International Journal of Astrobiology

cambridge.org/ija

Long-read sequencing reveals increased occurrence of genomic variants and adenosine methylation in *Bacillus pumilus* SAFR-032 after long-duration flight exposure onboard the International Space Station

Samantha M. Waters[1,2], S. Marshall Ledford[3], Amanda Wacker[4], Sonali Verma[2], Bianca Serda[5,6], Jordan McKaig[7], Joseph Varelas[1], Patrick M. Nicoll[2,8,9], Kasthuri Venkateswaran[10] and David J. Smith[2]

[1]Universities Space Research Association, Columbia, CA, USA; [2]NASA Ames Research Center, Space Biosciences Research Division, Moffett Field, CA, USA; [3]Vassar College, Poughkeepsie, NY, USA; [4]University of California San Diego, La Jolla, CA, USA; [5]Department of Biology, University of New Mexico, Albuquerque, NM, USA; [6]Current: Department of Biochemistry and Molecular Biology, MSU-DOE Plant Research Laboratory Michigan State University, East Lansing, MI, USA; [7]Department of Earth and Atmospheric Sciences, Georgia Institute of Technology, Atlanta, GA, USA; [8]Blue Marble Space Institute of Science, Moffett Field, CA, USA; [9]Current: Department of Psychology, University of Victoria, Victoria, BC, Canada and [10]Biotechnology and Planetary Protection Group, NASA Jet Propulsion Laboratories, California Institute of Technology, Pasadena, CA, USA

Research Article

Cite this article: Waters SM *et al* (2021). Long-read sequencing reveals increased occurrence of genomic variants and adenosine methylation in *Bacillus pumilus* SAFR-032 after long-duration flight exposure onboard the International Space Station. *International Journal of Astrobiology* **20**, 435–444. https://doi.org/10.1017/S1473550421000343

Received: 10 June 2021
Revised: 27 September 2021
Accepted: 10 October 2021
First published online: 16 November 2021

Key words:
Bacillus; ionizing radiation; ISS; methylation; sequencing

Author for correspondence:
Samantha M. Waters,
E-mail: Samantha.m.waters@gmail.com

Abstract

Bacillus pumilus SAFR-032, an endospore-forming bacterial strain, was investigated to determine its methylation pattern (methylome) change, compared to ground control, after direct exposure to space conditions onboard the International Space Station (ISS) for 1.5 years. The resulting ISS-flown and non-flown strains were sequenced using the Nanopore MinION and an in-house method and pipeline to identify methylated positions in the genome. Our analysis indicated genomic variants and m6A methylation increased in the ISS-flown SAFR-032. To complement the broader omics investigation and explore phenotypic changes, ISS-flown and non-flown strains were compared in a series of laboratory-based chamber experiments using an X-ray irradiation source (doses applied at 250, 500, 750, 1000 and 1250 Gy); results show a potentially higher survival fraction of ISS-flown DS2 at the two highest exposures. Taken together, results from this study document lasting changes to the genome by methylation, potentially triggered by conditions in spaceflight, with functional consequences for the resistance of bacteria to stressors expected on long-duration missions beyond low Earth orbit.

Introduction

The primary ATCG sequence of DNA, is a long-lived and stable molecule, which has allowed for its rise as the major informational tablet of billions of years of Earth's living history. Major genomic changes (insertions and deletions, gene duplications and hypermutational rates) may interrupt normal and nominal cellular functioning (Brüssow *et al*., 2004; Behe, 2010; Blank *et al*., 2014). However, terrestrial life is challenged by a number of externally driven environmental and metabolic influences, including, but not limited to, temperature, oxygen, starvation and cellular duplication (i.e. growth), which a cell must react to on molecular timescales. Cells have a number of transcriptional, posttranscriptional and translational mechanisms allowing for nuanced changes and plasticity when perturbed. A major phenotypic and transcriptional control mechanism in eukaryotes is that of DNA methylation and histone modifications, which may act to turn genomic regions 'on' or 'off', and are therefore essential for proper cellular functions (Holliday and Pugh, 1975; Holliday and Grigg, 1993; Reik, 2007; Lister *et al*., 2009; Gigante *et al*., 2019). Such modifications have the potential to persist for generations as heritable changes (i.e., epigenetics). In bacteria, DNA methylation is also present, regulating gene expression and guiding both stress response and DNA repair (Blyn *et al*., 1990; Casadesús and Low, 2006; Fang *et al*., 2012; Beaulaurier *et al*., 2015; Shaiwale *et al*., 2015; Blow *et al*., 2016; Cohen *et al*., 2016; Westphal *et al*., 2016; Nye *et al*., 2019). Although eukaryotic and bacterial genomes may contain multiple DNA methyltransferases (MTases), each with a different substrate specification for methylation of a variety of nucleic acid substrates, the two more widely studied modifications to DNA are to cytosines, C-5 methylation (m5C) and adenosines, methylation of the N-6 (m6A or 6 mA) (Casadesús and Low, 2006; Fang *et al*., 2012; Blow *et al*., 2016; Liu *et al*.,

CAMBRIDGE UNIVERSITY PRESS

2019a, 2019b). Despite decades of DNA methylation research, until recently, a comprehensive genomic determination of methylation states by high-throughput sequencing was prohibitive (Gupta, 2008; Flusberg *et al.*, 2010; Ritchie *et al.*, 2015; Lee *et al.*, 2016; Gigante *et al.*, 2019; Li and Tollefsbol, 2020). However, with the advent of long-read, direct sequencing technologies, it is now possible to pinpoint the effects of environmental stress conditions more robustly on global methylation patterns across a full genome (Liu *et al.*, 2019a, 2019b).

It is known that exposure to environmental stressors may lead to genomic changes in bacteria (Tenaillon *et al.*, 2004; Foster, 2005; Barrick *et al.*, 2009; Blaby *et al.*, 2012; Waters *et al.*, 2015; Khodadad *et al.*, 2017; Maddamsetti *et al.*, 2017). However, little is known about environmental stress as a potential modifier of bacterial methylomes. Work in *E. coli* with antibiotics has shown no substantial variation in the core methylome after exposure (Cohen *et al.*, 2016). Several decades ago, a single study showed that *E. coli* does alter its global methylome after exposures to both ionizing and non-ionizing radiation (Whitfield and Billen, 1972). In the study, there was an increase in adenosine methylation after exposure to ionizing radiation but not non-ionizing radiation (UV). A major consequence of long-duration spaceflight and solar system exploration is the protracted exposure to various types of background radiation, including solar particle events (SPE) and galactic cosmic radiation (GCR) that impart high energy destruction into biological systems (Simonsen *et al.*, 1990; Townsend, 2005; Maurer *et al.*, 2008; Nicholson, 2009; Nicholson *et al.*, 2009; Horneck *et al.*, 2010; Chancellor *et al.*, 2014). On Earth, atmospheric layers largely shield terrestrial life from biocidal ionizing and non-ionizing space radiation; for example, low-wavelength ultraviolet (UV) radiation, such as UV-C (100–280 nm), is attenuated by ozone in the middle stratosphere. Here we expand on decades of bacterial exposure research, which has previously shown survival assays and genomic alterations of a number of organisms exposed directly to the space environment in low-Earth orbit (LEO), by investigating the mutational and methylation pattern changes across the *Bacillus pumilus* SAFR-032 genome after a 1.5 year exposure onboard the International Space Station (ISS) compared to its ground control.

