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Virus-Like Particle Production in Atmospheric Eubacteria Isolates

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Abstract: Culturable eubacterial isolates were collected at various altitudes in Earth's atmosphere, including ~1.5 m above ground in Tallahassee, FL, USA; ~10.0 m above sea level over the mid-Atlantic ridge (~15° N); ~ 20 km above ground over the continental United States; ~20 km above sea level over the Pacific Ocean near southern California; and from the atmosphere of Carlsbad Cavern, Carlsbad Cavern National Park, NM, USA. Isolates were screened for the presence of inducible virus-like particles (VLP) through the use of mitomycin C and epifluorescent direct counts. We determined that 92.7% of the isolates carried inducible VLP counts in exposed versus non-exposed culture controls and that the relationship was statistically significant. Further statistical analyses revealed that the number of isolates that demonstrated VLP production did not vary among collection sites. These data demonstrate a high prevalence of VLP generation in isolates collected in the lower atmosphere and at extreme altitudes. They also show that species of eubacteria that are resistant to the rigors of atmospheric transport play a significant role in long-range atmospheric inter- and intra-continental dispersion of VLP and that long-range atmospheric transport of VLP may enhance rates of evolution at the microbial scale in receiving environments.

Keywords: direct counts; mitomycin C; bacteria; bacteriophages; atmosphere

1. Introduction

Wherever there is life, there are viruses. Viruses outnumber all other organisms combined, and it has been estimated that the current daily population on Earth is ~10^{31} [1]. Most of these viruses are believed to be bacteriophages, which are viruses that utilize bacteria as their hosts [2,3]. Bacteriophage densities in soil have been shown to occur at a range of ~10^3 to 10^8 g^{-1} [4–7] and in aquatic environments, one to two logs greater than the typical bacterial population of ~10^5 to 10^9 mL^{-1} [8,9]. It was recently demonstrated that in desert arid soil environments, concentrations of bacteria and virus-like particles (VLP) ranged from ~10^3 to 10^7 g^{-1} and that their concentration gradients were 'patchy' in nature [5]. The term VLP has been defined in many microbial ecology studies as including bacteriophages, genetic transfer agents (GTA), and membrane vesicles (MV) [10–12]. GTA are similar to bacteriophages in that they contain a capsid and tail yet typically transport host-associated genes (versus viral genes) [10]. MV bud from the cell and are generated throughout its life and during lyses and also typically transport host genes [10]. VLP significantly influence the evolution and diversity of prokaryotic communities and microbiogeochemical cycles in Earth's numerous aquatic and terrestrial environments through gene transfer [13–17].

Bacteriophages typically replicate through one of two main life cycles, the lytic or lysogenic cycles [18]. In the lytic cycle, the bacteriophage injects its genome into its host cytoplasm and pirates its host replication pathways to replicate itself. The end result of the lytic cycle is the rupture or lysis of the host cell and the release of the newly replicated bacteriophages. In the lysogenic cycle, bacteriophages integrate their genome into their host cell (integration into the cellular genome or the establishment of a stable plasmid within the host cytoplasm) and enter a non-lytic state known as a prophage. As a prophage, the viral genome is replicated with the host during cell division, and thus the bacteriophage genome is passed to host progeny. Bacteriophages of this type are known as temperate. Lysogeny is usually terminated by some form of host-cell stress caused by environmental factors such as ultraviolet (UV) damage, toxin exposure, or a change in nutrient conditions, at which time the prophage enters the lytic cycle [19,20]. During bacteriophage replication, host genes may be incorporated into the new phage and ultimately transferred to another host, a process known as transduction. The introduction of extraneous genetic material into a host cell by bacteriophage infection can cause phenotypic shifts, such as the acquired ability to produce toxins, to resist antibiotic exposure, and to resist infection by other bacteriophages [21–24]. Gram-negative bacteria have been shown to generate high numbers of GTA and MV in the absence of an inducible phage and, due to the virus-like size range of many of these types of genetic shuttles, can easily be confused with bacteriophages when enumerating with epifluorescent microscopy [10,25].

