Host-associated phages disperse across the extraterrestrial analogue Antarctica

Running title: Dispersal of Antarctic phage

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Abstract

Extreme Antarctic conditions provide one of the closest analogues of extraterrestrial environments. Since air and snow samples especially from polar regions yield DNA amounts in the lower picogram range, binning of prokaryotic genomes is challenging and renders studying the dispersal of biological entities across these environments difficult. Here, we hypothesized that dispersal of host-associated bacteriophages (adsorbed, replicating or prophages) across the Antarctic continent can be tracked via their genetic signatures and benefits our understanding of virus and host dispersal across long distances. Phage genome fragments (PGFs) reconstructed from surface snow metagenomes of three Antarctic stations were assigned to four host genomes, mainly Betaproteobacteria including *Ralstonia* spp. Betaproteobacteria of this genus have been found in Antarctic snow as well as on space-related equipment. We reconstructed the complete genome of a temperate phage with near-complete alignment to a prophage in the reference genome of *Ralstonia pickettii* 12D. PGFs from different stations were related to each other at the genus level and matched similar hosts. Metagenomic read mapping and nucleotide polymorphism analysis revealed a wide dispersal of highly identical PGFs, 13 of which appeared in seawater from the Western Antarctic Peninsula with up to 5538 km to the snow sampling stations. Our results suggest that host-associated phages, especially of *Ralstonia* sp. disperse over long distances despite harsh conditions of the Antarctic continent. Due to the additional identification of 14 phages associated with two *R. pickettii* draft genomes isolated from space equipment, we conclude implications for the spread of biological contaminants in extraterrestrial settings.

Importance

Host-associated phages of the bacterium *Ralstonia* identified in snow samples can be used to track microbial dispersal over thousands of kilometers across the Antarctic continent, which functions as an extraterrestrial analogue because of its harsh environmental conditions. Due to presence of this bacterial strain including genome-integrated prophages on space-related equipment, and the here demonstrated potential for dispersal of host-associated phages, our work has implications for Planetary Protection, a discipline in Astrobiology interested in preventing contamination of celestial bodies with alien biomolecules or forms of life.

Introduction

Due to harsh environmental conditions and isolation of Antarctica by the surrounding Southern Ocean's Circumpolar Current, this continent is considered an analogue for multipurpose space exploration (1, 2). For example, its McMurdo Dry Valleys are regarded as a close terrestrial analogue to Mars (3). Astrobiology model organisms found on Antarctica are highly adapted to stressful conditions and comprise prokaryotes such as spore-forming *Bacilli* (4, 5), but also microfungi (3, 6). Understanding endurance and dispersal of microorganisms under conditions that mimic those on extraterrestrial planets, i.e., high UV radiation, low temperature, and low nutrient availability, has important implications for Planetary Protection. For instance, the dispersal of microbes that hitchhike to a celestial body is currently not considered in Planetary Protection, a discipline in Astrobiology set out with the aim of preventing contamination of celestial bodies with foreign biomolecules or forms of life.

Among the potential candidates hitchhiking spacecraft are Betaproteobacteria of the genus *Ralstonia* (order *Burkholderiales*). These bacteria are able to thrive under oligotrophic conditions (7) and were reported to be ubiquitously present on space-related equipment including water systems of the International Space Station (ISS) (8, 9), and the Mir space station (10, 11). Likewise, they belonged to the microbial inventory of Mars Odyssey and Mars Phoenix lander facilities and can thus prevail under strict Planetary Protection regulations (12, 13). *Ralstonia picketii* strains were found to thrive in simulated microgravity compared to normal gravity (11) and demonstrated high resistance against different metal ions and UV-C radiation (8).

Ralstonia spp., (mainly *Ralstonia pickettii*), were previously found in in Antarctic soils (14), Antarctic snow (15-17), in snow over Tibetan Plateau Glaciers (18), and in the air of the Antarctic base Concordia (19). Interestingly, this genus has been regarded as an atmospheric traveler rather than being part of true snow microflora in Antarctica (15). Despite a report on bacterial activity at subzero temperatures in South pole snow (20), *Ralstonia* was not among active bacterial communities as inferred from cDNA-based 16S rRNA amplicon sequencing (15). This view was supported by a comprehensive Antarctic surface snow microbiome study that did not detect this bacterial genus (21), and by the fact that spatial variability of snow microbiomes in Antarctica is high (22). Although aerial dispersal is probably the major contributor to (micro-)biological input to remote regions (23), the role of bioaerosol transport to microbial ecology of isolated systems such as the Antarctic continent is poorly understood (24). Applying high throughput sequencing approaches to study bacterial dispersal over the Antarctic continent remains considerable challenging due to the low microbial biomass of atmosphere-derived samples regarding their DNA content (25), and resulting issues of recovering high quality assemblies from metagenomic reads (26).

In addition to the limited knowledge about how transport via aerosols and snow across Antarctica shapes microbial dispersal patterns, another open question relates to the distances that microbes can cover within the atmosphere of extreme environments. A study on the dispersal of airborne faecal coliforms showed a distribution over about just 175 m from a sewage outfall at Rothera Research Station (Antarctic Peninsula) and thus prolonged survival was considered unlikely (27). More stress-resistant microorganisms could, however, endure for much longer periods. Recently, L. A. Malard et al. (22) found high abundances of spore-forming *Bacilli* and suggested that long-term dispersal may seed continental Antarctic snow ecosystems. However, to date, the role of aerial dispersal in shaping patterns of microbial biogeography is supported by little empirical evidence (23).

Here, we follow the hypothesis that *Ralstonia* spp. prophages, which are geographically widespread entities (28), as well as replicating and adsorbed bacteriophages (all types further referred to as “host-associated” phages) can be used to study dispersal across the Antarctic continent. Extracting prokaryotic genomes from low DNA samples including air or precipitation is challenging, as low input libraries (1 pg) can result in the absence of bins (26). However, (pro)phage genomes or their fragments are much smaller than prokaryotic genomes and thus easier to identify, track and compare. In this study, genome-resolved metagenomics was applied to demonstrate dispersal of host-associated phage genome fragments (PGFs) from surface snow across the Antarctic continent over hundreds to thousands of kilometers. We detected PGFs belonging to the orders *Caudovirales* and *Tubulavirales* in this extraterrestrial analogue and additionally show that similar genome-integrated phages are frequent colonizers of space equipment. Therefore, we suggest that host-associated PGFs represent a useful tool to study spatial dispersal of bacteria and their phages in extreme environmental settings and further envision implications for the dispersal of microbiological

contaminations on spacecraft and celestial bodies that previously escaped Planetary Protection measures.