Methods

Bacterial strains, media, spore preparation and growth conditions

Bacillus pumilus SAFR-032, originally isolated from a spacecraft assembly room (Venkateswaran *et al.*, 2001; Link *et al.*, 2004; Gioia *et al.*, 2007), was previously exposed, in spore form, onboard the ISS on the European Technology Exposure Facility (EuTEF) for ∼1.5 years (∼February 2008–September 2009) with parallel ground controls (Vaishampayan *et al.*, 2012). The ISS-exposed spores were part of the UV-vacuum samples, meaning externally mounted on ISS in the EXPOSE facility and with full UV exposure (Vaishampayan *et al.*, 2012). This exposure resulted in nearly all spores being inactivated; however, samples of viable, 'first generation' vegetative cells were recovered, referred to as 56 T-2 in previous reports (Chiang *et al.*, 2019). For our follow-on study, both original, non-flown SAFR-032 and ISS-flown, surviving SAFR-032 spores were germinated (Difco nutrient broth media, 37 °C, 160 RPM, overnight [∼24 h., ∼7 generations]) and used to create vegetative stocks (−80 °C cell stocks in 20% glycerol) for this investigation: hereafter referred to as *non-flown DS1* and *ISS-flown DS2*. Spores of SAFR-032 non-flown DS1 and ISS-flown DS2 were then generated with a standard sporulation method (Schaeffer *et al.*, 1965). Spores were harvested by centrifugation ∼4 days after inoculation into spore prep media from vegetative cell stocks, washed 3 × in cold, sterilized ultra-pure water (PURELAB Chorus 1 System, Evoqua, Pittsburg, PA, USA), and resuspended in sterilized ultra-pure water in addition to 10 µg ml^{-1} filter-sterilized lysozyme (final concentration). The spore and lysozyme mix were incubated at 37 °C for 24 h to digest cellular remains. Spores were 3 × washed and harvested by centrifugation. Pellets were resuspended and stored in 10 mL sterile ultra-pure water at 4 °C. Concentrations of the spore stocks were determined by standard serial dilutions (1:10), plating and colony-forming unit (CFU) counts. Briefly, 100 µl of stocks were serially diluted into 900 µl of sterilized phosphate buffer solution (PBS) and vortexed. Dilutions were plated at 50 µl per dilution onto nutrient agar plates. Plates of DS1 and DS2 were incubated overnight at 37 °C. Concentrations of DS1 and DS2 spores were diluted to a final working concentration of ∼10^7 cells per mL for scanning electron microscopy (SEM) analysis and radiation exposure experimental coupons. These dilutions were chosen to ensure monolayers of spores, as layered biomass has been shown to have a shielding effect on radiation experiments (Khodadad *et al.*, 2017).

Spore coupon preparation

Spores were plated on aluminium coupons (Khodadad *et al.*, 2017), prepared with and without regolith (used for shadowing effects), for ionizing radiation exposures. A Mars regolith simulant, JSC MARS-1 (Allen *et al.*, 1998) was baked at 300 °C for 24 h to prevent contamination. Baked regolith was diluted to 10 mg ml^{-1} in sterilized, ultra-pure water. Coupons were baked at 300 °C for 24 h to prevent contamination and cooled in a sterile biosafety cabinet before application of spore aliquots. Spore stock (∼10^7 spores mL^{-1}) were mixed 1:1 with regolith solution; 5 µl of the solution was spotted onto coupons. For spore coupons without regolith, spore stock was mixed 1:1 with sterilized, ultra-pure water; 5µl of the solution was spotted onto coupons. After coupon preparation, the concentration of triplicate spots was further checked by individually washing spots off the coupons and plating dilutions for CFUs. For washing spots off the coupons, filter-sterilized PBS-Tween 20 (0.5% Tween 20 final concentration) was used at volumes 10–20µl to pipette individual spots off coupons. The results from washing spots show a recovery of spores and cells at concentrations equivalent to stocks used to prepare coupons.

Nucleic acid extraction

Three cultures of non-flown DS1 were inoculated from the freezer stocks into Difco media and grown overnight (37 °C, 160 RPM). Vegetative cells were pelleted and DNA was extracted using the Qiagen PowerViral DNA extraction kit (Hilden, Germany). Modifications of the protocols were as followed: DNA was eluted off the column with 50 µl of 37 °C heated HyClone Molecular Grade H_2O (Cytiva, Marlborough, MA, USA). Quantification of DNA preparations was done with a Qubit fluorometer using the dsDNA Broad Spectrum Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The ISS-flown DS2 was grown separately triplicated from vegetative stocks, cells harvested and DNA extracted and quantified; DS1 and DS2 were processed separately so as not to cross-contaminate the experimental set-up.

https://doi.org/10.1017/S1473550421000343 Published online by Cambridge University Press

Long-read sequencing, methylation determination and mutational analyses

DNA extractions were sequenced on Nanopore's MinION (Oxford, UK) platform using the following: FLO-MIN106 flow cells, SQK-LSK109 library preparation kit, and AmPure beads (Beckman Coulter, Indianapolis, IN, USA). The manufacturer's protocol was modified as follows: total DNA loaded per run was 200 ng; DNA was melted off of AmPure beads by incubating at 37 °C for 10–15 min; final elution of DNA off of beads was done with Molecular Grade H_2O incubated with beads at 37 °C for 10–15 min. Sequencing carried out in triplicate and run information (pores, length of run, total reads, etc.) is listed in Table 1. DS1 and DS2 extracted DNA were run on separate flow cells to prevent cross-contamination of reads. Runs were performed offline without base calling. For base calls, Nanopore's program Guppy v 3.1.5 was used. Reads were analysed for QC using MinIONQC (Lanfear *et al.*, 2019). Mapping was conducted using Graphmap (Sović *et al.*, 2016) using the *B. pumilus* SAFR-032 reference genome (3.7 Mbp), NC_009848.1 (Gioia *et al.*, 2007; Tirumalai *et al.*, 2013), and conversion of resulting sequence alignment map (SAM) to binary alignment map (BAM) files using SAMtools (Li *et al.*, 2009); mapping quality of the reads from each replicate run against the reference genome was performed with AlignQC (Weirather *et al.*, 2017) (Supplemental Figures S1–S6, Supplemental Table S1). Methylation calling of m6A modifications was done using mCaller (McIntyre *et al.*, 2019) and Nanopolish (Loman *et al.*, 2015) (Supplemental Figure S7); only positions with a minimum depth of 15 and more than 50% of reads at a position called as m6A were considered. The non-ISS-flown DS1 consensus of m6A calls was compared to the triplicate consensus of the ISS-flown DS2 runs (Fig. 1). m6A methylations were plotted as total counts in 40 000 bp regions plotted along the genome using R (Figures 1 and S1). Genomic variants were called using Nanopolish using a minimum depth cutoff of 15 and positions with more than 50% of reads agreeing with mutational change; calls were further scrutinized for quality using a BaseCalledFraction cutoff of 0.6 and SupportFraction of 0.8 (Table 2). The BaseCalledFraction is the fraction of called reads that support the variant and the SupportFraction is the fraction of event-space reads (i.e. fast5 data) that support the variant calls. Supplemental Table S2 shows all variants called, including those that were not consistent across all the replicate runs and the threshold reached for each variant across the replicate runs. Genomic variants were analysed to determine if they occurred within a known coding region and if so, what was the potentially corresponding amino acid change, if any. Sequencing data have been deposited in NASA's GeneLab (Ray *et al.*, 2019) under the identifier: GLDS-383 (DOI: 10.26030/dk3z-b805; https://doi.org/10.26030/dk3z-b805).

Scanning electron microscopy

Spore preparations were checked for quality and monolayers with SEM (Hitachi S-4800 Field Emission Scanning Electron Microscope, Tokyo, Japan), without sputter coating, by spotting diluted spores onto aluminium coupons (Supplemental Figure S8).

Ionizing radiation exposures

Exposure experiments were conducted using a Precision X-Rad160 (North Branford, CT, USA) with an aluminium filter. Spore coupons were exposed in parallel at 2500 Gy intervals with a dose rate ~120 cGy min^{-1}. Following each exposure, three spots were harvested for CFU counts. Non-flown DS1 and ISS-flown DS2 were exposed separately, so as not to cross-contaminate experiments. Exposure experiments for DS1 and DS2 without regolith included technical replicates ($n \geq 3$ spots) of biological duplicate experiments (two separate spore preparations used). Figure 2 depicts the plotted survival fractions of exposed to unexposed spores (N/N_0) for each total dose point: 250, 500, 750, 1000 and 1250 Gy. Survival fractions reported are the average CFUs of exposed replicates to the average CFUs of unexposed replicates. For Fig. 2A, the average survival fraction from the two biological duplicate experiments is shown; the individual survival fractions from the biological duplicated experiments are plotted in Figure S9. Due to the concern of terrestrial spores being deposited on the surface of Mars and potentially buried or shielded from radiation exposure by Martian surface dust, spores were additionally exposed to ionizing radiation mixed with a Martian regolith simulant (Fig. 2B). For Figures 2B and S5, exposed and unexposed values are the average CFUs from replicate spots ($n \geq 3$) determined after plating.

