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„Molecular mechanisms of *Deinococcus radiodurans* survivability in outer space “

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Contents

Abstract

Most living entities are not able to survive under vacuum (10^{-4} Pa), extensive UV and ionizing radiation, combined with temperature variations between -100 °C and +100 °C. These conditions are present at the low Earth orbit (LEO), the location of the International Space Station (ISS). However, some microorganisms manage to withstand such harsh environmental conditions for years. One example is the polyextremophile bacterium *Deinococcus radiodurans*, which was sent to the ISS for exposure between one and three years on the Japanese Exposure Module Kibō. Molecular analysis of the cells after exposure may help understanding how organisms can survive long term exposure to space conditions. Analysing tools include survival assays, cellular characterization by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), and interpretation of molecular adaptions via transcriptomic, proteomic and metabolomic approaches. Results of the conducted experiments showed that *D. radiodurans* indeed can survive long-term exposure outside the ISS and after a short lag phase of few hours in complex medium, it proliferates again. This indicates that an interplanetary travel of certain organisms might be possible. Results presented in this dissertation can be interesting for future space exploration and planetary protection, however, as it similarly important for areas involving stress response from extreme environmental conditions, such as vacuum, radiation and temperature cycles.

The present work comprises different stress response experiments on *D. radiodurans*. Apart from LEO exposure, several other experiments were conducted with simulated LEO conditions. *D. radiodurans* was exposed solely to vacuum, to vacuum and UVC radiation, and a combination of vacuum, UVC radiation and temperature cycles, mirroring conditions present outside the ISS. In addition, the response of *D. radiodurans* to growth under simulated microgravity conditions was studied.

An integrative extraction protocol for limited amounts of *D. radiodurans* was established to extract mRNA, extra- and intracellular proteins and polar metabolites simultaneously from each replicate. Transcripts were measured on an Illumina HiSeq, proteins in a shotgun proteomics approach on a LC-orbitrap and polar metabolites on a GC-TOF. All applied methods were used for quantitative approaches.

Neither simulated nor real space exposure caused visible damage to the cell wall in *D. radiodurans*. However, exposure experiments reduced survivability compared to control cells

and induced molecular alterations. All applied factors, except microgravity, induced the expression of genes related to DNA repair, especially the UvrABC excision repair mechanism appears as a major component of the repairment of nucleic acid damage after exposure to LEO conditions. Furthermore, proteins to alleviate oxidative stress, for instance catalases, peroxidases or related proteins, were higher abundant after exposure. Generally, the overall level of amino acids and organic acids was lower in *D. radiodurans* cells, which were exposed to harsh environmental conditions, as they might be used as intermediates in repair processes or as carbon energy source. Moreover, induced expression levels of different regulators were observed, for instance histidine kinases, which may be involved in activation of stress responses. Ultimately, statistical analysis showed that S-layer proteins of *D. radiodurans* are likely involved in stress response after LEO exposure.

In conclusion, multiple alterations on various molecular levels were observed after LEO or simulated LEO exposure. The combination of several experimental, methodological and bioinformatical strategies presented in this work provide new insights into complex stress response mechanisms, which are triggered through exposure to outer space conditions.

Zusammenfassung

Für die meisten Lebewesen stellen Umwelteinflüsse wie Vakuum (10-4 Pa), starke UV- und ionisierende Strahlung in Kombination mit Temperaturschwankungen zwischen -100 °C und +100 °C eine lebensfeindliche Umgebung dar. Solche Bedingungen gibt es an der Außenseite der Internationale Raumstation (ISS) in der niedrigen Erdumlaufbahn. Manche Mikroorganismen sind allerdings imstande diesen extremen Einflüssen zu trotzen. Das polyextremophile Bakterium *Deinococcus radiodurans* wurde für ein bis drei Jahre an dem japanischen Modul Kibō der niedrigen Erdumlaufbahn ausgesetzt. Eine anschließende Analyse der Zellen auf molekularer Ebene sollte helfen zu verstehen, wie Organismen eine lange Zeit unter Weltallbedinungen überstehen können. Dafür wurden neben zellbiologischen Untersuchungen mittels Rasterelektronenmikroskopie (SEM) und Transmissionselektronenmikroskopie (TEM) auch Analysen des Transkriptoms, Proteoms und Metaboloms durchgeführt. Die Ergebnisse der durchgeführten Experimente zeigten, dass *D. radiodurans* für einen längeren Zeitraum außerhalb der ISS überleben kann und sich nach einer kurzen Anpassungsphase von wenigen Stunden in komplexem Medium wieder vermehrt. Daher besteht zumindest die Möglichkeit interplanetarer Reisen bestimmter Organismen. Die in dieser Dissertation vorgestellten Ergebnisse können für zukünftige lange Weltraummissionen, sowie für Fragestellungen bezüglich dem Planetaren Schutz von Interesse sein. Da es sich bei den durchgeführten Experimenten um Stressreaktionen handelt, sind die Ergebnisse außerdem interessant für alle Bereiche, welche sich mit extremer Umgebungsbedingungen wie Vakuum, Strahlung und Temperaturzyklen befassen.

Die vorliegende Arbeit umfasst verschiedene Stressreaktionsexperimente an *D. radiodurans*. Neben dem Experiment in der niedrigen Erdumlaufbahn wurde *D. radiodurans* verschiedenen Einflüssen im Labor ausgesetzt, welche jene der niedrigen Erdumlaufbahn simulierten. Darunter befanden sich Vakuum, Vakuum mit UVC-Strahlung sowie eine Kombination aus Vakuum, UVC-Strahlung und Temperaturzyklen. Zusätzlich wurde die Auswirkungen von *D. radiodurans* auf Wachstum unter simulierter Mikrogravitation untersucht.

Um genügend Probenmaterial aus einer begrenzten Menge von D. radiodurans zu erhalten, wurde ein integratives Extraktionsprotokoll erstellt. Aus jedem Replikat wurden simultan mRNA, extra- und intrazelluläre Proteine und polare Metaboliten extrahiert. Die mRNA wurde auf einem Illumina HiSeq, Proteine in einem Shotgun-Proteomics-Ansatz auf einer LC-

Orbitrap und polare Metaboliten auf einem GC-TOF gemessen. Bei allen Ansätzen handelte es sich um quantitative Messungen.

Kein Stressreaktionsexperiment mit *D. radiodurans* verursachte sichtbare Schäden an der Zellwand. Es reduzierte sich allerdings die Anzahl der überlebenden Kulturen verglichen mit Kontrollzellen. Außerdem konnten molekulare Veränderungen festgestellt werden. Alle Stressfaktoren mit Ausnahme der Mikrogravitation induzierten die Expression von Genen, die mit DNA-Reparatur in Zusammenhang stehen. Ist *D. radiodurans* den Bedingungen der niedrigen Erdumlaufbahn ausgesetzt, so entstehen Schäden an Nukleinsäuren, welche insbesondere durch den UvrABC-Exzisionsreparaturmechanismus repariert werden. Auch Proteine, welche oxidativem Stress entgegenwirken, wie Katalasen oder Peroxidasen, waren im Anschluss an die Zeit unter Weltallbedingungen häufiger vorhanden. Weiters wurde eine Verminderung des Gesamtgehalts an Aminosäuren und organischen Säuren festgestellt, da sie als Zwischenprodukte bei Reparaturprozessen oder als Kohlenstoff-Energiequelle verwendet werden könnten. Außerdem wurden induzierte Expressionsniveaus regulatorisch wirkender Proteine beobachtet, beispielweise Histidin Kinasen, welche an der Aktivierung von Stressreaktionen beteiligt sein können. Außerdem zeigen statistische Analysen, dass S-Layer-Proteine vermutlich an der Stressreaktion beteiligt sind

Es wurde festgestellt, dass alle durchgeführten Stressreaktionsexperimente multiple Veränderungen auf verschiedenen molekularen Ebenen verursachen. Die Kombination mehrerer experimenteller und bioinformatischer Ansätze, die in dieser Arbeit vorgestellt werden, bieten neue Einblicke in komplexe Stressreaktionsmechanismen, welche durch einen längeren Aufenthalt unter Weltraumbedingungen induziert werden.

1 Introduction

1.1 Life Beyond Earth

The search for extra-terrestrial life has always fascinated humans and is still tremendously reflected in nowadays' research. Space exploration in twenty-first century aims to search for habitable environments on other worlds and discover areas where liquid water, energy sources and biologically important elements and organic molecules have been available. Mars gained an increased attention to send orbiters, landed and rover missions for various investigations. Opportunity, which was active on Mars since 2004, was able to collect compositional data of fractures in layered rocks of the Endeavour crater, which suggest formation of Al-rich smectites by aqueous leaching (Arvidson et al., 2014). Although Opportunity confirmed that diverse aqueous environments existed on the Martian surface billions of years ago, it could not test whether these environments had been habitable (Grotzinger, 2014). The Curiosity rover, which landed on the Martian surface in 2012, detected the key biogenic elements carbon, hydrogen, oxygen, sulphur, nitrogen and phosphorus in Yellowknife Bay, Gale Crater (Grotzinger et al., 2014). Furthermore, fine-grained sedimentary rocks were discovered, which indicated the existence of an ancient lake, and a probable biosphere based on chemolithoautotrophy had been suggested (Grotzinger et al., 2014). Even though various studies put forward a concept of life on Mars which might have been possible in the water rich Noachian era (<3.95-3.7 billion years ago) (Arvidson et al., 2014; Grotzinger, 2014; Grotzinger et al., 2014; Hassler et al., 2014), so far no organism, its remnants or valid organic biosignature was detected. Apart from Mars, there are other potentially habitable zones in our solar system, for instance, the Saturn moon Enceladus, where the spacecraft Cassini discovered potential hydrothermal reactions (Waite et al., 2017). Another object of astrobiological interest is Europa, one of the four Galilean satellites of Jupiter. The Galileo spacecraft already revealed that rising water or icy mush have burst through the surface of Europa, resulting in alterations on the icy surface of the moon (Kerr, 1997). Galileo also showed that the interior of Europa is electrically conductive, implying that most of the outer water layer is liquid (Kerr, 1997). The theory of a global ocean under its surface was later supported by an observation of the Hubble telescope. The wavelength distribution and the emissions near Europa's south pole fitted a cloud of water vapour, likely ejected from a source on the surface (Roth et al., 2014). The presence of liquid water on Enceladus and Europa, which is essential for life on Earth, makes these moons of a great interest to astrobiologists. Further research efforts are made towards the Saturn moon Titan, which hosts

lakes, rain, and clouds of methane and ethane. These liquids are probable homes for carbon-based life (McKay, 2016). Even the lower cloud layer of Venus (47.5-50.5 km) could host microbial life due to its moderate conditions of ~60 °C, 1 atm and presence of micron-sized sulfuric acid aerosols (Limaye et al., 2018).

On the 25th of August 2012, the space probe Voyager 1 was the first human made object to enter the interstellar space (Webber and McDonald, 2013). It will still take 40000 years until the spacecraft will be near another solar system. However, no probe is needed to detect habitable zones in other solar systems. The number of identified exoplanets (more than 4000 (nasa.gov, 2019)(nasa.gov, 2019)) is constantly increasing (Pearson et al., 2017). In 2018, data from NASA's Chandra X-ray observatory succeeded in discovering planets in one of the other $2*10^{12}$ galaxies (Conselice et al., 2016) outside the Milky way for the first time (Dai and Guerras, 2018). Although there is no confirmed extra-terrestrial life yet, it is very likely that in other habitable zones, life, as we know it, exists (Filkin, 1997). Habitable rocky planets presumably emerged 10-17 million years after the Big Bang, 13.8 billion years ago (Loeb, 2014).

However, it is still a big mystery how life on Earth, or anywhere else, originated. Several theories of abiogenesis, the formation of life from non-living material (Bailey, 1938), were postulated (Ricardo and Szostak, 2009). However, another possibility might be the interplanetary or interstellar exchange of microbes and organic material (Arrhenius, 1903). Reality may represent parts of both theories (Klyce, 2001). Although there is no evidence that an interplanetary exchange of microbes ever occurred, it is still interesting to test various conditions which hypothetically occur during such a journey (Olsson-Francis and Cockell, 2010). The lithopanspermia theory, which focuses on the transfer of organisms in rocks, puts emphasis on three main stages during the interplanetary transport which are challenging for every organism (Nicholson, 2009). The first stage, planetary ejection, involves extreme forces of acceleration, resulting in high pressure and temperature variations. During transit, the second stage, organisms are forced to cope with challenging conditions of outer space. Ultimately, throughout the third stage, atmospheric entry, high temperatures are present near the transporting rock’s surface, erasing the possibility of photosynthetic organisms near the surface to survive the impact (Cockell et al., 2007). All of these factors have been investigated in simulation experiments, concluding that there is a possibility that some simple organisms are able to survive ejection, transit and impact (Mastrapa et al., 2000; Fajardo-Cavazos et al., 2005; Horneck et al., 2008; Olsson-Francis and Cockell, 2010).

1.2 The International Space Station as an Astrobiological Experimental Platform

The second lithopanspermia stage, survival during the transit, can be studied via simulation experiments, but for valid results, it is necessary to expose the chosen organisms for a long period to outer space conditions. Short-term exposure to spaceflight environment, performed on *Cupriavidus metallidurans* (Leys et al., 2009), *Rhodospirillum rubrum* (Mastroleo et al., 2009), *Escherichia coli* (Li et al., 2015) and *Bacillus cereus* (Su et al., 2014), showed no decrease of viability, but changes at the molecular level. The International Space Station (ISS) provides a suitable environment for astrobiological experiments in the low Earth orbit. Between 2008 and 2015, the EXPOSE experiments concluded that not only sporulating bacteria, such as *B. subtilis* can survive an interplanetary travel, but also seeds and lichens, for example *Stichococcus* sp., *Trichoderma* sp. and *Acarospora* sp. (Onofri et al., 2012; Vaishampayan et al., 2012; Neuberger et al., 2014). *B. pumilus* (Vaishampayan et al., 2012) spores (2-3 spore layers) showed a survival rate of 10-40 % after 18 months of exposure to LEO conditions. Within the same exposure experiment, *B. subtilis* spores (5-10 spore layers) showed a survival rate of 6.1-55 % (Horneck et al., 2012; Nicholson et al., 2012). However, a follow-up experiment (Novikova et al., 2015) (EXPOSE-R) showed that even *Bacillus* sp spores are not able to survive in monolayers when exposed to LEO conditions. A third mission (EXPOSE-R2) was launched in July 2014, featuring the long term exposure experiment BIOMEX (BIOlogy and Mars EXperiment) (de Vera et al., 2019). The exposures of 46 species of bacteria, fungi and arthropods took place on the Russian module Zvezda. *Chroococcidiopsis* (Baqué et al., 2013), a desert cyanobacteria, was exposed to compare the survivability of dried biofilms to their planktonic counterparts under LEO and simulated Martian conditions. Confocal microscopy, PCR based assays and CFU counting revealed an overall higher resistance of biofilms when compared to the planktonic counterpart (Billi et al., 2017). EXPOSE-R2 included the exposure of *D. radiodurans* cells to Mars-like conditions in the LEO. CFU counts, 16s rRNA qPCR showed strong detrimental effects on the survival of *D. radiodurans*, however its carotenoid deinoxanthin, investigated by Raman spectroscopy, preserved its integrity after exposure. Therefore, it was concluded that deinoxanthin is a suitable, easily detectable biomarker for the search of Earth like organic pigment containing life on other planets (Leuko et al., 2017).

1.3 Tanpopo Space Mission

The Tanpopo (English: dandelion) mission was presented by Yamagishi et al. (2007) at the 26th International Symposium on Space Technology and Science (At Hamamatsu, Japan). It is a panspermia related long-term exposure experiment on the exposure facility of the Japanese experimental module Kibō (English: hope) of the ISS. It includes several experiments to capture and expose microbes. Preliminary tests revealed that several *Deinococcus* spp. are candidates for survival experiments on the ISS (Kawaguchi et al., 2013). Furthermore, these experiments confirmed that dried deinococci cells in monolayers are not able to survive harsh environmental conditions in the LEO. However, accumulated cells with several hundred µm thickness can survive. To examine their survivability after one to three years of exposure in the low Earth orbit, *D. radiodurans*, *D. aerius* and *D. aetherius* were exposed at the Japanese facility Kibō on the ISS (Kawaguchi et al., 2016).

1.4 *Deinococcus radiodurans*

In 1956, tests were performed to sterilize canned food using high doses of gamma radiation. However, a tin of meat spoiled and *D. radiodurans* was isolated (Anderson, 1956). First placed in the genus *Micrococcus*, after rRNA sequencing, it was given its own genus *Deinococcus*, closely related to the genus *Thermus* (Makarova et al., 2001). Apart from *Thermus* related genes, *D. radiodurans* spp. host genes similar to organisms from other kingdoms of life. These genes may have been acquired through interkingdom horizontal gene transfer and ultimately have led to differences in gene size and gene content between different deinococcal species (Jung et al., 2010).

Due to its high resistance to dehydration (Potts, 1994), UV (Sweet and Moseley, 1974) and ionizing radiation (Mattimore and Battista, 1996) *D. radiodurans* became a well-studied polyextremophile. Compared to *E. coli*, *D. radiodurans* is approximately 30 times more resistant to UV light and 50 times more resistant to gamma radiation (Sweet and Moseley, 1976). *D. radiodurans* is nonmotile, non-pathogenic (Makarova et al., 2001) spherical, pink/red bacterium that usually forms diplococci and tetracocci morphologies. It stains gram positive, although its rather unique cell envelope resemble a gram negative bacterium (Battista, 1997). It is an obligate aerobic chemoorganoheterotroph that does not form endospores. *D. radiodurans* is ubiquitous in nature, however its populations are minor compared to other bacteria which inhabit the same ecological niches. Therefore, from an evolutionary perspective, *D. radiodurans* may has traded the ability of efficient growth (reproduction) for efficient

survival (robustness). This results in low populations of *D. radiodurans* in mild natural habitats, where faster growing competitors are more prominent, however, under harsh environmental conditions for instance in deserts, desiccated *D. radiodurans* populations dominate competitors due to their ability to regrow after rehydration (Krisko and Radman, 2013).

The high radiation resistance does not provide any selective advantage to *D. radiodurans*, as no terrestrial habitats generate such a high level of radiation (Scott, 1983). Hence, even an extra-terrestrial origin of *D. radiodurans* was proposed (Pavlov et al., 2006). However, since its phylogeny is related to other terrestrial organisms, it is more likely that radiation resistance co-evolved with another adaptation, for instance desiccation, which is a common physiological stress. As 41 ionizing radiation sensitive strains of *D. radiodurans* showed similar susceptibility to desiccation (Mattimore and Battista, 1996), a relationship between desiccation and ionizing radiation resistance can be assumed. Nevertheless, not all desiccation resistant organisms are similarly resistant to radiation and vice versa. Radiation resistant *E. coli* strains are not resistant to desiccation (Harris et al., 2009) and desiccation resistant *Actinobacteria*, *Arthrobacter* and *Rhodococcus* are susceptible to radiation (Shukla et al., 2007). Therefore, alternative theories were proposed, for example the "adaptive radiation hypothesis", claiming that *D. radiodurans* radiation resistance originated under high natural ionizing radiation levels in deeply buried manganese-rich marine sediments (Sghaier et al., 2007).

The high resistance of *D. radiodurans* to various environmental factors is not caused by an efficient DNA protection mechanism. Studies showed that ionizing radiation induced double strand breaks (DSB) are caused with equal efficiency in all prokaryotic and eukaryotic cells (~0.05 DSB/Megabase/Gray irradiation) (Gladyshev and Meselson, 2008; Daly, 2009). *D. radiodurans* can repair hundreds to thousands of radiation induced DSB per cell, whereas other species can repair only a dozen (Cox and Battista, 2005). Consequently, researches anticipated the discovery of a novel, high sophisticated repair mechanism in *D. radiodurans*. Due to its properties, *D. radiodurans* was an early target for whole genome sequencing. The full genome of *D. radiodurans* R1 was published in 1999 (White et al., 1999) and revealed two large chromosomes (3.06 Mb) and two plasmids (223 kb). However, extensive analysis of genes/proteins involved in DSB repair in *D. radiodurans* concluded that the reason for its resistance is not a special, exceptional DNA repair mechanism (Makarova et al., 2000; Omelchenko et al., 2005; Slade et al., 2009). In addition, no proteins which are typically involved in DSB repair appear at higher concentrations in *D. radiodurans* compared to other

bacteria, such as *E. coli* (Blasius et al., 2008). In 2007, Daly et al. claimed that the high robustness of *D. radiodurans* is caused by protection of proteins rather than extraordinary DNA repair mechanisms (Daly et al., 2007). Radiation induced oxidative damage to proteins (protein carbonylation) inactivates their function, which leads to reduced DNA repair, proteostasis and structural maintenance in all organisms (Krisko and Radman, 2013). Experiments confirmed that protein carbonylation correlates with cell death and that the amount of gamma radiation and UVC radiation needed to saturate protein carbonylation in *E. coli* is significantly lower compared to *D. radiodurans* (Krisko and Radman, 2010). As a universal rule for all organisms, it can be assumed that dedicated proteins repair nucleic acids, but no living cell can function correctly with an oxidized proteome. It was even proposed that accumulated oxidative damage to proteins whose severity and complexity increase with time is the cause of aging (Krisko and Radman, 2019).

Experiments suggested a connection between radiation induced oxidative proteome damage and cell death. Radiation promotes the production of reactive oxygen species (ROS), which lead to protein carbonylation (Suzuki et al., 2010). Therefore, *D. radiodurans* requires abilities to avoid or reduce oxidative damage to proteins. Understanding and preventing radiation induced oxidation events in cells opens multiple biotechnological and medicinal opportunities. Concerning the environment, it could be used for bioremediation of contaminated sites (Brim et al., 2000; Choi et al., 2017). Interesting medical applications involve potential treatment of Parkinson (Drechsel and Patel, 2008) and Alzheimer (Benzi and Moretti, 1995) disease. Furthermore, studying protein protection mechanisms may help to delay the process of aging (Liochev, 2013) and contribute to cancer prevention (Waris and Ahsan, 2006).

Antioxidative elements associated to the cells are necessary to minimize damage through ROS. The prominent pink/red colour of *D. radiodurans* is caused by the carotenoid deinoxanthin, which is bond to the S-layer of the bacterium (Farci et al., 2016). *In vitro*, deinoxanthin has a stronger scavenging ability on H_2O_2 than other carotenes (lucopene and β-carotene) (Tian et al., 2007). However, mutations of the *crtB* (phytoene synthase) gene, which blocks the carotenoid synthesis pathway, negligibly increased sensitivity to ionizing radiation, desiccation and UV radiation (Tian et al., 2007; Zhang et al., 2007). These results imply that other ROS scavenging mechanisms can successfully replace carotenoids, carotenoids are less effective *in vivo* as *in vitro* or carotenoids and the membrane is not the primary target of ROS (Slade and Radman, 2011).

Nevertheless, *D. radiodurans* has a multitude of other antioxidant systems that potentially contribute to its exceptional resistance to various environmental conditions. Several enzymes support the cells to alleviate ROS damage. The expression of SodA (superoxide dismutase) is strongly induced in the early stages of irradiation injury recovery in *D. radiodurans* (Luan et al., 2014). However, similar peroxidases and catalases present in *D. radiodurans* can be found in *Shewanella oneidensis*, a radiosensitive bacterium (Peana et al., 2018). Thus, additional antioxidative stress mechanisms must be present in *D. radiodurans*. Mutants with a G6PDH deficiency are more sensitive to UV-induced oxidative stress compared to the wild type (Zhang et al., 2005). Hence, glucose metabolism might be involved in the defence strategy of *D. radiodurans*. A study showed that the DNA excision repair mechanism in *D. radiodurans* is facilitated by the pentose phosphate pathway, as it provides adequate metabolites (Zhang et al., 2003). Furthermore, the amount of proteins containing iron-sulphur clusters is considerably lower compared to radiation sensitive organisms, such as *S. oneidensis* (Ghosal et al., 2005), which encodes 65 % more proteins with iron-sulphur clusters. *D. radiodurans* is a proteolytic bacterium and thus contain conserved mechanisms for protein degradation and amino acid catabolism. The elevated levels of proteolytic activities after exposure to ionizing radiation (Daly et al., 2010) enable an efficient repair and finally survival through the high energy demanding recovery period (Omelchenko et al., 2005). To ensure enough energy from external resources, *D. radiodurans* relies on 90 ABC transporters dedicated to amino acid and peptide uptake (de Groot et al., 2009). Finally, radiation resistant bacteria show an extremely high Mn/Fe ratio compared to radiation sensitive bacteria. Divalent iron is a source of production of the most reactive ROS, the hydroxyl radical. It has been shown that radiation resistant bacteria contained up to 300 times more Mn^{2+} ions and about three times less Fe^{2+} ions than most radiation-sensitive bacteria (Daly et al., 2007).

The analysis of the ultrafiltrate of *D. radiodurans* resulted in the detection of manganese complexes, elevated concentrations of orthophosphate, nucleotides and their derivatives, as well as amino acids and peptides (Daly et al., 2010). Based on manganese antioxidants, present in *D. radiodurans*, Gupta et al. designed a Mn^{2+}-decapeptide-P_i complex, which was injected to mice. After 30 days, treated mice displayed 63 % less lethality compared to untreated mice (Gupta et al., 2016). Moreover, a bioinformatical approach revealed that the amount of proteins with Mn^{2+} binding abilities is considerably higher in *D. radiodurans* than in radiosensitive bacteria (Peana et al., 2018). However, the molecular background, especially the regulatory switches responsible for activating the different defence mechanisms in *D. radiodurans* need

to be thoroughly investigated. There might be multiple regulators responsible for activating repair and response mechanisms to environmental stress conditions in *D. radiodurans*. G-quadruplex DNA secondary structures might play a role in DNA damage response and in the regulation of expression of DNA repair proteins required for radioresistance in *D. radiodurans* (Mishra et al., 2019).

1.5 Systems Biology in Stress response studies

Systems biology describes molecular mechanisms which are operative in cells (Bruggeman et al., 2007) with high throughput technologies such as microarrays or next generation sequencing on genomics and transcriptomics level and mass-spectrometry based methods on proteomics and metabolomics level (Weckwerth, 2011). The combination of several –omics analysis can help understanding molecular cascades in specific organisms and how it responds to environmental changes in correlation to the phenotype.

The unbiased approach of systems biology is applied in several stress related studies in all kind of organisms to identify specific markers/indicators. A study regarding vaccine efficacy revealed early gene "signatures" that predicted immune responses in humans vaccinated with yellow fever vaccine YF-17D (Querec et al., 2009). Further details of signalling cascades, connecting transcriptional with translational and metabolic regulations, can be achieved by coupling systems biology approaches with machine learning algorithms in mutant experiments. This approach was used to identify protein targets in *Arabidopsis thaliana* to identify transcriptional regulators of primary and secondary metabolism under cold and high light stress (Furtauer et al., 2018).

Due to its extraordinary resistant properties, *D. radiodurans* was targeted by several system biology approaches. The wild type strain *D. radiodurans* R1 was shown to withstand gamma radiation up to 16000 Gy (Gray), however, *katA* (catalase A) mutants are 2 to 15 fold more sensitive and *sodA* (superoxide dismutase [Mn]) mutants are even 3-90 fold more sensitive to the same amount of radiation (Markillie et al., 1999). Due to different survival characteristics of certain mutants and wild type cells, early systems biology approaches targeted the proteome (Lipton et al., 2002) and transcriptome (Liu et al., 2003) of irradiated *D. radiodurans* cells. In both studies, *recA* (RecA, recombinase A) showed an increased abundance after cells were exposed to ionizing radiation. RecA is considered central in activating SOS response to extensive DNA damage after irradiation, as it catalyses the hydrolysis of ATP in the presence of single-stranded DNA for uptake of single-stranded DNA to duplex DNA (Cox, 2003). Unlike

E. coli, RecA in *D. radiodurans* binds the duplex DNA first and the homologous ssDNA substrate second (Kim et al., 2002). Furthermore, proteins/genes involved in DNA replication, repair and recombination appeared higher abundant after exposure to irradiation (Lipton et al., 2002; Liu et al., 2003). Especially the UvrABC endonucleotide excision repair mechanism plays a fundamental role after irradiation stress as many proteins involved in this mechanism were observed in high levels in irradiated cells (Lipton et al., 2002). The second UvrA protein, present in *D. radiodurans* might facilitate the export of damaged DNA pieces as it is attached to the cell membrane and has been linked to ABC transporter proteins (Bauche and Laval, 1999; White et al., 1999). Basu and Apte (2012) exposed *D. radiodurans* to gamma radiation, let cells recover for one hour and applied a proteomics approach to identify enhanced expression patterns. Results showed several DNA repair proteins, for instance RecA and the DNA damage response proteins DdrA and DdrB. Furthermore, proteins involved in oxidative stress alleviation, *e.g.,* KatA and SodA showed increased abundances after irradiation of cells.

2 Results and Discussion

Publication

Proteometabolomic response of Deinococcus radiodurans exposed to UVC and vacuum conditions: Initial studies prior to the Tanpopo space mission

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In order to find an ideal candidate for LEO exposure, it is necessary to perform several preliminary tests and evaluate chances of survival under simulated conditions, which mirror those at the ISS. In case of the Tanpopo space mission, several *Deinococcus* spp. were selected and exposed to heavy-ion beams, temperature cycles, vacuum and UV irradiation (Kawaguchi et al., 2013). Results of survival tests revealed that approximately 30 days exposure to ionizing radiation similar to LEO, does not affect desiccated *D. radiodurans* cells. On the contrary, UV radiation drastically decreased survival of cells. They only survived if several layers accumulated and the upper cells shielded the lower ones from harmful radiation.

Based on survival results, an experiment to evaluate molecular changes after exposure to UVC radiation and vacuum was planned. A 200 µm layer of dried *D. radiodurans* cells was prepared in aluminium plates with wells (2 mm diameter, 2 mm depth) equal to those used for the Tanpopo space mission. Subsequently, cells were exposed to $UVC_{254\ nm}$ dose 862 kJm^{-2} under reduced pressure (400 Pa). To evaluate the cellular integrity after exposure, dried cells were examined with a scanning electron microscope. Subsequently, cells were recovered in complex medium for 5 h to stimulate repair mechanisms. Primary metabolites and proteins were extracted according to Weckwerth et al. (2004) with slight modifications and measured on GC-TOF and LC-orbitrap, respectively. Although -omics experiments were performed on *D. radiodurans* before, especially after applying ionizing radiation to cells (Liu et al., 2003; Basu and Apte, 2012), several unique parameters were used in this setup. The sample holders with the dried deinococci cells were equal to those used in LEO and the applied environmental stress (UVC irradiation and vacuum) is similarly present during the Tanpopo mission.

Assays revealed an average survival rate of 65 % after exposure compared to dried, non-exposed controls. Scanning electron microscopy showed no visible differences between the two conditions. As a conclusion, UVC irradiation under vacuum conditions neither affected

morphology, nor cellular integrity of dehydrated cells. However, on a molecular level, exposed cells differ from control cells. Overall, primary metabolites appeared less abundant after exposure to UVC and vacuum conditions. As cells experienced severe damage through oxidation events, these metabolites may be utilized in various repair processes, for instance, DNA mismatch repair (Zhang et al., 2003). Regarding protein damage, Joshi et al. (2004) observed a selective degradation and re-synthesis of several proteins during the lag period after applying ionizing irradiation. Our data approved the hypothesis for post UV/vacuum recovery, as we found several aminoacyl ligases, necessary for protein translation, in higher abundances compared to the control cells. Many repair processes require energy which can be obtained via the TCA cycle. After exposure, the majority of proteins involved in the TCA cycle showed an increased abundance, in line with the results of Joshi et al. (2004).

D. radiodurans survives UVC and vacuum exposure, however, its nucleic acids are tremendously damaged. The comparative proteomics approach revealed several nucleic acid repair proteins which are more abundant after exposure. The UvrABC endonucleotide excision repair mechanism is certainly involved after application of UVC exposure. Although *D. radiodurans´* genome encodes for multiple polymerases, the level of PolA is significantly induced after exposure to UVC and vacuum. The importance of PolA in re-synthesis of fragmented DNA after ionizing radiation exposure of *D. radiodurans* has been described previously (Slade et al., 2009). PolA mutants are highly sensitive to ionizing radiation (Gutman et al., 1993) and show substantial defects in DNA synthesis and repair abilities (Zahradka et al., 2006).