Results

Reconstruction of MAGs from Antarctic snow metagenomic data with little evidence for adaptive immunity

The microbial community composition of surface snow samples collected close to three Russian Antarctic stations, Druzhnaja, Mirnii and Progress, based on 16S rRNA gene sequencing was described earlier (16) and showed that *Ralstonia* was the most dominant organism in snow collected at the Mirnii station, the second most dominant after *Janthinobacterium* at Druzhnaja, and the third most dominant (after *Flavobacteria* and *Hydrogenophaga*) around Progress station. We reconstructed four prokaryotic and one eukaryotic metagenome assembled genomes (MAG) from the three low-biomass snow metagenomes (Figure 1A). Two MAGs related to *Ralstonia* sp. and *Ralstonia pickettii* were recovered from Druzhnaja and Mirnii samples and had 86%/10% and 55%/0% completeness/contamination scores, respectively. MAGs of *Janthinobacterium lividum* with 92%/6% scores and of *Flavobacterium micromati* with 96%/0% scores were recovered from Druzhnaja and Progress, respectively. We also identified a MAG of a diatom (likely *Thalassiosira* sp.) in the Mirnii metagenome, which we used for normalizing some of our viral analysis (see below) but was not further characterized in this manuscript. Coverage of bacterial MAGs as performed by read mapping revealed coverage scores of 5.9, 7.1, 6.7 and 14.6 for the MAGs of *Ralstonia* sp., *Ralstonia pickettii*, *Janthinobacterium lividum* and *Flavobacterium micromati*, respectively.

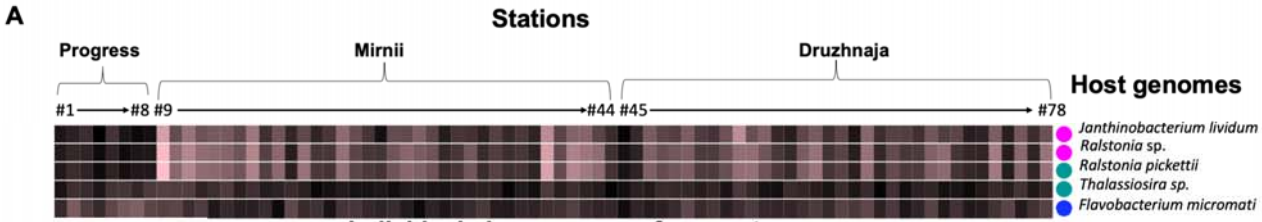
We searched for clustered regularly interspaced short palindromic repeats (CRISPR) spacers in the snow metagenome reads to link them to potential protospacers on PGFs. CRISPR arrays and *cas* genes were absent from both *Ralstonia* and the *Janthinobacterium lividum* MAGs as determined by CRISPRcasFinder. BLASTing of direct repeat (DR) sequences to NCBI's NR database also did not indicate *Ralstonia* or *Janthinobacterium* to be the host of the CRISPR array. Solely the genome of *Flavobacterium micromati* contained two CRISPR arrays with four spacers each (both evidence level 3= highly likely candidates). No CRISPR arrays were found on the *Ralstonia pickettii* draft genomes SSH4 and CW2 obtained from space equipment.

PGF-host analyses suggest shared hosts of Antarctic phages and a newly discovered temperate phage p12D

A total of 26 predicted PGFs, eight of them being putative PGFs, was found in Antarctic snow metagenomic data (Table S1). VIBRANT (29) additionally detected 52 PGFs, resulting in a total of 78 PGFs (Figure 1A). PGFs with minimum 75% of the genome covered with reads had coverages ranging between 1.9 and 17.5. Most PGFs were partial sequences according to viralComplete, only Antarcphage10_Mi_4716 and Antarcphage49_Dr_7823_circ were estimated to be of full length (Table S1), although annotations of Antarcphage10_Mi_4716 in comparison to *Ralstonia* phage p12J revealed missing genes and let us question its completeness (Figure 2). Out of 78 PGFs, 77 were categorized as lytic by VirSorter (30) and 75 were found to be of viral or unclassified source by CheckV (Table S1). 43.6%, 46.1% and 10.3% of identified PGFs originated from the Druzhnaja, Mirnii and Progress, respectively.

All 78 PGFs were analyzed in conjunction with reconstructed MAGs using VirHostMatcher. We defined a dissimilarity threshold of d2* value= 0.436, which corresponds to the lowest

dissimilarity value (=highest similarity) for PGFs matching the MAG of *Thalassiosira* sp. from the snow environment (Figure 1A) by assuming that the eukaryotic MAG does not match any of our extracted presumably prokaryote infecting PGFs. In total, 50 of the 78 PGFs matched a host MAG below the defined dissimilarity threshold, based on their shared k-mer patterns. Most PGFs (41) matched both MAGs of *Ralstonia* sp. and *Ralstonia pickettii* with dissimilarity d2* values <0.436 (Figure 1A). 38 and 14 PGFs matched *Janthinobacterium lividum* and *Flavobacterium micromati* MAGs, respectively. Five out of eight PGFs extracted from the Progress Station metagenomic snow sequences matched the MAG of *Flavobacterium micromati* recovered from this station. Seven PGF matches were shared between all prokaryotic MAGs (Figure S1). However, with 30 shared matches, the three MAGs belonging to the order *Burkholderiales* shared the most overlap (Figure 1B, Figure S1).