Results and discussion

Long-read sequencing of B. pumilus SAFR-032 after long-term exposure to low-earth orbit

Strains of *B. pumilus* SAFR-032 that were retrieved after the previous ISS bacterial exposure payload were sequenced using the Nanopore MinION (Table 1). The ground-control sample, non-flown DS1 and the experimental sample, ISS-flown DS2, were grown, DNA extracted and sequenced in triplicate. Triplicate sequencing allowed for increased confidence in mutational and methylation calls, as a replicate agreement in sequencing may indicate non-stochastic results. DS1 and DS2 samples were processed on separate days, to limit cross-contamination; additionally, the two were sequenced on separate flow cells. Read statistics in Table 1 show most runs resulted in over 1 gigabases of data with the exception of DS2 run 3. This run started with one order of magnitude fewer sequencing pores at the start of sequencing compared to the other runs. However, DS2 run 3 still resulted in a similar N50 compared to the other runs despite the decrease in sequencing pores and resulting reads. The coverage across the reference genome of *B. pumilus* SAFR-032 was >100× for each run with three runs (DS1 runs 1 & 3, DS2 run 1) having potentially >2000× coverage. N50 statistics show that MinION sequencing resulted in long reads with lengths over 7000 nt for most runs. There were also at least 0.5% of reads from each run over 20 000 nt in length, which is ~0.5% of the entire reference genome (~3.7 Mbp).

Mapping and quality of long-reads

Reads were mapped against the *B. pumilus* SAFR-032 genome using Graphmap (Sović *et al.*, 2016), a tool specific for quick and accurate mapping of long-reads. The alignments of reads against the reference genome were analysed and checked for quality using AlignQC (Weirather *et al.*, 2017) (Figures S1–S6, Table S1). All runs had >60% of reads map to the reference genome with >85% of bases correctly aligned (Table S1). The fraction of reads based on length bins that mapped did not fall with

https://doi.org/10.1017/S1473550421000343 Published online by Cambridge University Press

Table 1. Long-read statistics from Nanopore MinION sequencing runs of non-flown DS1 and ISS-flown DS2

Sample name	Sequencing run	Total sequences base called (Guppy)	N50	Total gigabases	Potential coverage of genome	Sequence maximum length	# Reads >10 kb	# Reads >20 kb	# Reads >50 kb
DS1	1	1 900 357	8530	10.6	2650	66 230	300 562	13 263	11
DS1	2	720 823	6092	2.86	715	39 541	32 560	386	–
DS1	3	2 121 781	6918	10.78	2695	44 033	172 910	3498	–
DS2	1	1 669 578	7505	8.6	2150	49 982	183 923	3330	–
DS2	2	430 537	7316	1.51	378	47 116	32 652	1017	–
DS2	3	153 860	7163	0.61	153	55 011	12 356	316	1

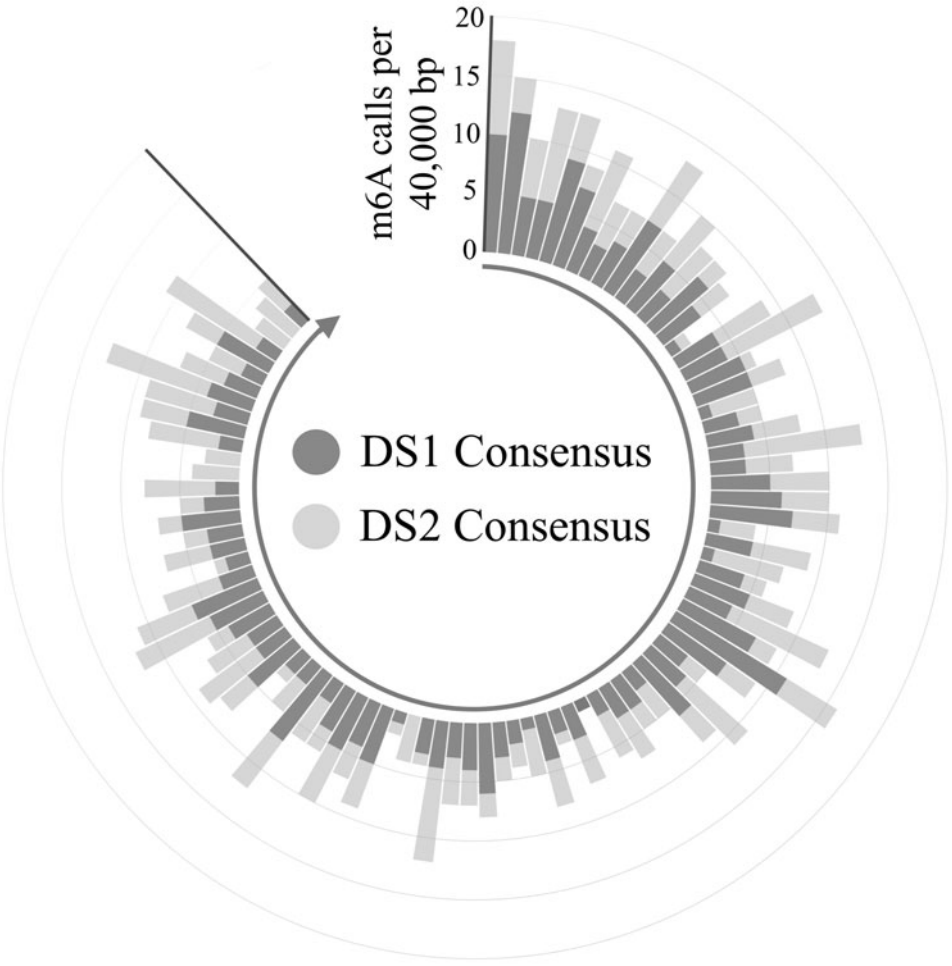

Fig. 1. Total m6A Nanopolish and mCaller consensus counts across the *Bacillus subtilis* SAFR-032 genome, binned at 40 kbp lengths, for non-flown DS1 versus ISS-flown DS2.

increasing length (Figures S1–S6), indicating that reads >4000 nt were not the result of chimaeras (left and right read passing through a sequencing pore and resulting in one long read) nor were more prone to errors than shorter reads of only a few thousand nucleotides. Base error type (mismatch, insertion, or deletion) percentages were mostly <5%, with the exception of DS2.2 runs 2 and 3 with slightly higher per cent insertion bases of 5.2 and 5.3, respectively. MinION has been shown to accurately sequence long-reads, discernable at the strain level, of *Bacillus* species from mock and mixed communities (Brown *et al.*, 2017; Sanderson *et al.*, 2018; Deshpande *et al.*, 2019; Dilthey *et al.*, 2019; Burton *et al.*, 2020; Leidenfrost *et al.*, 2020). These results further demonstrate and support the ability of MinION in sequencing *Bacillus* species and the accurate and fast mapping

https://doi.org/10.1017/S1473550421000343 Published online by Cambridge University Press

Table 2. Single-nucleotide polymorphisms (SNPs) present in ISS-flown DS2 and absent in non-flown DS1

Position on genome	Locus tag (NCBI; GenBank)	Mutation	Amino acid change	Annotated function(s) (NCBI)
105 751	BPUM_RS 00540; BPUM_0073	C→T	–	DNA integrity scanning protein DisA
927 703	BPUM_RS 04400; BPUM_0849	A→T	T133F	PqqD family protein
927 704	BPUM_RS 04401; BPUM_0849	C→T	T133F	PqqD family protein
927 705	BPUM_RS 04402; BPUM_0849	A→T	T133F	PqqD family protein
1 856 466	BPUM_RS 19070; BPUM_1804	A→T	–	S-layer family protein
2 088 546	BPUM_RS 10245; BPUM_2044	G→C	–	HAMP domain-containing protein
2 659 471	BPUM_RS 13160; BPUM_2646	C→T	–	NCS2 family permease
2 833 137	BPUM_RS14185; BPUM_2832	C→T	A307 V	Na + /H + antiporter subunit D
2 833 138	BPUM_RS14186; BPUM_2832	C→T	A307 V	Na + /H + antiporter subunit D
3 274 486	BPUM_RS16320; BPUM_3288	G→A	–	flagellar hook-basal body protein