Although VLP occur in all terrestrial and aquatic habitats examined thus far, most studies conducted to date have focused on their occurrence and role in aquatic environments. It has been shown that the bacteriophage component of VLP accounts for significant bacterial mortality in aquatic sediments and in the overlying water column [26]. In prophage studies, it has been shown that many bacteria harbor prophages and that it is not uncommon to find bacterial genomes that harbor multiple prophage genomes [27,28]. Relative to aquatic bacteriophage/host research, fewer studies have been conducted in terrestrial-soil environments, where it has been estimated that over 90% of prokaryotic diversity occurs [4,7,29]. Recent investigations indicate that lysogeny is more prevalent in soil microbial communities than in marine environments [7]. The lysogenic cycle is believed to be a strategic means of maintaining bacteriophage genomes in environments that contain low host numbers or other limiting environmental factors, such as low or no available water to facilitate movement from one host to the next [30,31]. Research has demonstrated the wide prevalence of lytic and lysogenic bacteriophages in extreme environments (cold-and hot-water environments), including those found in Earth's harsh desert environments [8,32–34].

The role that potential host bacteria play in moving VLP vast distances in the Earth's atmosphere is not clear. Host exposure to UV radiation is a known cause of VLP induction [35]. Since there is significant potential for UV exposure during atmospheric transport, bacterial cells that host VLP may not survive long-range transport or long-term suspension unless the host is UV-tolerant and/or is shielded via attachment to organic detritus or inorganic particulates. Host tolerance to UV exposure via cell pigmentation, high guanine and cytosine content in their genome, or genes that provide enhanced nucleic acid stabilization or repair may favor long-range dispersion or prolonged survival at altitude. Thus, UV-tolerant hosts may facilitate the movement of VLP-mediated genes around the planet. This may play a particularly important role in planetary-scale atmospheric-dispersion paths that result from natural phenomena such as volcanic eruptions and the more frequent large-scale dust storms that emanate from the continents of Australia, Africa, Eurasia, and the Americas [36]. In addition, the formation of VLP (organic or inorganic) colloids in the atmosphere could to be an important factor in the survival and persistence of bacteriophages outside the bacterial cell [37].

Bacterial species identified in volcanic soils have previously been identified at an altitude of 20 km in Earth's atmosphere, and dust storms that originate from Earth's deserts are known to move diverse groups of microorganisms vast distances throughout the troposphere, tropopause, and lower stratosphere [38–44]. Although these cited works have demonstrated bacterial and fungal dispersion on a global scale, no studies have been undertaken so far to address the potential for dispersed prokaryotes to facilitate the long-range movement of VLP through Earth's atmosphere.

In this work, we investigate the occurrence of inducible VLP in groups of eubacteria collected at various altitudes in Earth's atmosphere: a group collected at ~1.5 m above ground in the city of Tallahassee, Florida [45]; a group collected at ~10 m above sea level at a mid-Atlantic site while aboard the JOIDES (Joint Oceanographic Institutions for Deep Earth Sampling) Resolution during Ocean Drilling Program (ODP) Leg 209, when African dust was present in the atmosphere [46]; and a group comprised of isolates collected at an altitude of 20 km during three different flights conducted by NASA's Stratospheric and Cosmic Dust Program [47–49]. The remaining isolate was collected underground from the atmosphere of Carlsbad Cavern, Carlsbad Caverns National Park, NM, USA [50].

2. Experiments

2.1. Origin of Isolates

Table 1 lists the sites of collection and taxonomy data for the 41 isolates utilized in this study. Isolates were collected from four different atmospheric settings and grouped accordingly. The first group of bacteria (17 isolates) was obtained from an altitude of ~1.5 m from atop a concrete patio table in an open courtyard, located between two office buildings in Tallahassee (latitude 30.477377, longitude−84.294601), from 6 July 2010 to 15 July 2012 [45]. The second group of bacteria (14 isolates) was collected from the lower atmosphere (~10 m altitude) above the tropical mid-Atlantic ridge (~45° W, 14° N), when African dust was present in the atmosphere. Acquisition was made while aboard the JOIDES Resolution on Ocean Drilling Program Leg 209 between 25 May 2003 and 1 July 2003 [46]. The third group of bacteria (9 isolates) was obtained from an altitude of 20 km during three different NASA ER2 flights, two over the continental US and one over the Pacific Ocean off the coast of California. Collection data for these isolates have been described previously [47–49]. The remaining isolate was collected ~140 m underground from the atmosphere of Sand Passage, in Carlsbad Cavern on 27 September 2004 [50].

Table 1. Eubacteria isolate and sample site data.