Individual phage genome fragments

Station	Progress	Mirnii	Mirnii	Druzhnaja	Druzhnaja
Phylogenetic affiliation of host genome (species)	*Flavo-bacterium micromati*	*Thalassiosira* sp.	*Ralstonia pickettii*	*Ralstonia* sp.	*Janthino-bacterium lividum*
(family)	*(Flavobacteria ceae)*	(*Thalassiosira ceae)*	*(Burkholderiac eae)*	*(Burkholderia ceae)*	*(Oxalobacter aceae)*
Completeness (%)/ contamination (%)	96/0	n/a	55/0	86/10	92/6
Coverage	14.6	32.1	7.1	5.9	6.7

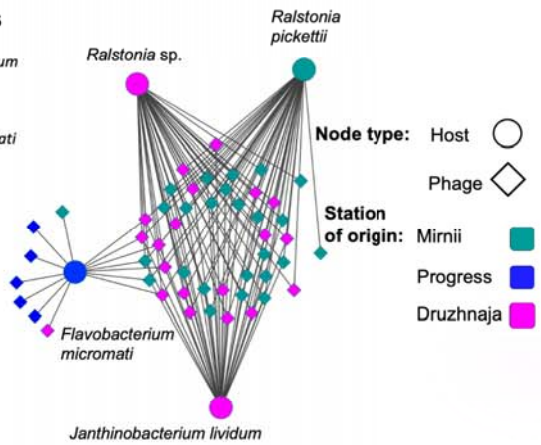


Figure 1: Host-phage pairings based on shared k-mer frequency patterns. A) Heatmap representing dissimilarity value d2* for matches between five host MAGs and 78 PGFs derived from three Antarctic snow metagenomes from the stations Progress, Mirnii, and Druzhnaja. The table shows MAG characteristics (phylogenetic affiliation, completeness, contamination, and coverage). **B)** Network of phage-host interactions based on k-mer frequency pattern reveals strong overlap between *Ralstonia* and *Janthinobacterium lividum* infecting PGFs. Here, only PGFs matching the host MAG below the defined threshold (see text) are shown. n/a= not available

Among the 78 PGFs, we detected a circular and thus complete 7.8 kb PGF termed Antarcphage49_Dr_7823_circ (Figure 2A, Figure S2). Since the Antarcphage49_Dr_7823_circ PGF from the Druzhnaja station was found in the chromosome of a *Ralstonia pickettii* 12D strain isolated from copper-contaminated sediment from a lake in Michigan with 99.88% identity (accession number: CP001644.1- 2272699-2280518, Figure 2A), we here propose the name p12D phage equivalent to *Ralstonia* p12J, a known phage infecting the strain *Ralstonia pickettii* 12J. Antarcphage49_Dr_7823_circ (from now on referred to as p12D) only matched the MAG of *Ralstonia* sp. and *Ralstonia pickettii* based on shared k-mer frequency patterns. Functional protein annotations provided evidence that p12D contained a gene for a resolvase domain containing protein/ site-specific recombinase, likely used for integration into the host genome, as well as for the zonular occludens toxin (Zot, PF05707) (Figure 2A, Figure S2, Table S2). The non-toxic component of Zot at the N-terminus represents a well-conserved protein in filamentous phages and has been used for phylogenetic analysis (31, 32). Consequent construction of a phylogenetic tree for Zot proteins from this study and respective references (Figure 2B, Figure S3) confirmed close identity of p12D to *Ralstonia* phage 1 NP-2014 and Antarcphage79_WAP_18.3. *Zot* of Antarcphage10_Mi_4716 was phylogenetically related to filamentous *Ralstonia* phage p12J. The tree shows four distinctive clusters for the Zot protein, reflecting the overall synteny of the gene order of the different *Ralstonia* phages very well.

Annotations against UniRef100 revealed that 68.8% of all annotated Antarctic PGF proteins remain hypothetical. The annotations taxonomically assigned 34.4% of all Antarctic PGF proteins to either *Ralstonia* or *Ralstonia pickettii*, and 11.6 % to phages of *Janthinobacterium* or *Janthinobacterium lividum*, potentially indicating lateral gene transfer between virus and host in their respective evolution (33, 34) or supporting that these PGF indeed represent prophages. Three of the Antarctic PGFs carried a site-specific integrase or resolvase domain,

and 4.2% of genes were related to phage structural proteins, e.g., head, tail, or capsid proteins (Table S3, Table S4).

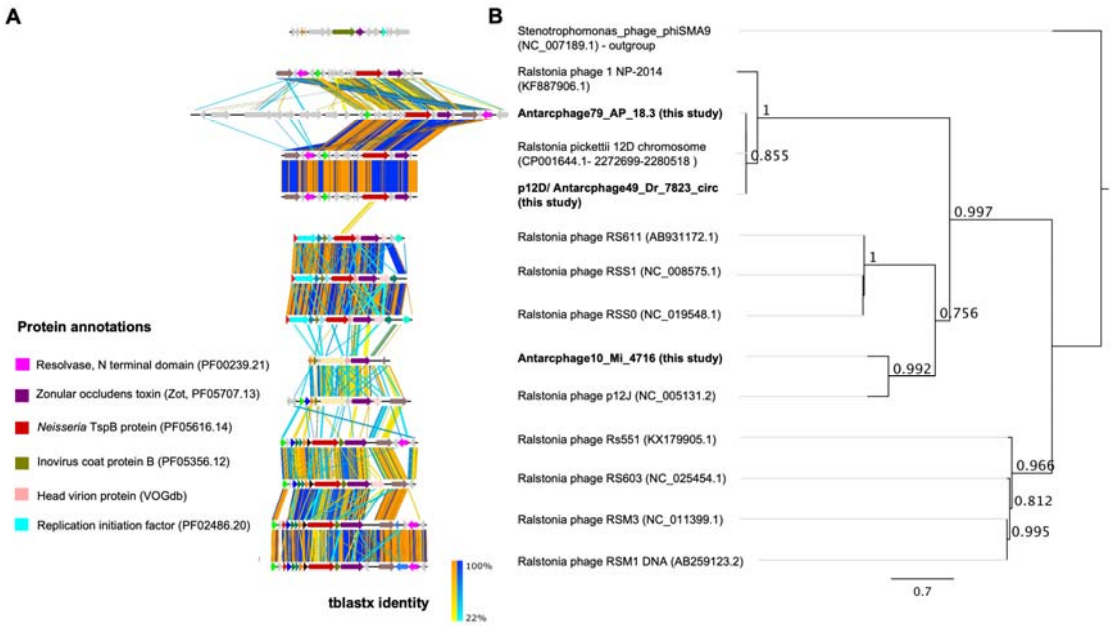


Figure 2: Phage genome comparisons, functional annotations and phylogenetic relationship based on zonular occludes toxin (Zot, Pfam-ID: PF05707). A) Synteny of known *Ralstonia* sp. phages from NCBI and Antarctic PGFs from this study with similar coding sequences (CDS) being in the same color if they occur on more than one phage genome. Functional annotations performed by DRAM-v (35) are given for all colored CDS where available. Vertical lines between sequences indicate regions of shared similarity shaded according to tBLASTx (orange gradient for matches in the same direction or blue gradient for inverted matches). Figure was created using Easyfig (36). **B)** The phylogenetic tree was built using FastTree 2.1.11 (37) in Geneious 11.1.5 (38) under default settings and shows four distinct clusters of Zot proteins based on their amino acid sequences (aligned with MUSCLE (39)).