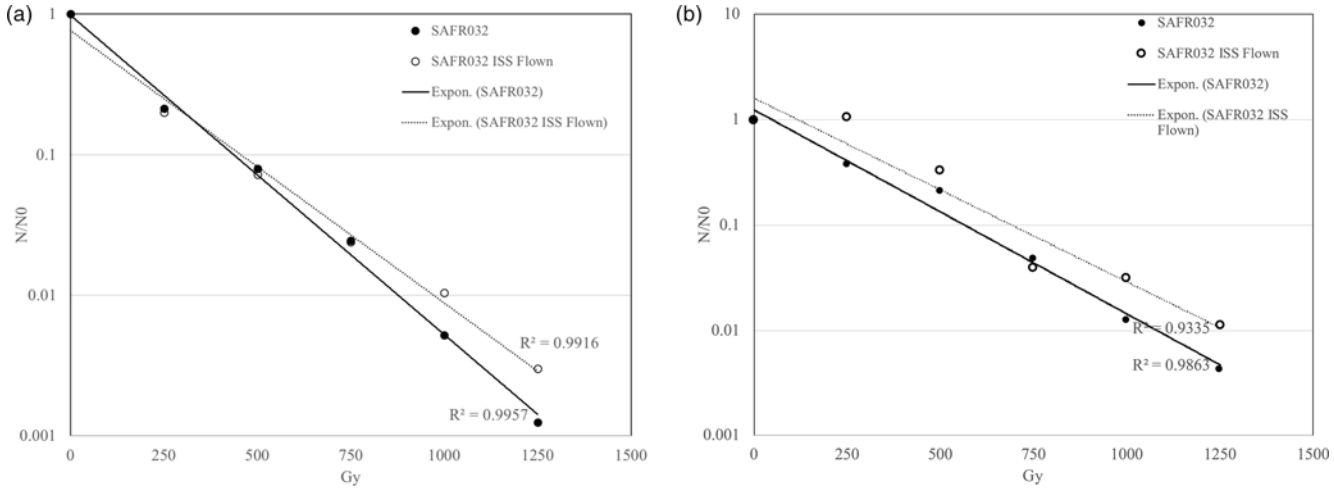

Fig. 2. Survival fractions of non-flown DS1 (filled) and ISS-flown DS2 (unfilled) spore ionizing radiation exposures: (a) spores and (b) spores mixed with a Mars regolith analogue. The fraction at 0 Gy for both datasets is 1.

of resulting long-reads against reference genomes using long-read specific programs such as Graphmap.

Genomic changes occurred in B. pumilus *SAFR-032 after long-term exposure to low-earth orbit*

Nanopolish, a software developed by Nanopore for analysing MinION long-read data, was used to make variant calls from the mapped reads to the reference genome (Table 2). Variations (mismatch, insertions and deletions) are reported that occurred in at least 50% of reads mapped at a position coverage of >15; results are further marked in Table S2 for their quality of (i) occurrence in each replicate sequencing run, (ii) BaseCalledFraction cutoff of >0.6 and (iii) SupportFraction of >0.8. There were 10 mutations in ISS-flown DS2 compared to non-flown DS1 that were identifiable in all DS2 sequencing replicates, and with the exception of positions 927 704 and 927 705, were all above cutoff values for quality (Table 2). Interestingly, 5 of the mutations occur in two separate codons and are the only mutations that result in an amino acid change in a coding region. Of the other 5 synonymous mutations, 4 were in the wobble position of their respective codons; while these mutations do not have a known effect on translation, it is conceivable these mutations might affect the structure of their DNA and/or RNA regions.

Additionally, five of the ten mutations were C to T transition mutations. The change in cytosine to thymine has been linked to methylation of cytosine (m5C) resulting in deamination to T (Selker and Stevens, 1985; Holliday and Grigg, 1993; Poole *et al.*, 2001; Walsh and Xu, 2006). Previously, SAFR-032's genome has been compared to genomes of other *Bacillus* species for the purpose of determining gene content that may be involved with its increased non-ionizing radiation resistance (Gioia *et al.*, 2007); Gioia *et al.*, identified a C-5 cytosine-specific DNA methyltransferase that was present in the genome of SAFR-032 but absent in other *Bacillus* species. Methylation analysis for m5C by DNA cytosine methyltransferase (dcm) is a feature of Nanopolish's methylation calling, but it is not included in mCaller. An initial screening of non-flown DS1 and ISS-flown DS2 did not reveal a level of m5C calls above the cutoff threshold described in the Methods section. The proportion of m5C called reads at a position to the non-methylated reads was < 0.10. This

https://doi.org/10.1017/S1473550421000343 Published online by Cambridge University Press

does not exclude the potential of m5C methylation in the SAFR-032 genome, as these results could be due to the inability of Nanopolish to detect (i) m5C positions due to its training for detection using solely *E. coli* reads and (ii) the presence of methylated cytosines outside of the known motif, CCWGG. For both non-flown DS1 and ISS-flown DS2, there were 56 variations from the reference genome that will need to be individually confirmed in the future and annotated as potential changes from the time of sequencing *B. pumilus* SAFR-032 to the present.

Long-term exposure to low-earth orbit increased m6A methylation across the B. pumilus *SAFR-032 genome*

Two independent methylation calling programs, mCaller and Nanoplish, were used to determine the m6A methylation pattern changes, if any, occurring between the ISS-flown DS2 and its ground control, non-flown DS1. Methylations were reported for positions with >15 × coverage and >50% reads having a methylated fraction. Most striking was the difference in absolute numbers of m6A calls between mCaller and Nanopolish. Nanopolish, markedly, in all replicates of both DS1 and DS2, called fewer m6A methylated positions than mCaller (Figure S7). The reason for the difference is unknown but potentially due to the training algorithms used as both Nanopolish and mCaller references indicated that their detection methods for the voltage differences of methylated bases were trained using *E. coli* sequences (Simpson *et al.*, 2017; McIntyre *et al.*, 2019). The consensus of both mCaller and Nanopolish m6A calls for non-flown DS1 and ISS-flown DS2 were therefore compared (Fig. 1). Results of the m6A consensus comparison reveal that ISS-flown DS2 displayed higher methylation across the genome compared to the non-flown DS1.

The modifications reported here by both mCaller and Nanopolish occurred at the palindromic GATC consensus motif for all m6A called. Because both of these programs were trained using *E. coli* sequences with GATC m6A modifications, these results were not surprising. It has been reported that Gram-negative bacterial genomes have a relatively consistent methylation pattern of m6A for GATC positions through various growth phases and after exposure to antibiotics (Shaiwale *et al.*, 2015; Cohen *et al.*, 2016; Westphal *et al.*, 2016; Liu *et al.*, 2020). Additionally, not all GATC positions are methylated, with some being heritably non-methylated (Blyn *et al.*, 1990; Casadesús and Low, 2006). This indicates that the GATC pattern across a genome could potentially change after a stress event and that this pattern change could be heritable. A search of the *B. pumilus* SAFR-032 genome for GATC reveals >17 000 occurrences; the results of the m6A methylation consensus calls from mCaller and Nanopolish show less than 10% (~1500) are potentially methylated in ISS-flown DS2.

ISS-flown DS2 was isolated from a nearly complete sample inactivation resulting from a 1.5 yr exposure to space and solar radiation (Vaishampayan *et al.*, 2012). It is very likely the surviving samples were shielded from UV radiation by overlying spore biomass in the sample, as direct UV exposure fully inactivates spores as reported elsewhere (Horneck *et al.*, 2001; Schuerger *et al.*, 2003; Schuerger and Nicholson, 2006; Osman *et al.*, 2008; Vaishampayan *et al.*, 2012; Khodadad *et al.*, 2017). However, protective effects from layering would not lessen ionizing radiation impacting ISS-flown DS2 during the 1.5 year exposure. It has been reported that when exposed to increased ionizing radiation, *E. coli* shows increased methylation of adenosine and cytosine across the genome (Whitfield and Billen, 1972); this study, conducted in 1972, is possibly the first report of increased genomic methylation after ionizing radiation exposure. However, this phenomenon is not found only in bacteria, as decades of cancer radiation biology research has shown a phenotype switching that occurs in cells exposed to ionizing radiation and that this is potentially partially due to increased and/or changed adenosine methylation in the genome (Miousse *et al.*, 2017; Chi *et al.*, 2018). Interestingly, DNA from HeLa cells also increases methylation after UV exposure (Low *et al.*, 1976); while the Whitfield and Billen study of *E. coli* showed the inverse: a decrease in global methylation after UV exposure (Whitfield and Billen, 1972). While still needing more work, there does seem to be an overlap in organisms, both eukaryotic and prokaryotic, in methylation of the genome and radiotolerance, as seen in the research looking at alkylation and radiation resistance in cells (Brendel *et al.*, 1970; Löser *et al.*, 2010; Ullmann *et al.*, 2021). Underlying reasoning may be that DNA repair mechanisms may mark DNA points with methyl groups after environmental stress. DNA is a target of cellular damage during exposure to both non-ionizing and ionizing radiation. As there was no detectable change in methylation after antibiotic exposure (Cohen *et al.*, 2016) using β-lactam and quinolones, antibiotics with mechanisms not targeting DNA, this may be specific to environmental insults that affect DNA.