To activate response processes and alter expression levels of certain proteins, several transcriptional regulators are necessary and act as activators or repressors on certain regions on the genome. Potential candidates that belong into this group are of utmost interest for stress response studies. We observed an increased abundance of a transcriptional regulator (DR_0997) belonging to the FNR/CRP family after exposure to UVC/vacuum. Cyclic AMP repressor proteins (CRP) are involved in several cellular pathways, including adaptation to starvation and other environmental stresses, in various bacteria (Green et al., 2014; Soberon-Chavez et al., 2017). An experiment showed that DR_0997 is involved in the stress response of *D. radiodurans* to ionizing radiation and UV radiation (Yang et al., 2016). On the other hand, we observed a decrease in abundance of another transcriptional regulator, which is part of the HTH_3 family (DR_2574). These regulators act as repressors of a variety of DNA damage

response proteins *in vitro* (Wang et al., 2015). These regulators, alongside others could be triggered via altered environmental conditions and therefore lead to a change in the proteome expression pattern to activate enzymatic and non-enzymatic stress response and repair mechanisms.

The experiments revealed several similarities between the response to UVC/vacuum and ionizing radiation on a molecular level in *D. radiodurans*. No specific single proteins are upregulated, it is rather a complex, multilayer process. Interesting protein targets, which are higher abundant during the repair processes were emphasized and the role of primary metabolites, which are potentially used for various repair processes, and as primary carbon source, was discussed. Furthermore, this preliminary study was used to establish the extraction procedure for samples exposed throughout the Tanpopo mission. It was shown that harsh environmental conditions affect dried deinococci cells to a high extent and differences between treated cells and dried controls can be observed via -omics based studies.

Publication

Proteomic and Metabolomic Profiling of Deinococcus radiodurans Recovering After Exposure to Simulated Low Earth Orbit Vacuum conditions

***Emanuel Ott**, Yuko Kawaguchi, Natalie Özgen, Akihiko Yamagishi, Elke Rabbow, Petra Rettberg, Wolfram Weckwerth and Tetyana Milojevic*

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Low Earth orbit exposure includes a multitude of challenging environmental conditions, including UV radiation, ionizing radiation, microgravity, temperature fluctuations and vacuum. To evaluate a possible survival of an organism outside the ISS, an exposure to the combined stress factors is necessary. However, looking at these factors individually likewise introduces interesting possibilities. Even though certain environmental conditions may not change the cells´ appearance, different changes are triggered on a molecular level, depending on the applied stress.

In the low Earth orbit, pressure is extremely low (below 10^{-4} Pa) and hence a factor that represents a serious threat to microorganisms, leading to potential severe changes on proteomic and genomic level. The lipid bilayer can undergo a conversion of bilayer sheets to spherical micelles, affecting cell membrane associated proteins like porins and membrane-bound cytochromes (Cox, 1993). Furthermore, DNA is prone to experience double strand breaks under vacuum, which resembles intense dehydration (Dose et al., 1992; Dose et al., 1995).

To assess effects caused by a single environmental factor, such as vacuum on *D. radiodurans* cells, an experimental setup which excludes other environmental influences is obligatory. In this experiment, *D. radiodurans* R1 cells were subjected to $8.7*10^{-5}$ Pa for a duration of 90 days in the ground-based Astrobiology Space simulation facility (Rabbow et al., 2016) at DLR (German Aerospace Centre), Cologne. Subsequent comparative metabolomic and proteomic analyses with control cells and inspection of RNA integrity were performed.

One cellular compartment that is heavily targeted by vacuum stress is the cell wall. *D. radiodurans* possesses a complex cell envelope with outer membranes, S-layers and ornithine-Gly containing mureins (Quintela et al., 1999). Due to thick cell walls, *D. radiodurans* is considered as a gram-positive bacterium, according to the staining, however, it includes a second membrane, which indicates a gram-negative bacterium (Battista, 1997). The external side of the cell wall is coated by an S-layer including several proteins and deinoxanthin, which

is the reason for the remarkable colour of *D. radiodurans*. The S-layer is extremely versatile, as protein expression is adapted to different environmental conditions by rearrangements of DNA (Pollmann et al., 2006).

Through proteomic and metabolomic measurements, we aimed to identify molecular responses, characteristic for vacuum exposed cells. Many results regarding repair mechanisms were comparable to what we observed in the previous study after applying UV/vacuum stress to *D. radiodurans*. Damaged exposed cells accumulate less primary metabolites as they are potentially used for repair processes (Zhang et al., 2003). Proteins which appeared higher abundant after vacuum exposure showed a high amount of protein-protein interactions, whereas proteins, which are less abundant show a fewer amount of protein-protein interactions. This result indicates that several connected pathways work together to alleviate cell damage caused by vacuum exposure. Many higher abundant proteins belong to nucleic acid repair mechanisms, such as the UvrABC endonuclease repair mechanism, including the proofreading polymerase PolA. Other proteins with induced levels were the topoisomerases GyrA and GyrB, which appeared higher abundant in previous vacuum exposure experiments on *B. subtilis* spores as well (Munakata et al., 1997; del Carmen Huesca Espitia et al., 2002).

ROS, which are produced extensively through vacuum exposure, entail dangers for various intracellular macromolecules. Consequently, *D. radiodurans* resort to enzymatic and non-enzymatic ways to reduce the amount of ROS during recovery in complex medium. On protein level, the amounts of peroxidases and catalases are increased after exposure. Polyamines, such as spermidine and putrescine may contribute to the non-enzymatic defence of *D. radiodurans* as they were described as a primordial form of stress molecules in bacteria, plants and animals (Rhee et al., 2007).

The activation of vacuum stress response in *D. radiodurans* is mediated by several regulators. A knockdown study performed by Im et al. (2013) revealed the importance of several histidine kinases in stress resistance to ionizing radiation and UV radiation. After vacuum exposure, we identified two type III histidine kinases DR_1227 and DR_0577 as more abundant during recovery. The upregulation of these histidine kinases may be connected to another protein, that showed a significant increased abundance during recovery, the S-layer protein DR_0774 (Kennan et al., 2015). Alongside SlpA (DR_2577), DR_0774 is a major component of the S-layer in the cell wall of *D. radiodurans* (Farci et al., 2014) and together with deinoxanthin, these

proteins were shown to be involved in cellular protection from UV radiation after desiccation (Farci et al., 2016).

Publication

Molecular response of Deinococcus radiodurans to simulated microgravity explored by proteometabolomic approach

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In press (19.11.2019) in Scientific Reports

In case of the first two publications, we used dried *D. radiodurans* cells and exposed them to harsh environmental conditions, which are present in outer space. This experiment involved an allegedly less harmful factor, which is ubiquitous in outer space. Evolution of all known life has been accompanied by gravity force, thus microgravity is rarely experienced by organism on Earth (Anken and Rahmann, 2002). Consequently, it is interesting to evaluate changes in the phenotype, but also on molecular level, induced by the loss of gravity. Naturally, taking into account future long-term space exploration missions, effects of microgravity on humans and plants are of utmost interest. However, effects on microorganisms are likewise important, as they will unavoidably join these missions. A 13-fold increased cell density and more resistance towards antibiotics were observed for *E. coli*, grown on the ISS (Zea et al., 2017). Furthermore, elevated levels of exopolymeric substances were observed for *Micrococcus luteus*, cultivated on the ISS (Mauclaire and Egli, 2010). Ultimately, *D. radiodurans* showed a faster recovery after radiation damage when exposed to microgravity (Kobayashi et al., 1996). To investigate possible molecular reactions triggered by microgravity, we performed proteomic and metabolomic measurements on *D. radiodurans* after growth under simulated microgravity.

To conduct this experiment, it was necessary to expose *D. radiodurans* for two days to microgravity, excluding short term exposure options, such as drop towers and parabolic flights. Consequently, a fast rotating 2-D clinostat was used to simulate reduced gravity for 48 h. The simulation of microgravity in a clinostat is based on the rotation around a horizontal axis perpendicular to gravity, assuming that cellular gravity-perception does no longer take place (Briegleb, 1992; Brungs et al., 2016; Hauslage et al., 2017). In case of agar plates, it is necessary to place a colony with a small diameter in the centre of the axis to guarantee the maximum simulation quality. For the experiment, the clinostat was placed in an incubator at 37 °C at 60 rpm, which correspond to ~0.0161 g residual acceleration for a colony diameter below 0.8 cm. Controls were placed in the same incubator to ensure same temperature and humidity conditions at static 1 g.

Obviously, growth of *D. radiodurans* under simulated microgravity caused less dramatic changes to the proteome compared to more severe environmental stress factors, for example, vacuum or radiation. Nevertheless, analysis of differently expressed proteins revealed several cellular pathways which responded to reduced gravity. Many microbial species, grown under low fluid shear environments (simulated microgravity) showed an increase in extracellular polymeric substances, cell aggregation, cell-cell contacts and biofilm formation (Searles et al., 2011; Kim et al., 2013; Wang et al., 2016). *D. radiodurans* does not produce biofilms, however several proteins associated with the extracellular milieu were present in higher abundances for cells grown under simulated microgravity. These include several transporter proteins, which are involved in transportation of polypeptides and DR_0774, a major component of the S-layer in the cell wall of *D. radiodurans*. DR_0774 was already observed as higher abundant after applying vacuum stress to *D. radiodurans* (Ott et al., 2019).

According to Uniprot, DR_0774 is annotated as type IV piliation system protein. In case of the biofilm producing *D. geothermalis*, adhesion threads are described as glycosylated type IV pili that are closely related to the type II protein secretion system (Saarimaa et al., 2006). In *D. radiodurans*, which lacks the ability to produce biofilms, Farci et al. (2014) described DR_0774 as a secretin like S-layer component of *D. radiodurans*. Together with SlpA, DR_0774 contributes to a complex that spans both the inner and the outer membrane (Farci et al., 2014). Thus, apart from being a structural pillar, which provides stability, these pillars may provide channels for trafficking. A higher abundance of these channels may contribute to increased extracellular trafficking, cell-cell contacts and other cell envelop associated events as a consequence of growth under simulated microgravity.

Cultivation under simulated microgravity may induce changes to expression patterns of different transcriptional regulators, which might be responsible for the observed measurements. Previous studies emphasized the altered expression of the Hfq regulon in various bacterial species (Wilson et al., 2007; Crabbe et al., 2011; Huang et al., 2015). Although this specific regulon is not present in *D. radiodurans* (Sun et al., 2002), other transcriptional regulators showed an induced expression. Four proteins were identified via a STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) search and uploaded to the SMART (Simple Modular Architecture Research Tool) database to annotate protein domains. Two of them are histidine kinases, which can be involved in controlling complex cellular processes, for instance as a result of adapting to environmental changes (Liu et al., 2017). The remaining two proteins

contained GGDEF and EAL domains. These domains are probably involved in modulating cell surface structures (Jenal, 2004) and extracellular protein production (Galperin et al., 2001).

Computationally derived results of this study suggest that growth under reduced microgravity induces gene expression of protein targets, involved in general stress response, cell envelope and the extracellular milieu. Further research is necessary to undoubtedly unravel the role of each identified target and why it responds to reduced gravity. Future steps include the creation of mutants and the exposure of *D. radiodurans* to hypergravity to search for expression patterns which correlate with the applied gravity force.

Publication (under review)

Molecular repertoire of Deinococcus radiodurans after 1 year of exposure to outer space

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Under Review (20.09.2019) in ISME

The final study included in the dissertation investigates molecular alteration in *D. radiodurans* after 1 y of exposure to outer space (LEO). Cells were placed in sample holders designed by the Tanpopo space mission team (Figure 1b). Wells located in round aluminium plates were filled with 1 µm of dried cell layers and covered with SiO_2 filters to cut off harmful UV radiation shorter than 200 nm (Yano et al., 2014). Dried *D. radiodurans* were shipped to the ISS on board the SpaceX Dragon commercial cargo spaceship attached to the SpaceX-Falcon 9 rocket, which launched on April 15th, 2015. They were manually attached to the exposed experiment handrail attachment mechanism (ExHAM), which was transferred to its final position on the Japanese exposure facility on May 26th (Figure 1a). *D. radiodurans* samples, used in this study, returned after one year on August 26th, 2016 on board the SpaceX Dragon C11.

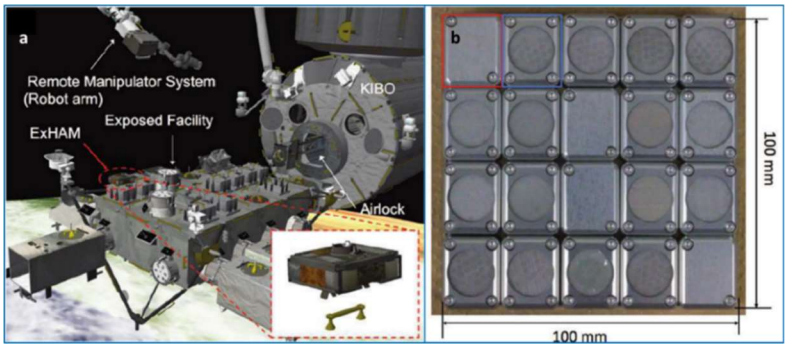

Figure 1: Exposure setup of dried *D. radiodurans* on the ISS (Figures from Kawaguchi et al. (2016)) (**a**) Japanese Exposure Facility KIBO of the ISS orbiting at an altitude of about 400 km. The exposed experiment handrail attachment mechanism (ExHAM) hosts the bacteria and can be maintained via the robotic arms of the remote manipulator system. (**b**) Exposure panel which is placed on the ExHAM. Each panel consists of 20 exposure units for round aluminium plates, containing 24 wells with the bacteria (blue rectangle). A passive dosimeter without samples is placed in every row (red rectangle).

Upon confirmation of survival and recovery in complex liquid medium for two hours, transcriptomic, proteomic and metabolomics measurements were performed. In addition, scanning electron microscopy (SEM) directly after exposure and transmission electron microscopy (TEM) before and after recovery were performed. To complement the study, a

simulation experiment, mirroring LEO conditions (vacuum, temperature cycles, UVC radiation) was performed at DLR, Cologne.

Survival assays confirmed the ability of *D. radiodurans* to survive 1 year in LEO, although survival rates were lower compared to control samples stored for the same period of time on Earth. SEM analysis revealed no changes in cellular integrity or morphological damage. However, the accumulation of nano-sized particles was detected on the surface of exposed cells. These spherical particles were not present on ground control samples and could be attributed to results of direct influence of outer space parameters on biological material (Maillard reactions). TEM observations further support the assumption of surface associated events, as the outer membrane of cells after exposure accumulate numerous vesicles.

The early stage of recovery after LEO exposure of *D. radiodurans* induces the expression of a variety of genes involved in different molecular mechanisms. Exposure to harsh conditions present in LEO causes damage to multiple cellular macromolecules, such as nucleic acids, lipids and proteins. Although *D. radiodurans* is able to efficiently deal with these induced damages, it still requires resources for the repair processes. Amino acids are a preferred carbon energy source (Slade and Radman, 2011) and as *D. radiodurans* is not able to utilize ammonia as nitrogen source (Venkateswaran et al., 2000), it relies on exogenous amino acids to cover the demand. A previous study showed an elevated level of proteolytic activity after exposure to ionizing radiation (Daly et al., 2010). In a comparable fashion, increased levels of several proteases were identified in *D. radiodurans* during recovery from LEO exposure. Moreover, the abundances of measured amino acids, notably glutamine and glutamate, were lower in exposed cells compared to control cells. One possible explanation for their reduced abundances might be the utilization as intermediates for repair processes. Similar results were observed for the LEO-simulation experiment, with glutamine and glutamate showing most reduction in abundance.

Measured proteomics data indicate that *D. radiodurans* focuses on nucleic acid repair during the early stage of recovery after LEO exposure. Especially the UvrABC endonuclease excision repair mechanism appears to be extremely important throughout this process. This flexible prokaryotic repair process is well suited to repair damage caused by UV radiation (Truglio et al., 2006). Indeed, an initial study to the Tanpopo space mission showed that although all factors present in outer space may pose a certain threat to *D. radiodurans*, UV radiation most negatively effects survivability (Kawaguchi et al., 2013). The transcriptomics analysis indicated

an increased expression of oxidative stress response proteins, such as catalase and *pdxT*, a gene involved in vitamin B6 synthesis, an efficient singlet oxygen quencher and potential antioxidant (Mooney et al., 2009). Compared to measured amino acids and organic acids, which appeared in lower abundances after LEO exposure, the level of the polyamine putrescine was higher. Putrescine may function as primordial form of stress molecules (Rhee et al., 2007). Moreover, many proteins and genes involved in putrescine biosynthesis appeared higher abundant after LEO exposure. Proteomic and transcriptomic approaches further revealed an increase of transporter proteins after space exposure. These additional transporters may enhance external nutrient uptake to support repair processes. Furthermore, they might be involved in withdrawal of vesiculated stress products, for instance damaged or misfolded proteins. This assumption is supported by TEM pictures, which show spherical-like structures associated with the outer membrane.

3 Concluding discussion and future perspectives

The title of this cumulative dissertation "Molecular mechanisms of *D. radiodurans* survivability in outer space" already implies that *D. radiodurans* possesses abilities to survive harsh conditions of outer space environment for a certain period. Although survival of *D. radiodurans* is reduced remarkably after being exposed on the ISS for one year, it is necessary to mention that further exposure up to three years does not decrease the survival rate much more (yet unpublished data). Exposure to extreme environmental conditions results in damages to intracellular macromolecules, which need to be addressed. The required response mechanisms were expected to be triggered once the dried, exposed cells recover in complex liquid medium. Therefore, cells from all conducted experiments (the only exception is the microgravity simulation), were put in recovering medium for a short period between 2-5 h. To gain a deep understanding of molecular mechanisms involved after recovery, cells were immediately harvested and homogenized once the desired timepoint was reached. Homogenization was typically followed by extraction of molecules for various -omics studies. Comparing preliminary simulation experiments with exposure to LEO reveals interesting similarities and differences in the corresponding -omics datasets (Figure 2).

Exposures to vacuum, UV/vacuum, LEO and, to a smaller extend, microgravity induce the expression of oxidative stress response proteins (*e. g.*, catalases, peroxidases). Although these proteins might not be unique to *D. radiodurans* and thus cannot explain its extraordinary resistance towards the extreme environmental challenges, they certainly help to alleviate ROS damage. In *D. radiodurans*, nucleic acids are similarly affected by ROS compared to other bacterial species, causing double-strand breaks, single strand breaks and damaged bases (Slade and Radman, 2011). Proteins involved in the UvrABC endonuclease excision repair mechanism appear higher abundant after all the applied stresses, emphasizing the importance of this multienzyme complex. The mechanism works on a vast array of DNA lesions, which can differ significantly in their chemical composition and molecular architecture (Van Houten and Snowden, 1993). After a DNA lesion is recognized, the DNA strand is cut on both sides of the lesion, the resulting oligonucleotide is removed, the gap is filled by DNA synthesis and the remaining nick is ligated (Goosen et al., 1998).

Another similarity observed after all applied stresses is a decrease in the relative amount of amino acids and organic acids after exposure during the early stages of repair. A possible explanation for this phenomenon might be the utilization of metabolites in repair processes

(Zhang et al., 2003). As *D. radiodurans* prefers amino acids as its energy carbon source and shows an increased proteolytic activity during repair processes after all applied stresses, this might result in a decreased amount at the snapshot impression after some hours of recovery.

Inspecting the genome of *D. radiodurans* makes it challenging to uncover genes, responsible for its resistance to multiple environmental factors. Research revealed an unusually high Mn/Fe ratio compared to radiation sensitive bacteria (Daly et al., 2007). Inspired by Mn-orthophosphate-peptide complexes present in *D. radiodurans*, synthetized complexes were injected into mice and increased their survivability against radiation damage (Gupta et al., 2016). However, *D. radiodurans* possesses another interesting property, which should be accentuated when considering its resistances. *D. radiodurans* is phylogenetically related to *Thermus thermophilus*, both are red pigmented, nonsporulating, aerobic, share a similar GC content and an A3β murein-type peptidoglycan (Henne et al., 2004). Nevertheless, they show a phenotypically difference in their gram staining. Bacteria belonging to the *Thermus* species have a typical gram-negative cell wall, stain gram-negative, but own features which typically appear in gram-positive bacteria (*e.g*., ornithine in the peptidoglycan, branched-chained fatty acids in the lipids) (Pask-Hughes and Williams, 1978; Donato et al., 1990). The *Deinococcus* spp. stains gram-positive, although their fatty acid profile resembles those of gram-negative bacteria and they have an outer cell membrane, which is a unique and defining characteristic of gram-negative species (Slade and Radman, 2011). The proteomics datasets after stress exposure showed higher abundances of proteins involved in the S-layer apparatus. SlpA, the major component of the S-layer, plays an important role in maintenance of cell envelope integrity in *D. radiodurans*. Deletion of *slpA* resulted in substantial alterations in cell envelope structure, defect in resistance to solvent and shear stresses and loss of the outer Hpi protein carbohydrate coat, which gives *D. radiodurans* its typical pink colour (Rothfuss et al., 2006). Another important protein for the cell envelope is the type IV piliation system protein DR_0774, which might work as a secretin like S-layer component (Farci et al., 2014). It is presumably involved in removing damaged intracellular macromolecules during recovery.

Results described in this cumulative dissertation cover three simulation experiments and wild type *D. radiodurans* exposed for one year outside the ISS. However, cells were exposed up to three years on the ISS and this long-term exposure experiment will probably unveil more mechanisms involved in repair after space exposure. Moreover, not only wild type *D. radiodurans* was exposed for a three-year period, but three mutants and *D. aerius* as well.

Mutations include genes responsible for DNA repair mechanisms, which are *recA*, *uvrA* and *pprA*. PprA plays a crucial role in a non-homologous end-joining pathway for the repair of radiation-induced DNA double strand breaks (Narumi et al., 2004). RecA is part of the bacterial SOS response and promotes DNA repair after exposure to extreme levels of ionizing radiation (Ngo et al., 2013). UvrA is part of the UvrABC excision repair mechanism and is responsible for initiating the repair by transporting UvrB to the damaged sites (Truglio et al., 2006). Based on our shotgun proteomic measurements, we expect the *uvrA* mutant to show most exciting results after exposure to LEO conditions, as all applied stresses (vacuum, UV and vacuum and LEO) induced the expression of genes related to the UvrABC excision repair mechanism.

Results of this cumulative dissertation can certainly support future space exploration missions and may be interesting for planetary protection issues. Even outside spacecrafts, certain microorganisms, for instance, *D. radiodurans* can maintain their cellular integrity and start to proliferate after getting in contact with the required nutrient resources at ambient conditions.

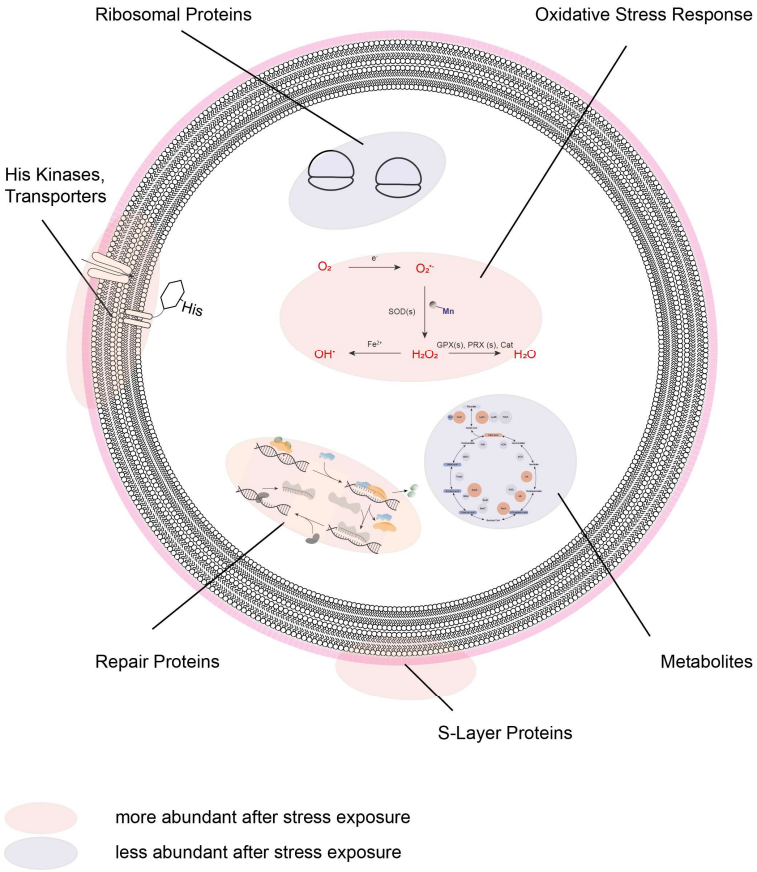

Figure 2: Graphical summary of important molecular alterations in *D. radiodurans* after exposure to real or simulated LEO conditions.

4 Results in form of publications and manuscripts

1. *Proteometabolomic response of Deinococcus radiodurans exposed to UVC and vacuum conditions: Initial studies prior to the Tanpopo space mission*

Emanuel Ott, *Yuko Kawaguchi, Denise Kölbl, Palak Chaturvedi, Kazumichi Nakagawa, Akihiko Yamagishi, Wolfram Weckwerth, Tetyana Milojevic*

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2. *Proteomic and Metabolomic Profiling of Deinococcus radiodurans Recovering After Exposure to Simulated Low Earth Orbit Vacuum conditions*

Emanuel Ott, *Yuko Kawaguchi, Natalie Özgen, Akihiko Yamagishi, Elke Rabbow, Petra Rettberg, Wolfram Weckwerth and Tetyana Milojevic*

Published in Frontiers in Microbiology (2019) DOI: 10.3389/fmicb.2019.00909

3. *Molecular response of Deinococcus radiodurans to simulated microgravity explored by proteometabolomic approach*

Emanuel Ott, *Felix M. Fuchs, Ralf Moeller, Ruth Hemmersbach, Yuko Kawaguchi, Akihiko Yamagishi, Wolfram Weckwerth and Tetyana Milojevic*

In press (19.11.2019) in Scientific Reports

4. *Molecular repertoire of Deinococcus radiodurans after 1 year of exposure to outer space*

Emanuel Ott, *Yuko Kawaguchi, Denise Kölbl, Elke Rabbow, Petra Rettberg, Maximilian Mora, Christine Moissl-Eichinger, Wolfram Weckwerth, Akihiko Yamagishi, Tetyana Milojevic*

Under Review (20.09.2019) in ISME

4.1 Proteometabolomic response of *Deinococcus radiodurans* exposed to UVC and vacuum conditions: Initial studies prior to the Tanpopo space mission

Emanuel Ott, Yuko Kawaguchi, Denise Kölbl, Palak Chaturvedi, Kazumichi Nakagawa, Akihiko Yamagishi, Wolfram Weckwerth, Tetyana Milojevic

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RESEARCH ARTICLE

Proteometabolomic response of *Deinococcus radiodurans* exposed to UVC and vacuum conditions: Initial studies prior to the Tanpopo space mission

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Abstract

The multiple extremes resistant bacterium *Deinococcus radiodurans* is able to withstand harsh conditions of simulated outer space environment. The Tanpopo orbital mission performs a long-term space exposure of *D. radiodurans* aiming to investigate the possibility of interplanetary transfer of life. The revealing of molecular machinery responsible for survivability of *D. radiodurans* in the outer space environment can improve our understanding of underlying stress response mechanisms. In this paper, we have evaluated the molecular response of *D. radiodurans* after the exposure to space-related conditions of UVC irradiation and vacuum. Notably, scanning electron microscopy investigations showed that neither morphology nor cellular integrity of irradiated cells was affected, while integrated proteomic and metabolomic analysis revealed numerous molecular alterations in metabolic and stress response pathways. Several molecular key mechanisms of *D. radiodurans*, including the tricarboxylic acid cycle, the DNA damage response systems, ROS scavenging systems and transcriptional regulators responded in order to cope with the stressful situation caused by UVC irradiation under vacuum conditions. These results reveal the effectiveness of the integrative proteometabolomic approach as a tool in molecular analysis of microbial stress response caused by space-related factors.

Introduction

The Gram positive bacterium *Deinococcus radiodurans* is extremely resistant to several environmental conditions, such as ionizing radiation [1], UV radiation [2], oxidation stress [3] and desiccation [4]. Such a multifaceted resistance of *D. radiodurans* ensures its potential to survive in the harsh outer space environment during interplanetary transfer. The Tanpopo, which means dandelion in Japanese, mission [5] includes a long-term exposure (separate

data collection and analysis, decision to publish, or preparation of the manuscript.

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experiments between one to three years) of *D. radiodurans* on the Japan Experimental Module of the International Space Station (ISS) in the low Earth orbit (LEO). It is performed in order to validate the panspermia theory [6]—the possible transfer of life between Earth and extra-terrestrial bodies. To ensure that *D. radiodurans* is suitable for a long term exposure experiment on the ISS, several preliminary exposure experiments have been performed by Kawaguchi, Yang [7]. During these experiments, the different parameters (heavy ion beam radiation, temperature cycles, vacuum and UVC radiation) were adapted to mirror LEO conditions and the following survival tests revealed that UVC radiation had the highest impact on cell survivability [7, 8]. It was shown that, even though *D. radiodurans* possesses high tolerance against UVC radiation, direct exposure of monolayers to LEO conditions results in no survival [5]. However, aggregated *Deinococci* cells exposed to UVC radiation showed that they should withstand solar UV radiation on the ISS for one year as multilayers of dehydrated cells, and survive, wherein upper cellular layers cover and protect underlying inner cells. Approximately 200 μm of cell layers are necessary to shield the inner layers of *D. radiodurans* efficiently from solar UV radiation. Based on these findings, massapanspermia has been proposed, implying that apart from rocks which shield the microbes against solar UV radiation (i.e., lithopanspermia), it is possible for cell-aggregates to function as a protective ark for interplanetary transfer of microbes, where upper layers shield lower layers from the harmful environment [7, 8]. Proving this theory is a part of the Tanpopo mission, as cell aggregates with different thicknesses of *D. radiodurans* are directly exposed to LEO conditions. These factors are microgravity, vacuum down to 10^{-7} Pa, solar UV radiation, galactic radiation, solar cosmic radiation, van Allen Belts and temperature cycles (from -120˚C up to 120˚C every 90 min) [9].

Complementing survivability studies, an approach to unravel the response to LEO conditions on a molecular level is desirable, as it might provide an explanation how it is possible for certain organisms to survive under such extreme conditions. A systems biology approach, especially the combination of several–omics analysis, improves the knowledge of microbial stress response mechanisms and explains how microorganisms respond to environmental changes on the molecular level. Environmental stresses can damage cells due to the formation of reactive oxygen species (ROS), which cause lipid peroxidation, protein oxidation and oxidative DNA damage. Exogenous factors can further interfere with genome integrity as they cause double strand breaks, primarily induced by vacuum and single strand breaks, primarily induced by UVC irradiation [3]. In addition to breaks, three major classes of bipyrimidine photoproducts (BPPs), cyclobutane pyrimidine dimers, pyrimidine 6–4 pyrimidone photoproducts and Dewar isomers, are formed if organic material is exposed to UVC radiation [10]. Although there is no evidence that the DNA damage repair mechanism is very different in *D. radiodurans* compared to *Escherichia coli* [11]. Despite the number of BPPs after UVC irradiation of 500 Jm^{-2} being comparable between *D. radiodurans* and *E. coli*, *D. radiodurians* is about 25 times more resistant to BPPs compared to *E. coli* [12, 13]. The reason for this higher resistance lies in the protection of intracellular proteins against UV induced oxidative damage [3]. However, as the amount of DNA damage caused by UVC irradiation and desiccation is severe, an efficient DNA repair mechanism is still important. Two separate nucleotide excision repair pathways act simultaneously to remove BPPs [14]. The pathways rely on the proteins UV DNA damage endonuclease (*uvsE*) and UvrABC system protein A (*uvrA*) [13]. Both pathways require the proofreading DNA polymerase I (*polA*), as mutants without the *polA* gene are extremely sensitive to UVC irradiation [15]. Another essential protein for enzymatic repair of DNA damage is RecA, which cleaves the repressor LexA that represses SOS response genes, like DNA repair enzymes [16]. After successful excision, recombinational repair is performed. The genome repair does not rely on a new pathway for double-strand break repair, caused by desiccation stress, but is rather a set of recombinational DNA repair functions which can be

observed in many other species [17]. Important proteins for the recombination process are gyrases (*gyrA* and *gyrB*), which cause negative supercoils to favor strand separation, DNA replication, transcription, recombination and repair [18]; PprA to stimulate the end-joining reaction catalyzed by DNA ligases [19] and the different single-stranded DNA binding proteins DdrA [20], DdrB [21], DdrC [22] and DdrD [22] for RecA independent genome reconstruction processes.

The aim of this study was to decipher the molecular response of *D. radiodurans* to space-related conditions of UVC radiation and vacuum using the experimental set-up of Tanpopo orbital project. Here we present an integrative proteometabolomic approach applied to reveal key components of the molecular mechanism of *D. radiodurans* survivability in response to UVC irradiation under vacuum conditions.