Cross-mapping and nucleotide variations reveal dispersal patterns of PGFs across Antarctica

Cross-mapping on the 78 PGFs with reads from the three snow samples and the WAP seawater sample (Figure 3A) demonstrated that PGFs derived from the Progress station could not be found in the other two snow metagenomes. By contrast, 16 different PGFs from Druzhnaja were present at Mirnii, and 27 Mirnii PGFs were present at Druzhnaja (Figure 3B). In addition, 4 and 9 PGFs from Mirnii and Druzhnaja were detected in the WAP dataset, respectively (Figure 3B), all of them having minimum 97% of their lengths covered with reads from the snow metagenomes (Table S1). Of 43 PGFs that were found at both stations (Mirnii and Druzhnaja) based on read mapping, 26 PGFs shared a viral cluster (VC), and 34 matched prokaryotic hosts based on k-mer frequencies between stations (Table S1). Interestingly, nine of the 13 PGFs occurring in WAP and Mirnii/Druzhnaja samples, contained identical single nucleotide polymorphisms (Table S5) or were even devoid of them pointing towards a common phage population before dispersal led to separation.

Only one of the DR sequences matched back to the MAG assigned *Flavobacterium micromati* from Progress station (Table S6). Matching of CRISPR spacers derived from CRISPR loci of unknown hosts (reconstructed from DR sequences) revealed that spacers from Mirnii matched 19, 14 and three PGFs from Druzhnaja, Mirnii and Progress, respectively, and one *Flavobacterium* sp. spacer from Progress matched a PGF from that station (Table S6, Figure S4). Out of these 37 spacer matches, ten matched protospacers of PGFs of unknown hosts and the remaining 27 were associated with PGFs (including p12D) that infect one or more of the four recovered prokaryotic MAGs according to VirHostMatcher. Since p12D was among the

PGF spacer targets and is a certain *Ralstonia* phage, this could indicate that the unknown host from which the CRISPR spacer were derived also belongs to the order *Burkholderiales* and uses adaptive immunity against its viruses.

Clustering of PGFs in VICTOR and vConTACT2 revealed that groups of two to four PGFs could be assigned to twelve distinct viral genera-forming viral clusters (Figure S5 & S6, Table S1, Figure 3A). Members of the same cluster were often recovered from different stations, for example, Druzhnaja and Mirnii, which are located 923 km apart (Figure 3B). The phylogenomic Genome-BLAST Distance Phylogeny (GBDP) tree yielded average support of 47% and 80% in the nucleic acid and amino acid-based analysis, respectively. OPTSIL clustering yielded 78 and 64 species and genus clusters at the nucleic acid level and 78 and 70 at the amino acid level, respectively.

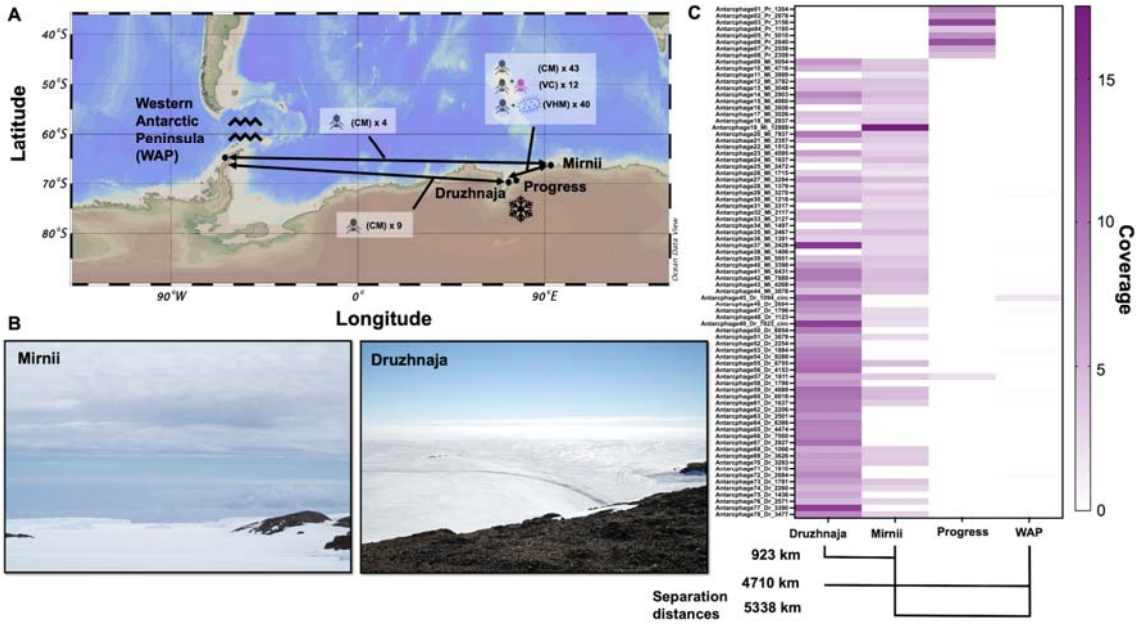


Figure 3: Dispersal distances of Antarctic PGFs. A) The map shows different stations and PGF whose presence was determined by cross mapping (CM) of reads and shared between two locations. Dispersal is only shown if at least one PGF occurred with equal or more than 75% of scaffold length of at least 1-fold coverage at two stations. Number of virus clusters

(VC) and virus-host cross matches (VHM) was determined for assembled PGFs from snow metagenomes (Druzhnaja, Mirnii, Progress). A VHM indicates that one PGF infects host MAGs from two stations. No VC and only one CM were observed for Progress-Druzhnaja and Progress-Mirnii. The Western Antarctic Peninsula (WAP) sample is derived from seawater (zigzag/wave symbol), the remaining samples are from snow (snowflake symbol). Map was built using Ocean Data View (40). **B)** Pictures of two sampling sites (photos: Anna Lopatina). **C)** Heatmap of read-normalized coverages based on CM of reads against 78 Antarctic PGFs and separation distances between stations. White areas indicate PGFs that were not present because they had less than 75% of scaffold length with least 1-fold coverage.