Isolate (Date Collected-ID)	Location	Altitude (m)	Taxonomy (GenBank Closest Neighbor)	GenBank Accession Number
07/06/10-A	Tallahassee	1.5	*Bacillus gibsonii*	HE604,338
07/06/10-B	Tallahassee	1.5	*Bacillus megaterium*	HE604,339
07/06/10-C	Tallahassee	1.5	*Bacillus* sp.	HE604,340
07/12/10-A	Tallahassee	1.5	*Bacillus* sp.	HE604,341
07/12/10-B	Tallahassee	1.5	*Bacillus* sp.	HE604,342
07/12/10-C	Tallahassee	1.5	*Enterobacteriaceae*	HE604,343
07/12/10-D	Tallahassee	1.5	*Enterobacteriaceae*	HE604,344
07/12/10-E	Tallahassee	1.5	*Bacillus* sp.	HE604,342
07/12/10-F	Tallahassee	1.5	*Bacillus* sp.	HE604,346
07/13/10-A	Tallahassee	1.5	*Bacillus* sp.	HE604,347
07/13/10-B	Tallahassee	1.5	*Bacillus* sp.	HE604,348
07/13/10-C	Tallahassee	1.5	*Exiguobacterium* sp.	HE604,349
07/13/10-D	Tallahassee	1.5	*Bacillus* sp.	HE604,350
07/14/10-A	Tallahassee	1.5	*Bacillus* sp.	HE604,345
07/14/10-B	Tallahassee	1.5	*Bacillus* sp.	HE604,342
07/15/12-E	Tallahassee	1.5	*Pseudomonas* sp.	HE995,774
07/15/12-G	Tallahassee	1.5	*Micrococcus* sp.	HE995,775
05/25/03-BY0	mid-Atlantic	10	*Actinobacteria*	AY857,677
05/25/03-BY1	mid-Atlantic	10	*Frigoribacterium*	AY857,767
05/26/03-BW0	mid-Atlantic	10	*Kocuria rosea*	AY857,672
05/27/03-BY0	mid-Atlantic	10	*Lentzea* sp.	AY857,673

06/01/03-BY0	mid-Atlantic	10	*Novosphingobium subarticum*	AY857,675
06/12/03-BY0	mid-Atlantic	10	*Brevibacterium casei*	AY857,665
06/12/03-BY1	mid-Atlantic	10	*Staphylococcus epidermis*	AY857,685
06/12/03-BY2	mid-Atlantic	10	*Liefsonia* sp.	AY857,676
06/15/03-BY0	mid-Atlantic	10	*Bacillus aminovorans*	AY857,666
06/19/03-BC2	mid-Atlantic	10	*Bacillus benzoevorans*	AY857,668
06/28/03-BP1	mid-Atlantic	10	*Gordonia terrae*	AY857,719
06/28/03-BY0	mid-Atlantic	10	*Pseudomonas* sp.	AY857,688
07/01/03-BW1	mid-Atlantic	10	*Bacillus aminovorans*	AY857,722
07/01/03-BW0	mid-Atlantic	10	*Bacillus* sp.	AY857,721
NASA1-1	Continental USA	20,000	*Bacillus luciferensis*	AY291,461
NASA1-71	Continental USA	20,000	*Bacillus sphaericus*	AY291,474
08/13/04-NASA2-8	Continental USA	20,000	*Micrococcaceae*	EU029,597
08/13/04-NASA2-25	Continental USA	20,000	*Staphylococcus* sp.	EU029,614
08/13/04-NASA2-33	Continental USA	20,000	*Micrococcus luteus*	EU029,622
08/13/04-NASA2-34	Continental USA	20,000	*Micrococcus thailand*	EU029,623
08/13/04-NASA2-43	Continental USA	20,000	*Brevibacterium* sp.	EU029,632
04/28/08-NASA-DS1	Pacific	20,000	*Bacillus endophyticus*	FJ649,336
04/28/08-NASA-DS3	Pacific	20,000	*Bacillus* sp.	FJ649,338
SPG PP1	Carlsbad Caverns	100 below surface	*Myceligenerans crystallogenes*	HE995776