Materials and methods

Cultivation and preparation of dehydrated *D. radiodurans* cells

D. radiodurans R1 (ATCC 13939) was cultured 15 h in mTGE medium (1%(w/V) tryptone, 0.6%(w/V) beef extract, 0.2%(w/V) glucose) at 30˚C in an incubator with shaking speed of 150 rpm until it reached the anaphase of the logarithmic phase. Liquid cultures of *D. radiodrans* R1 were washed in 10 mM phosphate buffer (PB). This step was repeated three times. Aluminum plates containing cylindrical wells (2.0 mm diameter, 2 mm depth) with flat floor were used as sample holders [8]. Twelve microliter of a cell suspension ($2.9^{*}10^{9}$ cells/mL) were dropped into 4 wells and dried up under $3.3^{*}10^{-2}$ atm in a desiccator at room temperature under sterile conditions. These steps were repeated 6 times. The amount of deinococcal cells was $3.5^{*}10^{7}$ cells per well corresponding to a multilayer of 200 μm thickness (S1 Fig). The cells were dried up under $3.3^{*}10^{-2}$ atm for 16 h.

UVC and vacuum exposure

A mercury lamp 254 nm was used to irradiate deinococcal cells in the vacuum chamber. The setup of the UVC-irradiation experiment was described previously [7]. The aluminum plates containing dehydrated cells of *D. radiodurans* were exposed to $UVC_{254\ nm}$ dose 862.0 kJ/m^2 under approximately 400 Pa. Control samples were only dehydrated cells kept in a desiccator at room temperature.

Survival assay

After the exposure to UVC and vacuum, cells were recovered from wells of aluminum plate using PB 10 mM. The cell suspension was serial diluted with PB 10 mM and the diluted cell suspension was dropped on mTGE agar plates [7]. The plates were incubated at 30˚C for 1.5 days. Surviving fractions were determined from the ratio of N/N_0, with N being the number of colony formation unit (cfu) of the irradiated cells and N_0 being the CFU of the control samples.

Scanning electron microscopy

The morphology and cellular integrity of the dehydrated cells of *D. radiodurans* deposited on aluminum plates were examined with a Zeiss Supra 55 VP scanning electron microscope. The dehydrated cells were coated with a thin Au/Pd layer (Laurell WS-650-23 spin coater). The imaging of dehydrated clustered cell layers and single cells was performed with the acceleration voltage of 5 kV.

Cultivation conditions

For cultivation of the dehydrated *D. radiodurans* cells, two wells were resuspended in 100 μL phosphate buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7) to inoculate 10 mL of mTGB medium. In total 4 biological replicates of the control non-irradiated cells and 4 biological replicates of the UVC/vacuum-irradiated cells were incubated at 30˚C with an agitation rate of 150 rpm for 5 hours. The growth of the cells was monitored by cell counting using a hemacytometer.

Integrative extraction of proteins and metabolites

Extraction and analysis of metabolites and proteins from one sample was performed according to Weckwerth, Wenzel [23] with slight modifications (for a detailed version of the extraction protocol see dx.doi.org/10.17504/protocols.io.j3bcqin). The cells were harvested (3000 g, 5 min, 4˚C), washed with 10 mM phosphate buffer three times and finally resuspended in ice-cold 1 mL MCW (methanol:chloroform:water 2.5:1:0.5). 0.5 g of FastPrepTM lysing matrix B (MP Biomedicals, Santa Ana, USA) was added to the mixture and the cells were homogenized with a FastPrepTM-24 Instrument (MP Biomedicals, Santa Ana, USA) at 3x4.5 m/s for 30 s with a 5 min cooldown on ice between the cycles. After centrifugation (21000 g, 15 min, 4˚C) the supernatant, which contained the metabolites was transferred into a new tube. The pellet, which contained the precipitated proteins was stored at 4˚C for the subsequent extraction. Phase separation was induced by adding 200 μL of water. The phases were separated in different tubes and dried in a vacuum concentrator.

Derivatisation and analysis of the metabolites with GC-TOF-MS

Polar metabolites were dissolved in 10 μL of 40 mg mL-1 methoxyamine-hydrochloride in pyridine through shaking at 650 rpm at 30˚C for 90 min. Subsequently, 40 μL of a silylation mix (1 mL N-methyl-N-trimethylsilyltrifluoroacetamid spiked with 30 μL of a mix of even-number alkanes (C10-C40)) was added and the mixture was incubated for 30 min at an agitation rate of 650 rpm at 37˚C. After centrifugation (14000 g, 2 min), the supernatant was transferred into a glass vial and 1 μL of it was injected into the GC (Agilent® 6890 gas chromatograph) in splitless injection mode.

For separation of the metabolites, an Agilent HP-5MS column (30 m length, 0.25 mm diameter and 0.25 μm film) was used. Further parameters were set as following: flow rate 1 mL min^{-1}; injection temperature 230˚C; column temperature started at 70˚C for one minute, then heated up to 330˚C in 9 min, where it was hold for 8 min; recorded masses in the LECO Pegasus® 4D GC×GC-TOF spectrometer were set between 40–700 m/z. Apart from the samples, a house intern standard mix of certain metabolites was measured to get level 1 identifications of common primary metabolites.

Identifications of the metabolites were based on matching the obtained MS-spectra and retention times with an in-house library (extended gmd database). Peak integration was performed with the LECO ChromaTOF® software. Metabolites which were also identified in the standard mix were considered a level 1 identification, the ones which were not present in the mix, but the retention index and the mass spectrum was similar to one of the database were considered a level 2 identification. The measured areas were normalized against the number of cells, used for the extraction.

Protein extraction

The pellets were suspended in 400 μL of a protein extraction buffer (100 mM NaCl, 100 mM Tris-HCl pH 7.5, 10% (v/v) Glycerol, 3% SDS (m/v)) and an equal amount of phenol

(saturated with Tris-HCl, pH 7.0, Roth) was added to the suspension. The mixture was vortexed, centrifuged (20000 g, 2 min, 4˚C) and the lower, phenolic phase was transferred into a new tube. To precipitate the proteins, five volumes of ice-cold 0.1 M ammonium acetate in methanol was added. After keeping the suspension at -20˚C overnight, it was centrifuged (5000 g, 30˚C, 4˚C) and the pellet was washed twice with methanol and once with acetone.

Protein quantification and In-gel digestion

Protein analysis was performed according to Chaturvedi, Ischebeck [24] with slight modifications. The pellet was dissolved in approximately 30 μL of urea buffer (6 M urea, 5% SDS). The proteins were quantified with a BCA (bicinchoninic acid) assay kit with a BSA standard. A total amount of 100 μg protein for each sample was mixed with 5x Laemmli buffer, heated at 95˚C for 5 min and applied on a SDS-polyacrylamide gel (separation gel 12%, stacking gel 5%). A voltage of 40 V was applied until the samples reached the interphase between the gels. Then the voltage was switched to 80 V until the bromophenol blue run approximately one centimeter into the separation gel. Gel staining was performed with 40% (V/V) methanol, 10% acetic acid (V/V), 0.1% (w/V) Coomassie R-250 in milliQ-water for 30 min, followed by four 20-min destaining (40% (V/V) methanol, 2% (V/V) acetic acid). Finally, the gel was washed in milliQ-water for half an hour and all protein lanes for each replicate were cut out of the gel.

For further analysis, the gel bands were cut into small pieces around 1 mm^3 and 1 mL 200 mM AmBic (ammonium bicarbonate) in 50% ACN (acetonitrile) solution was added to each replicate. The samples were incubated (37˚C, 30 min, agitation rate 650 rpm) and the supernatant was discarded. This process was repeated until the colour of the gel pieces completely disappeared. Afterwards, 500 μL of 50 mM AmBic in 5% ACN were added, incubated (37˚C, 15 min, agitation rate 650 rpm) and the supernatant was discarded. Finally, 500 μL of ACN were added to the gel pieces, incubated (37˚C, 10 min, agitation rate 650 rpm) and the supernatant was discarded. Gel pieces were air-dried and 12.5 ng/μL trypsin (Roche; in 25 mM AmBic, 10% ACN, 5 mM CaCl2) was added until all gel pieces were covered by the solution. Tryptic digestion took place at 37˚C for 16 h without shaking.

Peptide extractions and desalting

For the peptide extraction, 150 μL of 50% ACN with 1% formic acid were added to each tube, incubated for 5 min at room temperature, sonicated shortly in a low intensity ultrasound bath and the supernatant was transferred to a new tube. The procedure was repeated once. Ultimately, 100 μL 90% ACN with 1% formic acid were added, incubated 5 min at room temperature and the supernatant was transferred to the same tube again. Extracted peptides were dried down in a vacuum concentrator.

The peptides were suspended in 4% ACN, 0.25% formic acid and applied on C18-Bond Elut 96-well plates (Agilent Technologies). They were washed five times with 400 μL of water, whereby the first flow through was kept for another desalting step with graphite. Washed peptides were eluted with 400 μL methanol. Graphite spin column (MobiSpin Column F, MoBiTec) desalting with the first flow through was performed according to the manufacturer's manual (Thermo scientific, Pierce® graphite spin columns). The desalted eluates from the plates and the columns were combined for each sample and dried down in a vacuum concentrator.

Shotgun proteomics with HPLC nESI-MS/MS

Peptides were dissolved in 2% ACN with 0.1% formic acid to a theoretical concentration of 0.2 μg μL^{-1} based on the amount of protein which was loaded on the gel. 1 μg of each sample (4 biological replicates for UV and control) was applied on a C18 reverse phase column (Thermo

scientific, EASY-Spray 500 mm, 2 μm particle size). Separation was achieved with a 180 min gradient from 100% solution A (0.1% formic acid) to 40% solution B (90% ACN and 0.1% formic acid) with a flow rate of 300 nL min^{-1}. nESI-MS/MS measurements were performed on an Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) with the following settings: Full scan range 350–1800 m/z resolution 120000, max. 10 MS2 scans (activation type CID), repeat count 1, repeat duration 30 sec, exclusion list size 500, exclusion duration 30 sec, charge state screening enabled with rejection of unassigned and +1 charge states, minimum signal threshold 500.

Protein identification and LFQ (label free quantification)

For identification, a Uniprot database (last updated 2015-06-20) containing the annotation of 3088 proteins for *D. radiodurans* was used. The received Thermo raw files from the instrument were identified and quantified in MaxQuant (version 1.5.7.0) with the following parameters: first search peptide tolerance 20 ppm; main search peptide tolerance 4.5 ppm; ITMS MS/MS match tolerance 0.6 Da; a minimum of 7 amino acid were required for the peptide identification and a minimum of two peptides for the protein identification; a maximum of two missed cleavages were allowed; a maximum of five modifications (oxidation of methionine and acetylation of the N-term) were allowed per peptide; a retention time window of 20 min was used to search for the best alignment function and identifications were matched between runs in a window of 0.7 min; a revert decoy database was used to set a cut-off at a FDR of 0.01. LFQ with a minimum ratio of two was performed when at least one MS2 identification was present.

Statistical evaluation

Key metabolite pathways and protein abundance differences between the control cells and the cells exposed to UVC/vacuum conditions was analyzed with Perseus. PCAs and heatmaps were created with the R packages heatmaps.2 and ggplots. Cytoscape was used for the combined analysis of metabolomics and proteomics data. For all analysis, the LFQ intensity values which were calculated by MaxQuant, were used. First, the fold changes between the proteins were calculated. Proteins which weren't identified in at least three of the four replicates in at least one condition were excluded from the list. After z-transformation of the values, a Welch's T-test was performed between the two conditions. For all proteins with known annotations, different gene ontologies (cellular compartment, biological process, molecular function) and KEGG pathways were added as categorical columns. With these columns, a Fisher exact test ($p\text{-value} < 0.02$) was performed to identify gene ontologies/KEGG pathways with an unusual representation of proteins within the T-test.

Results

Effects of UVC/vacuum conditions on cellular integrity, growth and survivability of dehydrated *D. radiodurans*.

Survival assays after exposure to UVC irradiation under vacuum showed an average survival rate of $6.5^{*}10^{-1}$ (0.04 s.d.) compared to non-exposed control cells. In order to investigate cellular integrity after UVC irradiation under vacuum conditions, the surface of dehydrated clustered cell layers of *D. radiodurans* deposited on aluminum plates was examined with scanning electron microscopy (Fig 1 and S1 Fig). The observed typical morphology of diplococci and tetracocci of *D. radiodurans* is shown in Fig 1. In line with the extreme desiccation resistance of *D. radiodurans*, there was no detectable damage of cell surface and morphology of *D. radiodurans* observed after drying procedure under the control conditions (Fig 1A, 1C and 1E and

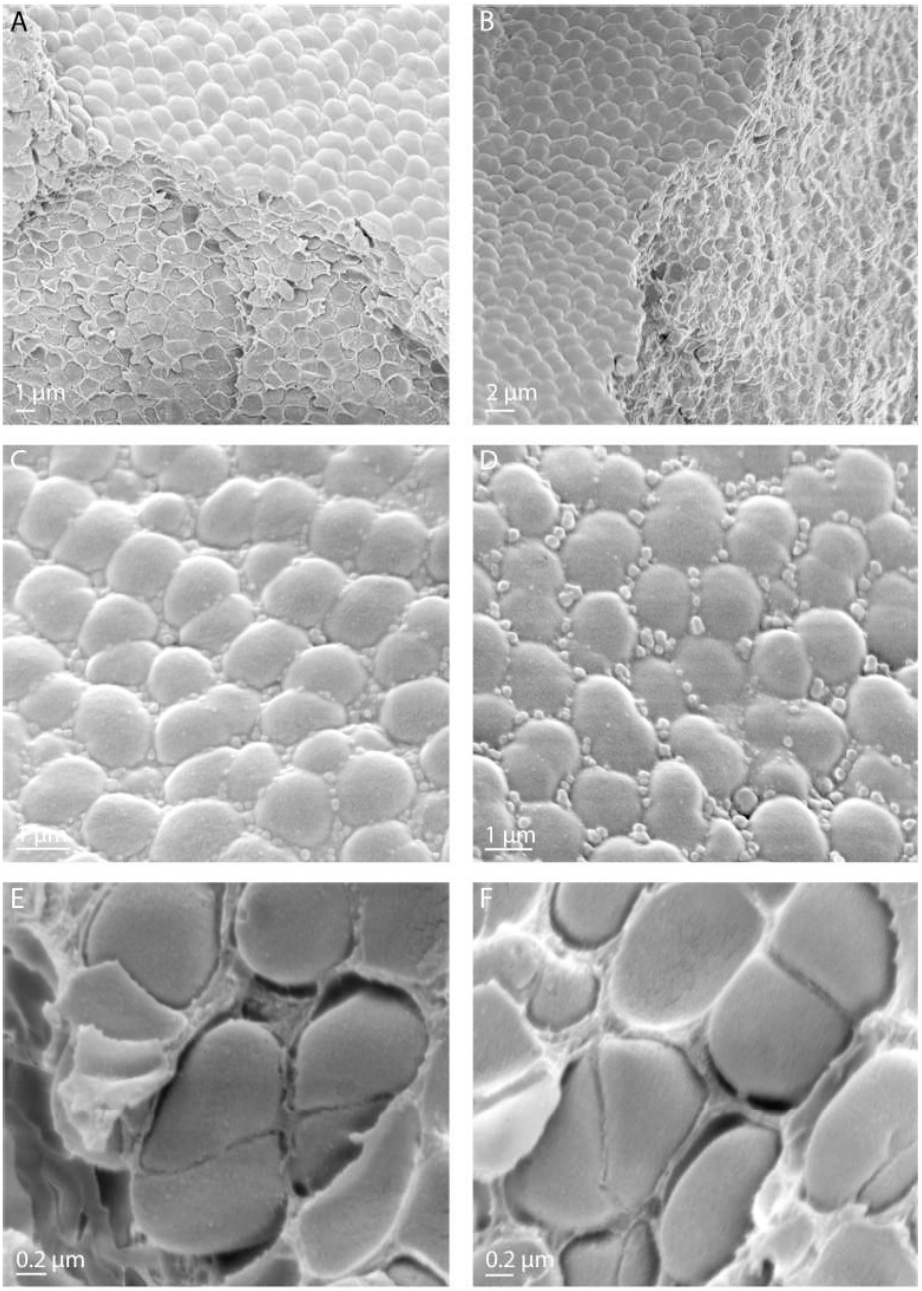

Fig 1. Scanning electron microscopy images of dehydrated cells of *D. radiodurans* deposited on aluminum plates and used in experimental set up of Tanpopo mission. (**A**, **B**) Scanning electron microscopy images, showing upper surface and inner content of multilayers of dehydrated *D. radiodurans* cells deposited on aluminum plates. (**C**, **D**) Higher magnification images displaying upper surface of multilayers of dehydrated cells of *D. radiodurans*. (**E**, **F**) Magnified images of tetracocci and diplococci of *D. radiodurans* taken from the inner part of dehydrated multilayers. (**A**, **C**, **E**) control cells of *D. radiodurans*; (**B**, **D**, **F**) cells of *D. radiodurans* exposed to UVC-vacuum conditions.

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S1 Fig). UVC irradiation under the vacuum conditions neither affected morphology, nor cellular integrity of dehydrated cells of *D. radiodurans* (Fig 1B, 1D and 1F and S1 Fig). Correspondingly, the analysis of survivability of cells using standard microbiological plating techniques and counts of colony forming units showed a relative survival rate of 65% for UVC/vacuum exposed cells compared to control conditions (S1 Table).

Functional analysis of identified proteins of *D. radiodurans*

The LC-Orbitrap Elite™ measurements identified 1661 proteins in at least one sample, comprising 54% of *D. radiodurans* genome. 59 proteins were only found in at least one of the UV irradiated replicates.

GO (Gene Ontology) annotations were assigned using the PANTHER (Protein ANalysis THrough Evolutionary Relationships, http://pantherdb.org, V 11.1) online tool with the latest GO database (released 2017-04-24). The tool was able to map 1452 Uniprot IDs and provide the corresponding GO annotation in case there was one. In total the molecular functions of 865, the biological processes of 954 and the cellular compartments of 332 were annotated on the second hierarchical level of gene ontology annotations (Fig 2). Regarding their biological process, the three most dominant categories were metabolic process (38%), cellular process (19%) and localization (4%). 47% of the proteins from the category metabolic process belonged to primary metabolic processes. Overall, the most dominant protein classes were transferases (21%), hydrolases (16%), oxidoreductases (15%) and nucleic acid binding (12%). Apart from that, 553 proteins could be assigned to at least one KEGG pathway.

Differences in the proteome between UVC/vacuum exposed and control cells

For quantitative analysis, only proteins which were identified in at least three out of four replicates in at least one of the conditions were used (1457 in total) (S2 Table). The LFQ intensities were z-scored and the PCA-scores (Fig 3) for all four biological replicates showed a clear separation between control and UVC treated cells on component 1.

A Welch's t-test (p-value < 0.05) identified 209 proteins as more abundant in the control cells and 357 in the cells exposed to UVC/vacuum conditions. With these proteins, a Fisher exact test for the KEGG categories was performed. The categories with an unusually high amount of proteins in one of the conditions are shown in Fig 4. Only categories with at least five identified proteins and a minimum enrichment factor of two in at least one condition are shown.

Annotations and overrepresentation studies provide an overview of pathways which might be affected by the applied stress condition. However, the majority of proteins ($> 99.2\%$; May 2017) in the gene ontology database are annotated based on automatic algorithmic sequence similarity search instead of manual curation. Therefore, a deeper comparison to the literature and described proteins is inevitable. Fig 5 shows boxplots of mainly manually curated proteins/genes related to DNA damage and oxidative stress response. Most of these proteins (8 out of 11 of selected DNA damage response proteins and 10 out of 11 of selected oxidative stress response proteins) show a significantly higher abundance in the UVC/vacuum exposed cells of *D. radiodurans*. Variances and number of outliers between the two conditions are similar for the chosen proteins.

Metabolomic analysis of *D. radiodurans*

Metabolite analysis from the same cells revealed 31 metabolites which were chosen for quantification. Analysis with GC-TOF usually leads to identification of primary metabolites

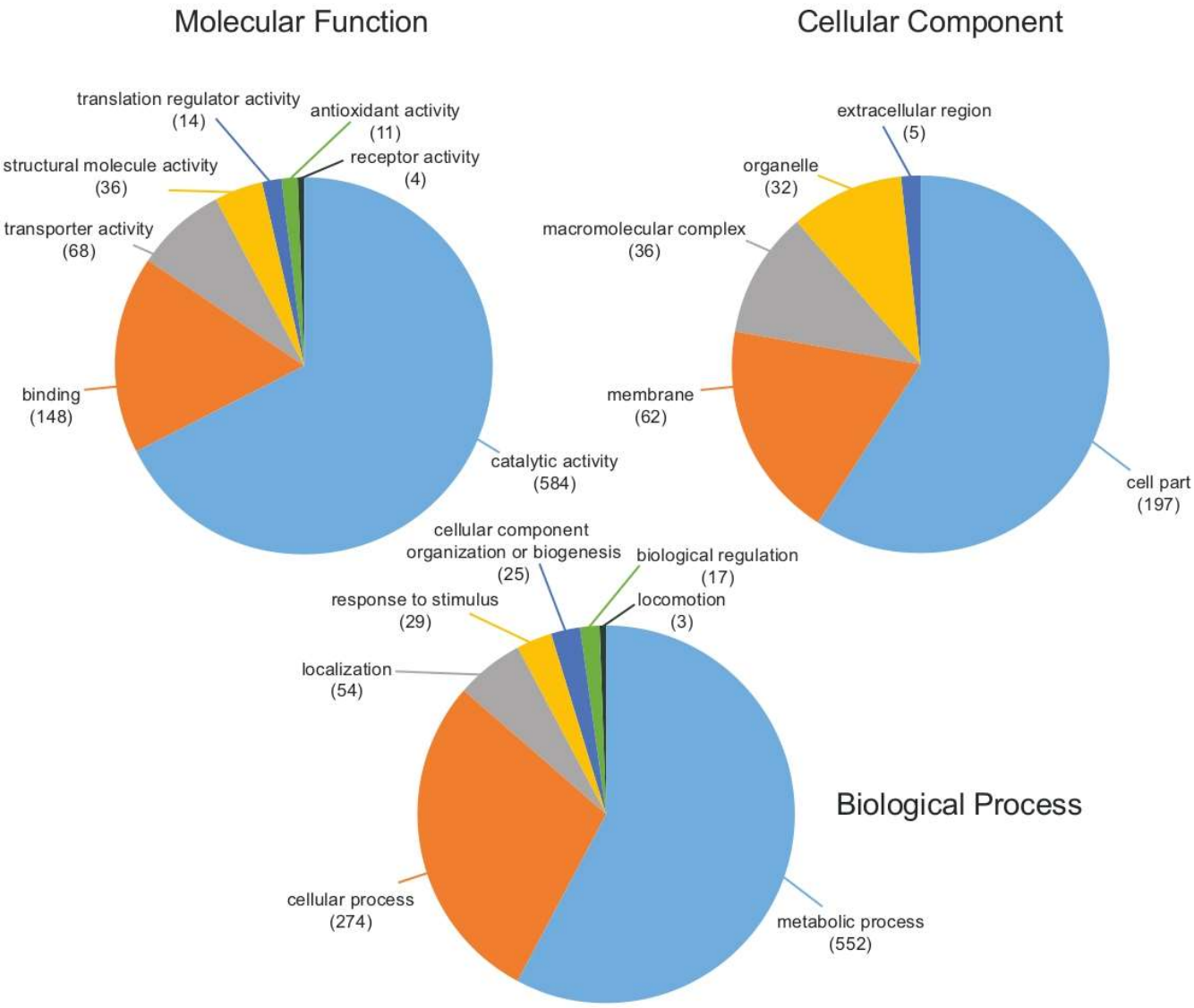

Fig 2. First two levels of gene ontology annotations of all proteins of *D. radiodurans*, which were identified in at least three out of four replicates in at least one condition.

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associated with the primary metabolism. For statistical analysis, only metabolites which were present in at least three out of four replicates in at least one of the conditions were used. The normalized areas were z-scored and compared with a Welch's t-test (p-value < 0.05). 24 metabolites, which abundances were considered different between the two conditions, were blotted as a heatmap (Fig 6). Six of them (O-Palmitoyl-L-Carnitine chloride, octadecanoic acid, ethanolamine, folic acid, mannosamine and cytidine-5-triphosphate disodium salt) were identified on level 2, all the others were identified on level 1 [25]. The majority of metabolites were more present in the control cells of *D. radiodurans* (S3 Table).

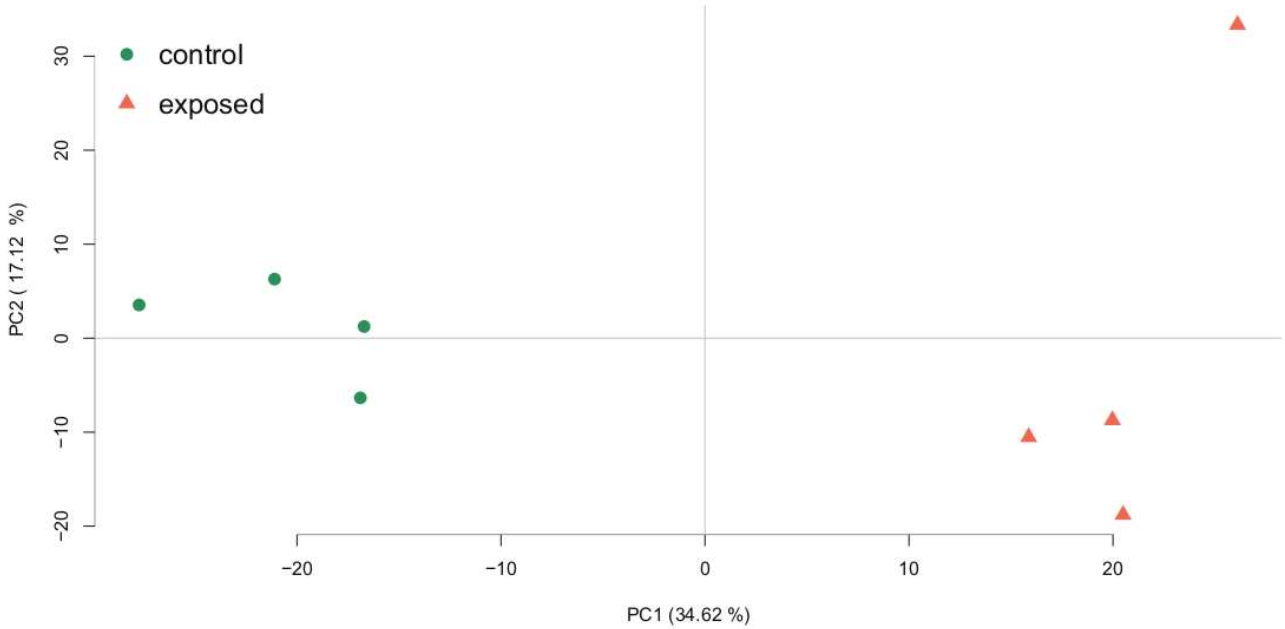

Fig 3. PCA score-plot of the z-scored label free quantification intensities. A clear separation can be observed on the PC1 level, which explains 34.62% of the data's variance, between the UVC/vacuum treated samples (red) and the control samples (green).

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Proteometabolic analysis of the TCA cycle

After exposure to stress conditions, additional energy is required to recover the cells. The TCA cycle provides large amounts of energy. Most TCA cycle related proteins showed a higher abundance in the UVC/vacuum exposed cells of *D. radiodurans* according to the LC-MS

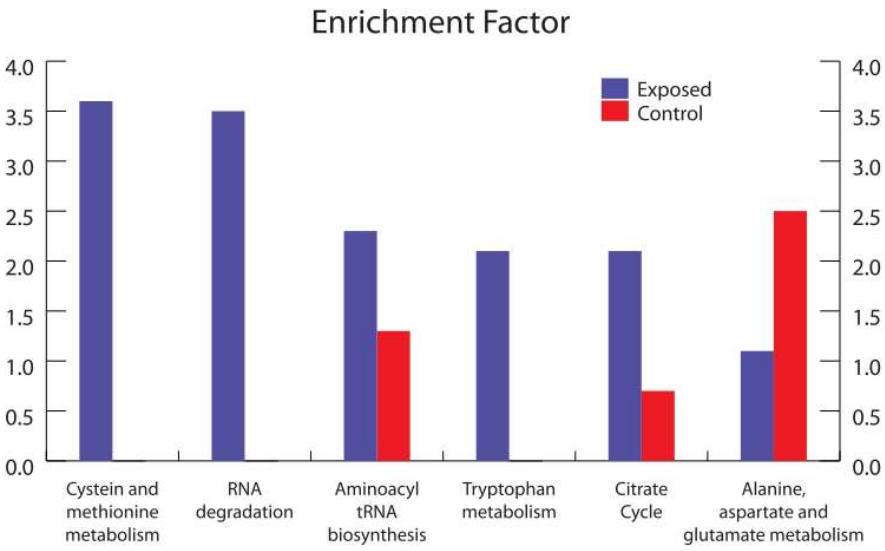

Fig 4. Bar plot of KEGG categories (x-axis) with corresponding enrichment factors (y-axis). Categories with a minimum enrichment factor of two for either UVC/vacuum (blue) treated or control (red) conditions are mapped. An enrichment factor of zero means that not a single protein in this category was upregulated in the displayed condition.

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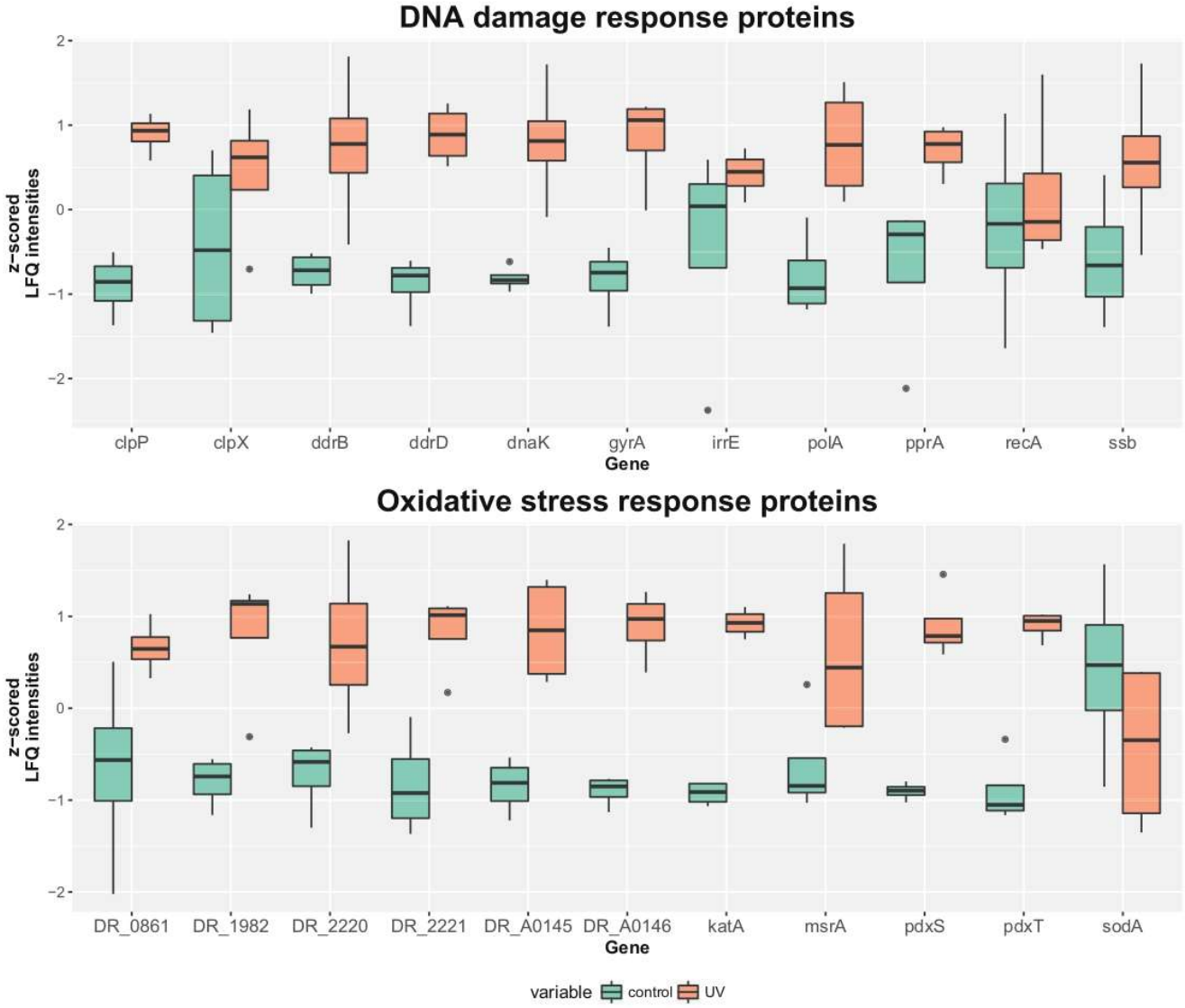

Fig 5. Boxplot of genes encoding important damage response proteins in *D. radiodurans* under the conditions of UVC/vacuum exposure. For every gene, the z-scored LFQ intensities are compared between the control and UVC/vacuum condition. The lower and the upper hinges correspond to the first and the third quartiles. The whiskers extend a maximum of 1.5 times the inter-quartile range. Outliers are indicated as dots. Proteins which are encoded by the mapped genes: Clp protease subunits (clpP and clpX), DNA damage response proteins (ddrB and ddrD), chaperone (dnaK), DNA gyrase subunit A (gyrA), radiation response metalloprotease (irrE), DNA polymerase (polA), DNA repair protein (pprA), recombinase (recA), single-stranded DNA-binding protein (ssb); catalase (katA), uncharacterized protein (DR_A0146), superoxide dismutase (sodA), phytoene dehydrogenase (DR_0861), Pyridoxal 5'-phosphate synthase (pdxS and pdxT), thioredoxin reductase (DR_1982), putative peroxidase (DR_A0145), peptide methionine sulfoxide reductase (msrA), tellurium resistance protein (DR_2220 and DR_2221).