Many PGFs formed VCs with no genomic relatedness to any known phages from the ProkaryoticViralRefSeq v94 database. Others were, however, like known *Ralstonia* PGFs (current family *Inoviridae*, order *Tubulavirales*) and hence clustered with those, such as p12D with *Ralstonia* phage 1 NP-2014, or Antarcphage10_Mi_4716 with *Ralstonia* phage PE226 and *Ralstonia* phage p12J (Figure 4, Table S2). Other Antarctic PGFs shared protein clusters with *Ralstonia* phages from space equipment based on vCONTACT2 or were related to phages of the *Caudovirales* order from the RefSeq database (Figure 4).

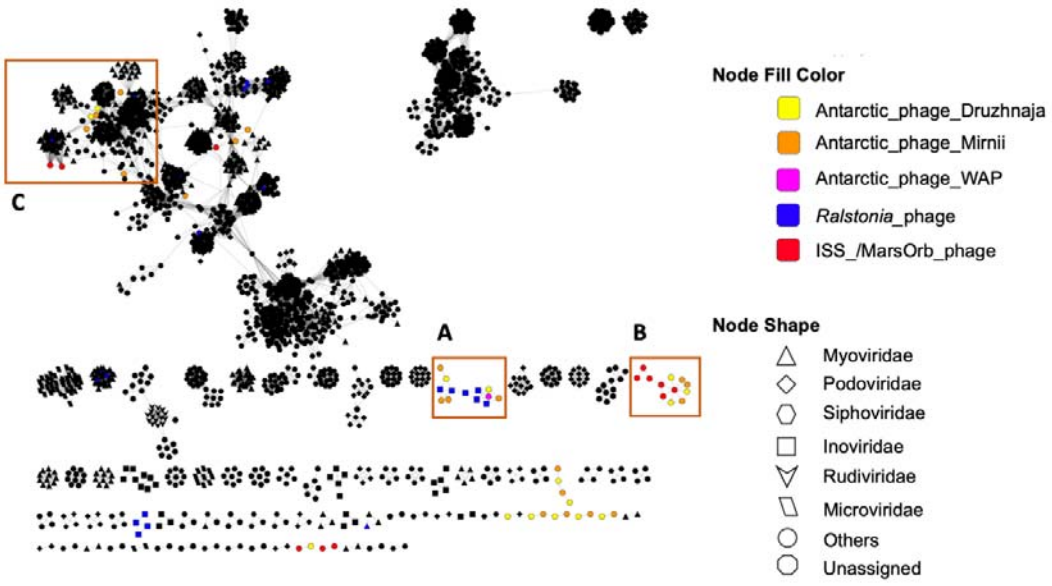


Figure 4: Phage network of Antarctic PGFs derived from Druzhnaja, Mirnii, Western Antarctic Peninsula (WAP) and space equipment (ISS/MarsOrb) clustered with viruses of the viral RefSeq database. Based on shared protein clusters, Antarctic PGFs from this study group with known *Ralstonia* phage of the family *Inoviridae* (A), with phages obtained from *Ralstonia* isolates from space equipment (B) or with known phages of the order *Caudovirales* (C). Black nodes refer prokaryotic viruses other than *Ralstonia* and PGFs from this study. Interactions show relatedness of genomes on viral genus or higher taxonomic level. "Others" refer to other known viral families not listed in the legend. Visualization was done using Cytoscape 3.8.2. (41). For details about viral clusters, please see Table S1.

In summary, our results support the idea of dispersal of host-associated phages between widely separated stations because a) cross-mapping revealed presence of Mirnii PGFs at Druzhnaja and vice versa, and presence of a temperate phage from snow in the WAP seawater dataset, b) PGFs in widely separated locations carry identical single nucleotide polymorphisms or lack nucleotide variations, c) some PGFs from Mirnii and Druzhnaja belong to the same genus/VC, and d) Mirnii and Druzhnaja PGFs share host MAGs according

to their k-mer frequency patterns. This was mainly true for PGFs affiliated to the host MAGs *Ralstonia* and *Janthinobacterium lividum* but rarely for PGFs of *Flavobacterium micromati*, which could indicate that certain bacterial species or their phage in Antarctic snow are more prone to atmospheric dispersal than others. And e) we found CRISPR spacers from two unidentified hosts (represented by two distinct CRISPR DR sequences) at the Mirnii station to match PGFs from Druzhnaja, Mirnii and Progress (Figure S4). This is particularly interesting because cross-mapping revealed little to no abundance of Progress PGFs at other stations, which could be related to the low biomass and/or little coverage of PGFs, but spacers from Mirnii suggest an infection history with PGF from that station, thus reflecting (past) dispersal of host and/or phage.

Ralstonia **phages occur across diverse ecosystems and on space equipment**

By BLASTing all recovered PGFs against the IMG/VR viral database, we found that p12D shared high identity (85.9 %) with a *Ralstonia pickettii* prophage (IMG/VR v.2 scaffold ID Ga0075447_10000781, genome ID: 3300006191) from a seawater metagenome of the WAP (Figure 2, Table S7), which was further validated by cross-mapping of WAP reads to p12D delivering 100% scaffold coverage with 90% identical reads. The WAP has 4710 km to the station Druzhnaja (Figure 3). The PGF Antarcphage79_WAP_18.3 was obtained by BLASTing the p12D scaffold against the WAP assembly but was assembled containing genomic regions extending the actual PGF. Based on vConTACT2, it was affiliated to the same viral genus as p12D, fostering the genomic relatedness between these two phages. Other PGFs recovered from the Antarctic snow metagenome showed hits to phages deposited at IMG/VR. Hits were related to phages originating from freshwater, wastewater, groundwater, or phages that were associated with plant root microbial communities, which is in accordance

with the fact that *Ralstonia* sp. is a frequent phytopathogen (42). Many of the matching entries in IMG/VR were identified as phages of *Ralstonia* due to matching CRISPR spacers of this host (mostly *R. solanacearum*, Table S7).

