may be contracted. Another particle type observed, with a capsid 115 nm in diameter and a 170-nm-long tail (25 nm in diameter), exhibits a distinct fiber-dense base plate completed by a single terminal fiber (Fig. 3e). The smallest *Myoviridae*-type particle type isolated from the sand has a single terminal fiber on a tail 135 nm long and 25 nm in diameter attached to a capsid 82 nm in diameter (Fig. 2f).

Other morphologies observed among the purified phage-like particles include two that were classified as *Podoviridae*, with long hexagonal capsids (Fig. 3a, b). One particle type observed seems not to have a distinctly discernable tail. Instead, the base plate, consisting of lateral fibers and a single straight fiber, is directly connected to an elongated 80 nm by 40 nm capsid (Fig. 3a). The other particle type displays an elongated head with icosahedral symmetry 110 nm by 40 nm in size and a noncontractile tail 135 nm long that is <15 nm wide (Fig. 2b).

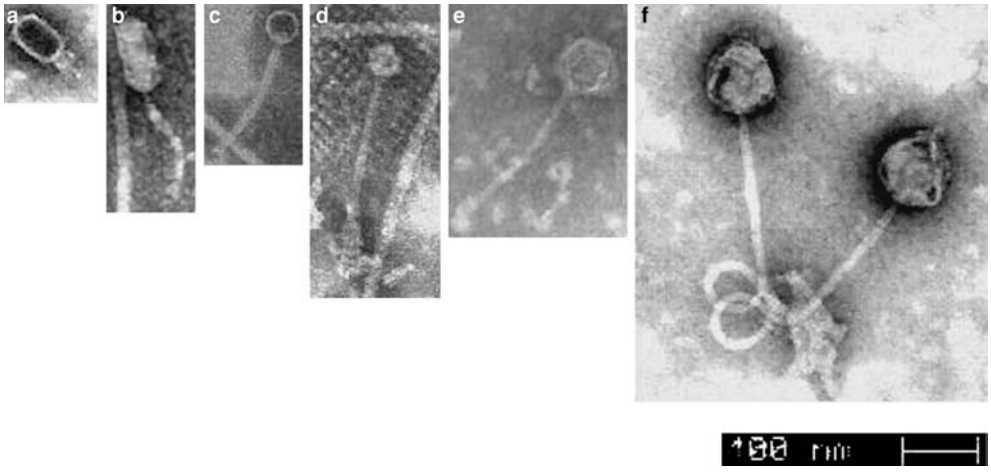

Fig. 3 Examples of phage particles of the *Podoviridae* (**a, b**) and *Siphoviridae* (**c**–**e**) family isolated from the Sahara Desert sample. Phage particles were isolated as described in the Materials and methods section and examined with a Philips 205 electron microscope on a Formvar carbon-coated copper grids after negative staining with phosphotungstate. *Scale bar* is 100 nm

Table 1 Virus-like particle morphotypes observed by electron microscopy

Morphotype (from Figs. 2, 3)	Culture media				Number of particles observed
	2x TGY + [a]	2x TGY −	0.25x TSB +	0.25x TSB −	
2.A	+	−	−	−	6
2.B	−	−	−	+	4
2.C	+	+	−	−	7
2.D	−	−	−	+	7
2.E	−	−	+	−	4
2.F	−	+	−	−	8
3.A	+	−	−	−	20
3.B	−	−	+	−	20
3.C	+	−	+	−	6
3.D	−	−	+	−	20
3.E	+	−	+	−	44
3.F	−	−	−	+	4

[a]The "+" and "−" symbols indicate incubation of the sand extractions in the presence and absence of Mitomycin-C, respectively (see Materials and methods for details)

Finally, four *Siphoviridae*-like particle types with noncontractile, long and narrow (< 15 nm) tails were observed. The shortest tail among the *Siphoviridae*-like morphotypes is 155 nm long with short terminal fibers in a rake-like structure and an icosahedral symmetric capsid 50 nm in diameter (Fig. 3c). Another particle type had a longer tail 190 nm in length, but a smaller capsid 45 nm in diameter. The other Siphoviridae-like phage particle types had capsids 75 nm in diameter with a 200 nm long tail (Fig. 3e) or a capsid 90 nm in diameter and a flexible tail over 460 nm in length (Fig. 3f).