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measurements. Accordingly, organic acids such as succinic acid, fumaric acid and malic acid were identified (at level 1) and quantified by GC-time of flight (TOF)-MS. Other metabolites were either not identified (limit of detection) or not abundant enough for quantification (limit of quantification).

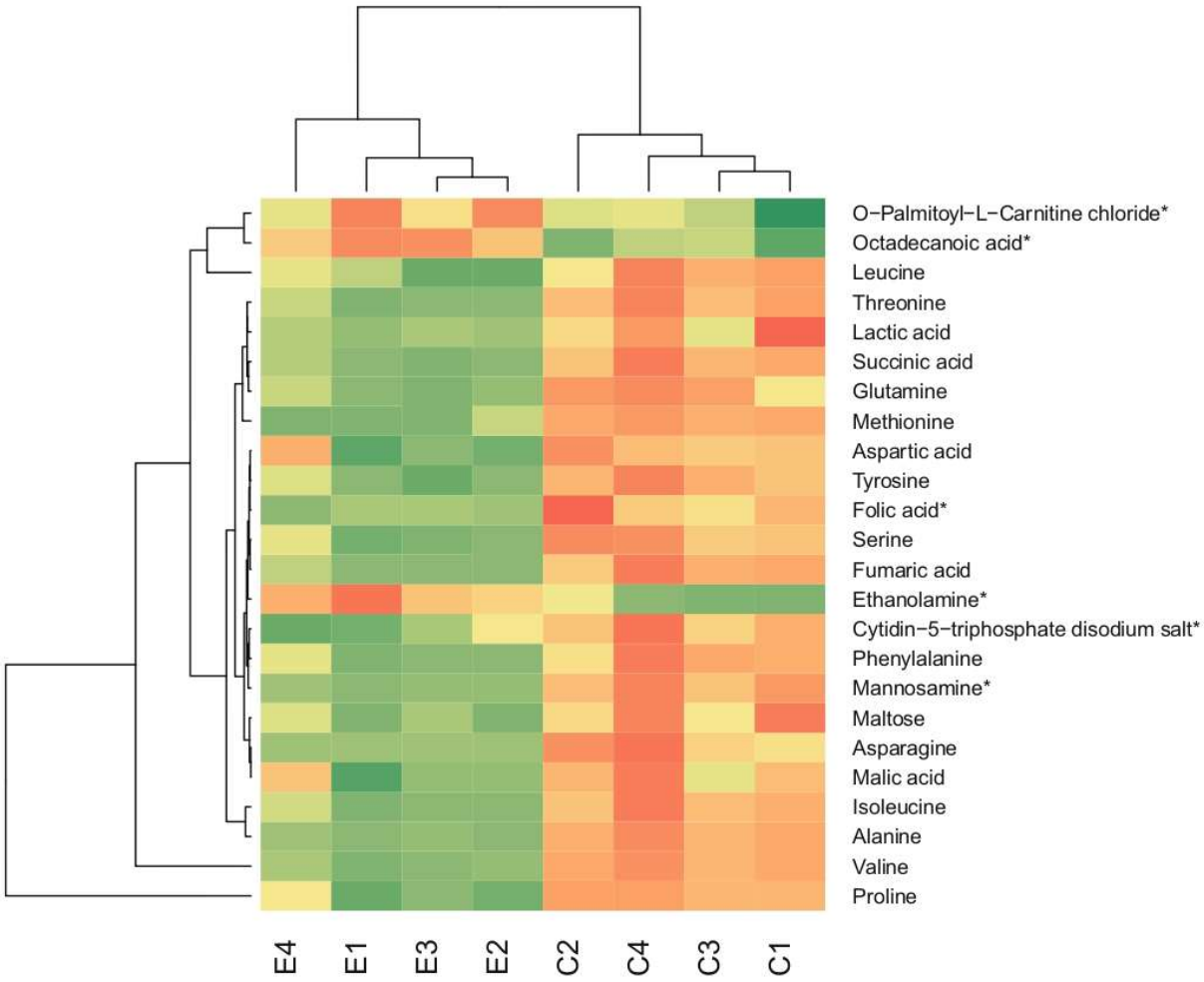

Fig 6. Heatmap of metabolites, which were considered different between cells of *D. radiodurans* exposed to UVC/vacuum and non-exposed control cells. Eucledian distance was used for calculating the dendrogram. *Identification was based on database research and not on a reference substance.

https://doi.org/10.1371/journal.pone.0189381.g006

Fig 7 shows a basic version of the TCA cycle of *D. radiodurans* according to the KEGG website including quantitative proteomics and metabolomics data. The pyruvate dehydrogenase complex, which is responsible for the connection between glycolysis and TCA cycle as it converts pyruvate to coenzyme A, consists of three subunits. The E1 component (aceE) shows a high abundance in the irradiated cells, whereas the dihydrolipoamide acetyltransferase (DR_0032) is more abundant in the control cells. However, according to the KEGG database, another acetyltransferase DR_0256 (S2 Table), which is more abundant in the irradiated cells, is also active in the pyruvate dehydrogenase complex. The third subunit, dihydrolipoamide dehydrogenase (DR_2370) is not significantly higher abundant in any of the two conditions. Further identified and quantified proteins, which are all part of the TCA cycle, are citrate synthase (gltA), aconitate hydratase (acn), isocitrate dehydrogenase (DR_1540), 2-oxoglutarate dehydrogenase (sucA), dihydrolipoamide succinyltransferase (DR_0083), succinate-CoA ligase (sucC), succinyl-CoA synthetase (sdhB), fumarate hydratase (fumC) and malate dehydrogenase (mdh).

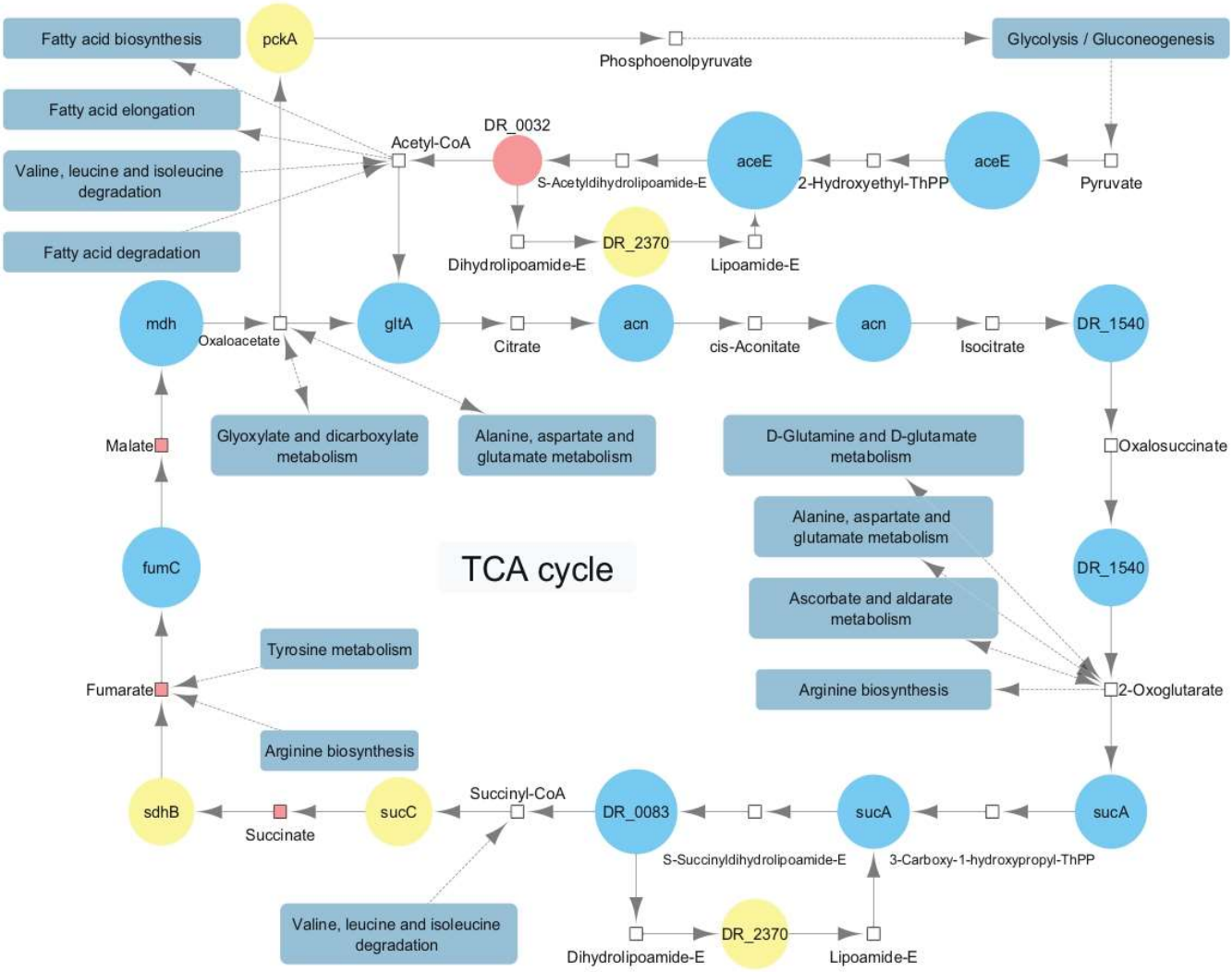

Fig 7. Main components of the TCA cycle in *Deinococcus radiodurans* connected to related pathways under the conditions of UVC/vacuum exposure. Metabolites are shown as rectangles. The areas of the proteins, which are shown as circles, correspond to the fold change between cells of *D. radiodurans* exposed to UVC/vacuum conditions and control non-exposed cells. The color shows whether the average protein or metabolite level was more abundant in the UVC/vacuum exposed cells (blue), the control cells (red), none of both conditions (yellow) or not measured/not abundant enough (colorless).

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Discussion

Overall alterations in the proteome of *D. radiodurans* after UVC/vacuum exposure

Proteomic analysis revealed that functional categories of cysteine, methionine and tryptophan metabolism, RNA degradation, aminoacyl-tRNA biosynthesis were overrepresented in *D. radiodurans* exposed to UVC/vacuum conditions compared with the control cells (Fig 4). A previous study [26] showed similar categories of differentially expressed genes after gamma-irradiation. In opposite, the proteins of alanine, aspartate and glutamine metabolism were downregulated in irradiated cells of *D. radiodurans*. Methionine and cysteine as sulfur-containing amino acids greatly contribute to the antioxidant defense system and are key constituents in the regulation

of cell metabolism. Apart from structural and catalytic role in proteins, cysteine chemistry is important to the enzymatic mechanism of the thiol-disulfide oxidoreductases of the thioredoxin superfamily, such as thioredoxins, glutaredoxin, and protein disulfide isomerase [27]. The levels of the proteins involved in cysteine biosynthesis, including thioredoxin reductase and thiosulfate sulfurtransferase, were highly upregulated in irradiated cells of *D. radiodurans* (S2 Table). Surface exposed methionines serve as potent endogenous antioxidants to protect other functionally essential residues from oxidative damage [28]. Methionines are readily oxidized to methionine sulfoxide by many ROS. Methionine sulfoxides can be then subsequently converted back to methionines with the help of methionine sulfoxide reductases Msr [28]. Significant upregulation (p-value 0.043) of MsrA was observed after the exposure of cells to UVC/vacuum conditions as one of the most obvious responses of *D. radiodurans* to oxidative damage (S2 Table, Fig 5).

The Fisher Exact test revealed a high enrichment factor for proteins connected to the TCA cycle and different amino acid pathways (Fig 4). Apart from that, RNA degradation enzymes, which dispose the damaged RNA, are enriched in the UVC irradiated under vacuum conditions cells of *D. radiodurans*. Finally, proteins related to the aminoacyl-tRNA biosynthesis are enriched in the irradiated cells too, indicating an increased demand of protein synthesis. These proteins catalyze the esterification of a specific amino acid to its appropriate tRNA to form an aminoacyl tRNA. In the ribosome, the amino acid is transferred from the corresponding tRNA to a growing peptide strain.

Energy metabolism

The Fisher Exact Test indicated proteins, which are more abundant in the cells of *D. radiodurans* exposed to UVC/vacuum, belonging to the TCA-cycle. Nearly all key enzymes of the TCA cycle are more abundant in the cells of *D. radiodurans* exposed to UVC/vacuum conditions. Citrate synthase, which is responsible for the condensation of acetyl-CoA and oxaloacetate to form citrate and CoA-SH as well as aconitate hydratase, which isomerize citrate into isocitrate and isocitrate dehydrogenase, which is allosterically activated by high ADP concentrations and 2-oxoglutarate dehydrogenase (both subunits) are significantly more abundant in the irradiated cells too. Apart from succinate-CoA ligase (hydrolyse of succinyl-CoA into succinate and CoA), all other key enzymes (succinate dehydrogenase for the oxidation of succinate into fumarate, fumarate hydratase, which catalyzes the trans addition of water to produce malate and malate dehydrogenase which oxidizes malate to oxaloacetate) were found in a significantly (p-value below 0.05) higher abundance in the cells of *D. radiodurans* exposed to UVC/vacuum conditions (Fig 7).

Apart from enzymes directly involved into the TCA cycle, all four proteins involved in the pathway that generates pyruvate from D-glyceraldehyde 3-phosphate were more abundant in the cells exposed to UVC/vacuum. Pyruvate dehydrogenase E1 which contributes to the transformation of pyruvate to acetyl CoA for the first step in the TCA cycle was as well upregulated in the UVC/vacuum exposed *D. radiodurans*. Glyceraldehyde 3-phosphate can be obtained from sugars or as a by-product in the tryptophan metabolism [29]. Several proteins of the tryptophan metabolism were significantly upregulated in the UVC/vacuum exposed cells.

Daly [30] reported a higher manganese to iron ratio in *D. radiodurans* compared to other bacteria. Manganese contributes to the resistance against various extreme environmental conditions through the formation of ROS scavenging complexes with orthophosphate and peptides [31]. After application of these complexes, a mouse model showed increased survivability after exposure to ionizing radiation [32]. Furthermore, manganese was proposed to influence glucose incorporation into the DNA after UV exposure [33]. It was shown that glucose is solely

metabolized by the pentose phosphate pathway, which augments the DNA excision repair system as it provides adequate metabolites for DNA mismatch repair [34]. Therefore, mutants which lack important pentose phosphate pathway genes, as glucose-6-phosphate-dehydrogenase (*zwf*) are more sensitive to conditions that induce DNA excision repair, such as UV irradiation [34]. Our data supports the assumption that *zwf* might participate in stress response, as it was significantly more abundant in the UVC/vacuum treated cells, which experienced DNA damage.

As there are many upregulated proteins which are directly or indirectly connected to the energy metabolism it can be assumed that more energy for regeneration is required for the UVC/vacuum exposed cells of *D. radiodurans* compared to the control cells. Joshi, Schmid [35] observed a degradation and resynthesis of several proteins after ionizing irradiation. The high abundance of various aminoacyl ligases in the irradiated cells indicates that the resynthesis most likely occur after the exposure to UVC/vacuum as well. The attachment of an amino acid to its tRNA which is catalyzed by these enzymes, is an energy demanding reaction which consumes one ATP per amino acid. At the same time the free amino acid pool in the UVC/vacuum exposed cells is lower than in the control cells also suggesting that protein resynthesis is a highly abundant process during recovery of the cells (Fig 6). The increased energy demand of the irradiated cells is also necessary to cope with the nucleic acid damage which is triggered by upregulation of a number of ribonucleases (S2 Table).

DNA damage response

D. radiodurans wild type strain is approximately 25 times more resistant to UVC irradiation than *E. coli* wild type [11]. Early experiments with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine revealed mutant strains of *D. radiodurans* which are more sensitive to UVC irradiation [36–38]. In 1994, Gutman, Carroll [39] confirmed a lower resistance to UVC in the *recA* and the *polA* [15] mutants. The IrrE mutant showed that the IrrE (also named *pprI*) gene function as a regulator for the expression of DNA repair and oxidative stress response proteins, like *recA* and *pprA* [40, 41]. *pprA*, which encodes a protein that can protect DNA ends from degradation and stimulate DNA-ligase activities, despite its function, seems to play a lesser role in UVC resistance, although it was upregulated in a previous UVC study [42]. Bauermeister, Bentchikou [43] showed in a comparison study that the UVC energy needed to kill 90% of a *D. radiodurans* culture was 1.5 times lower for the *pprA* mutant, 8 times lower for the *irrE* mutant and 20 times lower for the *recA* mutant compared to the wild type. In our proteomics analysis, *polA* (p-value 0.006) and *pprA* (p-value 0.027) were significantly more abundant in the irradiated cells, while *recA* and *irrE* levels showed no significant difference. Previous shotgun proteomic measurements of *Deinococci spp*. were primarily performed with a combination of two dimensional gel electrophoresis and MALDI-TOF after γ-irradiation. In a study conducted by Dedieu, Sahinovic [44] SSB, PprA, RecA, GyrA/B, UvrD, DdrB and DdrD showed upregulation after ionizing irradiation was applied on *Deinococcus deserti*. Another study [45] found only SSB and PprA among these proteins to be upregulated in *D. radiodurans* after γ-irradiation. However, in a transcriptional approach, Tanaka, Earl [22] showed an upregulation of *recA*, *gyrA/B* and also for the DNA damage response genes *ddrB* and *ddrD* in *D. radiodurans*. In our experiment, a higher abundance of PprA, GyrA/B (both p-values 0.002), DdrB (p-value 0.022) and DdrD (p-value $1.7^{*}10^{-4}$) was observed in irradiated cells, while RecA was present constitutively at high levels in both control and irradiated cells (Fig 5, S2 Table). As our cells were incubated in TGB medium for 5 h after exposure, this fits to a kinetic study [46], which showed that RecA was upregulated for two hours after irradiation, but changed back to basal expression after four hours. Different proteomics/transcriptomics experiments showed some

consistency in which DNA damage response proteins were upregulated [22, 44–46]. Differences can be explained due to a number of variable experimental parameters, e.g., dose and type of irradiation, cells dried or in suspension and recovery time. However, as shown in Fig 5, a lot of manually curated DNA damage response proteins were upregulated in our experiment, indicating that the severe DNA damage, which can be caused by UVC irradiation and desiccation stress, increases the synthesis rate of such proteins.

Contrary to the well-characterized radiation-induced damage, the strategies by which cells of *D. radiodurans* protect their DNA integrity in response to vacuum damage are poorly understood. Along with rapid dehydration of bacterial cells and changes in membrane permeability, DNA damage and mutagenesis have been previously described in microorganisms exposed to space vacuum [47]. Interestingly, the *gyrA* gene, coding for DNA gyrase subunit A has been reported to carry the majority of mutations induced by exposure of spores of *B. subtilis* to high and low vacuum [48, 49]. As suggested by our comparative proteomics analysis, GyrA protein (DR_1913) was upregulated (p-value 0.002) in UVC-irradiated cells of *D. radiodurans* under vacuum conditions, which can be also potentially attributed to the influence of vacuum.

Furthermore, the exposure of *D. radiodurans* to UVC/vacuum stress conditions triggered a suit of proteins involved in detoxification process and aimed to remove damaged nucleotides from the cell. The proteomics experiments revealed that UvrB, helixase subunit of the DNA excision repair endonuclease complex, was significantly more abundantly represented in cells of *D. radiodurans* in conditions of UVC/vacuum stress. The upregulated UvrB binds to DNA, searches it for potential lesions and interacts with other proteins to repair them [50]. MutT/nudix family protein (DR_0550) and MutS2 (DR_1976) involved in mismatch excision repair were upregulated in response to UVC/vacuum exposure. Some members of the Nudix family, such as MutT of *E. coli*, limit mutations by hydrolyzing oxidized nucleotide metabolism products, which are mutagenic once misincorporated into the genome [51, 52]. MutS2 in *D. radiodurans* is involved in ROS detoxification and the repair of ROS-induced DNA damage [53]. Thus, induction of Mut and Nudix family members may be one of the important protective responses to UVC/vacuum stress. Expression of the proteins (recQ and ruvABC) involved in recombinational DNA repair was also significantly induced (S2 Table).

The Mrr restriction system protein (DR_0508) that belongs to a yet unknown pathway showed high abundance (p-value 0.043) in UVC/vacuum exposed cells (S2 Table). Type IV restriction Mrr (methylated adenine recognition and restriction) endonucleases with specificity for methylated DNA have been reported to restrict DNA containing N6-methyladenine and also DNA with C5-methyl-cytosine residues [54]. Contrary to well-characterized Mcr restriction endonucleases, the physiological role of Mrr like nucleases in the cell has been less clarified. Recently, the Mrr restriction system was shown to implement into the peculiar piezophysiology of *E. coli*. Mrr endonuclease activity was linked to cellular filamentation and prophage induction in response to sub-lethal high-pressure shock in *E. coli* K12 [54, 55]. Hence, the observed up-regulation of Mrr restriction protein in *D. radiodurans* under the influence of UVC/vacuum conditions might assign a novel role for this less studied protein in response to space-related stress stimulus.

Molecular systems of stress response

Our comparative proteomic analysis revealed a number of differentially abundant proteins in UVC/vacuum exposed cells of *D. radiodurans* that belong to the functional machinery of general stress response and oxidative stress response. Proteins of general stress response function to protect and repair damage to cellular structures, such as DNA, the cell envelope and

proteins, and to provide microorganisms the ability to recuperate from the stress they experience. Overexpression of a number of chaperons occurred in UVC/vacuum exposed cells of *D. radiodurans*. Heat shock protein that belongs to HSP20 family (DR_1114) and chaperonins hslO (DR_0985) and groL (DR_0607), which are involved in various metabolic processes and responsible for protein folding, were upregulated in UVC/vacuum exposed cells (S2 Table). Chaperone proteins ClpB (Q9RVI3), DnaJ (Q9RUG2) and DnaK Q9RY23 were as well more abundantly represented in UVC/vacuum exposed cells of *D. radiodurans* (S2 Table). By binding to proteins, which are misfolded and damaged in response to various environmental stresses, these molecular chaperones can direct the misfolded proteins to the associated proteases for degradation. The elevated level of several proteases (Lon proteases Q9RXG4 and Q9RSZ5 and ATP-dependent Clp protease ClpA (DR_0588)) in irradiated cells indicates the involvement of quality monitoring and proteolytic regulation in response to combined UVC/vacuum stress.

Comparative proteomics analysis revealed a number of universal reactive oxygen species (ROS) scavengers, e.g., catalase, and redox active proteins (pyridoxal 5'-phosphate synthase, peroxidase, sulfoxide reductase MsrA, thioredoxin reductase) induced in cells of *D. radiodurans* exposed to UVC radiation under vacuum conditions, manifesting the upregulation of antioxidant defense mechanisms in response to these factors (Fig 5). The extreme resistance of *D. radiodurans* against radiation and oxidative damage relies on the high levels of constitutive catalase activity and superoxide dismutase (SOD) activity [3]. These enzymatic systems are devoted to the protection of cells against toxic reactive oxygen species. Out of three known catalases (DR1998, DRA0146, and DRA0259) in genome of *D. radiodurans*, our data show the elevated levels of two of them: catalase katA (p-value $1.1^{*}10^{-6}$) and predicted protein with catalase function DR_A0146 (p-value $4.3^{*}10^{-4}$) in UVC/vacuum exposed *D. radiodurans* (Fig 5). The sodA protein was constitutively represented in both irradiated and control cells (Fig 5). UVC irradiation under vacuum caused 2-fold elevated expression of the pyridoxine biosynthesis proteins PdxS and PdxT (Fig 5) which are singlet oxygen resistance proteins involved in the synthesis of vitamin B_6, an efficient singlet oxygen quencher and a potential antioxidant [56]. The upregulated upon UVC/vacuum-irradiation thioredoxin reductase/alkyl hydroperoxide reductase (DR_1982) (Fig 5) is encoded by the gene *trxB/ahpF*, which is a key determinant of thiol redox sensing antioxidant enzymatic system in *D. radiodurans*. Thioredoxin reduces oxidized cysteine sulfur groups in proteins and is subsequently reverted from its oxidized form by thioredoxin reductase in an NADPH-dependent manner [3, 57]. A putative iron-dependent peroxidase (DRA_0145), enzyme that may implement in defense against oxidative stress by providing protection against toxic hydroperoxides [58], was also among upregulated proteins in response to UVC/vacuum stress. This unique putative peroxidase has very few orthologs among bacteria [58] and is listed among predicted systems of protection against oxidative stress [59].

Among significantly upregulated proteins in response to UVC/vacuum irradiation was also the peptide methionine sulfoxide reductase MsrA (DR_1849) (Fig 5, S2 Table) that shares similarity with *E. coli*'s methionine sulfoxide reductase and performs repair of oxidized proteins reducing protein-bound methionine sulfoxide back to methionine via a thioredoxin-recycling process [59]. Reduction of oxidized methionine residues in proteins is essential mechanism for cells survival under oxidative stress [58] and loss of MsrA sensitizes *E. coli* to hydrogen peroxide [60]. The induction of the gene *msrA* has been reported after ionizing irradiation of *D. radiodurans* [22]. Thiosulfate sulfurtransferase (DR_0217), a rhodanese superfamily enzyme was as well more abundantly represented in exposed cells of *D. radiodurans*. Proteins containing a single rhodanese-like domain are generally considered to mediate different forms of stress response [51]. The level of thiosulfate sulfurtransferase has been earlier reported as significantly increased after ionizing irradiation [46].

Oxidative stress-responsive proteins within tellurium resistance operon TerB (DR2220) and TerD (DR2221) were upregulated in cells of *D. radiodurans* exposed to UVC/vacuum conditions (Fig 5, S2 Table). The homologous tellurium resistance proteins contribute to the resistance of *E. coli* to various damaging agents, such as heavy metal ions and UVC radiation, and to the maintenance of the intracellular reducing environment, possibly by directly reversing disulfide bonds [3]. Several reports suggest oxidative stress as major determinant of tellurite toxicity in tellurite sensitive organisms, including *D. radiodurans* [61]. The genes encoding tellurium resistance have been specifically upregulated in a-proteobacterium *Rhodospirillum rubrum* followed by space exposure at ISS in frames of MELiSSA project, as well as significant differentially expressed under the conditions of modeled microgravity [62, 63]. The genes encoding TerB and TerE tellurium resistance proteins in *D. radiodurans* were shown to respond to acute ionizing radiation [64]. Moreover, the genes encoding TerB and TerZ proteins were found to be upregulated immediately after gamma-irradiation of *D. radiodurans* [22], while tellurium resistance proteins TerB and TerD were also alleviated during gamma radiation in another study [46], implementing an adaptation to oxidative stress. Apart from tellurium resistance proteins, putative copper resistance protein (DR_A0299) with the predicted function of response to stress stimulus was found more abundant in UVC-irradiated cells of *D. radiodurans* (S2 Table). Such an observed involvement of tellurium resistance elements in the response to radiation or factors related to space environment may be part of a metal sensing stress response system, as well as inner membrane oxidative stress response.

The red-pigmented *D. radiodurans* encodes a set of genes involved in biosynthesis of carotenoids [3, 51]. Carotenoid pigments have also been shown to contribute in protection against oxidative stress damage. Our comparative proteomic data analysis shows that phytoene desaturase (DR_0861), enzyme of carotenoid biosynthetic pathway in *D. radiodurans*, was more abundantly represented (p-value 0.043) in UVC-vacuum stressed cells (Fig 5). The arrest of lycopene synthesis and the accumulation of phytoene along with enhanced sensitivity to acute ionizing radiation and oxygen stress have been reported to the colorless DR0861 gene knockout strain, while complementation of the mutant with a heterologous or homologous gene restored pigmentation and resistance [65]. Increased abundance of phytoene desaturase in UVC-irradiated cells of *D. radiodurans* indicates the contribution of the carotenoid synthesis pathway to the radioresistance and oxidative stress tolerance of *D. radiodurans*.

Other upregulated enzymes with a possible role in oxidative stress response were probable manganese-dependent inorganic pyrophosphatase ppaC (DR_2576) involved in oxidative phosphorylation and FrnE dithiol-disulfide isomerase (DR_0659) that catalyzes formation of protein disulfide bonds and is involved in sulfur metabolism. FrnE was induced in response to ionizing radiation [22]. This thioredoxin fold protein is included in predicted radiation and desiccation resistance regulon of *Deinococci* [66].

Transcriptional regulators

A number of transcriptional regulators and repressors have been identified in our proteomic analysis as constitutively expressed in both UVC-irradiated under vacuum conditions and control cells of *D. radiodurans*. The expression level of transcriptional regulators and repressors of TetR, MerR, GntR and AsnC families remained unaltered in irradiated cells of *D. radiodurans*, being constitutively represented under the control and UVC/vacuum conditions (S2 Table). The transcriptional regulator of FNR/CRP family (DR_0997) was significantly more abundantly represented in cells of *D. radiodurans* after UVC radiation under vacuum conditions. Cyclic AMP (cAMP) repressor proteins (CRP) act as global transcriptional regulators involved in many cellular pathways in various bacteria, including adaptation to starvation and

extreme conditions [67–70]. The genome of *D. radiodurans* encodes four predicted CRP family proteins, including DR_0997, DR_1646, DR_2362, and DR_0834 (64). Recently, the gene encoding DR_0997 was shown to regulate stress response of *D. radiodurans* on the transcriptional level and loss of the Dr_0997 gene sensitized *D. radiodurans* toward H_2O_2, ultraviolet radiation, ionizing radiation, and mitomycin C [70]. Interestingly, our comparative proteomic analysis showed the upregulation of DR_0997 along with the upregulation of several proteins, encoded by genes, which belong to CRP regulon [70] in *D. radiodurans* under UVC-vacuum combined stress conditions (S2 Table). Among them are Lon proteases (DR_0349 and DR_1974), DNA repair protein PprA (DR_A0346), UvrABC system protein B UvrB (DR_2275), catalase katA (DR_0146) and tellurium resistance protein TerB (DR_2220). Thus, DR_0997 might act as a positive regulator in response to combined UVC-vacuum stress in *D. radiodurans*.

DdrO, a transcriptional regulator of HTH_3 family (DR_2574) was 2.6-fold downregulated in UVC-irradiated cells of *D. radiodurans* under vacuum conditions (S2 Table). Acting as a transcriptional repressor of Radiation Desiccation Response (RDR), DdrO binds 17 bp palindromic sequence called Radiation Desiccation Response Motif (RDRM) in 21 RDRM-promoters of *D. radiodurans in vitro* [71] and represses a variety of DNA Damage Response (DDR) genes. We have also found that a number of RDR proteins comprising DdrO regulon were upregulated in UVC-irradiated cells of *D. radiodurans* under vacuum conditions. Among them are DNA gyrase B subunit GyrB (DR_0906), Tkt transketolase (DR_2256), RecQ helicase (DR_1289), UvrD superfamily I helicase (DR1775), urocanate hydratase (DRA0151) and FrnE uncharacterized DsbA-like thioredoxin fold protein (DR_0659) (S2 Table). Apparently, DdrO as a global master regulator serves to control reprogramming of microbial physiology in order to permit the adaptation of *D. radiodurans* to combined UVC-vacuum stress.

Metabolic regulation

Our approach focuses on the identification and quantification of polar, primary metabolites. These are involved in growth, development and reproduction—parameters which are affected by UVC/vacuum stress. The metabolite analysis showed a significantly reduced abundance of overwhelming majority of identified polar metabolites in the irradiated cells of *D. radiodurans* (Fig 6). As *D. radiodurans* is a bacterium with a proteolytic life-style, it uses amino acids as preferred carbon source [72, 73]. Ethanolamine was one of the very few metabolites, which were more abundant in the UVC-irradiated cells of *D. radiodurans* (Fig 6). Splitting ethanolamine into ammonia and acetaldehyde can serve as a cellular supply of reduced nitrogen as well as a precursor for acetyl CoA [74], sustaining the necessary levels of these compounds in irradiated cells. Interestingly, the elevated level of a palmitoyl-derivative of carnitine was observed in the UVC-vacuum exposed cells of *D. radiodurans* (Fig 6). Apart from its nutritional function, a quaternary amine compound carnitine has various physiological effects. As a compatible solute, carnitine is important osmoprotectant, and can also enhance thermotolerance, cryotolerance and barotolerance, impacting bacterial survival in extreme conditions [75]. At the same time, osmotic stress has been described as a part of stress response which microorganisms experience exposed to the outer space environment or to its individual simulated factors [62, 76–78] In this context, the observed upregulation of O-Palmytoyl-L-Carnitine chloride (Fig 6) may suggest the role of this quaternary amine compound responsible for adaptation to extreme conditions [70] in the protection of *D. radiodurans* against combined stress conditions of UVC and vacuum. Moreover, carnitine as a compatible solute might be potentially necessary to overcome damaging desiccation effects of vacuum [47, 79] by binding additional water molecules, helping to stabilize proteins and cell membranes, and thus preventing complete desiccation of the cell.