Altogether, 14 PGFs were found on space equipment, hereafter referred to as ISSphage (7) and MarsOrbphage (7) for PGFs extracted from the *Ralstonia pickettii* strains CW2 and SSH4 draft genomes from the ISS cooling system and Mars Odyssey Orbiter, respectively. Seven of these PGFs were identified as lysogenic and according to protein annotations (Table S3 & S4) mostly use proteins of the integrase family for integration into the host chromosome. VCONTACT revealed that some of the ISSphage4 formed a common genus cluster with 4 Antarctic PGFs, MarsOrbphage5 shared protein clusters with ISSphage3, and MarsOrbphage7 formed a genus cluster with several *Burkholderia* phages among other *Caudovirales* members (Table S1, Figure 4).

Discussion

Some Antarctic PGFs are temperate phages of the order *Tubulavirales*

CRISPR-Cas systems equip bacteria and archaea with a powerful defense system against invading mobile genetic elements including plasmids, phages and viruses. Only ca. 50% of bacteria rely on this adaptive immune system, and only 31% of genomes from public databases belonging to plant-pathogenic *Ralstonia solanacearum* contain CRISPR-Cas arrays (43). Upon exposure to a virulent phage under laboratory conditions, the CRISPR array of *Ralstonia solanacearum* strain CFBP2957 did not acquire new spacers from viral protospacers. This demonstrates the CRISPR system's inefficiency in viral eradication and suggests alternative antiviral mechanisms in this *Ralstonia* strain (43). More recently, a

publication reported that in the presence of CRISPR arrays, 27.9% of spacers from *Ralstonia* genomes targeted prophage elements (28). Overall, our study demonstrated the lack of CRISPR systems in *Ralstonia* from a low temperature environment, and further underpins that this genus might employ other strategies for defense against mobile genetic elements (43). J. L. Weissman et al. (44) suggested that psychrophilic and psychrotolerant microbes would disfavor CRISPR systems more than mesophilic species, due to lower viral decay rates, higher viral diversity, and greater dispersal in colder compared to warmer environments. In addition, frequent targeting of temperate phages by CRISPR spacers can be another reason for the loss of CRISPR systems (45). We assume that not all sequenced PGFs are prophages but could also have been sequenced during their adsorption or replicating stage in the lytic cycle.

Based on shared protein clusters, some Antarctic PGFs such as Antarcphage10_Mi_4716, Antarcphage48_Dr_1123, Antarcphage17_Mi_3026, and p12D clustered with known *Ralstonia* phages including *Ralstonia* phage p12J (NC_005131.2), *Ralstonia* phage PE226 (NC_015297.1) or *Ralstonia* phage 1 NP-2014 (NC_023586.1). These are filamentous phages of the *Inoviridae*, a family that has been recently called for re-classification to higher taxonomic ranks (32, 46). Inoviruses (order *Tubulavirales*) typically feature circular, single stranded DNA genomes of ~5-15 kb length, partake in chronic infection cycles and are globally abundant (32). As we sequenced dsDNA viruses, detection of ssDNA inoviruses shows that these must be either replicating phages or genome-integrated prophages. *Zot*, which was detected on p12D, Antarcphage10_Mi_4716 and Antarcphage79_WAP_18.3 is a typical gene in filamentous *Ralstonia* phages (47, 48). Filamentous phages confer little burden to their hosts or even serve them, e.g., by increasing their hosts' virulence and evolutionary fitness, and because virions can leave the host in a non-destructible way (31, 32). *Ralstonia* phages detected in Arctic viromes were shown to transduce genomic information of cold-shock proteins to their hosts (49), which would be an asset for microorganisms in polar

environments. Although we did not find cold shock genes on PGFs in this study, transduction of beneficial genes, which is common in extreme environments (50), could theoretically be another reason why *Ralstonia* hosts do not apply CRISPR defenses against their phages.

Some *Ralstonia* phages occur as non-integrative, episomal forms, e.g., RS603, a hybrid of RSM1/3 infecting the phytopathogen *Ralstonia solanacearum* (51), whose genome lacks a resolvase domain (Figure 2A), but many mesophilic *Ralstonia* also occur as lysogens (52). Lysogeny, a lifestyle during which the phage genome becomes integrated into the host chromosome, is a widespread phenomenon in low temperature environments (53-55) and likely attributed to prolonged starvation and low activity of host cells under harsh conditions. Low activity was previously also reported for *Ralstonia* (15). Since p12D and its counterpart from the Western Antarctic Peninsula (WAP) have a resolvase-domain containing protein functioning in integration/excision during lysogenisation (56, 57), we conclude that they must be temperate phages of *Ralstonia*.

PGFs travel long distances across the Antarctic continent

Many, i.e., 75 of the 78 Antarctic PGFs found in this study shared little protein clusters with known phages from public databases. This missing relatedness was previously reported for ssDNA viruses originating from Antarctic cryoconite holes and attributed to the isolation and extreme environmental conditions at the Antarctic continent (58). In fact, 28 of the 78 PGFs did not match any host MAG below a 0.436 threshold (Table S1), meaning that they were either false-positives PGFs or PGFs of non-identified host MAGs (ten of them had at least one spacer match).

Aeolian transport of viruses over polar environments, especially attached to snowflakes, has been scarcely investigated to date. Former investigations, mainly conducted at low latitudes, demonstrated intercontinental transport of microbes by winds (59), and that highly identical phages can be found in distantly related areas and in various ecosystems around the globe (60-63). C. M. Bellas et al. (64) recently reported the presence of near-identical phage genomes being spread by up to 4000 km in cryoconite holes of Svalbard, Greenland, and the Alps. Our data show that despite the isolation of the Antarctic continent, and under no consideration of anthropogenic dispersal (65), bacteria and phage distribution via snow over extensive distances across Antarctica is possible. We cannot be certain about the nature of the PGFs (prophage or lytic phage) by metagenomic predictions alone. While the *Inoviridae* fraction of the dataset is likely to occur as prophages or episomal forms, frequent CRISPR spacer matches against PGFs and PGFs assigned to lytic categories by VIBRANT and VirSorter (category 1-3) support that host-associated, lytic phages during adsorption stage were present as well. We thus commend further experiments that involve cultivation attempts and sequencing of metaviromes (free phages) to reveal the extent of lysogenic or chronic compared to lytic infection styles in Antarctic snow.