It has been reported that the ISS-flown SAFR-032 space-exposed surviving strains have an increased UV survival phenotype compared to controls (Vaishampayan *et al.*, 2012). However, ISS-flown DS2 was most likely shielded from UV while still being exposed to penetrating ionizing radiation (~130–180 mGy, (Horneck *et al.*, 2012)). Here, non-flown DS1 and ISS-flown DS2 spores were exposed to increasing ionizing radiation and the survival fraction in relation to unexposed spores were plotted (Figures 2 and S9). This was repeated for two separate spore preparations (i.e. biological duplicates) and in both experiments ISS-flown DS2 appears to have a less steep kill slope as determined by the exponential trendline. This difference in ionizing radiation survival fraction between non-flown DS1 and ISS-flown DS2 occurs at the two highest exposures 1000 and 1250 Gy, where DS2 has a survival fraction twice that of DS1 for both biological replicates. For the first exposure experiment, DS2 has a survival fraction of 0.0187 at 1000 Gy and 0.0051 at 1250 Gy compared to DS1 survival fraction of 0.0094 at 1000 Gy and 0.0021 at 1250 Gy. For the second exposure experiment, DS2 had a survival fraction of 0.0021 at 1000 Gy and 0.0009 at 1250 Gy compared to DS1 survival fractions at 1000 Gy of 0.0010 and at 1250 Gy of 0.0004. The average spores per spot at the beginning of the experiments do not explain this difference. For DS1, the first experiment has an average of 1.6×10^6 spores/spot and the second experiment has an average of 6.4×10^6 spores/spot; DS2 averaged 5×10^5 spores/spot for the first experiment and 4.6×10^6 spores/spot for the second. In both experiments, DS1 averages at the start of the experiment were slightly higher than DS2, but the survival fractions for DS1 drop below those of DS2 at the two highest exposures.

Spores were also tested in the presence of a Martian regolith simulant (Fig. 2B). With regolith present during exposure, ISS-flown DS2 also showed an elevated tolerance to higher doses with a survival fraction of 0.0313 at 1000 Gy and 0.0112 at 1250 Gy compared to DS1 survival fractions of 0.0124 at 1000 Gy and 0.0042 at 1250 Gy (DS1 and DS2 averaged 6×10^5 and 3.9×10^5 spores/spot at the start of the experiment). While regolith has been reported to mitigate the lethal effects of non-ionizing radiation exposure by shielding spores of *Bacillus* species (Osman *et al.*, 2008), ionizing radiation is not blocked by thin

https://doi.org/10.1017/S1473550421000343 Published online by Cambridge University Press

layers of regolith and may actually have an increased microbiocidal affect when regolith is present (Horneck *et al.*, 2001; Nicholson, 2009; Moeller *et al.*, 2010). However, this data set, the survival fractions of DS1 and DS2 are 0.004 and 0.011, respectively, in the presence of regolith at 1250 Gy. This is elevated from the survival fractions without regolith. This could be due to larger regolith grains providing more protection to some spores (Fig. S9C), but is unclear as this was experiment was not repeated on multiple different spore preparations. More studies are needed in the future to determine the full extent of an elevated survival phenotype and if methylation of the genome plays a role.

It is important to note that GATC methylated positions are not typically associated with *Bacillus* species due to a number of historic reasons. First, much of what was previously known of methylation in bacterial genomes revolved around the Gram-(−) organisms, specifically, *E. coli*, where methylation is an important component in the detection and destruction of foreign DNA by restriction-modification (RM) mechanisms, which when compared to its Gram-(+) counterpart, *Bacillus subtilis*, reveals a lack of the full RM machinery (Lenhart *et al.*, 2012). Second, the lack of available tools to determine global methylation states of bacterial genomes has limited the widespread study of comparative methylation motifs and genome patterns across bacteria, until recently (Blow *et al.*, 2016). In 2020, Nye *et al.*, using a third-generation sequencing platform, PacBio's SMRT, studied the global m6A patterns in the genome of *B. subtilis* and identified a previously undescribed methyltransferase, DnmA, which methylates adenosines at non-palindromic sites (Nye *et al.*, 2020). An additional, and possibly more nuanced, reason behind the limited knowledge of methylation patterns in Gram-(+) organisms compared to Gram-(−) is the application of the well-studied model organism being considered canonical for all; in other words, what happens in *B. subtilis* must be true for all the other *Bacillus* species. In direct confrontation to this, evidence of m6A methylation at palindromic GATC sites in other *Bacillus* species has existed for several decades (Hattman *et al.*, 1978; BUENO *et al.*, 1986; Dingman, 1990). Most notable, it has been shown that even among the strain types of *Bacillus velezensis* UCMB5140 and UCMB5021 there is a difference in methylation type and pattern (Reva *et al.*, 2019). The differences of methylation type become more dramatic when making cross-species comparisons in *Bacillus* species (Reva *et al.*, 2020). As previously reported, *B. pumilus* SAFR-032 has an increased survival phenotype for non-ionizing radiation exposure compared to *B. subtilis* (Gioia *et al.*, 2007), which may be due to differences in methylation (Liu *et al.*, 2020; Reva *et al.*, 2020) and genetic features (Gioia *et al.*, 2007).

It is clear that for high-throughput Nanopore MinION identification of methylation modifications, programs need to be more robustly trained on different (i) methylation sequence motifs and (ii) bacterial sequences outside of *E. coli.* A literature review shows that genome-wide methylation modification research is dominated by the use of PacBio's SMRT platform (Flusberg *et al.*, 2010; Fang *et al.*, 2012; Lluch-Senar *et al.*, 2013; Krebes *et al.*, 2014; Beaulaurier *et al.*, 2015; Zautner *et al.*, 2015; Cohen *et al.*, 2016; Westphal *et al.*, 2016; Couturier and Lindås, 2018; Hagemann *et al.*, 2018; Nicholson *et al.*, 2018; Payelleville *et al.*, 2018; Zhao *et al.*, 2018; Forde *et al.*, 2019; Chhotaray *et al.*, 2020; Coy *et al.*, 2020; Estibariz *et al.*, 2020; Liu *et al.*, 2020; Nye *et al.*, 2019, 2020; Reva *et al.*, 2020) compared to Nanopore's Minion (Rand *et al.*, 2017; Jain *et al.*, 2018; Giesselmann *et al.*, 2019; Gigante *et al.*, 2019; Miga *et al.*, 2019; Zhang *et al.*, 2021); of particular note, the organisms that have been studied using Nanopore's Minion tend to be eukaryotic. And with a few exceptions, bacterial methylomes are dominated by reads from the SMRT platform. There could be a number of factors influencing individual research projects use of SMRT over the MinION (Szopa-Comley, 2013), but it seems that MinION methylation data is often paired with bisulphate sequencing, which excludes investigation of methylated adenosines, the predominant methylation type in bacteria. Additionally, MinION methylation detection seems to be limited to the ability of researchers to train their own datasets using already developed software or to develop in-house methylation calling tools (Rand *et al.*, 2017; Gießelmann *et al.*, 2018; McIntyre *et al.*, 2019; Liu *et al.*, 2019a, 2019b). These together could explain the over-representation of methylation calls in eukaryotic organisms with the MinION platform as the Nanopolish methylation caller has three cytosine methylation presets for 'cpg', 'dcm' and 'gpc' compared to only 'dam' for adenosine methylation; cytosine methylation is the major studied methylation type in eukaryotes. We also report on the results of the eventalign command output, required by both mCaller and Nanopolish, as a potential limitation for MinION usage (Supplemental Information: Discussion).

Conclusion

It was expected that after exposure to the effects of radiation in LEO there would be mutational differences between the ISS-flown and non-ISS flown strains. Most striking was the increase of methylated adenosines (m6A) across the genome in the ISS-flown strain. This is only the second bacterial species that has been reported to increase adenosine methylation after ionizing radiation exposure. The mechanism is unclear as to why methylation may increase, but it could be a possible marking of damaged DNA points after certain types of environmental exposures that affect DNA.

Global methylation changes in bacteria after increased exposure to radiation could lead to adaptive phenotypic changes to extreme environments, such as the surface of Mars. While decades of research supports the near-complete biocidal effect of UV radiation on bacterial cells, shielding acts as a mitigating factor for survival. Shielding, either from natural environmental factors, such as soil deposition, or inside the man-made craft, eliminates non-ionizing radiation, but does not prevent penetrating, ionizing radiation. Chronic ionizing radiation exposure is of high relevance to space biology and exobiology. Future work that looks outside the biocidal effects and more to the potential adaptive repercussions of exposure survivors could generate products for alleviating human health risks associated with spaceflight. Our results indicate the usefulness of bacterial ionizing radiation experiments in studying methylomic and phenotypic changes as a proxy for animal exposure experiments. Additionally, results here support the use of the Nanopore MinION platform for methylome research. More work is still needed to understand the mechanism(s) that are responsible for increased adenosine methylation after ionizing radiation exposure and if such a response is ubiquitous in terrestrial organisms.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1473550421000343.