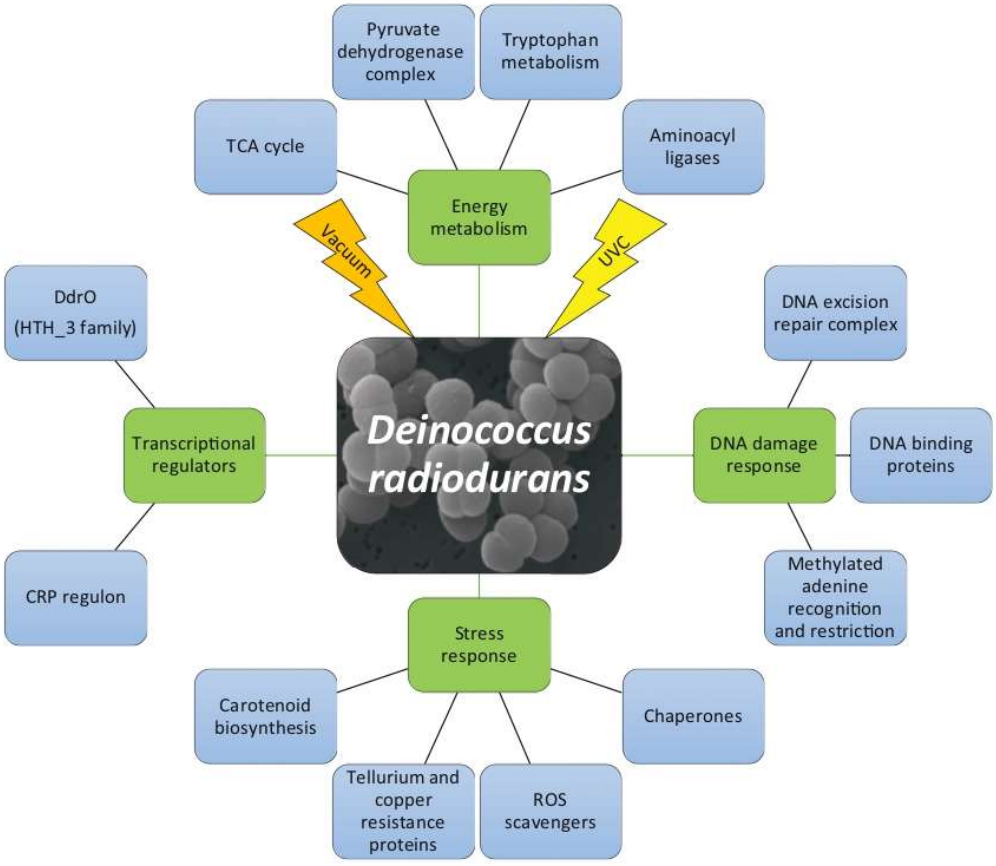

Fig 8. Molecular response of *D. radiodurans* experienced under UVC and vacuum conditions. First two levels of molecular pathways are represented which are affected by UVC irradiation under vacuum conditions.

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The amount of octadecanoic (stearic) acid, which has been described as a minor component of *D. radiodurans* [75], was significantly increased in vacuum/UVC-irradiated cells of *D. radiodurans* (Fig 6). The surface-active compound stearic acid was identified in biosurfactants of several bacterial species [76, 77]. Decreased levels of stearic acid associated with the dramatic reduction in biofilm formation of *Streptococcus sanguinis nox* mutant [78], suggesting its involvement in stress-related reactions. Stearic acid can potentially be involved in covering the cells of *D. radiodurans* by a layer less permeable to water, thereby preserving the structural integrity of cell membranes in conditions of vacuum-induced dehydration. Although the cells of *D. radiodurans* do not naturally produce stearic acid in big quantities under non-stressed conditions [75], the observed accumulation of this biofilm-associated compound may potentially lead to the high survival of *D. radiodurans* in dry multilayers under UVC/vacuum combined stress.

Our study shows that response to UVC/vacuum combined stress and the enzymatic repair caused by the damage after ionizing radiation have overlapping molecular components in *D. radiodurans*. The combination of proteomic with metabolomic analysis of cells after UVC-irradiation under vacuum condition reveals that the response is a multilayer process (Fig 8). It requires a high amount of energy in order to initiate stress defense mechanisms necessary to alleviate cell damage.

Supporting information

S1 Fig. Scanning electron micrographs showing multilayers of dehydrated cells of *D. radiodurans* deposited on aluminum plates and used in experimental set up of Tanpopo mission. A, control cells of *D. radiodurans* dried in aluminum plates in accordance to Kawaguchi et al., 2016. B, dried cells of *D. radiodurans* after exposure to UVC/vacuum conditions. Shown is the upper surface of dehydrated *D. radiodurans* multilayers.
(TIF)

S1 Table. Individual and average relative survival rates for four control and irradiated replicates.
(XLSX)

S2 Table. Raw proteomic data.
(XLSX)

S3 Table. Raw metabolomic data.
(XLSX)

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References

1. Dainton FS. On the existence of free atoms and radicals in water and aqueous solutions subjected to ionizing radiation. The Journal of physical and colloid chemistry. 1948; 52(3):490–517. PMID: 18904932

2. Krisko A, Radman M. Biology of Extreme Radiation Resistance: The Way of Deinococcus radiodurans. Cold Spring Harbor Perspectives in Biology. 2013; 5(7):a012765. https://doi.org/10.1101/cshperspect.a012765 PMID: 23818498
3. Slade D, Radman M. Oxidative stress resistance in Deinococcus radiodurans. Microbiol Mol Biol Rev. 2011; 75(1):133–91. https://doi.org/10.1128/MMBR.00015-10 PMID: 21372322
4. Potts M. Desiccation tolerance of prokaryotes. Microbiol Rev. 1994; 58(4):755–805. PMID: 7854254
5. Yamagishi A, YOKOBORI S-i, Hashimoto H, Hajime Y, HIGASHIDE M, TABATA M, et al. Tanpopo: astrobiology exposure and micrometeoroid capture experiments—proposed experiments at the Exposure Facility of ISS-JEM. Transactions of the Japan Society for Aeronautical and Space Sciences, Aerospace Technology Japan. 2014; 12(ists29):Tk_49–Tk_55.
6. Arrhenius S. Die Verbreitung des Lebens im Weltenraum. Die Umschau. 1903; 7:481–6.
7. Kawaguchi Y, Yang Y, Kawashiri N, Shiraishi K, Takasu M, Narumi I, et al. The possible interplanetary transfer of microbes: assessing the viability of Deinococcus spp. under the ISS Environmental conditions for performing exposure experiments of microbes in the Tanpopo mission. Orig Life Evol Biosph. 2013; 43(4–5):411–28. https://doi.org/10.1007/s11084-013-9346-1 PMID: 24132659
8. Kawaguchi Y, Yokobori S, Hashimoto H, Yano H, Tabata M, Kawai H, et al. Investigation of the Interplanetary Transfer of Microbes in the Tanpopo Mission at the Exposed Facility of the International Space Station. Astrobiology. 2016; 16(5):363–76. https://doi.org/10.1089/ast.2015.1415 PMID: 27176813
9. Horneck G, Rettberg P. Complete course in astrobiology. Weinheim: Wiley-VCH; 2007. xx, 413 p. p.
10. Rastogi RP, Richa, Kumar A, Tyagi MB, Sinha RP. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. J Nucleic Acids. 2010; 2010:592980. https://doi.org/10.4061/2010/592980 PMID: 21209706
11. Krisko A, Radman M. Protein damage and death by radiation in Escherichia coli and Deinococcus radiodurans. Proc Natl Acad Sci U S A. 2010; 107(32):14373–7. https://doi.org/10.1073/pnas.1009312107 PMID: 20660760
12. Moeller R, Douki T, Rettberg P, Reitz G, Cadet J, Nicholson WL, et al. Genomic bipyrimidine nucleotide frequency and microbial reactions to germicidal UV radiation. Arch Microbiol. 2010; 192(7):521–9. https://doi.org/10.1007/s00203-010-0579-3 PMID: 20454780
13. Tanaka M, Narumi I, Funayama T, Kikuchi M, Watanabe H, Matsunaga T, et al. Characterization of pathways dependent on the uvsE, uvrA1, or uvrA2 gene product for UV resistance in Deinococcus radiodurans. J Bacteriol. 2005; 187(11):3693–7. https://doi.org/10.1128/JB.187.11.3693-3697.2005 PMID: 15901692
14. Minton KW. DNA repair in the extremely radioresistant bacterium Deinococcus radiodurans. Mol Microbiol. 1994; 13(1):9–15. PMID: 7984097
15. Gutman PD, Fuchs P, Minton KW. Restoration of the DNA damage resistance of Deinococcus radiodurans DNA polymerase mutants by Escherichia coli DNA polymerase I and Klenow fragment. Mutat Res. 1994; 314(1):87–97. PMID: 7504195
16. Slade D, Lindner AB, Paul G, Radman M. Recombination and replication in DNA repair of heavily irradiated Deinococcus radiodurans. Cell. 2009; 136(6):1044–55. https://doi.org/10.1016/j.cell.2009.01.018 PMID: 19303848
17. Cox MM, Keck JL, Battista JR. Rising from the Ashes: DNA Repair in Deinococcus radiodurans. PLoS Genetics. 2010; 6(1):e1000815. https://doi.org/10.1371/journal.pgen.1000815 PMID: 20084105
18. Reece RJ, Maxwell A. DNA gyrase: structure and function. Crit Rev Biochem Mol Biol. 1991; 26(3–4):335–75. https://doi.org/10.3109/10409239109114072 PMID: 1657531
19. Narumi I, Satoh K, Cui S, Funayama T, Kitayama S, Watanabe H. PprA: a novel protein from Deinococcus radiodurans that stimulates DNA ligation. Mol Microbiol. 2004; 54(1):278–85. https://doi.org/10.1111/j.1365-2958.2004.04272.x PMID: 15458422
20. Harris DR, Tanaka M, Saveliev SV, Jolivet E, Earl AM, Cox MM, et al. Preserving genome integrity: the DdrA protein of Deinococcus radiodurans R1. PLoS Biol. 2004; 2(10):e304. https://doi.org/10.1371/journal.pbio.0020304 PMID: 15361932
21. Norais CA, Chitteni-Pattu S, Wood EA, Inman RB, Cox MM. DdrB protein, an alternative Deinococcus radiodurans SSB induced by ionizing radiation. J Biol Chem. 2009; 284(32):21402–11. https://doi.org/10.1074/jbc.M109.010454 PMID: 19515845
22. Tanaka M, Earl AM, Howell HA, Park MJ, Eisen JA, Peterson SN, et al. Analysis of Deinococcus radiodurans's transcriptional response to ionizing radiation and desiccation reveals novel proteins that contribute to extreme radioresistance. Genetics. 2004; 168(1):21–33. https://doi.org/10.1534/genetics.104.029249 PMID: 15454524

23. Weckwerth W, Wenzel K, Fiehn O. Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. Proteomics. 2004; 4(1):78–83. https://doi.org/10.1002/pmic.200200500 PMID: 14730673

24. Chaturvedi P, Ischebeck T, Egelhofer V, Lichtscheidl I, Weckwerth W. Cell-specific analysis of the tomato pollen proteome from pollen mother cell to mature pollen provides evidence for developmental priming. J Proteome Res. 2013; 12(11):4892–903. https://doi.org/10.1021/pr400197p PMID: 23731163

25. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, et al. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics. 2007; 3(3):211–21. https://doi.org/10.1007/s11306-007-0082-2 PMID: 24039616

26. Luan H, Meng N, Fu J, Chen X, Xu X, Feng Q, et al. Genome-wide transcriptome and antioxidant analyses on gamma-irradiated phases of deinococcus radiodurans R1. PLoS One. 2014; 9(1):e85649. https://doi.org/10.1371/journal.pone.0085649 PMID: 24465634

27. Foloppe N, Nilsson L. The glutaredoxin -C-P-Y-C- motif: influence of peripheral residues. Structure (London, England: 1993). 2004; 12(2):289–300.

28. Levine RL, Moskovitz J, Stadtman ER. Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. IUBMB life. 2000; 50(4–5):301–7. https://doi.org/10.1080/713803735 PMID: 11327324

29. Sprenger GA. Aromatic Amino Acids. In: Wendisch VF, editor. Amino Acid Biosynthesis ~ Pathways, Regulation and Metabolic Engineering. Berlin, Heidelberg: Springer Berlin Heidelberg; 2007. p. 93–127.

30. Daly MJ. A new perspective on radiation resistance based on Deinococcus radiodurans. Nat Rev Micro. 2009; 7(3):237–45.

31. Daly MJ, Gaidamakova EK, Matrosova VY, Kiang JG, Fukumoto R, Lee DY, et al. Small-molecule antioxidant proteome-shields in Deinococcus radiodurans. PLoS One. 2010; 5(9):e12570. https://doi.org/10.1371/journal.pone.0012570 PMID: 20838443

32. Gupta P, Gayen M, Smith JT, Gaidamakova EK, Matrosova VY, Grichenko O, et al. MDP: A Deinococcus Mn2+-Decapeptide Complex Protects Mice from Ionizing Radiation. PLoS One. 2016; 11(8): e0160575. https://doi.org/10.1371/journal.pone.0160575 PMID: 27500529

33. Zhang YM, Wong TY, Chen LY, Lin CS, Liu JK. Induction of a futile Embden-Meyerhof-Parnas pathway in Deinococcus radiodurans by Mn: possible role of the pentose phosphate pathway in cell survival. Appl Environ Microbiol. 2000; 66(1):105–12. PMID: 10618210

34. Zhang YM, Liu JK, Wong TY. The DNA excision repair system of the highly radioresistant bacterium Deinococcus radiodurans is facilitated by the pentose phosphate pathway. Molecular Microbiology. 2003; 48(5):1317–23. PMID: 12787358

35. Joshi B, Schmid R, Altendorf K, Apte SK. Protein recycling is a major component of post-irradiation recovery in Deinococcus radiodurans strain R1. Biochem Biophys Res Commun. 2004; 320(4):1112–7. https://doi.org/10.1016/j.bbrc.2004.06.062 PMID: 15249204

36. Moseley BE. The isolation and some properties of radiation-sensitive mutants of Micrococcus radiodurans. J Gen Microbiol. 1967; 49(2):293–300. https://doi.org/10.1099/00221287-49-2-293 PMID: 6077931

37. Moseley BE, Copland HJ. Isolation and properties of a recombination-deficient mutant of Micrococcus radiodurans. J Bacteriol. 1975; 121(2):422–8. PMID: 1112771

38. Moseley BE, Evans DM. Isolation and properties of strains of Micrococcus (Deinococcus) radiodurans unable to excise ultraviolet light-induced pyrimidine dimers from DNA: evidence for two excision pathways. J Gen Microbiol. 1983; 129(8):2437–45. https://doi.org/10.1099/00221287-129-8-2437 PMID: 6415229

39. Gutman PD, Carroll JD, Masters CI, Minton KW. Sequencing, targeted mutagenesis and expression of a recA gene required for the extreme radioresistance of Deinococcus radiodurans. Gene. 1994; 141 (1):31–7. PMID: 8163172

40. Hua Y, Narumi I, Gao G, Tian B, Satoh K, Kitayama S, et al. PprI: a general switch responsible for extreme radioresistance of Deinococcus radiodurans. Biochemical and Biophysical Research Communications. 2003; 306(2):354–60. PMID: 12804570

41. Lu H, Gao G, Xu G, Fan L, Yin L, Shen B, et al. Deinococcus radiodurans PprI switches on DNA damage response and cellular survival networks after radiation damage. Mol Cell Proteomics. 2009; 8 (3):481–94. https://doi.org/10.1074/mcp.M800123-MCP200 PMID: 18953020

42. Pogoda de la Vega U. Residual Restoration of DNA Lesions in Deinococcus Radiodurans Mutants Indicate Presence of a Bypass UV-repair Process2008.

43. Bauermeister A, Bentchikou E, Moeller R, Rettberg P. Roles of PprA, IrrE, and RecA in the resistance of Deinococcus radiodurans to germicidal and environmentally relevant UV radiation. Arch Microbiol. 2009; 191(12):913–8. https://doi.org/10.1007/s00203-009-0522-7 PMID: 19882142

44. Dedieu A, Sahinovic E, Guérin P, Blanchard L, Fochesato S, Meunier B, et al. Major soluble proteome changes in Deinococcus deserti over the earliest stages following gamma-ray irradiation. Proteome Science. 2013; 11:3-. https://doi.org/10.1186/1477-5956-11-3 PMID: 23320389

45. Zhang C, Wei J, Zheng Z, Ying N, Sheng D, Hua Y. Proteomic analysis of Deinococcus radiodurans recovering from gamma-irradiation. Proteomics. 2005; 5(1):138–43. https://doi.org/10.1002/pmic.200300875 PMID: 15593145

46. Basu B, Apte SK. Gamma radiation-induced proteome of Deinococcus radiodurans primarily targets DNA repair and oxidative stress alleviation. Mol Cell Proteomics. 2012; 11(1):M111 011734.

47. Horneck G, Klaus DM, Mancinelli RL. Space microbiology. Microbiol Mol Biol Rev. 2010; 74(1):121–56. https://doi.org/10.1128/MMBR.00016-09 PMID: 20197502

48. del Carmen Huesca Espitia L, Caley C, Bagyan I, Setlow P. Base-change mutations induced by various treatments of Bacillus subtilis spores with and without DNA protective small, acid-soluble spore proteins. Mutat Res. 2002; 503(1–2):77–84. PMID: 12052506

49. Munakata N, Saitou M, Takahashi N, Hieda K, Morohoshi F. Induction of unique tandem-base change mutations in bacterial spores exposed to extreme dryness. Mutation research. 1997; 390(1–2):189–95. PMID: 9150768

50. Goosen N, Moolenaar GF. Repair of UV damage in bacteria. DNA Repair (Amst). 2008; 7(3):353–79.

51. Makarova KS, Aravind L, Wolf YI, Tatusov RL, Minton KW, Koonin EV, et al. Genome of the Extremely Radiation-Resistant Bacterium Deinococcus radiodurans Viewed from the Perspective of Comparative Genomics. Microbiology and Molecular Biology Reviews. 2001; 65(1):44–79. https://doi.org/10.1128/MMBR.65.1.44-79.2001 PMID: 11238985

52. Fowler RG, Schaaper RM. The role of the mutT gene of Escherichia coli in maintaining replication fidelity. FEMS Microbiol Rev. 1997; 21(1):43–54. PMID: 9299701

53. Zhang H, Xu Q, Lu M, Xu X, Wang Y, Wang L, et al. Structural and functional studies of MutS2 from Deinococcus radiodurans. DNA Repair (Amst). 2014; 21:111–9.

54. Tesfazgi Mebrhatu M, Wywial E, Ghosh A, Michiels CW, Lindner AB, Taddei F, et al. Evidence for an evolutionary antagonism between Mrr and Type III modification systems. Nucleic Acids Research. 2011; 39(14):5991–6001. https://doi.org/10.1093/nar/gkr219 PMID: 21504983

55. Aertsen A, Michiels CW. Mrr instigates the SOS response after high pressure stress in Escherichia coli. Molecular Microbiology. 2005; 58(5):1381–91. https://doi.org/10.1111/j.1365-2958.2005.04903.x PMID: 16313623

56. Bilski P, Li MY, Ehrenshaft M, Daub ME, Chignell CF. Vitamin B6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. Photochem Photobiol. 2000; 71(2):129–34. PMID: 10687384

57. Obiero J, Pittet V, Bonderoff SA, Sanders DA. Thioredoxin system from Deinococcus radiodurans. J Bacteriol. 2010; 192(2):494–501. https://doi.org/10.1128/JB.01046-09 PMID: 19933368

58. Omelchenko MV, Wolf YI, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, et al. Comparative genomics of Thermus thermophilus and Deinococcus radiodurans: divergent routes of adaptation to thermophily and radiation resistance. BMC Evolutionary Biology. 2005; 5:57-. https://doi.org/10.1186/1471-2148-5-57 PMID: 16242020

59. Ghosal D, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Venkateswaran A, et al. How radiation kills cells: survival of Deinococcus radiodurans and Shewanella oneidensis under oxidative stress. FEMS Microbiol Rev. 2005; 29(2):361–75. https://doi.org/10.1016/j.femsre.2004.12.007 PMID: 15808748

60. Moskovitz J, Rahman MA, Strassman J, Yancey SO, Kushner SR, Brot N, et al. Escherichia coli peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. J Bacteriol. 1995; 177(3):502–7. PMID: 7836279

61. Anaganti N, Basu B, Gupta A, Joseph D, Apte SK. Depletion of reduction potential and key energy generation metabolic enzymes underlies tellurite toxicity in Deinococcus radiodurans. Proteomics. 2015; 15(1):89–97. https://doi.org/10.1002/pmic.201400113 PMID: 25331933

62. Mastroleo F, Van Houdt R, Leroy B, Benotmane MA, Janssen A, Mergeay M, et al. Experimental design and environmental parameters affect Rhodospirillum rubrum S1H response to space flight. Isme j. 2009; 3(12):1402–19. https://doi.org/10.1038/ismej.2009.74 PMID: 19571896

63. Mastroleo F, Van Houdt R, Atkinson S, Mergeay M, Hendrickx L, Wattiez R, et al. Modelled microgravity cultivation modulates N-acylhomoserine lactone production in Rhodospirillum rubrum S1H

independently of cell density. Microbiology. 2013; 159(Pt 12):2456–66. https://doi.org/10.1099/mic.0.066415-0 PMID: 24025602

64. Liu Y, Zhou J, Omelchenko MV, Beliaev AS, Venkateswaran A, Stair J, et al. Transcriptome dynamics of Deinococcus radiodurans recovering from ionizing radiation. Proc Natl Acad Sci U S A. 2003; 100.
65. Xu Z, Tian B, Sun Z, Lin J, Hua Y. Identification and functional analysis of a phytoene desaturase gene from the extremely radioresistant bacterium Deinococcus radiodurans. Microbiology. 2007; 153(Pt 5):1642–52. https://doi.org/10.1099/mic.0.2006/002857-0 PMID: 17464079
66. Makarova KS, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, et al. Deinococcus geothermalis: The Pool of Extreme Radiation Resistance Genes Shrinks. PLOS ONE. 2007; 2 (9):e955. https://doi.org/10.1371/journal.pone.0000955 PMID: 17895995
67. Shimizu K. Regulation Systems of Bacteria such as Escherichia coli in Response to Nutrient Limitation and Environmental Stresses. Metabolites. 2013; 4(1):1–35. https://doi.org/10.3390/metabo4010001 PMID: 24958385
68. Green J, Stapleton MR, Smith LJ, Artymiuk PJ, Kahramanoglou C, Hunt DM, et al. Cyclic-AMP and bacterial cyclic-AMP receptor proteins revisited: adaptation for different ecological niches(). Current Opinion in Microbiology. 2014; 18(100):1–7.
69. Soberon-Chavez G, Alcaraz LD, Morales E, Ponce-Soto GY, Servin-Gonzalez L. The Transcriptional Regulators of the CRP Family Regulate Different Essential Bacterial Functions and Can Be Inherited Vertically and Horizontally. Front Microbiol. 2017; 8:959. https://doi.org/10.3389/fmicb.2017.00959 PMID: 28620358
70. Yang S, Xu H, Wang J, Liu C, Lu H, Liu M, et al. Cyclic AMP Receptor Protein Acts as a Transcription Regulator in Response to Stresses in Deinococcus radiodurans. PLoS One. 2016; 11(5):e0155010. https://doi.org/10.1371/journal.pone.0155010 PMID: 27182600
71. Wang Y, Xu Q, Lu H, Lin L, Wang L, Xu H, et al. Protease Activity of PprI Facilitates DNA Damage Response: Mn(2+)-Dependence and Substrate Sequence-Specificity of the Proteolytic Reaction. PLOS ONE. 2015; 10(3):e0122071. https://doi.org/10.1371/journal.pone.0122071 PMID: 25811789
72. Murray RGE. The Family Deinococcaceae. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K- H, editors. The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. New York, NY: Springer New York; 1992. p. 3732–44.
73. He Y. High cell density production of Deinococcus radiodurans under optimized conditions. J Ind Microbiol Biotechnol. 2009; 36(4):539–46. https://doi.org/10.1007/s10295-008-0524-5 PMID: 19137334
74. Garsin DA. Ethanolamine utilization in bacterial pathogens: roles and regulation. Nat Rev Micro. 2010; 8(4):290–5.
75. Meadows JA, Wargo MJ. Carnitine in bacterial physiology and metabolism. Microbiology. 2015; 161 (6):1161–74. https://doi.org/10.1099/mic.0.000080 PMID: 25787873
76. Zhang X, Fang X, Liu C. Genomic and Proteomic Analysis of Escherichia coli After Spaceflight Reveals Changes Involving Metabolic Pathways. Archives of Medical Research. 2015; 46(3):181–5. https://doi.org/10.1016/j.arcmed.2015.03.007 PMID: 25846064
77. Li T, Chang D, Xu H, Chen J, Su L, Guo Y, et al. Impact of a short-term exposure to spaceflight on the phenotype, genome, transcriptome and proteome of Escherichia coli. International Journal of Astrobiology. 2015; 14(3):435–44.
78. Wilson JW, Ramamurthy R, Porwollik S, McClelland M, Hammond T, Allen P, et al. Microarray analysis identifies Salmonella genes belonging to the low-shear modeled microgravity regulon. Proceedings of the National Academy of Sciences. 2002; 99(21):13807–12.
79. Frosler J, Panitz C, Wingender J, Flemming HC, Rettberg P. Survival of Deinococcus geothermalis in Biofilms under Desiccation and Simulated Space and Martian Conditions. Astrobiology. 2017; 17 (5):431–47. https://doi.org/10.1089/ast.2015.1431 PMID: 28520474

4.2 Proteomic and Metabolomic Profiling of *Deinococcus radiodurans* Recovering After Exposure to Simulated Low Earth Orbit Vacuum conditions

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Proteomic and Metabolomic Profiling of *Deinococcus radiodurans* Recovering After Exposure to Simulated Low Earth Orbit Vacuum Conditions

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The polyextremophile, gram-positive bacterium *Deinococcus radiodurans* can withstand harsh conditions of real and simulated outer space environment, e.g., UV and ionizing radiation. A long-term space exposure of *D. radiodurans* has been performed in Low Earth Orbit (LEO) in frames of the Tanpopo orbital mission aiming to investigate the possibility of interplanetary life transfer. Space vacuum (10^{-4}–10^{-7} Pa) is a harmful factor, which induces dehydration and affects microbial integrity, severely damaging cellular components: lipids, carbohydrates, proteins, and nucleic acids. However, the molecular strategies by which microorganisms protect their integrity on molecular and cellular levels against vacuum damage are not yet understood. In a simulation experiment, we exposed dried *D. radiodurans* cells to vacuum (10^{-4}–10^{-7} Pa), which resembles vacuum pressure present outside the International Space Station in LEO. After 90 days of high vacuum exposure, survival of *D. radiodurans* cells was 2.5-fold lower compared to control cells. To trigger molecular repair mechanisms, vacuum exposed cells of *D. radiodurans* were recovered in complex medium for 3 and 6 h. The combined approach of analyzing primary metabolites and proteins revealed important molecular activities during early recovery after vacuum exposure. In total, 1939 proteins covering 63% of *D. radiodurans* annotated protein sequences were detected. Proteases, tRNA ligases, reactive oxygen species (ROS) scavenging proteins, nucleic acid repair proteins, TCA cycle proteins, and *S*-layer proteins are highly abundant after vacuum exposure. The overall abundance of amino acids and TCA cycle intermediates is reduced during the recovery phase of *D. radiodurans* as they are needed as carbon source. Furthermore, vacuum exposure induces an upregulation of Type III histidine kinases, which trigger the expression of *S*-layer related proteins. Along with the highly abundant transcriptional regulator of FNR/CRP family, specific histidine

kinases might be involved in the regulation of vacuum stress response. After repair processes are finished, *D. radiodurans* switches off the connected repair machinery and focuses on proliferation. Combined comparative analysis of alterations in the proteome and metabolome helps to identify molecular key players in the stress response of *D. radiodurans*, thus elucidating the mechanisms behind its extraordinary regenerative abilities and enabling this microorganism to withstand vacuum stress.

Keywords: *Deinococcus radiodurans*, high vacuum exposure, dehydration, proteomics, metabolomics, molecular stress response

INTRODUCTION

With future long-term space explorations in mind, understanding the molecular mechanisms of survival in outer space becomes increasingly important. The vacuum and radiation-filled outer space provides hostile conditions to any form of life. However, there are some organisms that developed survival strategies for extreme environments on Earth that may also be favorable for their viability in outer space, most prominently the desiccation-resistant spores of *Bacillus subtilis* (Horneck et al., 2012) or *tardigrades* (Jönsson et al., 2008) in their multi-resistant tun state. Desiccation by space vacuum exposure (pressure below 10^{-4} Pa) is one of the most harmful factors to microorganisms in outer space, leading to severe changes on a proteomic and genomic level (Cox, 1993). One of the primary targets of dehydration is the lipid bilayer which can undergo a conversion of bilayer sheets to spherical micelles, subsequently affecting cell membrane associated proteins like porins and membrane-bound cytochromes as well (Cox, 1993). Furthermore, Maillard reactions can lead to amino-carbonyl reactions, causing cross-linking of proteins to other proteins, sugars and nucleic acid components (**Supplementary Figure S1**) (Cox, 1993). The resulting polymerization of biomolecules can alter crucial cell functions by changing membrane permeability, impeding enzyme function (Horneck et al., 2010) and subsequently major biosynthesis as well as transport and repair pathways. Desiccation-induced disturbance of the mitochondrial electron transport chain in combination with the disruption of protein function by Maillard reactions lead to an intracellular build-up of ROS in *Zea mays* (Billi and Potts, 2002; França et al., 2007). The accumulation of ROS ultimately results in a destructive biochemical cascade, reinforcing lipid peroxidation, denaturation of proteins and nucleic acid damage with severe consequences on overall cell metabolism (Hansen et al., 2006; Garcia, 2011).

Upon dehydration, DNA is prone to experience double strand breaks (DBS), as detected in spores of *B. subtilis* and in the gram-positive bacterium *D. radiodurans* after simulated outer space vacuum (10^{-6} Pa) and real outer space vacuum treatment (Dose et al., 1992, 1995). This observation is supported by various subsequent studies with DNA repair deficient mutants that exhibited decreased survival during high vacuum conditions (Horneck et al., 1995; Munakata et al., 1997). Moreover, a transcriptomic analysis of *B. subtilis* spores subjected to 1.5 years of outer space and simulated Mars conditions conducted by Wayne et al. indicated a DNA response unique to vacuum desiccation as a single factor (Nicholson et al., 2012). In this study, spores of *B. subtilis* were exposed to outer space on aluminum coupon stack triplets and were subsequently compared with spores subjected to simulated Martian environment. Spores retrieved from the middle and lower and therefore UV-shielded layer of the space vacuum aluminum coupons exhibited an overall much stronger and broader DNA damage response compared to the samples exposed to the UV-shielded Martian environment (Nicholson et al., 2012). The only differing parameter in terms of space related stress between both conditions was the surface pressure subjected on spores (3 Pa simulated Martian atmosphere vs. 10^{-4} Pa), thus highlighting the importance of high vacuum as an environmental factor (Nicholson et al., 2012). Interestingly, the DNA damage response of spores after UV-shielded space exposure differed from the classic DNA damage response. It was lacking elevation of *lexA* expression, a master regulator of the classic DNA damage response in *B. subtilis* (Nicholson et al., 2012). This suggests that vacuum desiccation may trigger a DNA damage response unique to outer space related stress factors (Nicholson et al., 2012).