2.2. Isolate Collection Assay

The Tallahassee isolates were collected using a small portable membrane-filtration unit (Fisher Scientific 110 V vacuum pump, product #13-310-485, and a PVC (PolyVinylChloride) two-place manifold, housed in a large plastic toolbox) [45]. Presterilized filter housings containing 47-mm-diameter, 0.2-µm pore-size cellulose acetate filter membranes were used for all air samples (Fisher Scientific, Atlanta, GA, USA, Catalog #09-74030G). The filter housings were removed from their respective sterile bags and placed on the analytical filter manifold. The housing lid was removed and vacuum applied using a vacuum pump. Filtration flow rates ranged from 8.7 to ~17.4 L min^{-1}. After filtration, the housings and lids were replaced in their respective bags, and the bags were sealed with tape. R2A medium (Fisher Scientific, Atlanta, GA, USA) was utilized in the following manner for microbial culture. The sample filters were placed whole on R2A-medium plates, sample side up, and were incubated in the dark at ambient temperature (36 °C). The ODP Leg 209 isolates were collected and identified in a similar manner using the same membrane filtration unit in addition to a high-volume filtration unit that was positioned atop the JOIDES Resolution derrick (the two 1 July 2003 isolates were obtained from a high-volume filter. The remaining included in this report were collected using the low-volume filtration unit and filters described above). Specific collection and identification details for these ODP isolates are given in Griffin et al., 2006 [46]. The NASA high-altitude isolates were collected via a deployed aluminum flag coated with glycerol and a modified flag, as previously described, at an altitude of 20,000 m during NASA ER2 missions [47–49]. The Carlsbad Cavern isolate was obtained using a break-down (all of the equipment minus the toolbox-like housing) version of the low-volume membrane filtration unit described above, and the filter was plated and incubated at ambient cave temperature for initial growth. After growth, the plates were transported to St. Petersburg, FL, USA, for isolation of colony-forming units (CFUs) [50]. Once isolated from their respective environments, CFUs were cryogenically stored at −70 °C and identified using the following protocol. Isolates were grown overnight in tryptic soy broth (TSB, Fisher Scientific, Atlanta, GA, USA). All isolates that grew on R2A also grew in TSB. Genomic DNA was extracted from the isolates using the Qiagen Plant and Tissue DNeasy Kit, as per the Gram + extraction protocol described in the kit. Universal 16S rRNA polymerase-chain reaction (PCR) primers [51] and amplicon sequencing

were utilized as previously described for identification. A GenBank Blast search [52] was used for amplicon/isolate identification. GenBank accession numbers for each isolate are listed in Table 1.

2.3. Mitomycin C Induction Plate Setup

In a separate 96-well micro-plate, 100 μL of 4 mg mL^{-1} mitomycin C were added to the top well in each column. This quantity was serial diluted to the seventh well of each column using 90 μL of sterile H_2O and 10 μL of supernatant from the previous well. The eighth and last well was inoculated with sterile H_2O (negative control). Using a multi-channel pipette, 45 μL from each one of these wells was transferred to matching wells on another 96-well micro-plate. In these wells, the mitomycin dilution-series concentration ranged from 18 μg at 10^0 to 18 pg at the 10^{-6} dilution (rows/wells A through G, and the H well was utilized as a negative control for each column/isolate, see Table 2). One-hundred and fifty-five μL of sterile tryptic soy broth (TSB) was then added to each well, followed by the addition of 50 μL of isolate culture (overnight culture grown in TSB at room temperature, one isolate per micro-plate column). Two columns were used for each isolate, one that contained TSB and the isolate seed and the other that contained TSB, the isolate seed and the described mitomycin C-dilution series. The micro-plate was then incubated overnight at room temperature on a table-top orbitor (set at less than 100 orbits per minute). The visual observation of reduction to turbidity in wells with mitomycin dilution-series concentration was marked as indicative of the occurrence of induction (Table 2).

Table 2. Results from Mitomycin C experiments. Columns marked with and "X" indicate turbidity and no visible clearing of the wells. Unmarked columns indicate visible clearing. Columns A through H represent the dilution series 10^0 (A = 18 μg mitomycin C) through 10^6 (G = 18 pg mitomycin C) and the negative control well (H), respectively. Column A wells were used for direct counts. No clearing was noted in any of the matched control wells utilized as an additional control.

Collection Location	Samples ID	A	B	C	D	E	F	G	H
Tallahassee samples	070,610 A		X	X	X	X	X	X	X
	070,610 B			X	X	X	X	X	X
	070,610 C					X	X	X	X
	071,210 B				X	X	X	X	X
	071,210 A		X	X	X	X	X	X	X
	071,210 C		X	X	X	X	X	X	X
	071,210 D					X	X	X	X
	071,210 E			X	X	X	X	X	X
	071,210 F		X	X	X	X	X	X	X
	071,310 A		X	X	X	X	X	X	X
	071,310 B				X	X	X	X	X
	071,310 C				X	X	X	X	X
	071,310 D					X	X	X	X
	071,410 A				X	X	X	X	X
	071,410 B			X	X	X	X	X	X
	071,512 E			X	X	X	X	X	X
	071,512 G			X	X	X	X	X	X
Atlantic samples	052,503 BY0			X	X	X	X	X	X
	052,503 BY1			X	X	X	X	X	X
	052,603 BW0					X	X	X	X
	052,703 BY0			X	X	X	X	X	X
	060,103 BY0					X	X	X	X
	061,203 BY0		X	X	X	X	X	X	X
	061,203 BY1		X	X	X	X	X	X	X
	061,203 BY2				X	X	X	X	X