Acknowledgements. The authors would like to acknowledge Jon Rask for his regolith samples used.

Financial support. This work was funded by the NASA Postdoctoral Fellowship Program (S.M.W.), NASA Space Biology and NASA Planetary Protection research grants (D.J.S. and K.V.), the Blue Marble Institute Training Program (P.N. and S.V.) and the Space Life Sciences Training Program (B.M.S., J.M., A.W., & S.M.L.). The research described in this publication was carried out in part at the Jet Propulsion Laboratory, California Institute of Technology, under a contract with NASA (K.V.). Government sponsorship is acknowledged.

Conflicts of interests. None.

References

Allen CC, Morris RV, Jager KM, Golden D, Lindstrom DJ, Lindstrom MM and Lockwood JP (1998) Martian regolith simulant JSC Mars-1. Presented at the Lunar and planetary science conference, p. 1690.

Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK, Schneider D, Lenski RE and Kim JF (2009) Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* **461**, 1243–1247.

Beaulaurier J, Zhang X-S, Zhu S, Sebra R, Rosenbluh C, Deikus G, Shen N, Munera D, Waldor MK, Chess A, Blaser MJ, Schadt EE and Fang G (2015) Single molecule-level detection and long read-based phasing of epigenetic variations in bacterial methylomes. *Nature Communications* **6**, 7438.

Behe MJ (2010) Experimental evolution, loss-of-function mutations, and "the first rule of adaptive evolution". *The Quarterly Review of Biology* **85**, 419–445.

Blaby IK, Lyons BJ, Wroclawska-Hughes E, Phillips GC, Pyle TP, Chamberlin SG, Benner SA, Lyons TJ, de Crécy-Lagard V and de Crécy E (2012) Experimental evolution of a facultative thermophile from a mesophilic ancestor. *Applied and Environmental Microbiology* **78**, 144–155.

Blank D, Wolf L, Ackermann M and Silander OK (2014) The predictability of molecular evolution during functional innovation. *Proceedings of the National Academy of Sciences of the USA* **111**, 3044–3049.

Blow MJ, Clark TA, Daum CG, Deutschbauer AM, Fomenkov A, Fries R, Froula J, Kang DD, Malmstrom RR and Morgan RD (2016) The epigenomic landscape of prokaryotes. *PLoS Genetics* **12**, e1005854.

Blyn LB, Braaten BA and Low DA (1990) Regulation of pap pilin phase variation by a mechanism involving differential dam methylation states. *The EMBO Journal* **9**, 4045–4054.

Brendel M, Khan N and Haynes R (1970) Common steps in the repair of alkylation and radiation damage in yeast. *Molecular and General Genetics MGG* **106**, 289–295.

Brown BL, Watson M, Minot SS, Rivera MC and Franklin RB (2017) MinION™ nanopore sequencing of environmental metagenomes: a synthetic approach. *Gigascience* **6**, gix007.

Brüssow H, Canchaya C and Hardt W-D (2004) Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews* **68**, 560–602.

Bueno A, Villanueva JR and Villa TG (1986) Methylation of spore DNA in Bacillus coagulans strain 26. *Microbiology* **132**, 2899–2905.

Burton AS, Stahl SE, John KK, Jain M, Juul S, Turner DJ, Harrington ED, Stoddart D, Paten B and Akeson M (2020) Off earth identification of bacterial populations using 16S rDNA nanopore sequencing. *Genes* **11**, 76.

Casadesús J and Low D (2006) Epigenetic gene regulation in the bacterial world. *Microbiology and Molecular Biology Reviews* **70**, 830–856.

Chancellor JC, Scott GB and Sutton JP (2014) Space radiation: the number one risk to astronaut health beyond low earth orbit. *Life* **4**, 491–510.

Chhotaray C, Wang S, Tan Y, Ali A, Shehroz M, Fang C, Liu Y, Lu Z, Cai X and Hameed HA (2020) Comparative analysis of whole-genome and methylome profiles of a smooth and a rough *Mycobacterium abscessus* clinical strain. *G3: Genes, Genomes, Genetics* **10**, 13–22.

Chi H-C, Tsai C-Y, Tsai M-M and Lin K-H (2018) Impact of DNA and RNA methylation on radiobiology and cancer progression. *International Journal of Molecular Sciences* **19**, 555.

Chiang AJ, Mohan GBM, Singh NK, Vaishampayan PA, Kalkum M and Venkateswaran K (2019) Alteration of proteomes in first-generation cultures of *Bacillus pumilus* spores exposed to outer space. *Msystems* **4**.

Cohen NR, Ross CA, Jain S, Shapiro RS, Gutierrez A, Belenky P, Li H and Collins JJ (2016) A role for the bacterial GATC methylome in antibiotic stress survival. *Nature Genetics* **48**, 581–586.

Couturier M and Lindås A-C (2018) The DNA methylome of the hyperthermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*. *Frontiers in Microbiology* **9**, 137.

Coy SR, Gann ER, Papoulis SE, Holder ME, Ajami NJ, Petrosino JF, Zinser ER, Van Etten JL and Wilhelm SW (2020) SMRT Sequencing of *Paramecium bursaria* chlorella virus-1 reveals diverse methylation stability in adenines targeted by restriction-modification systems. *Frontiers in Microbiology* **11**, 887.

Deshpande SV, Reed TM, Sullivan RF, Kerkhof LJ, Beigel KM and Wade MM (2019) Offline next-generation metagenomics sequence analysis using MinION detection software (MINDS). *Genes* **10**, 578.

Dilthey AT, Jain C, Koren S and Phillippy AM (2019) Strain-level metagenomic assignment and compositional estimation for long reads with MetaMaps. *Nature Communications* **10**, 1–12.

Dingman DW (1990) Presence of N6-methyladenine in GATC sequences of *Bacillus popilliae* and *Bacillus lentimorbus* KLN2. *Journal of Bacteriology* **172**, 6156.

Estibariz I, Ailloud F, Woltemate S, Bunk B, Spröer C, Overmann J, Aebischer T, Meyer TF, Josenhans C and Suerbaum S (2020) In vivo genome and methylome adaptation of cag-negative *Helicobacter pylori* during experimental human infection. *Mbio* **11**.

Fang G, Munera D, Friedman DI, Mandlik A, Chao MC, Banerjee O, Feng Z, Losic B, Mahajan MC and Jabado OJ (2012) Genome-wide mapping of methylated adenine residues in pathogenic *Escherichia coli* using single-molecule real-time sequencing. *Nature Biotechnology* **30**, 1232–1239.

Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J and Turner SW (2010) Direct detection of DNA methylation during single-molecule, real-time sequencing. *Nature Methods* **7**, 461–465.

Forde BM, McAllister LJ, Paton JC, Paton AW and Beatson SA (2019) SMRT Sequencing reveals differential patterns of methylation in two O111: H-STEC isolates from a hemolytic uremic syndrome outbreak in Australia. *Scientific Reports* **9**, 1–11.

Foster PL (2005) Stress responses and genetic variation in bacteria. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **569**, 3–11.

Gießelmann P, Brändl B, Raimondeau E, Bowen R, Rohrandt C, Tandon R, Kretzmer H, Assum G, Galonska C, Siebert R, Ammerpohl O, Heron A, Schneider SA, Ladewig J, Koch P, Schuldt BM, Graham JE, Meissner A and Müller F-J (2018) Repeat expansion and methylation state analysis with nanopore sequencing. *bioRxiv*, 480285. https://doi.org/10.1101/480285.

Giesselmann P, Brändl B, Raimondeau E, Bowen R, Rohrandt C, Tandon R, Kretzmer H, Assum G, Galonska C and Siebert R (2019) Analysis of short tandem repeat expansions and their methylation state with nanopore sequencing. *Nature Biotechnology* **37**, 1478–1481.

Gigante S, Gouil Q, Lucattini A, Keniry A, Beck T, Tinning M, Gordon L, Woodruff C, Speed TP, Blewitt ME and Ritchie ME (2019) Using long-read sequencing to detect imprinted DNA methylation. *Nucleic Acids Research* **47**, e46–e46. https://doi.org/10.1093/nar/gkz107.