Snow PGFs were additionally found and carried identical single nucleotide polymorphisms in the seawater metagenome from the WAP being 4710 km or even 5338 km apart from Druzhnaja and Mirnii, respectively (Figure 3B), implying long distance transport. From seawater, a transmission route via sea spray aerosols to snow that is blown over ice surfaces (66) can be assumed. Aerosolization of bacteria from the sea surface is highly taxon-specific but seems to work well for *Ralstonia pickettii* (67) and also viruses (68). Alternatively, a transport via aerosols to clouds and precipitating snow is possible. In the latter scenario, bioaerosols including microbial cells would act as ice nucleation particles (69), transferring

microbes to ice clouds where they might induce their own precipitation (70, 71). The abundance results from this study support isolation of Progress-derived PGFs and point towards a decreasing gradient of abundances from Druzhnaja to Mirnii to the WAP for many PGFs (Table S1). Thus, general dispersal patterns of microbes across Antarctica seem governed by westward drift and are probably mediated by the prevailing Southern Hemisphere westerly winds (72). On short spatial-temporal scales, dispersal seems more complex and probably shaped by multifactorial dependencies such as the different potential of a species to become airborne (67), meteorological conditions or the local geography. For instance, while Druzhnaja is located ~50 km into the continent, the stations Mirnii and Progress are near the coast and more exposed to the sea in summer when ice breaks occur.

Implications for dispersal of viruses and microbes in extraterrestrial settings

We assume that the transferability of using PGFs to study microbial dispersal in space analogues such as the Antarctic continent is likely applicable to other celestial bodies like Mars. Three celestial bodies in our solar system (Mars, Europa, and Enceladus) have environmental conditions that could favor microbial life (reviewed by M. G. Netea et al. (73)). Most microbial isolates (85-95%) obtained from spacecraft and assembly facilities are associated with humans and consequently unmanned spacecraft contain fewer and less diverse microbes than their manned counterparts (74). Since *Ralstonia* spp. including *Ralstonia pickettii* can be human pathogens (75) and are able to thrive under harsh and oligotrophic conditions (7), they might contribute to the transmission of viruses to extraterrestrial environments, particularly via manned missions.

We found evidence for viral signatures associated with two *Ralstonia pickettii* strain draft genomes previously obtained from space equipment of a spacecraft assembly clean room and

from water systems of the ISS. This result in conjunction with the result that temperate *Ralstonia* phages (and other viruses) can undergo long-range dispersal in association with their hosts across the extraterrestrial analogue Antarctica suggests that contaminations of space equipment with particularly persistent microbes such as *Ralstonia* should receive more focus during microbiological monitoring in the framework of Planetary Protection. However, the field of astrovirology has so far generally found little attention (76). The contribution of lysogenic and episomal phage ('hidden hitchhikers') to overall viral loads on spacecraft and associated equipment has been overlooked despite early work reporting on alterations in prophage induction during spaceflight (77-79), tobacco mosaic virus to survive space flight equivalent proton irradiation (80), and the occurrence of phages and human-related circoviruses in clean rooms (81).

Planetary Protection aims preventing the spread of biological contaminants (forward contamination) to space shuttles and stations as well as extraterrestrial environments of the solar system. However, this policy largely ignores the potential of escaped biological contaminants to heavily disperse on foreign celestial bodies once being released, for instance after crash landings as happened for the Schiaparelli module of the ExoMars program in 2016 (82). Our results show that host-associated PGFs are not only suitable indicators for tracking long-distance dispersal in space analogues but also demonstrate that the release of contaminants that previously escaped Planetary Protection measures could spread far across extraterrestrial ecosystems and, in the worst-case scenario, confound future life detection missions.

Material and Methods

Metagenomic and genomic data processing

We analyzed publicly available metagenomic data sets, which correspond to Antarctic surface snow collected around three Russian stations (Druzhnaja, Mirnii, Progress) and are deposited at NCBI's Sequence Read Archive (SRA) as Bioproject PRJNA674475 (Table S8) and MG-RAST under project accession mgp13052 including taxon abundance data. DNA extraction for these metagenomes was carried out with DNA Blood & Tissue kit (Qiagen, Hilden, Germany), whose protocol causes removal of free viruses. Sequencing libraries were prepared with MiSeq reagent kit v.2 (Illumina, USA), targeting mainly dsDNA viruses. Metagenomic data of a seawater sample from the Western Antarctic Peninsula (WAP) was also obtained from SRA (accession number SRR5591034). Raw shotgun sequencing reads of the three snow metagenomes and the WAP dataset were quality-trimmed using BBDuk (https://github.com/BioInfoTools/BBMap/blob/master/sh/bbduk.sh) from the BBTools package (83) and Sickle (84) resulting in read counts between 1.31 – 2.65 Mio. for the three snow samples. Assembly of reads was done using MetaSPAdes version 3.13 (85). Scaffolds <1 kbps in length were removed. Draft genome sequences of *Ralstonia pickettii* strains SSH4 and CW2 isolated from space equipment were obtained from Genbank accession numbers JFZG00000000 and JFZH00000000 (9). Strains SSH4 and CW2 were isolated pre-flight from the surface of the Mars Odyssey Orbiter during assembly and from a water sample taken in-flight from the ISS cooling system, respectively.

Reconstruction of microbial genomes from metagenomes and CRISPR prediction

Binning of metagenome-assembled genomes (MAGs) from the three stations Druzhnaja, Mirnii and Progress was performed using Emergent self-organizing maps (ESOMs, (86)), ABAWACA (https://github.com/CK7/abawaca) and MaxBin 2.0 (87). Cross-mapping for use

of MaxBin 2.0 was conducted using Bowtie2 in --sensitive mode (88). Aggregation of bins was performed using DASTool (89) and bins were curated using GC base content, coverage and taxonomy in uBin (90), which also uses an universal marker gene set for bacteria and archaea to estimate genome quality (contamination and completeness) (91). Read coverage of recovered MAGs was obtained from read mapping using Bowtie2 in sensitive mode to the individual bins, followed by mismatch filtering (2% mismatch allowance, depending on read length).