Bacteriophage genome diversity and size estimation

To verify the potential viability of the extracted virus-like particles and characterize them further, we isolated the nucleic acids from our enriched phage preparations and separated them by pulsed field gel electrophoresis (Fig. 4).

As expected, no observable bands were seen on gels for the phage preparation obtained by direct extraction, as no phages were observable by electron microscopy (not shown). However, two distinct bands of double-stranded DNA were visible in the preparations using the extraction with incubation in 2x TGY media containing Mitomycin-C, with a high molecular weight band estimated to be 270 kb, and a lower molecular weight band estimated to be approximately 80 kb in length (Fig. 4, lane A). Using 0.25x TS media plus Mitomycin-C treatment, two distinct bands of double-stranded DNA were visible and estimated to be 60 and 45 kb in length (Fig. 4, lane B).

Discussion

Bacteriophages are being increasingly recognized for their importance in microbial ecology (reviewed in Fuhrman 1999; Wommack and Colwell 2000). However, most studies of their presence and diversity have been conducted in aquatic environments. In aquatic habitats, bacteriophages have been found to outnumber their bacterial hosts by up to 100-fold. Viruses of Archeae have also been extensively studied and can be isolated from the same extreme environments, such as deep sea thermal vents, as their hosts (Geslin et al. 2003; Forterre 2001). These studies have demonstrated that viruses per se can continue to exist in these extreme environments

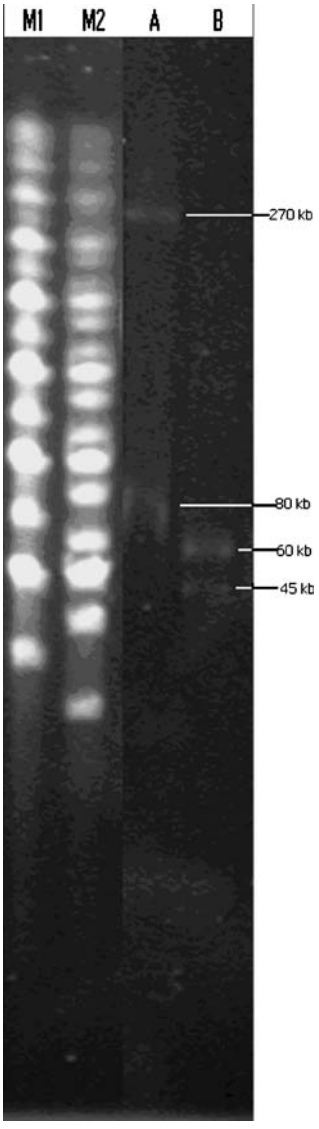

Fig. 4 Pulsed-field gel of phage DNA genomes from the Sahara Desert sample. The nucleic acids were extracted from the purified phages as described in Materials and methods and subjected to electrophoresis through a Bio-Rad CHEF Mapper apparatus. The gels were stained with ethidium bromide and photographed under UV light. Lanes M2 and M1 display the migration of pulsed-field electrophoresis MidRange molecular weight markers II and I (Bio-Rad), respectively, and lanes A and B represent phages isolated after incubation of the sand from the Sahara Desert in 2x TGY and 0.25x TS, respectively, with Mitomycin-C

and have thus evolved strategies to survive under these seemingly harsh conditions. It has recently been demonstrated that bacteriophages can also survive in extreme aquatic thermal environments, such as hot springs, and be recovered after filtration (Breitbart et al. 2004; Peng et al. 2001). The efficacy of recovery of phages from soil has been examined through the use of enrichment cultures (Rice et al. 2001) and via an assessment of extraction of exogenous phages from soil samples where the viral population was inoculated prior to their recovery (Williamson et al. 2003). However, their presence in extreme terrestrial environments, such as the surface sands of the Sahara Desert, has not, as yet, been demonstrated.