Gioia J, Yerrapragada S, Qin X, Jiang H, Igboeli OC, Muzny D, Dugan-Rocha S, Ding Y, Hawes A, Liu W, Perez L, Kovar C, Dinh H, Lee S, Nazareth L, Blyth P, Holder M, Buhay C, Tirumalai MR, Liu Y, Dasgupta I, Bokhetache L, Fujita M, Karouia F, Eswara Moorthy P, Siefert J, Uzman A, Buzumbo P, Verma A, Zwiya H, McWilliams BD, Olowu A, Clinkenbeard KD, Newcombe D, Golebiewski L, Petrosino JF, Nicholson WL, Fox GE, Venkateswaran K, Highlander SK and Weinstock GM (2007) Paradoxical DNA repair and peroxide resistance gene conservation in *Bacillus pumilus* SAFR-032. *PLoS One* **2**, e928.

Gupta PK (2008) Single-molecule DNA sequencing technologies for future genomics research. *Trends in Biotechnology* **26**, 602–611.

Hagemann M, Gärtner K, Scharnagl M, Bolay P, Lott SC, Fuss J, Huettel B, Reinhardt R, Klähn S and Hess WR (2018) Identification of the DNA methyltransferases establishing the methylome of the cyanobacterium *Synechocystis* sp. PCC 6803. *DNA Research* **25**, 343–352.

Hattman S, Keister T and Gottehrer A (1978) Sequence specificity of DNA methylases from *Bacillus amyloliquefaciens* and *Bacillus brevis*. *Journal of Molecular Biology* **124**, 701–711.

Holliday R and Grigg G (1993) DNA methylation and mutation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **285**, 61–67.

Holliday R and Pugh JE (1975) DNA Modification mechanisms and gene activity during development. *Science* **187**, 226–232.

Horneck G, Rettberg P, Reitz G, Wehner J, Eschweiler U, Strauch K, Panitz C, Starke V and Baumstark-Khan C (2001) Protection of bacterial spores in space, a contribution to the discussion on panspermia. *Origins of Life and Evolution of the Biosphere* **31**, 527–547.

Horneck G, Klaus DM and Mancinelli RL (2010) Space microbiology. *Microbiology and Molecular Biology Reviews* **74**, 121–156.

Horneck G, Moeller R, Cadet J, Douki T, Mancinelli RL, Nicholson WL, Panitz C, Rabbow E, Rettberg P and Spry A (2012) Resistance of bacterial endospores to outer space for planetary protection purposes—experiment PROTECT of the EXPOSE-E mission. *Astrobiology* **12**, 445–456.

Jain M, Koren S, Miga KH, Quick J, Rand AC, Sasani TA, Tyson JR, Beggs AD, Dilthey AT, Fiddes IT, Malla S, Marriott H, Nieto T, O'Grady J, Olsen HE, Pedersen BS, Rhie A, Richardson H, Quinlan AR, Snutch TP, Tee L, Paten B, Phillippy AM, Simpson JT, Loman NJ and Loose M (2018) Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nature Biotechnology* **36**, 338–345.

Khodadad CL, Wong GM, James LM, Thakrar PJ, Lane MA, Catechis JA and Smith DJ (2017) Stratosphere conditions inactivate bacterial endospores from a Mars spacecraft assembly facility. *Astrobiology* **17**, 337–350.

Krebes J, Morgan RD, Bunk B, Spröer C, Luong K, Parusel R, Anton BP, König C, Josenhans C and Overmann J (2014) The complex methylome of the human gastric pathogen *Helicobacter pylori*. *Nucleic Acids Research* **42**, 2415–2432.

Lanfear R, Schalamun M, Kainer D, Wang W and Schwessinger B (2019) MinIONQC: fast and simple quality control for MinION sequencing data. *Bioinformatics* **35**, 523–525.

Lee H, Gurtowski J, Yoo S, Nattestad M, Marcus S, Goodwin S, McCombie WR and Schatz MC (2016) Third-generation sequencing and the future of genomics. *BioRxiv*, 048603.

Leidenfrost RM, Pöther D-C, Jäckel U and Wünschiers R (2020) Benchmarking the minion: evaluating long reads for microbial profiling. *Scientific Reports* **10**, 1–10.

Lenhart JS, Schroeder JW, Walsh BW and Simmons LA (2012) DNA Repair and genome maintenance in *Bacillus subtilis*. *Microbiology and Molecular Biology Reviews* **76**, 530–564.

Li S and Tollefsbol TO (2020) DNA Methylation methods: global DNA methylation and methylomic analyses. *Methods* **187**, 28–43.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G and Durbin R (2009) The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078–2079.

Link L, Sawyer J, Venkateswaran K and Nicholson W (2004) Extreme spore UV resistance of *Bacillus pumilus* isolates obtained from an ultraclean spacecraft assembly facility. *Microbial Ecology* **47**, 159–163.

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z and Ngo Q-M (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* **462**, 315–322.

Liu H, Begik O, Lucas MC, Ramirez JM, Mason CE, Wiener D, Schwartz S, Mattick JS, Smith MA and Novoa EM (2019a) Accurate detection of m 6 A RNA modifications in native RNA sequences. *Nature Communications* **10**, 1–9.

Liu L, Zhang Y, Jiang D, Du S, Deng Z, Wang L and Chen S (2019b) Recent advances in the genomic profiling of bacterial epigenetic modifications. *Biotechnology Journal* **14**, 1800001.

Liu G, Jiang Y-M, Liu Y-C, Han L-L and Feng H (2020) A novel DNA methylation motif identified in *Bacillus pumilus* BA06 and possible roles in the regulation of gene expression. *Applied Microbiology and Biotechnology* **104**, 3445–3457.

Lluch-Senar M, Luong K, Lloréns-Rico V, Delgado J, Fang G, Spittle K, Clark TA, Schadt E, Turner SW, Korlach J and Serrano L (2013) Comprehensive methylome characterization of *Mycoplasma genitalium* and *Mycoplasma pneumoniae* at single-base resolution. *PLoS Genetics* **9**, e1003191–e1003191.

Loman NJ, Quick J and Simpson JT (2015) A complete bacterial genome assembled de novo using only nanopore sequencing data. *Nature Methods* **12**, 733–735.

Löser DA, Shibata A, Shibata AK, Woodbine LJ, Jeggo PA and Chalmers AJ (2010) Sensitization to radiation and alkylating agents by inhibitors of poly (ADP-ribose) polymerase is enhanced in cells deficient in DNA double-strand break repair. *Molecular Cancer Therapeutics* **9**, 1775.

Low M, Read EL and Borek E (1976) Methylation of DNA in HeLa cells after ultraviolet irradiation. *International Journal of Radiation Oncology*Biology*Physics* **1**, 289–294.

Maddamsetti R, Hatcher PJ, Green AG, Williams BL, Marks DS and Lenski RE (2017) Core genes evolve rapidly in the long-term evolution experiment with *Escherichia coli*. *Genome Biology and Evolution* **9**, 1072–1083.

Maurer RH, Fraeman ME, Martin MN and Roth DR (2008) Harsh environments: space radiation. *Johns Hopkins APL Technical Digest* **28**, 17.

McIntyre AB, Alexander N, Grigorev K, Bezdan D, Sichtig H, Chiu CY and Mason CE (2019) Single-molecule sequencing detection of N 6-methyladenine in microbial reference materials. *Nature Communications* **10**, 1–11.

Miga KH, Koren S, Rhie A, Vollger MR, Gershman A, Bzikadze A, Brooks S, Howe E, Porubsky D and Logsdon GA (2019) Telomere-to-telomere assembly of a complete human X chromosome. *BioRxiv*, 735928.

Miousse IR, Kutanzi KR and Koturbash I (2017) Effects of ionizing radiation on DNA methylation: from experimental biology to clinical applications. *Null* **93**, 457–469.

Moeller R, Rohde M and Reitz G (2010) Effects of ionizing radiation on the survival of bacterial spores in artificial Martian regolith. *Icarus* **206**, 783–786.

Nicholson WL (2009) Ancient micronauts: interplanetary transport of microbes by cosmic impacts. *Trends in Microbiology* **17**, 243–250.

Nicholson WL, Schuerger AC and Race MS (2009) Migrating microbes and planetary protection. *Trends in Microbiology* **17**, 389–392.

Nicholson TL, Brunelle BW, Bayles DO, Alt DP and Shore SM (2018) Comparative genomic and methylome analysis of non-virulent D74 and virulent Nagasaki *Haemophilus parasuis* isolates. *PLoS One* **13**, e0205700.