The mutagenicity of space vacuum was first reported in the Spacelab1 experiment in 1984 (Horneck et al., 1984). Spores of histidine deficient *B. subtilis* that were exposed to vacuum (1.2×10^{-4} Pa) showed a tenfold increase of histidine revertant mutants compared to samples kept on 1 atm (1.0×10^{5} Pa) (Horneck et al., 1984). Additionally, it was possible to show that vacuum increases the susceptibility of *B. subtilis* to ultraviolet radiation by a factor of 1,2 to 9,1 (Horneck et al., 1984). Further investigations of *B. subtilis* spores from strains subjected to high-vacuum (1×10^{-3} Pa) exposure by Munakata et al. (1997) indicate that mutation frequencies increase proportional to vacuum exposure time. In this study a 5′-CA to 5′TT tandem double base exchange located at codon 84 of the *gyrA* Gene was identified, which occurred in 55 to 62% of nalidixic acid-resistant mutant spores of the strains HA101 (*hisH101, metB101, leuA8*) and TKJ6312 (*uvrA10, spl-1*), respectively (Munakata et al., 1997). Strikingly, out of more than 500 mutants obtained after various treatments, this specific double base exchange mutation was reported to only arise in spores that were vacuum treated (Munakata et al., 1997).

In our study we focus on the vacuum-stress response of *D. radiodurans*, a gram-positive microorganism highly resistant against various extreme environmental conditions. In contrast

to *B. subtilis* it does not resort to spore formation to sustain under extreme conditions (Dose et al., 1992). With its highly effective and fast DNA repair mechanism (Zahradka et al., 2006; Slade et al., 2009), in combination with its ROS-scavenging capacities by intracellular antioxidant complexes (manganese (Mn^{2+}), orthophosphate (P_i) and peptides) (Daly et al., 2004, 2007, 2010), it can withstand extended periods of time of extreme dryness (Mattimore and Battista, 1996; Fredrickson et al., 2008) and ionizing radiation with an acute dosage of 5,000 grays (Gy) with almost no loss in viability (Moseley and Mattingly, 1971; Ito et al., 1983). This not only qualifies *D. radiodurans* as a model organism to unravel the different molecular mechanisms for withstanding detrimental outer space conditions, it also makes it a compelling candidate for biotechnological advances under extreme conditions. Thus, *D. radiodurans* can be considered for future biotechnological applications such as bioremediation of radioactive waste on earth or as a biotechnical tool for future space exploration missions. Presently, multi-resistance of *D. radiodurans* has been exploited in a few successful biotechnological attempts. For instance, a recombinant *D. radiodurans* strain for bioremediation was engineered and shown to be effective (Appukuttan et al., 2006). This strain expresses the non-specific uranium precipitating acid phosphatase *phoN* (Appukuttan et al., 2006) and the mercury (Hg) (II) resistance gene *merA*, respectively (Brim et al., 2000). The latter gene encodes for an enzyme capable of reducing toxic Hg (II) into less toxic volatile elementary Hg (Brim et al., 2000). Gaining insight into the molecular basis of its multi-stress resiliency will further aid in the development of effective sterilization techniques for space ships to not only prevent disease outbreak but also unwanted contamination of extraterrestrial environments during space missions. In respect to its highly effective coping mechanisms with ROS induced stress, *D. radiodurans* can be used as a model to study the molecular mechanisms of cancer and aging (Slade and Radman, 2011). An experiment involving treatment of human cell lines with *D. radiodurans* ultrafiltrate indicated that the manganese complexes also aid other organisms against harmful radiation and ROS (Daly et al., 2010).

In ionizing-radiation-resistant bacteria, *S*-layer proteins might play an important role in response to radiation damage (Gentner and Mitchel, 1975). The *S*-layer, which is the first line of defense against environmental factors, appears to be extremely versatile. It is assumed that protein expression is adapted to different stress factors through rearrangements of DNA (Pollmann et al., 2006). These proteins are anchored to the cell surface via non-covalent interactions and are proposed to interact with the pink carotenoid deinoxanthin within *D. radiodurans* cell envelope (Ghedira et al., 2016). This interaction helps to protect *D. radiodurans* from UV radiation under desiccation conditions (Farci et al., 2016).

Overall, these characteristics make *D. radiodurans* an excellent candidate for studies involving outer space survival and interplanetary space travel. However, data of *D. radiodurans* susceptibility and molecular response to outer space parameters is sparse. In our study, we aim to investigate the molecular response of *D. radiodurans* to outer space vacuum as the sole factor. Therefore, we subjected *D. radiodurans* cells to 8.7×10^{-5} Pa (space simulating vacuum) for a duration of 90 days in the ground-based Astrobiology Space simulation facility at DLR Cologne, to simulate outer space vacuum conditions (Rabbow et al., 2016). Subsequent metabolomic and proteomic analyses as well as an inspection of RNA integrity were performed with dehydrated cells of *D. radiodurans* exposed to space-simulating vacuum. The experiment was conducted as a preliminary investigation prior to the Tanpopo mission, where *D. radiodurans* was kept under LEO conditions at the International Space Station (ISS) for a period of 1 to 3 years (Kawaguchi et al., 2016; Yamagishi et al., 2018). Herewith, we hope to contribute to studies based on outer space response of *D. radiodurans* (Pogoda de la Vega et al., 2007; Bauermeister et al., 2011), in order to provide an in depth understanding of the molecular response to vacuum as single factor.

MATERIALS AND METHODS

Cultivation and Preparation of Dehydrated *D. radiodurans* Cells

Dehydrated *D. radiodurans* R1 cell layers with thickness of 1.4 mm were deposited in wells of a round aluminum plate as described previously (Ott et al., 2017). Briefly, *D. radiodurans* was cultured 15 h in TGB medium (1%(w/v) tryptone, 0.6%(w/v) beef extract, 0.2%(w/v) glucose) at 30°C in an incubator with shaking speed of 150 rpm until it reached the mid-exponential phase. Liquid cultures of *D. radiodurans* were washed in 10 mM phosphate buffer (PB): 10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7. This step was repeated three times. Aluminum plates containing cylindrical wells (2.0 mm diameter, 2 mm depth) with flat floor were used as sample holders (Kawaguchi et al., 2016). Twelve microliters of a cell suspension (2.9×10^9 cells/mL) were dropped into 4 wells and dried up under 3.3×10^3 Pa in a desiccator at room temperature (RT) under sterile conditions. These steps were repeated 6 times. The cells were dried up under 3.3×10^3 atm for 16 h. Subsequently, cells were exposed to vacuum that mirror LEO conditions outside the ISS as close as possible (8.7×10^{-5} Pa) at 21°C for 90 days, whereas the controls remained in a desiccator at 21°C (**Supplementary Figure S2**).

Recovery of Dehydrated *D. radiodurans* Cells

After exposure to vacuum, cells were recovered from wells of aluminum plates using 10 mM PB followed by incubation with TGB medium at 80 rpm at 32°C. OD_{600} measurements were performed at zero time point t_0 and the harvesting time points t_{3h} and t_{6h} (**Figure 1A**). To get a single, comparable value for each condition, the growth per hour between t_0 and the harvesting timepoint was calculated (**Figure 1B**). Additionally, the growth of vacuum exposed and control cells was monitored using colony formation units (CFU) counting to evaluate the loss of cell viability induced by the vacuum exposure (**Figure 1C**). For CFU counts, t_0 exposed and control cells were put on TGB agar plates

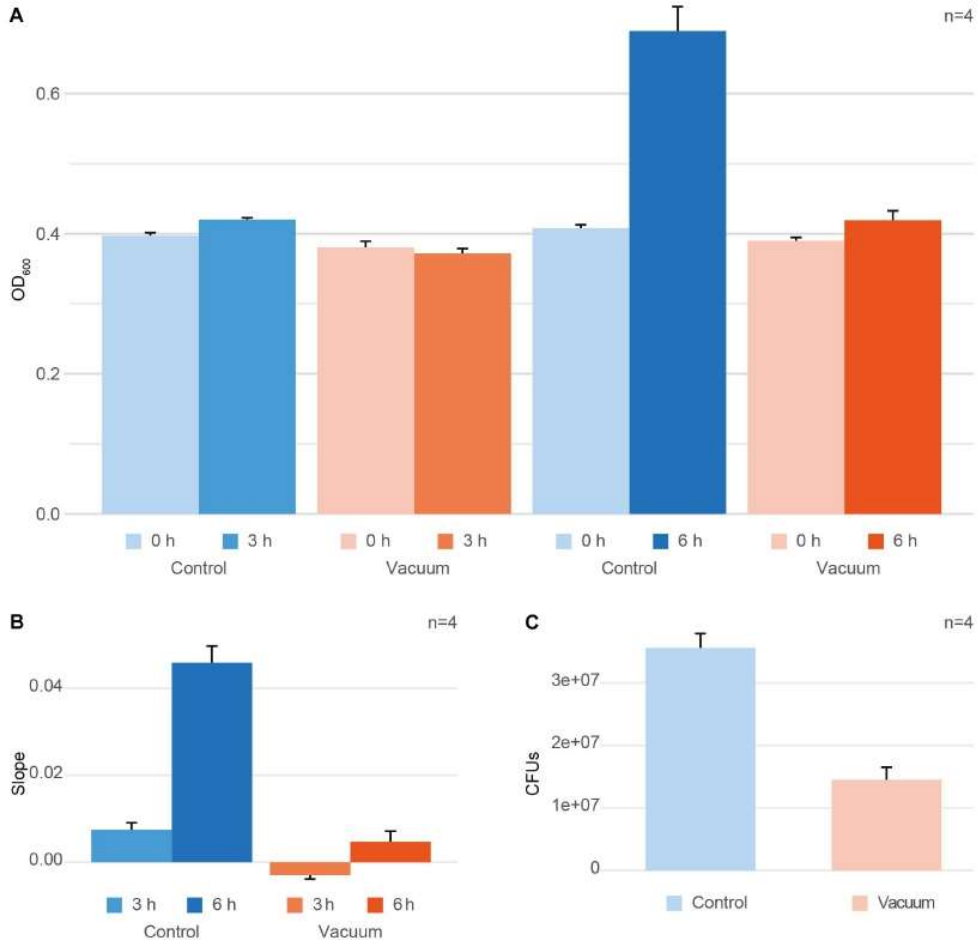

FIGURE 1 | Effect of vacuum on survival and growth of *D. radiodurans*. **(A)** OD_{600} measurements at t_0 (inoculation timepoint) and the harvesting timepoint for the control cells and vacuum exposed cells. **(B)** Changes in OD_{600} values between t_0 and the harvesting timepoint per hour of cultivation shown as ratio per hour. **(C)** Colony forming units (CFUs), counted for the control cells and vacuum exposed cells. In case with CFU, samples for plating were picked at t_0. Error bars always show the standard error based on the measurements of four replicates. Error bars at t_0 represent the measurement error of the instrument.

in different dilutions and were incubated for 2 days at 30°C until colonies achieved a countable size.

RNA Integrity

To determine suited timepoints for the extraction of metabolites and proteins, which allow insights in the early molecular response to the vacuum conditions, RNA integrity was evaluated. For RNA integrity measurements, two replicates for 3 h and two replicates for 6 h growth in a complex liquid medium were prepared (**Supplementary Figures S3, S6**).

To harvest the cells, the cultures were centrifuged (2000 *g*, 5 min, 4°C). The pellet was washed with 5 mL PB, 1.8 mL PB and 900 µL H_2O with vortex and centrifuge steps (2000 *g*, 5 min, 4°C) in between. The pellet was stored at −20°C.

Silica beads and 1 mL TRIzol (Thermo Fisher Scientific) were added to the pellets and homogenization was performed with a FastPrepTM-24 Instrument (MP Biomedicals; 2*60 s, 6.5 m/s). After bead beating, the mixture was incubated for 15 min at RT. 200 µL chloroform was added and samples were centrifuged (21000 *g*, 2 min, 4°C). The upper, polar phase containing RNA was transferred into a new tube.

To precipitate the RNA, 1,5 ml of 100% ethanol was added to the polar phase of each sample and incubated at RT for 10 min. The RNA supernatant containing ethanol was centrifuged (12000 *g*, 2 min, 4°C) using EconoSpin® Silica-Membrane Mini Spin Columns. Membrane-bound RNA was washed with 750 µL 90% and subsequently 70% ethanol and centrifuged between each washing step (12000 *g*, 2 min, 4°C). The column was centrifuged afterwards with maximum speed for 1 min to evaporate ethanol residuals. The RNA was eluted using 50 µL RNAase free H_2O (6000 *g*, 2 min, 4°C), after incubation for 1 min at RT. RNA integrity was evaluated by performing a 1% Agarose Gel Electrophoresis. Into each slot 1000 ng of RNA were loaded.

Simultaneous Extraction of Proteins and Metabolites

For the integrative extraction of proteins and metabolites a modified protocol according to Weckwerth et al. (2004) was used (Valledor et al., 2014). The sample extraction procedure is illustrated in **Supplementary Figure S4**.

The content of 14 wells with the vacuum exposed cells and 14 wells with the control cells were resuspended in 15 mL PB each. The suspensions were used to inoculate eight 250 mL flasks containing 30 mL of TGB medium for exposed and the control conditions. Cultures were incubated at 80 rpm at 32°C for 3 h and 6 h, respectively. 4 replicates for the vacuum exposed and 4 replicates for the control cells were incubated for 3 h. Additionally, 4 replicates for the vacuum exposed and 4 replicates for the control cells were incubated for 6 h. Cells were harvested as described in the previous paragraph "RNA integrity" and homogenization settings remained the same. Instead of TRIzol, 1 mL of ice-cold MCW (methanol:chloroform:water; 2.5:1:0.5) was used as solvent. After homogenization, samples were incubated 15 min on ice and centrifuged (21000 *g*, 4 min, 4°C). The supernatant, which contained metabolites, was transferred into a new tube for the subsequent purification of primary metabolites. The pellet, containing proteins and nucleic acids, was washed with 1 mL methanol, centrifuged and air-dried (21000 *g*, 4 min, 4°C). TRIzol was added to the dried pellet and it was additionally homogenized in the bead beater (30 s, 6.5 m/s). After bead beating, the mixture was incubated for 15 min at RT. Chloroform (200 µL) was added and samples were centrifuged (21000 *g*, 2 min, 4°C). The lower, apolar phase was transferred into new tubes for protein purification. The apolar phase (~550–600 µL) was washed once more with 550 µL H_2O, centrifuged (21000 *g*, 2 min, 4°C) and transferred into new tubes. Finally, 1.5 mL 0.1 M NH_4Ac in methanol with 0.5% beta-mercaptoethanol was added and proteins were precipitated over night at −20°C.

Shotgun Proteomics

Protein quantification and in-solution digestion

Protein pellets from the extraction step described above in 2.4 were centrifuged (21000 *g*, 15 min, 4°C), the supernatants were discarded, the pellets were washed two times with 1.8 mL ice-cold methanol and one time with 1.8 mL ice-cold acetone. For each washing step, the pellets were ultrasonicated for 5 min, centrifuged (21000 *g*, 15 min, 4°C) and the supernatants were discarded. After the final washing steps, pellets were air dried.

Pellets were resuspended in 40 µL 8.8 M urea in 50 mM HEPES on a shaker for 30 min at 750 rpm. After centrifugation (21000 *g*, 5 min, RT), a BCA (bicinchoninic acid assay) was performed to determine the protein concentration against different BSA concentrations (**Supplementary Figure S5**).

For digestion, 60 µg proteins of each sample were used. With the urea/HEPES buffer sample volumes were filled up to 15 µL. As a reduction step, samples were adjusted to 5 mM dithiothreitol (DTT) and incubated for 45 min on a thermoshaker at 37°C at 700 rpm. Afterwards, samples were alkylated by adjusting the iodoacetamide (IAA) concentration to 10 mM, followed by incubation for 60 min in dark on a thermoshaker at RT at 700 rpm. Alkylation was stopped by adjusting the DTT concentration to 10 mM DTT (total sample volume was 29.3 µL) and samples were further incubated for 15 min on a thermoshaker at RT. Before digestions, 29.3 µL 20% acetonitrile (ACN) 100 mM ammonium bicarbonate and 58.6 µL 10% ACN 25 mM ammonium bicarbonate and 10 mM $CaCl_2$ were added to the samples. Three microliter of trypsin beads (Promega) were added to digest proteins. Samples were incubated at 37°C at 10 rpm for 16 h.

Desalting and peptide quantification

To stop digestion, samples were put on ice. To desalt samples a C18 spec plate (Agilent) connected to a water-jet pump was used. The C18 membrane was activated with 2 × 800 µL methanol and washed with 2 × 800 µL H_2O without incubation time in between. Samples were acidified by adding 10 µL 20% formic acid, centrifuged (21000 *g*, 2 min, 4°C), loaded on the C18 material and incubated for 10 min at RT. Peptides were washed on the C18 material with 2 × 800 µL water and finally eluted with 3 × 200 µL methanol. Samples were dried down in a speedvac.

To determine digestion efficiency and to normalize the peptide amount throughout all samples, a colorimetric peptide quantification assay (Pierce) was performed (**Supplementary Figure S5**) after resuspending samples in 100 µL 2% ACN 0.1% formic acid. Samples were further diluted to a peptide concentration of 50 ng/µL.

HPLC nESI-MS/MS measurement and data analysis

For shotgun proteomics measurements, 5 µL of each sample were injected into an nHPLC-Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany), measurement settings were described before (Ott et al., 2017). Data analysis was performed with Maxquant (Cox and Mann, 2008). The minimum peptide length for identification was set to 7 amino acids and one unique peptide was required for protein identification (FDR 1%, based on target decoy database). For identification, measured spectra were compared to the *D. radiodurans* FASTA file from Uniprot (October 2018, 3085 sequences in the database). Further settings: 20 ppm first search peptide tolerance, 4.5 ppm main search peptide tolerance, maximum of 2 missed cleavages, maximum number of 5 modifications per peptide [variable: oxidation (M) and acetylation of protein *N*-term, fixed: carbamidomethylation (C)], label free quantification of samples. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD011868.

Derivatization and Analysis of Metabolites With GC-BT-TOF-MS

For metabolite measurements, 300 µL of H_2O was added to the supernatants after cell homogenization to achieve a phase separation. Samples were centrifuged (21000 *g*, 2 min, RT), and the upper, polar phase was transferred into a new tube and 3 µL of 10 mM PGP (Phenyl β-D-glucopyranoside) was added as internal standard. Samples were carefully dried in a speedvac. Before measurement, methoximation and silylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamid to add trimethylsilyl (TMS)

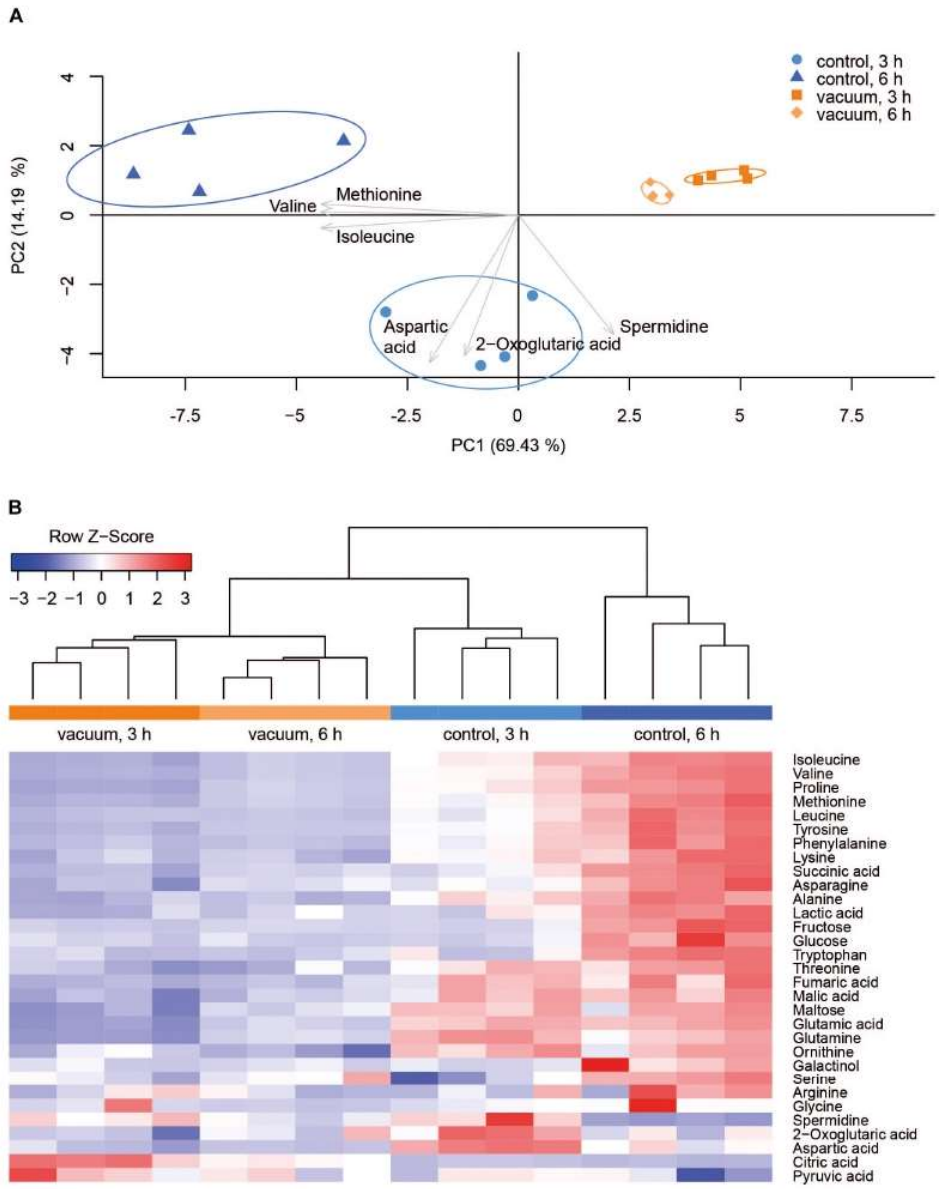

FIGURE 2 | Metabolic response of *D. radiodurans* to vacuum (targeted approach). **(A)** Principal component analysis of 32 metabolites. The plot was created in R with the pca3d package. All data was *z*-scored, and the most impactful loadings are indicated as arrows. **(B)** Heatmap of measured metabolites with a corresponding dendrogram created in R with the heatmap.2 package. Metabolite data was *z*-scored before plotting and the dendrogram was drawn by the hclust function.

residues was performed as described elsewhere (Weckwerth et al., 2004). Measurement of polar metabolites was performed on a GC-BT-TOF-MS (Leco) instrument. Separation of metabolites was achieved on an Agilent 7890B gas chromatograph on a Restek Rxi-5 ms (30 m length, 0.25 mm diameter and 0.25 µm film) in split 10 mode with helium as the carrier gas. The following settings were applied: flow rate 1 mL min^{-1}, injection temperature 230°C, column temperature start at 70°C for one minute, then heated up to 330°C in 9 min and hold for 8 min, ion source temperature at 250°C, acquisition rate 10 spectra s^{-1}, recorded masses 50–600 m/z.

ChromaTOF (Leco) was used for peak integration. For our targeted approach, metabolites were identified based on a house-intern quality control mix, containing several primary metabolites of interest. For data processing, first areas <10000 were removed from the dataset, then every sample was normalized to the area of the internal standard. Areas of the blank (medium extract without cells) was subtracted from

each measured substance and all derivatives of each metabolite were summed up. Finally, metabolites of every sample were normalized to the corresponding OD_{600} values.

The untargeted approach included a library search of all integrated peaks. Peaks with a similarity higher than 700‰ were annotated. As reference libraries, two GMD (Golm Metabolome Database) libraries and one NIST (National Institute of Standards and Technology) library were used. The hit with the highest similarity was chosen as annotation. Normalization was performed as described for the targeted approach.

Statistical Evaluation of Data

Statistically, all data from proteomics and metabolomics measurements were treated the same. To avoid miscalculation of missing values, an ANOVA was only performed if the protein/metabolite was present in all replicates. However, the applied methods also allowed analyses of proteins and metabolites uniquely represented in a single sample. In case of metabolite data, the already normalized (to the OD-measurement) intensities were used, for proteins, LFQ (label free quantification) intensities, which were calculated by Maxquant (Cox and Mann, 2008) were used. Each metabolite and protein was *z*-scored over all samples. Subsequently, samples were separated in 4 groups (3 h control, 6 h control, 3 h vacuum and 6 h vacuum) and an ANOVA was performed to identify significant differences between these groups. To evaluate the reasons for the significance in the ANOVA, a *post hoc* test was performed. For both statistical tests the Perseus software (Tyanova et al., 2016) was used. All figures were created with the *z*-scored data (except fold change figures). Most figures were created in R (R Development Core Team, 2018) with corresponding packages: Boxplots, bar charts and scatter plot (ggplot2 Wickham, 2016), heatmaps (heatmap.plus Day, 2012), PCA (pca3d Weiner, 2017). The spider plot was created in Microsoft® Excel and the TCA figure in Adobe Illustrator®.

RESULTS

Effect of Vacuum on Survival of *D. radiodurans*

After exposure to vacuum conditions, dehydrated cells of *D. radiodurans* were recovered in complex medium and their survival was evaluated by CFU counts. Additionally, OD_{600} measurements of microbial cultures were performed with the recovering cells. The same measurements were applied towards non-exposed control dehydrated cells of *D. radiodurans*, which were stored in a desiccator at ambient temperature. Cells were harvested at 3 h and 6 h of recovery of the vacuum-exposed and control *D. radiodurans* cultures. At t_{3h}, exposed cells showed a minimal decrease in OD_{600} values compared to t_0, whereas the control cells revealed a marginal increase (not significant, **Figure 1A**). The OD_{600} values measured after 6 h of recovery in a complex medium indicated an active increase in culture density in control cells, while the cell density of vacuum-exposed cells was only slightly affected (**Figure 1A**). The control non-exposed cells showed a small increase of OD_{600} values after 3 h and a much higher increase after 6 h, while the vacuum-influenced cells displayed a delay in the increase of OD_{600} values. The change in OD_{600} per hour (slope) is significantly higher for t_{6h} control samples compared to all other conditions (**Figure 1B**). Additionally, CFU-counts for vacuum-exposed cells showed a 2.5-fold lower survival rate compared to the control non-exposed cells (**Figure 1C**).

Metabolic Response to Vacuum

Primary metabolites were measured in all four replicates after 3 h and 6 h of recovery of vacuum exposed and control cells. Results of the targeted analysis are presented in **Figure 2** and **Supplementary Table S1**. On the Principal Component Analysis (PCA) (**Figure 2A**), all four data sets are clearly separated at the PC1 level, which explains approximately 70% of the variance. A general tendency is that control cells show much larger variation between 3 and 6 h of recovery than vacuum treated cells. Cells, which recovered for 3 h after vacuum exposure and control cells after 6 h of cultivation in a complex medium, were most different. According to the heatmap (**Figure 2B**), most amino acids are less present in vacuum exposed cells, especially after 6 h of recovery. In case of the untargeted approach, 252 peaks were successfully annotated. The number was reduced to 112, as only annotations present in at least 70% of the samples were used for further analyses (**Figure 3** and **Supplementary Table S2**).

Most TCA cycle intermediates show an identical pattern (**Figure 2B**), although 2-oxoglutaric acid appears most abundant in control cells after 3 h of recovery. Two other exceptions are citric acid and pyruvic acid, being most abundant in vacuum exposed cells after 3 h of recovery. The slightly higher abundances of pyruvic acid and citric acid after vacuum exposure indicate that the input to the TCA cycle is very similar in control cells and in vacuum exposed samples. The main difference is the conversion rate of intermediates and products.

Spermidine is more represented after 3 h compared to 6 h in control as well as in vacuum exposed cells (**Figure 2**). Furthermore, the spermidine content from control cells was 2.7-fold reduced between 3 h and 6 h, whereas cells exposed to the damaging high vacuum showed only 1.3-fold reduction (**Supplementary Table S1**). In our untargeted approach, we were able to identify cadaverine (4TMS) and putrescine (4TMS) which were most present in vacuum exposed samples at t_{3h} of recovery. Other interesting candidates are nicotinamide (1TMS), indole 3 pyruvic acid, lumichrome and some not yet identified metabolites. Unknown 14 shows m/z values characteristic for carbohydrates and Unknown 25 is connected to carboxylic acids (**Figure 3**).

Shotgun Proteomics Analysis

In total, 1939 proteins from *D. radiodurans* were identified in at least one sample, which represents a coding sequence coverage of approximately 63%. Out of these, 1166 proteins were quantified in each replicate and subsequently used for statistical analysis. The ANOVA (p-value ≤ 0.05) revealed 375 proteins as significantly different between the data sets. Differences between proteins from the vacuum exposed and control cells at both timepoints were determined by a *post hoc* test. After 3 h, 15

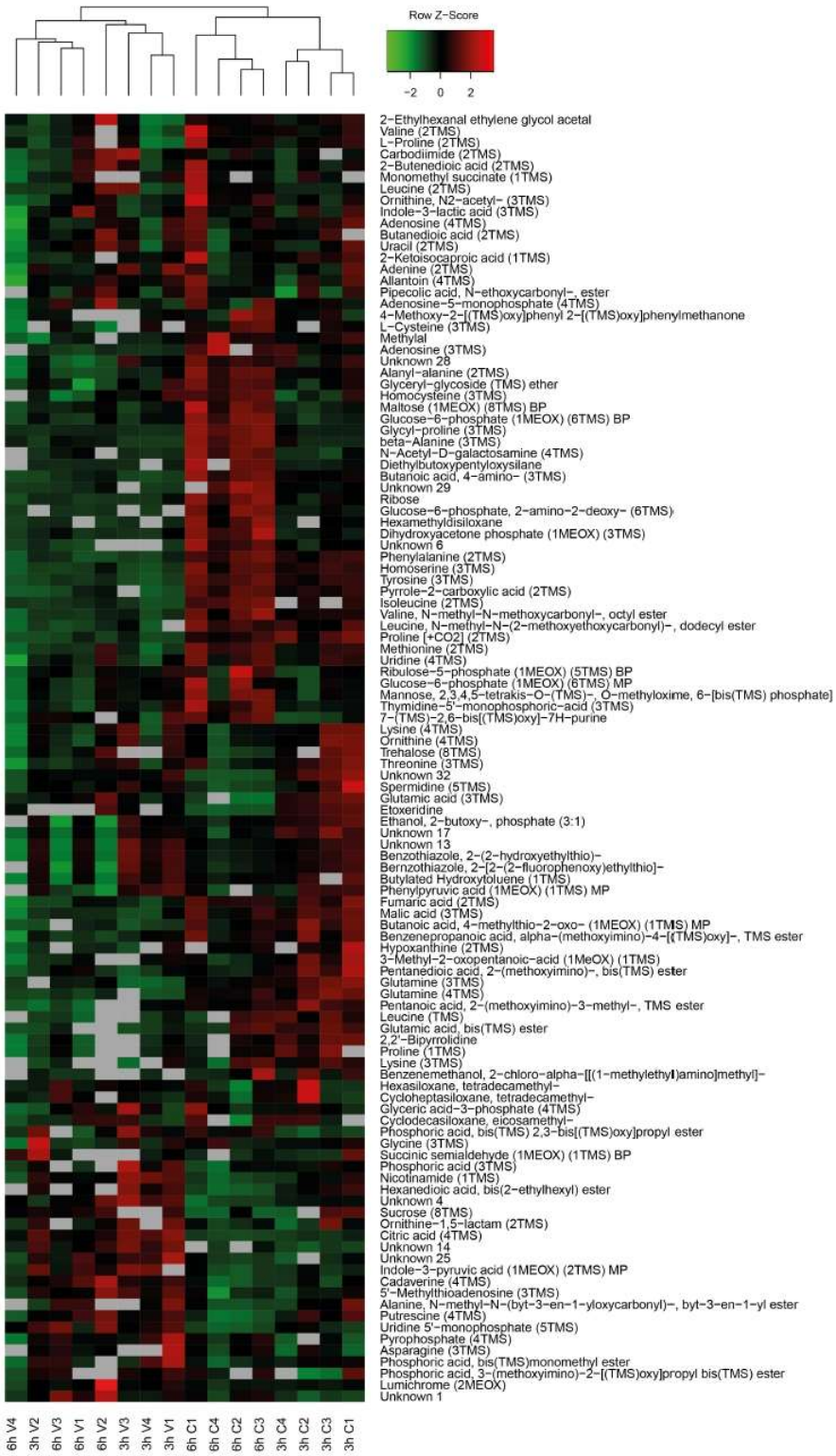

FIGURE 3 | Heatmap of untargeted metabolites with the corresponding dendrogram. Data was *z*-scored before plotting.

proteins were more abundant in the vacuum exposed compared to the control cells; 86 proteins were less abundant. After 6 h, 107 proteins were more abundant in the vacuum exposed cells compared to the control cells; 105 proteins were less abundant.