	061,503 BY0				X	X	X	X
	061,903 BC2				X	X	X	X
	062,803 BP1	X	X	X	X	X	X	X
	062,803 BY0				X	X	X	X
	070,103 BWO				X	X	X	X
	070,103 BW1		X	X	X	X	X	X
NASA samples	NASA 1–1			X	X	X	X	X
	NASA 1–71				X	X	X	X
	NASA 2–43				X	X	X	X
	NASA 2–25	X	X	X	X	X	X	X
	NASA 2–8	X	X	X	X	X	X	X
	NASA 2–33			X	X	X	X	X
	NASA 2–34				X	X	X	X
	NASA DS1				X	X	X	X
	NASA DS3				X	X	X	X
Carlsbad	SPG PP1			X	X	X	X	X

2.4. Epifluorescent Direct Counts

The first well of each column in the 96-well plate was utilized for VLP direct counts (Table 2). The direct-count protocol utilized follows a modification of a previously published protocol [53]. In short, 100 to 10 μL of post-mitomycin C culture were filtered (in duplicate) through Whatman Anodisc 0.02-μm-pore-size, 25-mm-diameter, glass-fiber filters (Whatman, # 6809-60 02, Fisher Scientific, Massachusetts, US). Some samples were centrifuged at 14,000 rpm for 10 min to limit bacteria carryover that may interfere with obtaining accurate VLP direct counts. For staining, the filters were placed sample side up, on top of a drop of diluted SYBR Gold nucleic-acid stain (Molecular Probes, Eugene) OR: 97.5 μL of 0.02 μm filtered H_2O + 2.5 μL of a 1/10 dilution of SYBR Gold) and incubated at room temperature in the dark for ~10 to 15 min. The filters were then removed from the drop of diluted SYBR Gold, excess stain was removed by blotting the back of the filter on tissue paper, and the filter was then placed on a glass slide. Twenty-seven microliters of antifade solution (990.0 μL 50% 1X PBS/50% Glycerin + 10.0 μL 10% P-phenylene diamine) were placed on a coverslip, and the coverslip placed over the filter. The coverslip was lightly pressed to expel any trapped air, and the slide was then refrigerated in the dark (for a maximum of 2 days) until being counted by epifluorescent microscopy. Fifteen fields per slide/duplicate were counted at 1000 × (oil) using a Carl Zeiss Inc. Axioskop 40 epifluorescent microscope (Jena, Germany). The average numbers of viral-like particles (VLP) were obtained by averaging both field and sample counts (considering filter-apparatus diameter, volume eluted, volume filtered, and ocular grid area at 1000 × magnification).

2.5. Statistics

SPSS 21 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A Kolmogorov–Smirnov Z test was conducted to check data normality. Spearman's rho was utilized to determine correlations when the data were not normally distributed. Data were subjected to a Kruskal–Wallis test to compare significant differences in concentrations of VLP mL^{-1} in different atmospheric settings.

3. Results

Most of the bacteria isolated from the various atmospheric environments were gram-positive bacteria (36/41 = 87.9%). In the Tallahassee and Atlantic groups, gram-positive bacteria represented 82.4 and 85.7% of the isolates, respectively. In contrast, the isolates collected at extreme altitudes in the NASA studies were all gram positives. All of the isolates included in this study were Firmicutes, Actinobacteria, or Proteobacteria. In the Tallahassee isolate group, 76.5% were Firmicutes, 17.6%

were Proteobacteria, and 5.9% were Actinobacteria. In the Atlantic group, 42.9% were Firmicutes, 42.9% were Actinobacteria, and 14.2% were Proteobacteria. In the NASA group, 55.5% were Firmicutes and 44.5% were Actinobacteria. The lone isolate from Carlsbad Cavern was an Actinobacteria.