Clustered regularly interspaced short palindromic repeats (CRISPR) arrays, which represent the prokaryotic adaptive immunity, were searched in host MAGs using CRISPRcasFinder and only those with evidence level 3 or 4 were considered (92). CRISPR loci consist of repeats interspaced with short DNA sequences (spacers) obtained from invading mobile genetic elements such as phages and thus provide a record of past infection events. Since CRISPR arrays might get lost during the binning processes, e.g., due to fragmentation in assembly of strain variants, absence of CRISPR arrays in CRISPRcasFinder was further investigated by reconstructing CRISPR systems from raw reads using Crass (93) and BLASTing the obtained direct repeat (DR) sequences, which are phylogenetically well-conserved (94), against the NCBI non-redundant database (release 1st March 2021) using a BLASTn --short algorithm (95) with subsequent filtering at 80% similarity and e-value threshold of 10e-05 for hits with potential hosts. DR sequences were also BLASTed against the MAGs and were used to extract CRISPR spacers from reads using MetaCRAST with settings -d 3 -l 60 -c 0.99 -a 0.99 -r (96), which were in turn matched against PGFs applying the same BLASTn and filtering step as mentioned above. In most cases, BLASTing the DR sequences against the NR database did not reveal the host's identity. Nevertheless, spacers derived from unknown hosts were considered as well, as their matches to PGFs increased our confidence that these

represent true mobile genetic elements, and matches can be used to infer infection patterns between stations.

PGF detection, host allocation and viral clustering

Phage genome fragments (PGFs) were identified from metagenome assemblies using a combination of bioinformatic tools and the classification of predicted viral genome (fragments) as “putative viruses” and “viruses” was done as previously described (97). VIBRANT v.1.2.1 with default settings was used to find additional PGFs (29). We did not set the usual cut-off length of 10 kb for PGFs (98), since many known *Ralstonia* phages and inoviruses have genome sizes <10 kb (32, 52), and because the low biomass Antarctic snow samples were overall very limited in PGFs compared to other ecosystems. VirSorter v1 (30) and VIBRANT aided in (pro)phage detection in draft genomes of *R. pickettii* from space equipment, and VirSorter contigs were taken for downstream analyses. CheckV (99) was used to determine the type of identified PGF, and viralComplete (100) applied to predict completeness and closely related phage. Pairwise comparisons of Antarctic PGFs and clustering was done using nucleic acid- and amino acid-based VICTOR (101). The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME including Subtree Pruning and Regrafting (SPR) postprocessing (102) using the distance formula D0. Branch support was inferred from 100 pseudo-bootstrap replicates each. Trees were rooted at the midpoint (103) and visualized with FigTree v1.4.4 (104). Taxon boundaries at the species and genus level were estimated with the OPTSIL program (105), using the recommended clustering thresholds (101) and an F value (fraction of links required for cluster fusion) of 0.5 (106).

Clustering of PGFs from Antarctic snow and space equipment was further substantiated via vConTACT2 v.0.9.19 (107, 108) in combination with the ProkaryoticViralRefSeq database (v94, (109)) followed by visualization of viral clusters (VC) in Cytoscape v. 3.8.2 (41). Virus-host matches were determined using the tool VirHostMatcher (110) with d_{2*} oligonucleotide frequency dissimilarity measures for a k-mer length of six as these settings should result in highest host prediction accuracy (110). Viral genus clusters were further determined using VIRIDIC (111). Venn diagrams for overlapping PGF-host matches between MAGs were calculated using the VIB-ugent webtool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Mapping of reads to assembled PGFs and nucleotide polymorphism analysis

We assume that presence of a PGF at two locations confirmed by read mapping represents dispersal. To determine if an assembled PGF from a single sample occurred in other metagenomes even if not being assembled, we performed read mapping (Bowtie2) following the previously published guidelines: that reads should map to a PGF with at least 90% identity and more than 75% of scaffold should have a coverage of at least 1x (98). Bowtie2 (88) settings were: --ignore-quals –mp = 1,1 –np = 1 –rdg = 0,1 –rfg = 0,1 --score-min = L,0,-0.1 as previously suggested (112). To detect the coverage of a PGF by reads after mapping we used an inhouse script *Calcopo* (https://github.com/ProbstLab/viromics/blob/master/calpoco) to calculate the coverage per nucleotide and then the percentage of positions in the genome covered. Mean coverage of PGFs was calculated using calc_coverage_v3 (https://github.com/ProbstLab/uBin-helperscripts/blob/master/bin/04_01calc_coverage_v3.rb) and normalized to sequencing depth to compare between samples. Analysis of nucleotide polymorphisms was conducted for the 13 PGFs that underwent long range dispersal, i.e., PGF being present in the WAP sample and

at least one of the snow metagenomes according to read mapping (Table S1). Variation analysis was performed in Geneious 11.1.5 (38) by applying default settings to the read-mappings generated as explained above.

Gene prediction and annotations

Open reading frames on PGFs were detected using Prodigal in meta mode (113). Functional and taxonomic annotations of predicted proteins of PGFs were performed by DIAMOND searches with a e-value of 10e-05 (114) against FunTaxDB (90) and by using DRAM-v (35). For the full-length genome of the PGF p12D, annotations were improved using HHpred against PDB, Pfam, UniProt-SwissProt-viral and NCBI_Conserved Domains ((115, 116), https://toolkit.tuebingen.mpg.de/tools/hhpred) with a probability threshold of 70%. Sequences of PGFs were BLASTed against IMG/VR 2.0 (117) with an e-value cut-off of 10e-05 to find related phages from other metagenomic datasets.

Synteny of *Ralstonia* phage and phylogenetic comparison of the *zonular occludens toxin* gene

Synteny of known *Ralstonia* sp. phages from NCBI and all Antarctic PGFs that were identified herein and carried the zonular occludens toxin (*zot*, Pfam-ID: PF05707) was performed with tBLASTx comparisons using Easyfig v.2.2.5 (36) on .gbk files generated by Prokka (118). A phylogenetic tree for the MUSCLE-aligned (39) amino acid and nucleic acid sequences of *zot* was constructed using the FastTree (37) algorithm in Geneious 11.1.5 (38).

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Author Contribution statement

J.R. designed the study, wrote the manuscript, and carried out the analyses with input from T.L.V.B.; A.L. and K.S. generated the raw data and performed the sampling; A.J.P conceptualized the project, provided supervision, resources, and was involved in data interpretation; All authors edited drafts of the manuscript.

Author Disclosure Statement

For all authors, no competing financial interests exist.

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