After 6 h of recovery we observed a similar amount of proteins that were more abundant in the vacuum treated cells compared to proteins which were less abundant (**Figure 4A**). Proteins with a *p*-value below 0.05 were divided into two groups and uploaded to the String database. The String database maps protein-protein interactions, connects interacting proteins with nodes and calculate the enrichment of those nodes (Szklarczyk et al., 2015). For proteins, which were less abundant after the vacuum exposure, no significant enrichment of nodes (*p*-value 0.260) was detected, whereas proteins, which were higher abundant after the vacuum exposure showed a high enrichment of nodes (*p*-value $6.02^{*}10^{-7}$) (**Figure 4B**). According to the String database, proteins which were higher abundant after 6 h of recovery in a complex medium, highly interact with each other. These proteins may work together to alleviate cell damage caused by the vacuum treatment. Proteins which were less abundant do not show a significant number of interactions. An enrichment analysis of proteins which were higher abundant identified several overrepresented KEGG pathways (Kanehisa and Goto, 2000). Proteins belonging to groups such as citrate cycle, nucleotide excision repair, aminoacyl-tRNA biosynthesis, microbial metabolism in diverse environments (**Figure 4C**) were more abundant after the exposure. Ribosomal proteins did not show a significant increase or decrease.

Proteomics analysis showed that after 6 h of cultivation in complex medium a lot of TCA cycle enzymes are more abundant in vacuum exposed cells (**Figure 5**). Furthermore, we observed an increase of some proteases (**Figure 6B**) during the recovery phase after vacuum treatment. Many t-RNA ligases (**Figure 6A**) that produce aminoacyl-tRNA (aa-tRNAs) were higher abundant at 6 h of recovery of vacuum exposed cells. These aa-RNAs are usually used by the ribosome for protein synthesis. However, as shown in **Figure 4C**, the enrichment analysis did not reveal a high abundance for ribosomal proteins after the vacuum exposure. It can be assumed that the non-proliferating, vacuum exposed cells after 6 h of recovery do not synthesize a lot of proteins, but still produce high amounts of aa-RNAs. An increase in proteins such as peroxidase DR_A0145, catalase KatA, several proteins involved in the UvrABC nucleotide excision repair machinery and polymerase PolA was observed during the first hours of recovery (**Supplementary Table S3**). To initiate all defense lines, intercellular signal cascades are undoubtedly important for a fast and efficient regulation of stress response. With our proteomics approach, we measured the abundances of several histidine kinases (**Figure 7**) at t_{6h} from vacuum exposed and control cells and identified several histidine kinases that were higher abundant in the vacuum exposed cells.

DISCUSSION

Deinococcus radiodurans is well known for its extraordinary resistance to radiation and desiccation. It was identified that ionizing radiation sensitive mutants are more vulnerable to desiccation (Mattimore and Battista, 1996), indicating that these effects trigger similar stress response mechanisms in the cell. Previously, it was shown that *D. radiodurans* can handle desiccation exceptionally well, but the exposure to high vacuum tremendously decreased survival of cells (Saffary et al., 2002). Our study supports these findings, as the number of colony forming units is reduced 2.5-fold (**Figure 1C**) after exposure of dehydrated *D. radiodurans* cells to high vacuum compared to dehydrated non-exposed control cells. Vacuum-induced desiccation causes severe dehydration, promoting Maillard reactions of carbohydrates, proteins and nucleic acids which result in cross linking and errors in polymerization (**Supplementary Figure S1**). These give rise to functional changes, such as altered enzyme activity, changes in membrane permeability, and alteration of genetic information (Horneck et al., 2010). Survivors of dehydration stress which are recovered in a cultivation medium undergo a prolonged lag phase (Bucker et al., 1972). **Figure 1A** shows that at 6 h of recovery, OD_{600} values of the control cells nearly doubled. Cells exposed to high vacuum remained in a growth arrest phase for a longer period and therefore, the OD_{600} did not increase noticeably after 6 h. Apparently, during the growth arrest phase, cells are mostly engaged in repairing the damage caused by vacuum.

Although *D. radiodurans* can shield proteins from ROS induced damage (Daly et al., 2010), the amount of double strand breaks (DSB) is similar in all prokaryotic cells (Krisko and Radman, 2010). In addition to DSB, desiccation generates single strand breaks and base damage (Slade and Radman, 2011). Several repair pathways, e.g., base and nucleotide excision repair, mismatch repair and recombinational repair are used to fix damaged nucleic acids. Therefore, many proteins involved in these pathways appear least abundant in control cells at t_{6h} of recovery. This includes proteins involved in the UvrABC endonuclease repair (UvrA, UvrB, and UvrC), the endonuclease MutS2, the polymerase PolA and the gyrases GyrA and GyrB (topoisomerase). In previous studies, GyrA was identified to carry the majority of mutations induced by high and low vacuum in *B. subtilis* spores (Munakata et al., 1997; del Carmen Huesca Espitia et al., 2002). The higher abundance during the early phase of recovery indicates that various nucleic acid repair processes are ongoing. In *D. radiodurans*, RecA dependent DNA damage response is regulated by the transcriptional repressors LexA and LexA2 (DR_A0074). At t_{3h} we observed a higher abundance in LexA2 in vacuum exposed samples compared to control samples. However, RecA levels were lower in vacuum exposed samples. In a study with *lexA2* deficient mutants of *D. radiodurans* an increased amount of RecA was observed (Satoh et al., 2006). Therefore, we conclude that an increased level of LexA2 results in a low abundance of RecA, which delays DNA repair. The nucleic acid repair system in *D. radiodurans* is extremely efficient and our proteomics data shows an increased abundance of many repair related proteins in the early stages of recovery (**Figure 8**). However, there is no documented evidence that proteins typically involved in DSB repair appear higher abundant or with a higher specific activity in *D. radiodurans* compared to *E. coli*

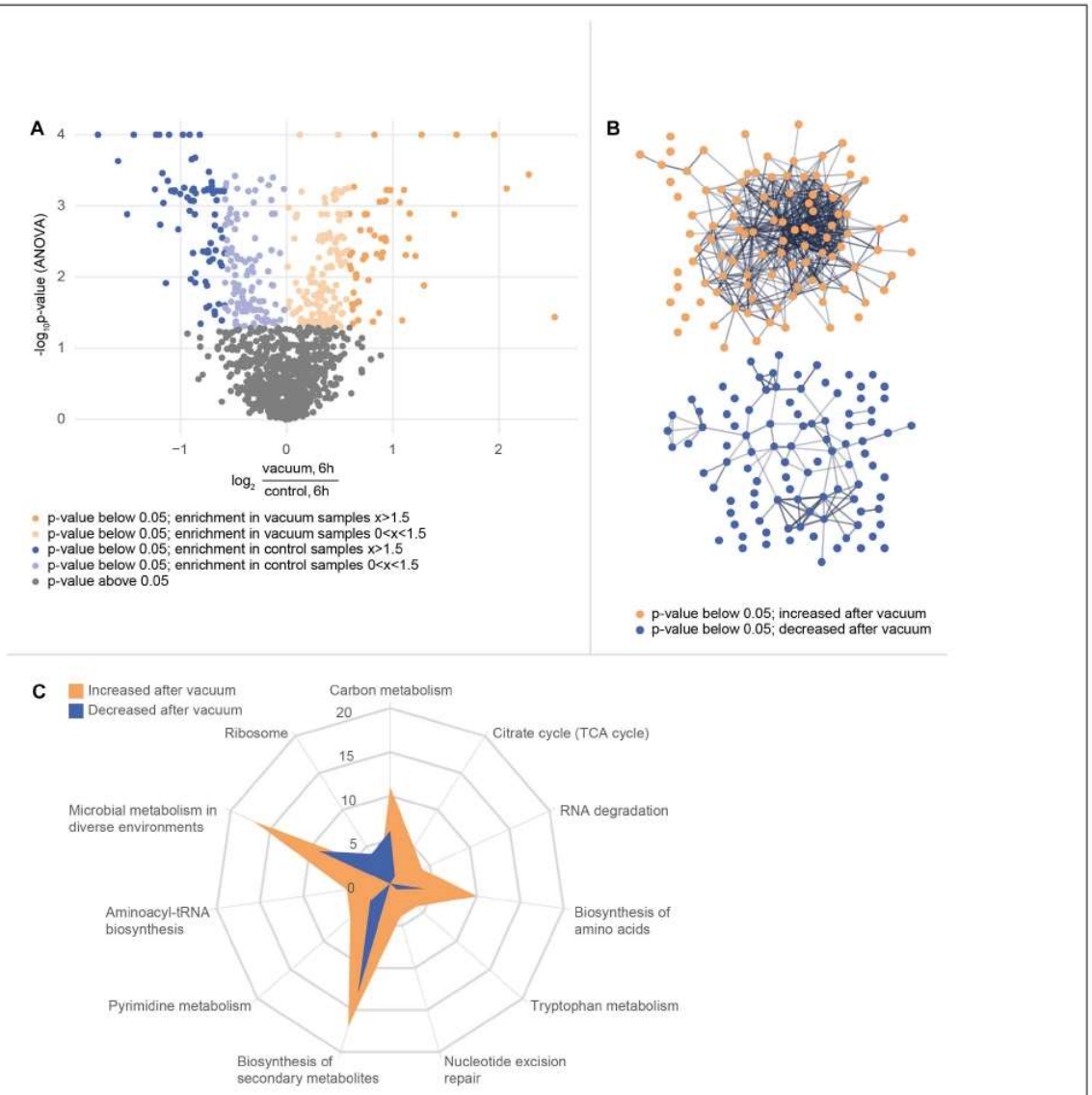

FIGURE 4 | Proteomics analysis of the vacuum exposed and control cells after 6 h of recovery in a complex medium. **(A)** Volcano plot of all 1166 proteins that were identified in every replicate of every condition and timepoint. The *y*-axis plots the negative $\log_{10}$ corrected *p*-value (*q*-value) of the ANOVA. The *x*-axis shows the $\log_2$ fold change. All proteins with a fold change below 1.5 are indicated in brighter colors. **(B)** STRING database analysis from selected proteins. Proteins with a corrected *p*-value below 0.05 were divided into two groups. A *post hoc* test confirmed if there is a difference at the 6 h timepoint between control and vacuum exposed cells. 107 proteins were identified as higher abundant in vacuum exposed cells (orange group) and 105 proteins as less abundant (blue group). The STRING database was able to map 104 of the higher abundant ones and 98 of the lower abundant ones. Nodes are uploaded proteins and edges are interactions between proteins. The null hypothesis tests if the number of interactions could be assigned to any random set of proteins. The p-value for the orange set is $6.0^{*}10^{-7}$, the one for the blue set is 0.26. **(C)** KEGG pathway annotations were added to the uploaded proteins. The number of proteins from both sets of proteins which belong to several KEGG pathways is shown in the spider plot.

(Daly, 2009), although *E. coli* is about 30 times more susceptible to DSB than *D. radiodurans* (Slade and Radman, 2011).

Role of Primary Metabolites and Energy Modulation After Vacuum Stress

Polyamines like spermidine and putrescine were postulated to be exploited by organisms from bacteria to plants and animals as a primordial form of stress molecules (Rhee et al., 2007). The exposure to oxidative stress induces polyamine synthesis, which leads to an expression of genes involved in ROS scavenging and repairing damage. In *E. coli*, transcription of catalases and other oxidative stress response proteins are induced by transcriptional regulons like RpoS and OxyR. The expressions of *rpoS* (starvation response) and *oxyR* (ROS response) are induced

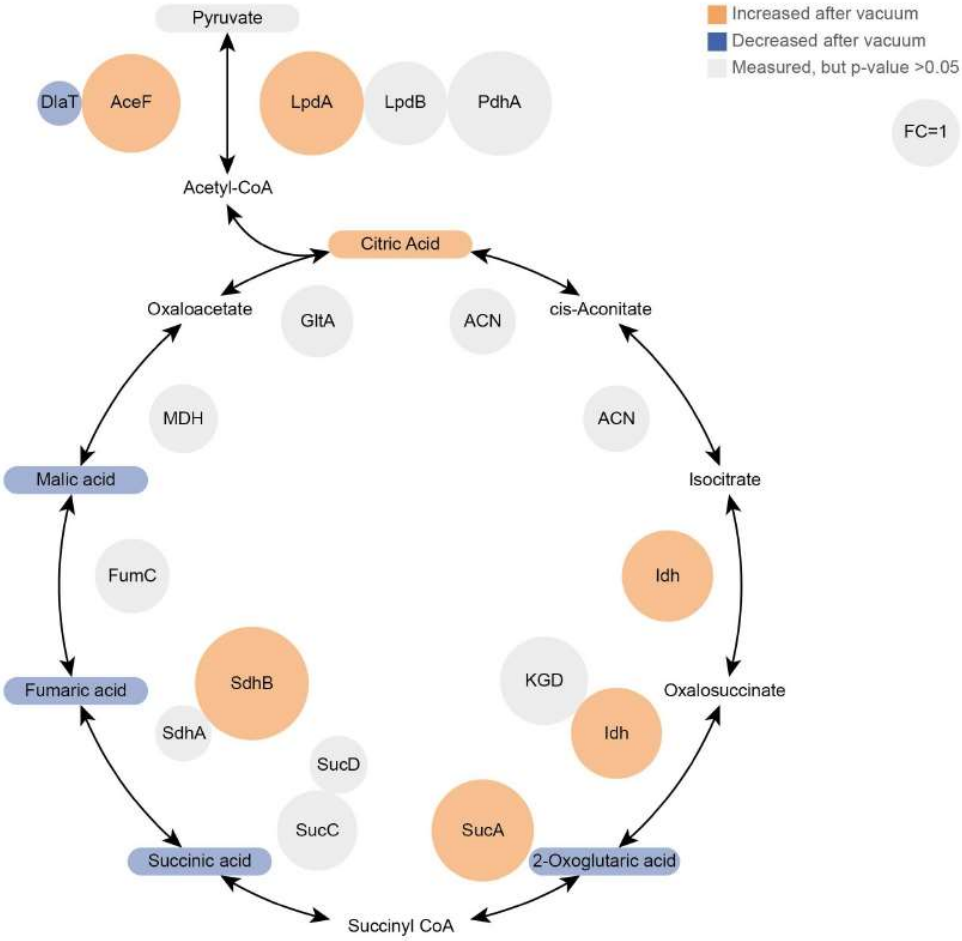

FIGURE 5 | Proteomics and Metabolomics comparison of the TCA cycle between vacuum exposed and the control cells after 6 h of recovery in a complex medium. Metabolites are rounded rectangles and proteins are circles. Molecules in orange were more abundant after the vacuum exposure (ANOVA and *post hoc* test), blue molecules were less abundant. Gray ones were measured, but no statistical difference was calculated. In addition, the sizes of protein circles mirror their fold changes (vacuum 6 h/control 6 h).

by polyamines in *E. coli* (Jung and Kim, 2003). Dehydrated cells exposed to high vacuum conditions and the control dehydrated cells showed an increase in spermidine after 3 h of recovery in a complex medium. The proteomics analysis revealed carboxynorspermidine decarboxylase, an enzyme that catalyzes the formation of spermidine from carboxyspermidine as significantly more abundant in t_{3h} compared to t_{6h}. This indicates that polyamines are used as a general stress response during recovery from vacuum- and dehydration-induced stress.

Nicotinamide, a precursor of nicotinamide-adenine-dinucleotide (NAD), showed an increase in the early recovery phase (t_{3h}) after vacuum exposure compared to the other conditions. NAD can be used as coenzyme for redox reactions and as substrate for NAD consuming enzymes, for instance ADP-ribose transferases (Gazzaniga et al., 2009). Lumichrome, a derivative of riboflavin, which is responsible for various extracellular processes in bacteria, such as quorum sensing signaling and extracellular electron transfer (Rajamani et al., 2008). In addition, riboflavin serves as precursor for flavin adenine dinucleotide (FAD), which is involved in redox reactions (Moreno-Hagelsieb et al., 2015). These two metabolites may contribute to oxidative stress response mechanisms in *D. radiodurans* after exposure to vacuum (**Figure 8**).

Cells, which were exposed to vacuum are metabolically less diverse during the growth arrest phase. We assume that in vacuum exposed cells, TCA cycle intermediates (2-oxoglutaric acid, fumaric acid, succinic acid, malic acid) and amino acids (**Figures 2B**, **5**) are more rapidly enzymatically converted to support repair processes and therefore appear less abundant. In control cells, however, primary metabolites are not directly used, but rather produced as intended by the cells at logarithmic phase in optimal growth conditions. It can be concluded that vacuum

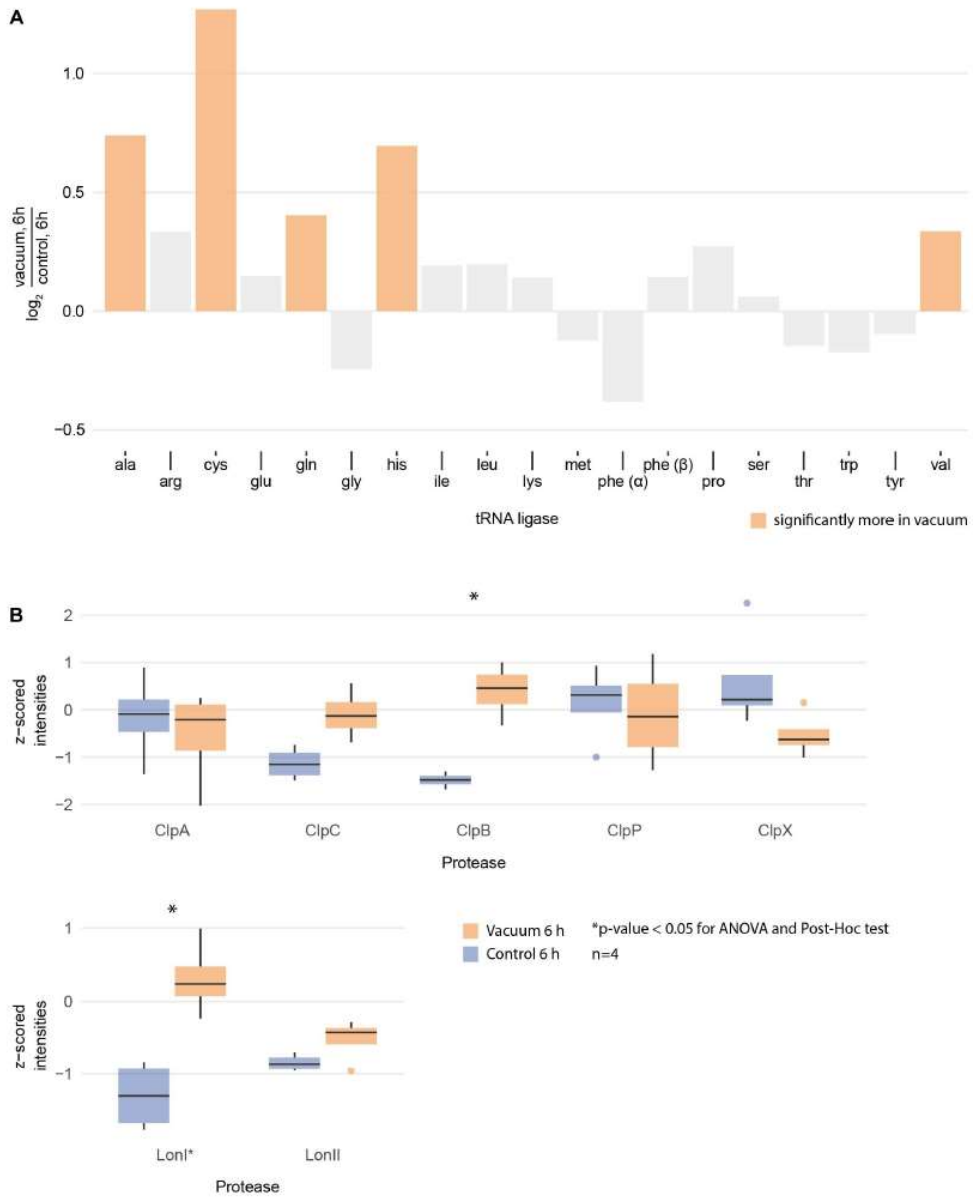

FIGURE 6 | (A) Logarithmic fold change of all measured tRNA ligases between vacuum exposed and the control cells after 6 h of recovery in complex medium. The ones with statistically significant differences (ANOVA and *post hoc* test) are high lightened in color. **(B)** Normalized intensities of measured Clp and Lon proteases after 6 h of recovery between the vacuum exposed and the control cells. Statistically significant differences are indicated with a*.

treatment leads to an increase of TCA cycle intermediate conversion (**Figure 8**).

After exposure to desiccation stress many organisms showed an induction of proteins involved in the TCA cycle to produce the necessary amount of energy to alleviate cell stress (Riedel and Lehner, 2007; Gruzdev et al., 2012; Kocharunchitt et al., 2012). TCA cycle intermediates serve as precursors for amino acids, which are the preferred carbon source for *D. radiodurans* (Venkateswaran et al., 2000). Lon and Clp proteases presumably degrade damaged proteins to deliver more amino acids (Servant et al., 2007). However, the amount of amino acids after vacuum exposure is lower compared to control cells. We therefore looked for the correlation between the observed elevation in proteases and the decrease in amino acid abundance in recovering cells after the vacuum exposure. Several studies uncovered roles of aa-tRNAs as substrates in biochemical processes apart from protein synthesis (Raina and Ibba, 2014). Firstly, aa-RNAs can attach amino acids to the amino-terminus of damaged proteins

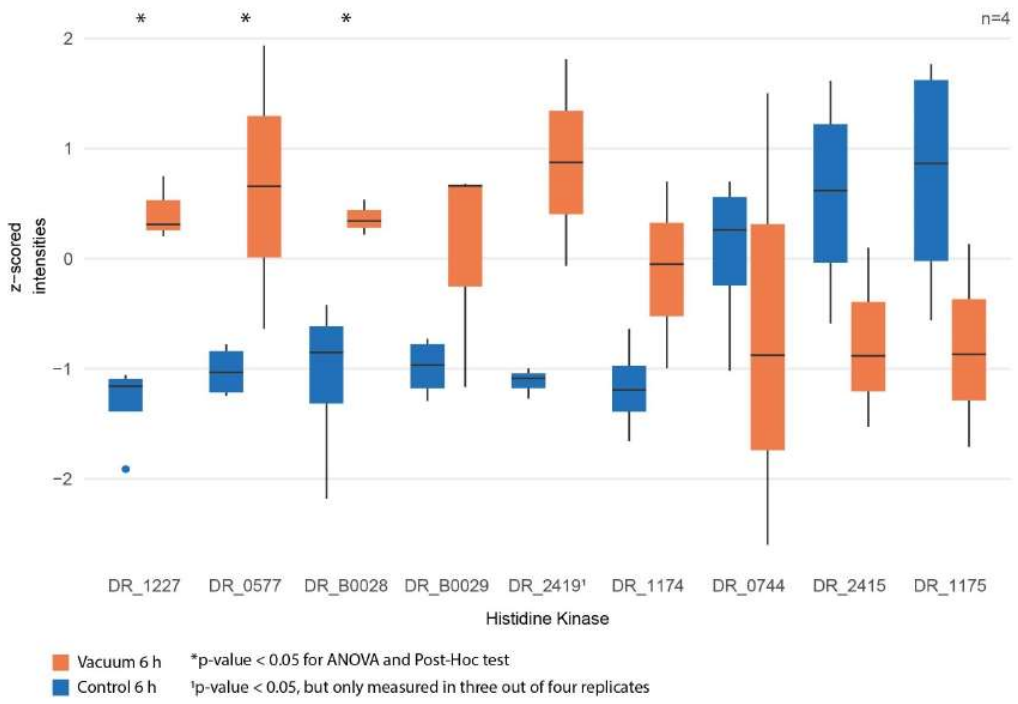

FIGURE 7 | Normalized intensities of measured histidine kinases between vacuum exposed and control cells after 6 h of recovery in a complex medium. Statistically significant differences are indicated with a*.

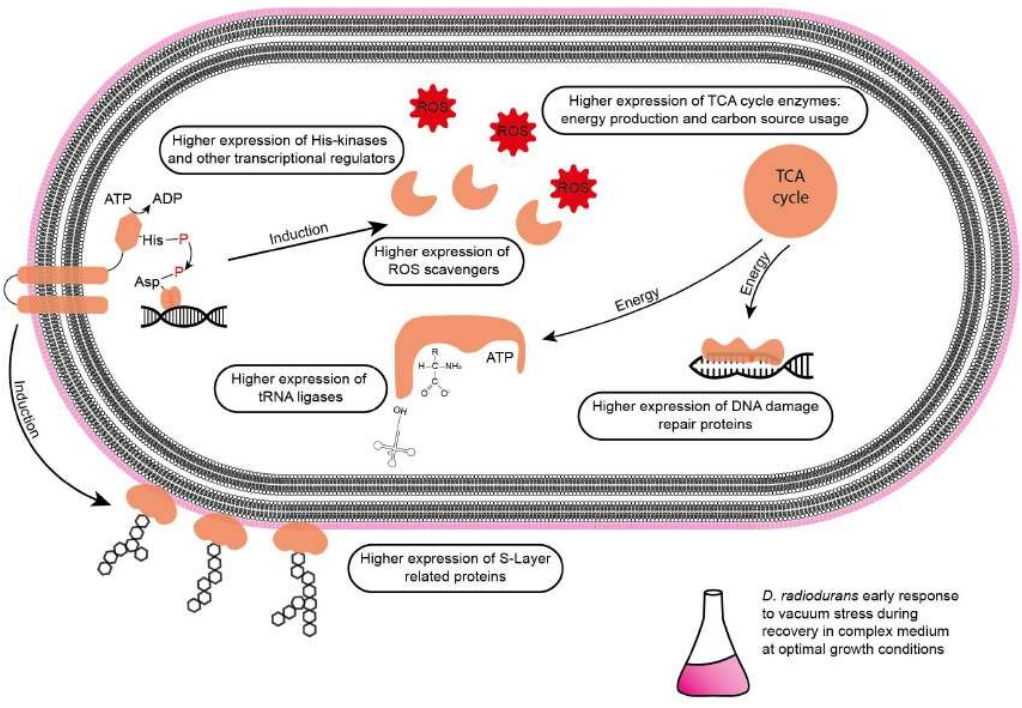

FIGURE 8 | Summary of affected molecular components in *D. radiodurans* during the early stage of recovery from vacuum stress in a complex medium.

as recognition sites for proteases (Mogk et al., 2007; Raina and Ibba, 2014). Furthermore, in *Streptomyces viridifaciens*, the antibiotic valanimycin is produced by transferring the seryl residue from seryl-tRNA to the hydroxyl group of isobutylhydroxylamine (Garg et al., 2006; Banerjee et al., 2010). Moreover, aminoacyl-tRNAs were shown to be involved in the formation of peptidoglycans as structural components of cell walls and membrane phospholipid modification (Shepherd and Ibba, 2013). Thus, we propose that the cell wall of *D. radiodurans* is one of the primary targets of vacuum-induced stress and that as a result, aa-tRNAs are recruited in order to recycle amino acids from the TCA cycle to aid in the reconstruction of damaged cell wall and membrane components (**Figure 8**).

Regulation of the Vacuum-Induced Stress Response

Desiccation and high vacuum put cells under very stressful conditions, but even over a period of 90 days, *D. radiodurans* can survive and proliferate again, if the appropriate cultivation conditions are provided. Nevertheless, a growth arrest phase is necessary before proliferation of the vacuum exposed cells can be initiated. Our data indicates that ROS are eliminated, and macromolecules are repaired during this phase (**Figure 8**). Defective two-component signal transduction systems like histidine kinases and response regulators can increase susceptibility of *D. radiodurans* to various stress factors. In a knockdown study it was shown that some mutants that lack specific histidine kinases are less resistant to extreme conditions like ionizing radiation and UV radiation (Im et al., 2013). Our statistical analysis revealed that DR_1227 and DR_0577 were more abundant at 6 h after the vacuum exposure compared to the control cells at the same time point (**Figure 7**). These proteins belong to the less known type III histidine kinases (Kim and Forst, 2001) and might play an important role in the response to vacuum stress in *D. radiodurans*. Type III histidine kinases are usually part of chemotaxis signal transduction systems, but also appear in genomes that completely lack chemotaxis genes (Adebali et al., 2017). These histidine kinases always appear together with a putative marker gene for bacterial type IV pilus-based twitching motility (Kennan et al., 2015) (DR_0774), which might be regulated by them. In *D. radiodurans* this protein was identified as important part of the *S*-layer in the cell wall (Farci et al., 2014) alongside SlpA (DR_2577) (Farci et al., 2016). The role of these histidine kinases in the regulation of vacuum stress response needs to be further thoroughly elucidated.

To identify regulatory proteins that might be of special importance to vacuum response exclusively, only those which were higher expressed after 3 h and 6 h of recovery of the vacuum exposed cells were considered (**Table 1**). Out of these proteins, the histidine kinase DR_B0028, the type IV piliation system protein DR_0774, and the FNR/CRP transcriptional regulator DR_0997 are candidates that could contribute to the regulation of vacuum stress response. The histidine kinases DR_B0028 and DR_B0029 are thought to be co-regulated with an operon that encodes an antisigma factor-regulation system which is known to be involved in stress response in other bacteria (Hecker and Volker, 1998; Makarova et al., 2001). The aforementioned DR_0774 is a component of the *S*-layer in the cell wall. The *S*-layer coating on the external side of the cell wall together with the carotenoid deinoxanthin are involved in cellular protection from extreme environmental conditions, especially UV radiation after desiccation (Farci et al., 2016).

FNR/CRP transcriptional regulators respond to a broad spectrum of intracellular and exogenous signals such as cAMP, anoxia, redox state, oxidative and nitrosative stress, 2-oxoglutarate, temperature (Körner et al., 2003). Apart from that, one of their family members, the DR_0997 protein also responds to high vacuum, according to our data (**Table 1**).

The results provided in this study are based on chromatographic separations coupled to mass spectrometers. These methods can be very powerful if misinterpretation is avoided. Bottom up/shotgun proteomics provides the possibility to relatively quantify several proteins from one organism, which is similar to combining multiple western blots. However, low abundant proteins might be under the limit of detection and as a result are not recognized by the detector (Zhang et al., 2013; Takáč and Šamaj, 2015). Furthermore, although the abundance is measured, the activity of a certain protein must be verified via enzymatic assays. GC-MS metabolomics based on reference

TABLE 1 | Proteins which are more abundant in the vacuum exposed cells of *D. radiodurans* at 3 h and 6 h of recovery in a complex medium.

			The average *z*-scored intensities			
Protein IDs	**Protein Annotation**	**ANOVA (*q*-value)**	**3h C**	**6h C**	**3h V**	**6h V**
Q9RRY8	Uncharacterized protein	0.0004	−0.48	−1.23	1.13	0.58
Q9RVA2	Uncharacterized protein	0.0006	0.03	−1.36	1.11	0.23
Q9RY64	50S ribosomal protein L21	0.0013	−0.28	−1.22	1.06	0.44
Q9RXP1	Uncharacterized protein DR_0269	0.0006	0.09	−1.44	1.00	0.34
Q9RW95	Probable type IV piliation system protein	0.0006	−0.41	−1.22	0.97	0.66
Q9RVN0	Transcriptional regulator, FNR/CRP family	0.0001	0.32	−1.54	0.96	0.26
Q9RZS7	Uncharacterized protein	0.0077	−0.81	−0.75	0.88	0.69
Q9RZT5	Sensor histidine kinase/response regulator	0.0045	−0.48	−1.08	0.81	0.75

The table shows Uniprot IDs, the corresponding protein annotations, the corrected p-values from the ANOVA and the average z-scored intensities for control (C) and vacuum (V) conditions. A color gradient runs from the lowest (saturated blue) to the highest (saturated red) value.

substances avoids false positive identifications and allows exact relative and absolute quantification, while untargeted approaches combined with library searches offer many more identifications. In general, these studies provide important initial insights after applying a certain environmental stress to an organism but should be later investigated by targeted application of genetic and cell biological methods.

CONCLUSION

It is generally accepted that vacuum induced dehydration of cells leads to Maillard reactions, which support the formation of ROS. Consequently, macromolecules are severely altered. Furthermore, the cell wall is affected, as metabolite transport through the membrane can be disrupted. In addition, the *S*-layer of cells can be damaged. Our study shows that high vacuum stress induces a prolonged growth arrest phase in *D. radiodurans* (**Figure 1**). This condition of suppressed growth is mirrored in our molecular analysis. The comparison of vacuum treated cells after 3 h of recovery towards corresponding control cells showed only minor variations on a molecular scale, as samples are still in growth arrest phase. However, at 6 h of recovery after the vacuum exposure *D. radiodurans* cells portray a completely different picture. Overall, at this time point we observed important differences between vacuum exposed and control cells (**Figures 4–8**). Combining these results, it is possible to decipher molecular key components, which are necessary for an efficient repair after the vacuum exposure (**Figure 8**). In all conditions (except the control cells at 6 h of recovery, which are already in the proliferation phase), higher abundances of ROS scavenging proteins, e.g., peroxidases and catalases, were observed (**Figure 8** and **Supplementary Table S1**). Moreover, the amounts of nucleic acid damage repair proteins, tRNA ligases, proteases and proteins associated to the *S*-layer were increased. The higher expression rates of these proteins might be controlled by specific histidine kinases and transcriptional regulator of FNR/CRP family, which appeared to be higher abundant as well. Many of these molecular processes require ATP for being active, which is produced in the TCA cycle. Throughout the early stages of repair, *D. radiodurans* needs a large quantity of ATP and uses its preferred carbon source, amino acids, as energy resource, which was indicated by the low quantity of extracted metabolites and TCA cycle intermediates from the vacuum treated cells. This study gives insights how *D. radiodurans* cope with the vacuum conditions on a molecular scale, but in addition, it shows interesting opportunities for future mutant-based studies, as important marker proteins are emphasized. As high vacuum causes severe damage to the cell wall, mutant studies with *S*-layer proteins, e.g., SlpA or DR_0774 together with the putative regulatory type III histidine kinases appears very attractive. A combination of shotgun proteomics with imaging techniques could help to define the roles of these proteins in restoring the cell wall after the vacuum exposure.