The number of wells that were observed to clear or not clear for each isolate is illustrated in Table 2 and listed in Table 3. Table 3 also lists the VLP counts for all the samples (control and mitomycin C treated). No clearing of wells was observed for five of the isolates (three from the Tallahassee group and one each from the Atlantic and NASA groups). In these samples, VLP mL^{-1} ranged from 7.3×10^3 to 6.1×10^6 in wells containing mitomycin C, and from 0 to 6.9×10^5 in control wells (no mitomycin C). For the remaining isolates, exposure to mitomycin C resulted in VLP mL^{-1} counts that ranged from 0 to 5.6×10^7 versus control counts that ranged from 0 to 4.1×10^7.

Table 3. Bacteriophage production (virus-like particles, VLP) per epifluorescent direct count.

Collection Location	Isolate ID	Wells Cleared [a]	Mitomycin VLP mL^{-1}	Control Wells VLP mL^{-1}	VLP Production
Tallahassee samples	070610 A	1	5.60×10^6	2.39×10^6	3.21×10^6
	070610 B	2	1.31×10^5	3.07×10^5	-1.76×10^5
	070610 C	4	1.28×10^7	0.00×10^1	1.28×10^7
	071210 B	3	1.61×10^5	7.30×10^4	8.80×10^4
	071210 A	0	7.30×10^3	0.00×10^1	7.30×10^3
	071210 C	0	6.13×10^6	6.94×10^5	5.44×10^6
	071210 D	4	4.46×10^6	1.90×10^6	2.56×10^6
	071210 E	2	1.99×10^5	1.02×10^5	9.70×10^4
	071210 F	0	9.86×10^5	1.83×10^5	8.03×10^5
	071310 A	1	4.67×10^5	1.31×10^5	3.36×10^5
	071310 B	3	8.98×10^5	1.10×10^5	7.88×10^5
	071310 C	3	5.60×10^7	4.11×10^6	5.19×10^7
	071310 D	4	2.10×10^7	2.04×10^5	2.08×10^7
	071410 A	3	8.32×10^5	0.00×10^0	8.32×10^5
	071410 B	3	1.18×10^6	1.09×10^6	9.00×10^4
	071512 E	2	2.19×10^4	7.30×10^3	1.46×10^4
	071512 G	2	5.84×10^4	4.38×10^4	1.46×10^4
Atlantic samples	052503 BY0	2	4.91×10^6	4.02×10^5	4.51×10^6
	052503 BY1	2	1.66×10^6	8.03×10^5	8.57×10^5
	052603 BW0	4	4.46×10^7	4.38×10^4	4.46×10^7
	052703 BY0	2	3.60×10^6	3.21×10^5	3.28×10^6
	060103 BY0	4	1.18×10^6	5.40×10^5	6.40×10^5
	061203 BY0	1	8.07×10^6	0.00×10^0	8.07×10^6
	061203 BY1	0	3.25×10^6	2.92×10^5	2.96×10^6
	061203 BY2	3	7.04×10^6	5.84×10^5	6.46×10^6
	061503 BY0	4	9.24×10^6	4.75×10^5	8.77×10^6
	061903 BC2	4	5.95×10^5	1.10×10^6	-5.05×10^5
	062803 BP1	1	1.72×10^6	3.37×10^5	1.38×10^6
	062803 BY0	4	1.68×10^6	7.23×10^5	9.57×10^5
	070103 BWO	4	2.59×10^6	0.00×10^0	2.59×10^6
	070103 BW1	2	0.00×10^0	3.20×10^6	-3.20×10^6
NASA samples	NASA 1-1	3	6.79×10^6	0.00×10^0	6.79×10^6
	NASA 1-71	4	4.85×10^6	2.23×10^6	2.62×10^6
	NASA 2-43	4	1.41×10^7	5.84×10^4	1.40×10^7
	NASA 2-25	0	3.62×10^6	4.38×10^4	3.58×10^6
	NASA 2-8	1	1.53×10^6	8.18×10^5	7.12×10^5

	NASA 2-33	3	5.50×10^6	1.90×10^6	3.60×10^6
	NASA 2-34	1	3.58×10^6	0.00×10^0	3.58×10^6
	NASA DS1	4	1.42×10^6	1.02×10^5	1.32×10^6
	NASA DS3	4	4.10×10^6	5.84×10^5	3.52×10^6
Carlsbad Cavern	SPG PP1	3	2.40×10^6	8.32×10^5	1.57×10^6

[a] Wells cleared = number of wells that cleared in the dilution series experiments with mitomycin C.