Nye TM, Jacob KM, Holley EK, Nevarez JM, Dawid S, Simmons LA and Watson Jr ME (2019) DNA Methylation from a type I restriction-modification system influences gene expression and virulence in *Streptococcus pyogenes*. *PLoS Pathogens* **15**, e1007841.

Nye TM, van Gijtenbeek LA, Stevens AG, Schroeder JW, Randall JR, Matthews LA and Simmons LA (2020) Methyltransferase DnmA is responsible for genome-wide N6-methyladenosine modifications at non-palindromic recognition sites in *Bacillus subtilis*. *Nucleic Acids Research* **48**, 5332–5348.

Osman S, Peeters Z, La Duc MT, Mancinelli R, Ehrenfreund P and Venkateswaran K (2008) Effect of shadowing on survival of bacteria under conditions simulating the Martian atmosphere and UV radiation. *Applied and Environmental Microbiology* **74**, 959–970.

Payelleville A, Legrand L, Ogier J-C, Roques C, Roulet A, Bouchez O, Mouammine A, Givaudan A and Brillard J (2018) The complete methylome of an entomopathogenic bacterium reveals the existence of loci with unmethylated adenines. *Scientific Reports* **8**, 12091.

Poole A, Penny D and Sjöberg B-M (2001) Confounded cytosine! tinkering and the evolution of DNA. *Nature Reviews Molecular Cell Biology* **2**, 147–151.

Rand AC, Jain M, Eizenga JM, Musselman-Brown A, Olsen HE, Akeson M and Paten B (2017) Mapping DNA methylation with high-throughput nanopore sequencing. *Nature Methods* **14**, 411–413.

Ray S, Gebre S, Fogle H, Berrios DC, Tran PB, Galazka JM and Costes SV (2019) GeneLab: omics database for spaceflight experiments. *Bioinformatics* **35**, 1753–1759.

Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* **447**, 425–432.

Reva ON, Swanevelder DZ, Mwita LA, Mwakilili AD, Muzondiwa D, Joubert M, Chan WY, Lutz S, Ahrens CH and Avdeeva LV (2019) Genetic, epigenetic and phenotypic diversity of four *Bacillus velezensis* strains used for plant protection or as probiotics. *Frontiers in Microbiology* **10**, 2610.

Reva ON, Larisa SA, Mwakilili AD, Tibuhwa D, Lyantagaye S, Chan WY, Lutz S, Ahrens CH, Vater J and Borriss R (2020) Complete genome

https://doi.org/10.1017/S1473550421000343 Published online by Cambridge University Press

sequence and epigenetic profile of *Bacillus velezensis* UCMB5140 used for plant and crop protection in comparison with other plant-associated *Bacillus* strains. *Applied Microbiology and Biotechnology* **104**, 7643–7656.

Ritchie MD, Holzinger ER, Li R, Pendergrass SA and Kim D (2015) Methods of integrating data to uncover genotype–phenotype interactions. *Nature Reviews Genetics* **16**, 85–97.

Sanderson ND, Street TL, Foster D, Swann J, Atkins BL, Brent AJ, McNally MA, Oakley S, Taylor A and Peto TE (2018) Real-time analysis of nanopore-based metagenomic sequencing from infected orthopaedic devices. *BMC Genomics* **19**, 714.

Schaeffer P, Millet J and Aubert J-P (1965) Catabolic repression of bacterial sporulation. *Proceedings of the National Academy of Sciences* **54**, 704–711.

Schuerger AC and Nicholson WL (2006) Interactive effects of hypobaria, low temperature, and CO_2 atmospheres inhibit the growth of mesophilic *Bacillus* spp. Under simulated Martian conditions. *Icarus* **185**, 143–152.

Schuerger AC, Mancinelli RL, Kern RG, Rothschild LJ and McKay CP (2003) Survival of endospores of *Bacillus subtilis* on spacecraft surfaces under simulated Martian environments:: implications for the forward contamination of Mars. *Icarus* **165**, 253–276.

Selker EU and Stevens JN (1985) DNA methylation at asymmetric sites is associated with numerous transition mutations. *Proceedings of the National Academy of Sciences* **82**, 8114–8118.

Shaiwale NS, Basu B, Deobagkar DD, Deobagkar DN and Apte SK (2015) DNA adenine hypomethylation leads to metabolic rewiring in *Deinococcus radiodurans*. *Journal of Proteomics* **126**, 131–139.

Simonsen LC, Nealy JE, Townsend LW and Wilson JW (1990) Radiation Exposure for Manned Mars Surface Missions.

Simpson JT, Workman RE, Zuzarte P, David M, Dursi L and Timp W (2017) Detecting DNA cytosine methylation using nanopore sequencing. *Nature Methods* **14**, 407–410.

Sović I, Šikić M, Wilm A, Fenlon SN, Chen S and Nagarajan N (2016) Fast and sensitive mapping of nanopore sequencing reads with GraphMap. *Nature Communications* **7**, 11307.

Szopa-Comley A (2013) DNA Sequencing: Latest Developments In Next-Generation Sequencing.

Tenaillon O, Denamur E and Matic I (2004) Evolutionary significance of stress-induced mutagenesis in bacteria. *Trends in Microbiology* **12**, 264–270.

Tirumalai MR, Rastogi R, Zamani N, Williams EO, Allen S, Diouf F, Kwende S, Weinstock GM, Venkateswaran KJ and Fox GE (2013) Candidate genes that may be responsible for the unusual resistances exhibited by *Bacillus pumilus* SAFR-032 spores. *PLoS One* **8**, e66012.

Townsend LW (2005) Implications of the space radiation environment for human exploration in deep space. *Radiation Protection Dosimetry* **115**, 44–50.

Ullmann R, Becker BV, Rothmiller S, Schmidt A, Thiermann H, Kaatsch HL, Schrock G, Müller J, Jakobi J, Obermair R, Port M and Scherthan H (2021) Genomic adaption and mutational patterns in a HaCaT subline resistant to alkylating agents and ionizing radiation. *International Journal of Molecular Sciences* **22**. https://doi.org/10.3390/ijms22031146.

Vaishampayan PA, Rabbow E, Horneck G and Venkateswaran KJ (2012) Survival of *Bacillus pumilus* spores for a prolonged period of time in real space conditions. *Astrobiology* **12**, 487–497.

Venkateswaran K, Satomi M, Chung S, Kern R, Koukol R, Basic C and White D (2001) Molecular microbial diversity of a spacecraft assembly facility. *Systematic and Applied Microbiology* **24**, 311–320.

Walsh C and Xu G (2006) Cytosine methylation and DNA repair. *DNA Methylation: Basic Mechanisms*, 283–315.

Waters SM, Zeigler DR and Nicholson WL (2015) Experimental evolution of enhanced growth by *Bacillus subtilis* at low atmospheric pressure: genomic changes revealed by whole-genome sequencing. *Applied and Environmental Microbiology* **81**, 7525–7532.

Weirather JL, de Cesare M, Wang Y, Piazza P, Sebastiano V, Wang X-J, Buck D and Au KF (2017) Comprehensive comparison of Pacific Biosciences and Oxford Nanopore Technologies and their applications to transcriptome analysis. *F1000Research* **6**.

Westphal LL, Sauvey P, Champion MM, Ehrenreich IM and Finkel SE (2016) Genomewide dam methylation in *Escherichia coli* during long-term stationary phase. *MSystems* **1**.

Whitfield BL and Billen D (1972) *In vivo* methylation of *Escherichia coli* DNA following ultraviolet and X-irradiation. *Journal of Molecular Biology* **63**, 363–372.

Zautner AE, Goldschmidt A-M, Thürmer A, Schuldes J, Bader O, Lugert R, Groß U, Stingl K, Salinas G and Lingner T (2015) SMRT sequencing of the *Campylobacter coli* BfR-CA-9557 genome sequence reveals unique methylation motifs. *BMC Genomics* **16**, 1–12.

Zhang Z, Wang H, Wang Y, Xi F, Wang H, Kohnen MV, Gao P, Wei W, Chen K and Liu X (2021) Whole-genome characterization of chronological age-associated changes in methylome and circular RNAs in moso bamboo (*Phyllostachys edulis*) from vegetative to floral growth. *The Plant Journal* **106**, 435–453.

Zhao L, Song Y, Li L, Gan N, Brand JJ and Song L (2018) The highly heterogeneous methylated genomes and diverse restriction-modification systems of bloom-forming *Microcystis*. *Harmful Algae* **75**, 87–93.

https://doi.org/10.1017/S1473550421000343 Published online by Cambridge University Press