The majority of soil microorganisms live, in their natural ecosystem, in biofilm communities attached to surfaces and covered with an extracellular coating of polysaccharides (Davey and O'Toole 2000; Donlon 2002), which provides a measure of protection of the microorganisms from extreme conditions that can occur in certain environments (e.g., radiation, desiccation, extremes of pH, etc.) (Elasri et al. 2000). Some bacteria can be isolated and subsequently cultivated under laboratory conditions from the surface sands of the Sahara Desert, known to be exposed to desiccation and extremes of ultraviolet light irradiation and temperature variation (Heulin et al. 2003). The bacterial population of the surface sands used in this study has been estimated to be on the order of 10^3–10^4 bacteria/g of sand by a combination of cultivation methods and 16S rDNA analyses from total DNA isolated from samples of surface sands (T. Heulin, personal communication).

We attempted to isolate bacteriophages from this extreme terrestrial habitat and to begin to examine their diversity via an observation of their morphology and genome size.

A direct extraction method, using mechanical disruption to free bacteria and phages from the soil matrix, followed by digestion of free DNA and filtration of debris did not allow the isolation of bacteriophage particles in sufficient quantities for observation by electron microscopy and did not allow the visualization of distinct DNA bands on agarose gels.

Bacteriophages can be found as free virions as well as be located intracellularly, either as prophages or present in the cytoplasm of pseudolysogenic bacteria (Ripp and Miller 1998; Elasri and Miller 1999). In an effort to augment the detection of bacteriophages from the surface sand samples, we included an incubation step following sonication in two different types of culture media in order to enhance recovery of phages from pseudolysogenic bacteria. This step was included in an attempt to allow phages present in pseudolysogenic bacteria to complete a lytic cycle and be released from the cell. Moreover, we also treated samples with Mitomycin-C, a mutagenic agent known to induce lytic growth of some prophages via induction of RecA-mediated enhancement of repressor cleavage and inactivation (Canchaya et al. 2003). These extraction protocols did allow the recovery of a variety of observable phage particle morphologies.

Under electron microscopy, these phage particle morphologies corresponded to the three major families of tailed bacteriophages: *Myoviridae*, *Siphoviridae* and *Podoviridae*. Among the phage particles observed and classified as *Siphoviridae*-type bacteriophages, one (Fig. 3c) had a morphology similar to coliphage T1. T1 phage is a Siphovirus with a capsid of 60 nm in diameter and a tail of 140–160 nm in length and 7 nm in diameter

and is one of the most desiccation resistant phages known (Drexler 1988).

It was somewhat surprising to find such a diversity of *Myoviridae*-type bacteriophages, as their complex contractile tails, consisting of potentially fragile proteins, might not be expected to be well adapted to such a dry habitat. It is possible that these bacteriophages can survive and be protected from the effects of harsh environmental conditions either through an intracellular location in pseudolysogens and/or via being embedded within a biofilm (Elasri et al. 2000). However, as *Myoviridae*-type bacteriophage members can be temperate (Lucchini et al. 1999), it is also possible that these phages were present as prophages within lysogens.

The purification of phage nucleic acids and their separation by molecular weight using pulsed-field gel electrophoresis revealed four potential bacteriophage genomes that likely represent the most abundant intact bacteriophages in our extraction from sand. The high molecular weight nucleic acid band probably corresponds to a *Myoviridae*-type bacteriophage, which are known to have large genomes (Ackermann and DuBow 1987). *Siphoviridae* and *Podoviridae*, which are generally smaller and have tails of lesser complexity than Myoviridae-type phages, generally have genomes of 100 kb and less and would likely correspond to the lower molecular weight bands observed (Ackermann and DuBow 1987).

The presence of large potential phage DNAs suggest their presence as intact, encapsidated genomes, and implies that the protocol we developed can allow for the recovery of potentially viable virions of the endogenous phage population. It is nonetheless interesting to note that we observed fewer discernible phage genomes as bands on agarose gels than as phage particle types by electron microscopy. These genomes and phage particle types represent the most abundant phages that were extractable from the sand. It is possible that some of the phage particle types have identical genome sizes despite a different apparent morphology or are not stable once extracted, their genomes being released from the virions and subjected to various levels of degradation in the crude phage preparations.

In conclusion, we were able to establish the presence of bacteriophages on the surface sand of the Sahara Desert, despite such extreme environmental factors as high UV irradiation, responsible for a large majority of bacteriophage decay in aquatic habitats (Noble and Fuhrman 1997). Further research will permit us to define the parameters necessary for an efficient quantitative recovery of the total phage population in the sand of the Sahara Desert and a characterization of their potential mechanism(s) of survival.

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