VLP were detected in almost all samples, with the exception of 070,103BW1 where they were not observed in the well containing mitomycin C, and in samples 071,210B, 071,410A, 061,203BYO, 070,103BWO, and NASA 1-1 in the control wells. In the Tallahassee group, the VLP counts in the mitomycin C wells ranged over four orders of magnitude from ~10^3 to ~10^7 VLP mL^{-1} (average 6.5×10^6). For the control wells, the VLP counts ranged over six orders of magnitude from 0 to ~10^6 VLP mL^{-1} (average of 6.6×10^5). In the Atlantic isolate group, VLP mL^{-1} counts in the wells containing mitomycin C ranged over seven orders of magnitude from 0 to ~10^7 (average 6.4×10^6), and control counts ranged over six orders of magnitude from 0 to ~10^6 mL^{-1} (average 6.3×10^5).

The NASA group VLP counts in the wells with mitomycin C ranged over one order of magnitude from ~10^6 to ~10^7 mL^{-1} (average of 5.0×10^6), and the control wells ranged over six orders of magnitude from 0 to ~10^6 VLP mL^{-1} (average 6.4×10^5). The Carlsbad Cavern isolate produced 2.4×10^6 VLP mL^{-1} in the presence of mitomycin C and 8.3×10^5 VLP mL^{-1} in the control well.

The differences between the average of VLP mL^{-1} counts in the mitomycin C wells with respect to the control reactions by sites and bacterial groups (Firmicute, Proteobacteria, Actinobacteria) are illustrated in Figure 1. VLP mL^{-1} counts for the Firmicute and Proteobacteria isolates in all groups were within one order of magnitude. The Atlantic Actinobacteria isolates produced the highest overall VLP counts at an average of 1.0×10^7 VLP mL^{-1}. Within the Atlantic isolate group, the Actinobacteria and Firmicute average VLP counts were higher than those observed with the Proteobacteria (within one order of magnitude). The lowest overall counts that were above the minimum detection limit occurred with the Tallahassee Actinobacteria isolate at 5.8×10^4 VLP mL^{-1}. This count level was around two orders of magnitude lower than the average counts observed with Firmicute (avg. 7.7×10^6 VLP mL^{-1}) and Proteobacteria (avg. 3.5×10^6 VLP mL^{-1}) isolates at that site. However, with the NASA Firmicutes and Actinobacteria isolates, the average VLP mL^{-1} in the mitomycin C and for control wells were ~10^6 and ~10^5, respectively. Bacteriophage production in the absence of mitomycin C ranged from 4.4×10^4 to 6.9×10^5 VLP mL^{-1} for the Actinobacteria, from 5.9×10^5 to 8.4×10^5 for the Firmicutes, and from 6.3×10^5 to 8.7×10^5 for the Proteobacteria.

Statistical Analysis

The Kolmogorov–Smirnov test results demonstrated that the VLP counts, in the wells containing mitomycin C and in the control wells, from Atlantic at 0.05% (N = 14, $p = 0.093$, 0.252) and NASA at 0.05% (N = 9, $p = 0.722$, 0.444) groups, were normally distributed. However, in the Tallahassee isolate group, the VLP counts in the mitomycin C and control wells were not normally distributed at 0.05% (N = 17, $p = 0.044$, 0.049). Overall, the concentrations of VLP in wells containing mitomycin C (first/top well) were significantly different than what was observed in the control wells (matched first/top well) at 0.05% (N = 41, $p = 0.023$, $r_s = 0.354$). If the Atlantic isolate 070,103BW1 is removed (no VLP detected after exposure to mitomycin C) as an outlier, the strength of the association increases from weak to moderate at 0.01% (N = 40, $p = 0.005$, $r_s = 0.428$). A weak positive correlation was observed between the concentrations of VLP counts in the presence of mitomycin C and the number of wells that cleared (r_s of 0.295 of $p = 0.064$) for all isolates. Statistical analysis showed no significant difference between the concentrations of VLP counts in the presence of mitomycin C (p-Values of 0.163) and in the control wells (p-Values of 0.648), based on isolate origin (Tallahassee, Atlantic, NJ, USA, or NASA).

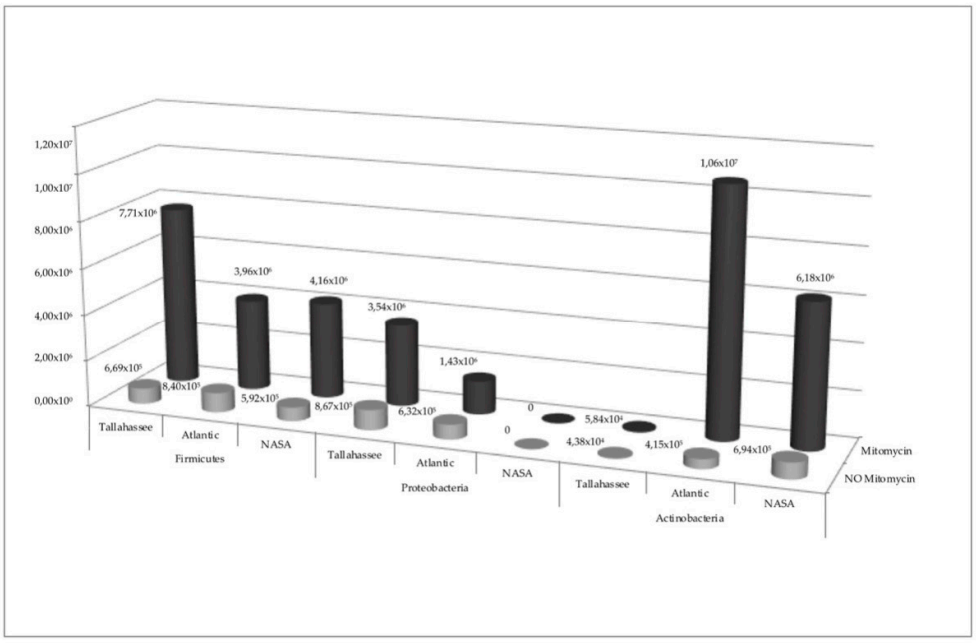

Figure 1. Average of VLP mL^{-1} obtained in the mitomycin C and control wells by sites and bacterial groups.

4. Discussion

The objective of this study was to measure the concentration of mitomycin C-inducible VLP in groups of eubacteria collected at various altitudes in Earth's atmosphere. Our data indicate that there are no differences in the concentrations of VLP mL^{-1} among the various sites of origin (Tallahassee = terrestrial near ground, Atlantic = aquatic near surface, and NASA = high altitude). These data could imply that environmental stress factors associated with atmospheric transport, such as UV exposure and desiccation, may not directly influence the generation of VLP in communities of airborne eubacteria. Observations in aquatic environments have demonstrated the detrimental effects of environmental stress on extracellular VLP decay and production [30,53]. The stresses associated with atmospheric transport could preselect for bacteriophage carriers that have the genetic characteristics to provide enhanced protection of the cells' nucleic acids or that are carriers of prophages that resist induction in an atmospheric setting.

Induction of VLP by mitomycin C was observed in 38 out of 41 (92.7%) of the isolates in this study. These data were similar to those reported by Mercanti et al., 2011 [54] for strains of *Lactobacillus*, but were much higher than those observed by Weinbauer and Suttle (1996) and Jiang and Paul (1998) [13,30] in aquatic environments at 43% and 47%, respectively; or by Williamson et al., 2007 [7] in soil microbial communities at 33.0%. This high prophage prevalence rate in eubacteria isolates versus those observed in other environments indicates that the atmosphere may quickly select for carriers.

The presence of mitomycin C appeared to suppress VLP production with the 070,103 BW1 isolate. The absence of induction by mitomycin C with isolates could be due to suppression, resistance to the induction agent, or the absence of prophages. Five of the isolates demonstrated an increase in phage production in the presence of mitomycin C, but clearing of the wells was not noted. These data indicate that those isolates were resistant to lysis or that there was a rapid transition to an infection-resistant state. The presence of VLP was observed in 34 out of 41 (83.0%) of the control samples. These data indicate that it is possible that culturable eubacteria may serve as effective long-range

atmospheric VLP vectors, and thus both host and VLP elements may influence the genetic diversity of downwind environments.

5. Conclusions

A high prevalence rate of VLP at lower and extreme altitudes in Earth's atmosphere was detected in this study. It may be possible that, once injected into the atmosphere, the stress of that environment quickly selects against eubacteria that are not capable of high VLP production.

Data also suggest that this may be a significant mechanism for long-range atmospheric inter-and intra-continental dispersion of viable VLP-related genes and thus could be a driver of evolution in downwind environments.

Additional sampling and testing are required to confirm these hypotheses.

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