AUTHOR CONTRIBUTIONS

EO, YK, NÖ, and ER performed the experiments. All authors provided the editorial input, made substantial contributions to the acquisition, analysis, and interpretation of data described in this manuscript, and critically reviewed the report and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00909/full#supplementary-material

REFERENCES

Adebali, O., Petukh, M. G., Reznik, A. O., Tishkov, A. V., Upadhyay, A. A., and Zhulin, I. B. (2017). Class III histidine kinases: a recently accessorized kinase domain in putative modulators of type IV pilus-based motility. *J. Bacteriol.* 199:e00218-17. doi: 10.1128/JB.00218-17

Appukuttan, D., Rao, A. S., and Apte, S. K. (2006). Engineering of *Deinococcus radiodurans* R1 for bioprecipitation of uranium from dilute nuclear waste. *Appl. Environ. Microbiol.* 72, 7873–7878. doi: 10.1128/aem.01362-06

Banerjee, R., Chen, S., Dare, K., Gilreath, M., Praetorius-Ibba, M., Raina, M., et al. (2010). tRNAs: cellular barcodes for amino acids. *FEBS Lett.* 584, 387–395. doi: 10.1016/j.febslet.2009.11.013

Bauermeister, A., Moeller, R., Reitz, G., Sommer, S., and Rettberg, P. (2011). Effect of relative humidity on *Deinococcus radiodurans*' resistance to prolonged desiccation, heat, ionizing, germicidal, and environmentally relevant UV radiation. *Microb. Ecol.* 61, 715–722. doi: 10.1007/s00248-010-9785-4

Billi, D., and Potts, M. (2002). Life and death of dried prokaryotes. *Res. Microbiol.* 153, 7–12. doi: 10.1016/s0923-2508(01)01279-7

Brim, H., McFarlan, S. C., Fredrickson, J. K., Minton, K. W., Zhai, M., Wackett, L. P., et al. (2000). Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments. *Nat. Biotechnol.* 18, 85–90. doi: 10.1038/71986

Bucker, H., Horneck, G., Facius, R., Schwager, M., Thomas, C., Turcu, G., et al. (1972). Effects of simulated space vacuum on bacterial cells. *Life Sci. Space Res.* 10, 191–195.

Cox, C. S. (1993). Roles of water molecules in bacteria and viruses. *Orig. Life Evol. Biosph.* 23, 29–36. doi: 10.1007/bf01581988

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372. doi: 10.1038/nbt.1511

Daly, M. J. (2009). A new perspective on radiation resistance based on *Deinococcus radiodurans*. *Nat. Rev. Microbiol.* 7, 237–245. doi: 10.1038/nrmicro2073

Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Kiang, J. G., Fukumoto, R., Lee, D.-Y., et al. (2010). Small-molecule antioxidant proteome-shields in *Deinococcus radiodurans*. *PLoS One* 5:e12570. doi: 10.1371/journal.pone.0012570

Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Leapman, R. D., et al. (2007). Protein oxidation implicated as the primary determinant of bacterial radioresistance. *PLoS Biol.* 5:e92. doi: 10.1371/journal.pbio.0050092

Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Venkateswaran, A., et al. (2004). Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. *Science* 306, 1025–1028. doi: 10.1126/science.1103185

Day, A. (2012). *heatmap.plus: Heatmap with more Sensible Behavior*. Available at: https://CRAN.R-project.org/package=heatmap.plus (accessed October 29, 2012).

del Carmen Huesca Espitia, L., Caley, C., Bagyan, I., and Setlow, P. (2002). Base-change mutations induced by various treatments of *Bacillus subtilis* spores with and without DNA protective small, acid-soluble spore proteins. *Mutat. Res.* 503, 77–84. doi: 10.1016/s0027-5107(02)00093-3

Dose, K., Bieger-Dose, A., Dillmann, R., Gill, M., Kerz, O., Klein, A., et al. (1995). ERA-experiment "space biochemistry". *Adv. Space Res.* 16, 119–129. doi: 10.1016/0273-1177(95)00280-r

Dose, K., Bieger-Dose, A., Labusch, M., and Gill, M. (1992). Survival in extreme dryness and DNA-single-strand breaks. *Adv. Space Res.* 12, 221–229. doi: 10.1016/0273-1177(92)90176-x

Farci, D., Bowler, M. W., Kirkpatrick, J., McSweeney, S., Tramontano, E., and Piano, D. (2014). New features of the cell wall of the radio-resistant bacterium *Deinococcus radiodurans*. *Biochim. Biophys. Acta* 1838, 1978–1984. doi: 10.1016/j.bbamem.2014.02.014

Farci, D., Slavov, C., Tramontano, E., and Piano, D. (2016). The S-layer protein DR_2577 binds deinoxanthin and under desiccation conditions protects against UV-radiation in *Deinococcus radiodurans*. *Front. Microbiol.* 7:155. doi: 10.3389/fmicb.2016.00155

França, M. B., Panek, A. D., and Eleutherio, E. C. A. (2007). Oxidative stress and its effects during dehydration. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 146, 621–631. doi: 10.1016/j.cbpa.2006.02.030

Fredrickson, J. K., Li, S. M., Gaidamakova, E. K., Matrosova, V. Y., Zhai, M., Sulloway, H. M., et al. (2008). Protein oxidation: key to bacterial desiccation resistance? *ISME J.* 2, 393–403. doi: 10.1038/ismej.2007.116

Garcia, A. H. (2011). Anhydrobiosis in bacteria: from physiology to applications. *J. Biosci.* 36, 939–950. doi: 10.1007/s12038-011-9107-0

Garg, R. P., Gonzalez, J. M., and Parry, R. J. (2006). Biochemical characterization of VlmL, a Seryl-tRNA synthetase encoded by the valanimycin biosynthetic gene cluster. *J. Biol. Chem.* 281, 26785–26791. doi: 10.1074/jbc.M603675200

Gazzaniga, F., Stebbins, R., Chang, S. Z., McPeek, M. A., and Brenner, C. (2009). Microbial NAD metabolism: lessons from comparative genomics. *Microbiol. Mol. Biol. Rev.* 73, 529–541. doi: 10.1128/MMBR.00042-08

Gentner, N. E., and Mitchel, R. E. J. (1975). Ionizing radiation-induced release of a cell surface nuclease from *Micrococcus radiodurans*. *Radiat. Res.* 61, 204–215. doi: 10.2307/3574039

Ghedira, K., Othman, H., Saied, T., Baccar, Z. M., Hosni, F., and Hamzaoui, A. H. (eds). (2016). "Insights into ionizing-radiation-resistant bacteria S-layer proteins and nanobiotechnology for bioremediation of hazardous and radioactive waste," in *Management of Hazardous Wastes*, (Chapters: IntechOpen).

Gruzdev, N., McClelland, M., Porwollik, S., Ofaim, S., Pinto, R., and Saldinger-Sela, S. (2012). Global transcriptional analysis of dehydrated *Salmonella enterica* serovar Typhimurium. *Appl. Environ. Microbiol.* 78, 7866–7875. doi: 10.1128/aem.01822-12

Hansen, J. M., Go, Y.-M., and Jones, D. P. (2006). Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annu. Rev. Pharmacol. Toxicol.* 46, 215–234. doi: 10.1146/annurev.pharmtox.46.120604.141122

Hecker, M., and Volker, U. (1998). Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the sigmaB regulon. *Mol. Microbiol.* 29, 1129–1136. doi: 10.1046/j.1365-2958.1998.00977.x

Horneck, G., Bucker, H., Reitz, G., Requardt, H., Dose, K., Martens, K. D., et al. (1984). Life sciences: microorganisms in the space environment. *Science* 225, 226–228. doi: 10.1126/science.225.4658.226

Horneck, G., Eschweiler, U., Reitz, G., Wehner, J., Willimek, R., and Strauch, K. (1995). Biological responses to space: results of the experiment "Exobiological Unit" of ERA on EURECA I. *Adv. Space Res.* 16, 105–118. doi: 10.1016/0273-1177(95)00279-n

Horneck, G., Klaus, D. M., and Mancinelli, R. L. (2010). Space microbiology. *Microbiol. Mol. Biol. Rev.* 74, 121–156. doi: 10.1128/MMBR.00016-09

Horneck, G., Moeller, R., Cadet, J., Douki, T., Mancinelli, R. L., Nicholson, W. L., et al. (2012). Resistance of bacterial endospores to outer space for planetary protection purposes—experiment PROTECT of the EXPOSE-E mission. *Astrobiology* 12, 445–456. doi: 10.1089/ast.2011.0737

Im, S., Song, D., Joe, M., Kim, D., Park, D. H., and Lim, S. (2013). Comparative survival analysis of 12 histidine kinase mutants of *Deinococcus radiodurans* after exposure to DNA-damaging agents. *Bioprocess Biosyst. Eng.* 36, 781–789. doi: 10.1007/s00449-013-0904-8

Ito, H., Watanabe, H., Takehisa, M., and Iizuka, H. (1983). Isolation and identification of radiation-resistant cocci belonging to the genus *Deinococcus* from sewage sludges and animal feeds. *Agric. Biol. Chem.* 47, 1239–1247. doi: 10.1271/bbb1961.47.1239

Jönsson, K. I., Rabbow, E., Schill, R. O., Harms-Ringdahl, M., and Rettberg, P. (2008). Tardigrades survive exposure to space in low Earth orbit. *Curr. Biol.* 18, R729–R731.

Jung, I. L., and Kim, I. G. (2003). Transcription of ahpC, katG, and katE genes in *Escherichia coli* is regulated by polyamines: polyamine-deficient mutant sensitive to H2O2-induced oxidative damage. *Biochem. Biophys. Res. Commun.* 301, 915–922. doi: 10.1016/s0006-291x(03)00064-0

Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 28, 27–30. doi: 10.1093/nar/28.1.27

Kawaguchi, Y., Yokobori, S., Hashimoto, H., Yano, H., Tabata, M., Kawai, H., et al. (2016). Investigation of the interplanetary transfer of microbes in the Tanpopo mission at the exposed facility of the international space station. *Astrobiology* 16, 363–376. doi: 10.1089/ast.2015.1415

Kennan, R. M., Lovitt, C. J., Han, X., Parker, D., Turnbull, L., Whitchurch, C. B., et al. (2015). A two-component regulatory system modulates twitching motility in *Dichelobacter nodosus*. *Vet. Microbiol.* 179, 34–41. doi: 10.1016/j.vetmic.2015.03.025

Kim, D., and Forst, S. (2001). Genomic analysis of the histidine kinase family in bacteria and archaea. *Microbiology* 147(Pt 5), 1197–1212. doi: 10.1099/00221287-147-5-1197

Kocharunchitt, C., King, T., Gobius, K., Bowman, J. P., and Ross, T. (2012). Integrated transcriptomic and proteomic analysis of the physiological response of *Escherichia coli* O157:H7 Sakai to steady-state conditions of cold and water activity stress. *Mol. Cell. Proteomics* 11:M111.009019. doi: 10.1074/mcp.M111.009019

Körner, H., Sofia, H. J., and Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol. Rev.* 27, 559–592. doi: 10.1016/s0168-6445(03)00066-4

Krisko, A., and Radman, M. (2010). Protein damage and death by radiation in *Escherichia coli* and *Deinococcus radiodurans*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14373–14377. doi: 10.1073/pnas.1009312107

Makarova, K. S., Aravind, L., Wolf, Y. I., Tatusov, R. L., Minton, K. W., Koonin, E. V., et al. (2001). Genome of the extremely radiation-resistant bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. *Microbiol. Mol. Biol. Rev.* 65, 44–79. doi: 10.1128/MMBR.65.1.44-79.2001

Mattimore, V., and Battista, J. R. (1996). Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. *J. Bacteriol.* 178, 633–637. doi: 10.1128/jb.178.3.633-637.1996

Mogk, A., Schmidt, R., and Bukau, B. (2007). The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies. *Trends Cell Biol.* 17, 165–172. doi: 10.1016/j.tcb.2007.02.001

Moreno-Hagelsieb, G., Gutiérrez-Preciado, A., Torres, A. G., Merino, E., Bonomi, H. R., Goldbaum, F. A., et al. (2015). Extensive identification of bacterial riboflavin transporters and their distribution across bacterial species. *PLoS One* 10:e0126124. doi: 10.1371/journal.pone.0126124

Moseley, B. E. B., and Mattingly, A. (1971). Repair of irradiated transforming deoxyribonucleic acid in wild type and a radiation-sensitive mutant of *Micrococcus radiodurans*. *J. Bacteriol.* 105, 976–983.

Munakata, N., Saitou, M., Takahashi, N., Hieda, K., and Morohoshi, F. (1997). Induction of unique tandem-base change mutations in bacterial spores exposed to extreme dryness. *Mutat. Res.* 390, 189–195. doi: 10.1016/s0165-1218(97)00020-7

Nicholson, W. L., Moeller, R., and Horneck, G. (2012). Transcriptomic responses of germinating *Bacillus subtilis* spores exposed to 1.5 years of space and simulated Martian conditions on the EXPOSE-E experiment PROTECT. *Astrobiology* 12, 469–486. doi: 10.1089/ast.2011.0748

Ott, E., Kawaguchi, Y., Kölbl, D., Chaturvedi, P., Nakagawa, K., Yamagishi, A., et al. (2017). Proteometabolomic response of *Deinococcus radiodurans* exposed to UVC and vacuum conditions: initial studies prior to the Tanpopo space mission. *PLoS One* 12:e0189381. doi: 10.1371/journal.pone.0189381

Pogoda de la Vega, U., Rettberg, P., and Reitz, G. (2007). Simulation of the environmental climate conditions on Martian surface and its effect on *Deinococcus radiodurans*. *Adv. Space Res.* 40, 1672–1677. doi: 10.1016/j.asr.2007.05.022

Pollmann, K., Raff, J., Merroun, M., Fahmy, K., and Selenska-Pobell, S. (2006). Metal binding by bacteria from uranium mining waste piles and its technological applications. *Biotechnol. Adv.* 24, 58–68. doi: 10.1016/j.biotechadv.2005.06.002

R Development Core Team (2018). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rabbow, E., Parpart, A., and Reitz, G. (2016). The planetary and space simulation facilities at DLR cologne. *Microgravity Sci. Technol.* 28, 215–229. doi: 10.1007/s12217-015-9448-7

Raina, M., and Ibba, M. (2014). tRNAs as regulators of biological processes. *Front. Genet.* 5:171. doi: 10.3389/fgene.2014.00171

Rajamani, S., Bauer, W. D., Robinson, J. B., Farrow, J. M. III, Pesci, E. C., Teplitski, M., et al. (2008). The vitamin riboflavin and its derivative lumichrome activate the LasR bacterial quorum-sensing receptor. *Mol. Plant Microbe Interact.* 21, 1184–1192. doi: 10.1094/mpmi-21-9-1184

Rhee, H. J., Kim, E. J., and Lee, J. K. (2007). Physiological polyamines: simple primordial stress molecules. *J. Cell. Mol. Med.* 11, 685–703. doi: 10.1111/j.1582-4934.2007.00077.x

Riedel, K., and Lehner, A. (2007). Identification of proteins involved in osmotic stress response in *Enterobacter sakazakii* by proteomics. *Proteomics* 7, 1217–1231. doi: 10.1002/pmic.200600536

Saffary, R., Nandakumar, R., Spencer, D., Robb, F. T., Davila, J. M., Swartz, M., et al. (2002). Microbial survival of space vacuum and extreme ultraviolet irradiation: strain isolation and analysis during a rocket flight. *FEMS Microbiol. Lett.* 215, 163–168. doi: 10.1111/j.1574-6968.2002.tb11386.x

Satoh, K., Ohba, H., Sghaier, H., and Narumi, I. (2006). Down-regulation of radioresistance by LexA2 in *Deinococcus radiodurans*. *Microbiology* 152(Pt 11), 3217–3226. doi: 10.1099/mic.0.29139-0

Servant, P., Jolivet, E., Bentchikou, E., Mennecier, S., Bailone, A., and Sommer, S. (2007). The ClpPX protease is required for radioresistance and regulates cell division after gamma-irradiation in *Deinococcus radiodurans*. *Mol. Microbiol.* 66, 1231–1239. doi: 10.1111/j.1365-2958.2007.06003.x

Shepherd, J., and Ibba, M. (2013). Direction of aminoacylated transfer RNAs into antibiotic synthesis and peptidoglycan-mediated antibiotic resistance. *FEBS Lett.* 587, 2895–2904. doi: 10.1016/j.febslet.2013.07.036

Slade, D., Lindner, A. B., Paul, G., and Radman, M. (2009). Recombination and replication in DNA repair of heavily irradiated *Deinococcus radiodurans*. *Cell* 136, 1044–1055. doi: 10.1016/j.cell.2009.01.018

Slade, D., and Radman, M. (2011). Oxidative stress resistance in *Deinococcus radiodurans*. *Microbiol. Mol. Biol. Rev.* 75, 133–191. doi: 10.1128/mmbr.00015-10

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., et al. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43, D447–D452. doi: 10.1093/nar/gku1003

Takáč, T., and Šamaj, J. (2015). Advantages and limitations of shot-gun proteomic analyses on Arabidopsis plants with altered MAPK signaling. *Front. Plant Sci.* 6:107. doi: 10.3389/fpls.2015.00107

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., et al. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods* 13, 731–740. doi: 10.1038/nmeth.3901

Valledor, L., Escandon, M., Meijon, M., Nukarinen, E., Canal, M. J., and Weckwerth, W. (2014). A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms. *Plant J.* 79, 173–180. doi: 10.1111/tpj.12546

Venkateswaran, A., McFarlan, S. C., Ghosal, D., Minton, K. W., Vasilenko, A., Makarova, K., et al. (2000). Physiologic determinants of radiation resistance in *Deinococcus radiodurans*. *Appl. Environ. Microbiol.* 66, 2620–2626. doi: 10.1128/aem.66.6.2620-2626.2000

Vizcaino, J. A., Csordas, A., del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., et al. (2016). 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* 44, D447–D456. doi: 10.1093/nar/gkv1145

Weckwerth, W., Wenzel, K., and Fiehn, O. (2004). Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. *Proteomics* 4, 78–83. doi: 10.1002/pmic.200200500

Weiner, J. (2017). *pca3d: Three Dimensional PCA Plots*. Available at: https://CRAN.R-project.org/package=pca3d (accessed February 2, 2017).

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag. doi: 10.1002/pmic.200200500

Yamagishi, A., Kawaguchi, Y., Hashimoto, H., Yano, H., Imai, E., Kodaira, S., et al. (2018). Environmental data and survival data of *Deinococcus aetherius* from the exposure facility of the Japan experimental module of the international space station obtained by the Tanpopo mission. *Astrobiology* 18, 1369–1374. doi: 10.1089/ast.2017.1751

Zahradka, K., Slade, D., Bailone, A., Sommer, S., Averbeck, D., Petranovic, M., et al. (2006). Reassembly of shattered chromosomes in *Deinococcus radiodurans*. *Nature* 443, 569–573. doi: 10.1038/nature05160

Zhang, Y., Fonslow, B. R., Shan, B., Baek, M.-C., and Yates, J. R. III (2013). Protein analysis by shotgun/bottom-up proteomics. *Chem. Rev.* 113, 2343–2394. doi: 10.1021/cr3003533

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.3 Molecular response of *Deinococcus radiodurans* to simulated microgravity explored by proteometabolomic approach

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Molecular response of *Deinococcus radiodurans* to simulated microgravity explored by proteometabolomic approach

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Regarding future space exploration missions and long-term exposure experiments, a detailed investigation of all factors present in the outer space environment and their effects on organisms of all life kingdoms is advantageous. Influenced by the multiple factors of outer space, the extremophilic bacterium *Deinococcus radiodurans* has been long-termly exposed outside the International Space Station in frames of the Tanpopo orbital mission. The study presented here aims to elucidate molecular key components in *D. radiodurans*, which are responsible for recognition and adaptation to simulated microgravity. *D. radiodurans* cultures were grown for two days on plates in a fast-rotating 2-D clinostat to minimize sedimentation thus simulating reduced gravity conditions. Subsequently, metabolites and proteins were extracted and measured with mass spectrometry-based techniques. Our results emphasize the importance of certain signal transducer proteins, which showed higher abundances in cells grown under reduced gravity. These proteins activate a cellular signal cascade, which leads to differences in gene expressions. Proteins involved in DNA replication and proteins connected to the extracellular milieu and the cell envelope showed an increased abundance under simulated microgravity. Focusing on the expression of these proteins might present a strategy of the cells to adapt to microgravity conditions.

Exploration of hostile environments by humans is a dangerous, yet essential and rewarding task with numerous factors to consider. It is of utmost importance to evaluate all environmental factors independently to elaborate their impact on all kind of organisms. Apart from ionizing and solar UV radiation, vacuum, and extreme temperature fluctuations, microgravity is another omnipresent environmental parameter to be considered. As gravity force was always present since the dawn of life, it has influenced the development of all organisms[1]. Numerous studies suggest that microgravity influence proteinaceous cellular components, depending on different cell types[2,3]. In humans, microgravity exposure causes a redistribution of blood toward the head, altered responses of baroreceptor, nervous and endocrine systems, which lead to space motion sickness[4]. In addition, the blood supply to the eye is altered depending on the duration of the flight, which impacts vascularization[5]. Apart from direct effects to animals, microgravity can alter host-symbiont/parasite interactions. A spaceflight experiment was performed with the squid *Euprymna scolopes* and its beneficial symbiont *Vibrio fischeri*. Transcriptomics analyses revealed that under spaceflight conditions, genes associated with oxidative stress response were enriched if the symbiont was absent[6]. Indeed, microgravity can alter cellular interactions between eukaryotes hosts and their associated microbes[7]. Incubation of *E. scolopes* and *V. fischeri* in a high aspect ratio rotating wall vessel resulted in suppression of the host´s innate immune response and acceleration of bacteria-induced apoptosis[8].

Q1 Q2 Q3 Q4

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Evolution forced complex organisms to develop systems for fluid regulation, gravity sensing, spatial orientation and locomotion. The STS (Space Transportation System)-95 Space Experiment showed that microgravity does not affect seed germination of pea and maize. However, the orientation of stem elongation, growth and development are strongly affected[9]. On the molecular level, there are indications, that gravity-dependent signal pathways might be controlled by mechano- (gravi-) sensitive ion channels and cascades of ubiquitous second messengers[10]. According to multiple studies, signal amplification, gravity sensing and graviorientation are closely connected to cytoskeletal elements[11–13] in plants. Single cells may be able to sense changes in gravity and convert them into biochemical signals[14]. These signals may lead to changes in the protein production capability and thus cause increased virulence[15] or different biofilm production[16]. Investigating infecting organisms under simulated microgravity has revealed an increased production of the heat-labile enterotoxin in *Escherichia coli* and tumour necrosis factor-alpha in the infected murine macrophages[17]. In a similar fashion, simulated microgravity supports the invasive potential of *Salmonella enterica* and enhances the production of tumour necrosis-factor alpha in infected epithelial cells[17]. *Staphylococcus epidermidis* showed an increased mutation of genes connected to resistance to the antibiotic rifampicin 122 h of growth on the ISS[18]. A spaceflight study performed on *E. coli* suggests a connection between increased antibiotic resistance and induction of 50 stress-response genes[19]. Spaceflight induced changes in the proteome of *Pseudomonas aeruginosa* were analysed in a study conducted by Crabbé *et al.*[20]. 28 proteins were identified as differentially expressed with Hfq as a global transcriptional regulator. As Hfq was also differentially expressed in spaceflight-grown *S. enterica*, it represents a spaceflight induced regulator acting across bacterial species[21]. The Δhfq mutant of *V. fischeri* confirmed that Hfq impacts regulatory processes under low-shear-modelled microgravity (LSMMG) differently than under normal gravity conditions[22].

Furthermore, in *S. enterica* many ribosomal proteins showed decreased abundance after the spaceflight[21]. In another study, *Rhodospirillum rubrum* showed differentially expressed ribosomal and stress response proteins[23]. Onboard the ISS (International Space Station), *E. coli* cultures showed a 13-fold increase in final cell counts compared to ground control cells[24]. Moreover, *E.coli* was able to grow in presence of normally inhibitory levels of antibiotics such as gentamicin sulphate[24]. The cultures onboard the ISS showed an increase in cell envelope thickness, outer membrane vesicles and tended to form clusters[24]. This aggregation of cells might be associated with effects, observed in biofilm forming bacteria after exposure to microgravity. *Micrococcus luteus*, grown on the ISS, showed an increased production of exopolymeric substances compared to the 1 g ground control strain[25].

The Gram-positive bacterium *Deinococcus radiodurans* possess some remarkable properties which makes it an ideal candidate for various space-related studies. It is extremely resistant to ionizing radiation[26], UV radiation[27] and desiccation[28]. *D. radiodurans* was used in the latest Low Earth orbit exposure mission outside the ISS: the Japanese Tanpopo mission[29]. Many studies were performed to elucidate the mechanisms behind its extraordinary survival regarding ionizing radiation and other reactive oxygen species (ROS) producing environmental factors[30–32]. However, little is known about the molecular response of *D. radiodurans* to microgravity. It was shown that the recovery of *D. radiodurans* after radiation damage is enhanced when subjected to microgravity[33]. Nevertheless, a high-resolution molecular approach which indicates key components that are responsible for gravity sensing and signal transmission is missing for *D. radiodurans*.

In this study, the effects of simulated microgravity on *D. radiodurans* were investigated by growing single cells to colonies during the incubation on a fast-rotating 2-D clinostat (Fig. 1). The 2-D clinostat was used in several microgravity simulation experiments, including *Arabidopsis*[34] seedlings and *V. natriegens*[35]. Since space experiments require an extraordinary effort due the planning, cost and experiment design, various ground-based approaches have been developed and are applied over the last centuries[36]. In order to quite simply achieve microgravity (free fall) on Earth only a few methods exist, such as sounding rockets, drop-tower to parabolic flights[37]. Unfortunately, these platforms only grant a little time frame of microgravity in the range of seconds to minutes, in which experiments can be conducted. Thus, facilities have been developed aiming to simulate microgravity for longer periods of time to grow cells for several generations. To simulate a continuous free fall, the rotating wall vessel uses a chamber, completely filled with cells in culture medium. It is rotating around an axle and thus subjecting the cells to a continuous free fall[38]. A random positioning machine consists of two frames (inner and outer frame), rotating independently from each other in random directions. As a consequence, the gravity vector is averaged to zero over time for samples that are located directly in the middle of the machine[39]. The principle of a 2-D clinostat is based on the rotation around a horizontal axis perpendicular to gravity, assuming that cellular gravity-perception does no longer takes place[40–42].In case of agar-based incubation experiments to grow colonies or biofilms, the quality of simulation is strongly limited to the diameter the colony can form. As the diameter increases, residual acceleration increases. Thus, small diameters ($r \leq 0.5$ cm), and localization of the colony exactly in the centre of rotation within the clinostat were considered. None of these methods achieve gravitational unloading and fluid convection and shear stress are not completely erased, however sedimentation is avoided through the omnilateral gravistimulation[15]. Due to these limitations, results from cells incubated in real microgravity might be similar, but not identical to cells incubated under simulated microgravity[43].

Following incubation on fast rotating 2-D clinostats, the proteome and metabolome of microgravity-grown cells and 1 g control cells of *D. radiodurans* were analysed. A bottom-up proteomics approach was used as it enables a relative quantitative comparison of a multitude of proteins which might be affected by microgravity. Metabolomics based on a well-established GC-MS approach allowed quantification of polar metabolites. This was performed as a preliminary experiment to real space exposure of *D. radiodurans* in frames of the Tanpopo space mission[29,44,45]. Multiple molecular stress response studies were performed on this well-studied extremophile, resulting in valid annotations for many proteins. Due to its high resistance to various environmental conditions, it is likely that *D. radiodurans* or microorganisms with similar properties are targets for spaceflight missions. Consequently, a proper understanding of how *D. radiodurans* adapt and respond to microgravity as space-environmental conditions is desirable. These results can contribute to understanding how cells react to reduced gravity without other, more influential environmental factors present in Low Earth orbit.

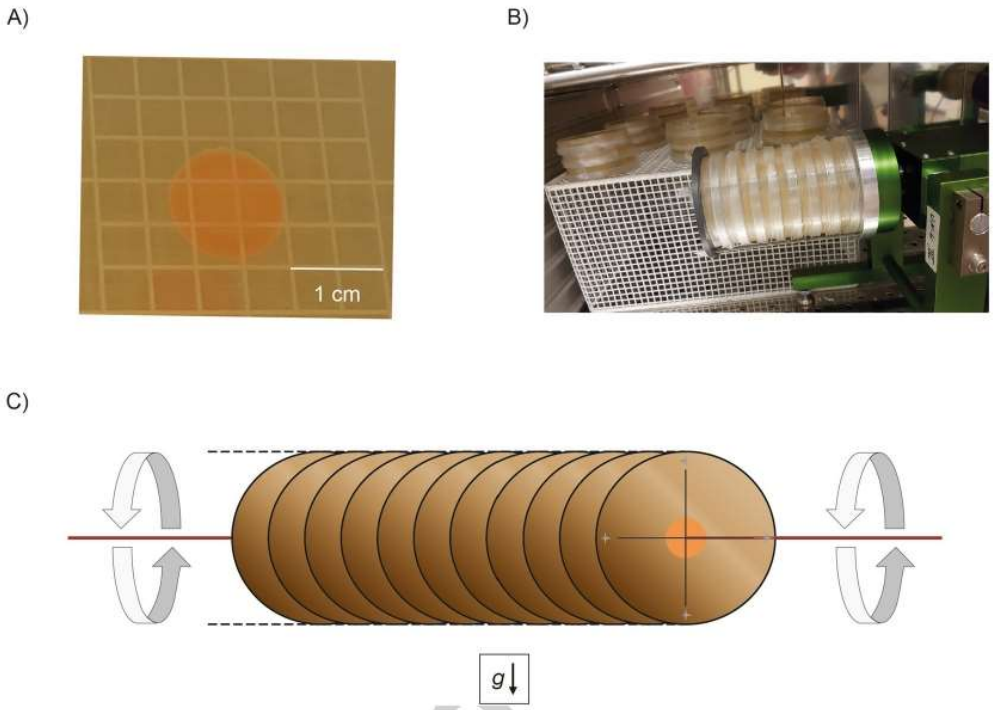

Figure 1. Principle of a 2-D Clinostat: TGB-agar plates are inoculated with 10 µl of an overnight PBS-washed *D. radiodurans* culture using a grid petri dish (**A**). After air-drying for 2 min, plates are sealed with Parafilm, mounted on a 2-D clinostat and secured by a lid and four butterfly screws. The clinostat is aligned so that the axis of rotation is parallel to the ground (**B**). Incubation takes place at 37 °C at 60 rpm, rotating counterclockwise. The scheme in (**C**) represents the principle of a 2D-clinostat: During the entire duration of the experiment, growing *D. radiodurans* cells are exactly placed on the axis of rotation, attached to the centre of a TGB-agar petri dish. Residual acceleration decreased to ~≤0,016 g, when assuming a final colony diameter of 0.8 cm.

Results

Proteomic response to microgravity. Out of the 3085 protein entries in the Uniprot FASTA file for *D. radiodurans*, 2168 were identified in at least one replicate (Table S1). A *Welch's* t-test identified 119 proteins as significantly different abundant between cells grown under clinorotation (Fig. 1) and the static 1 g control cells (Fig. S1, Table S1). Out of these, 46 were less abundant and 73 were more abundant when grown in simulated microgravity. Subsequently, plotting the proteins which were identified in every replicate (1618 proteins) on a PCA (principal component analysis) was performed (Fig. 2A). Cells exposed to simulated microgravity showed a decreased spreading on PC1, which explains 32.92% of the variance in the data.

Proteins which showed an increased abundance after growth in simulated microgravity: proteins which showed a reduced abundance were uploaded to the GO (gene ontology) classification tool PANTHER (Protein Analysis Through Evolutionary Relationships) to categorize protein classes[46]. The protein class search algorithm identified 40 hits for higher abundant proteins and 17 hits for less abundant proteins. This analysis showed that hydrolases and transferases are more abundant in *D. radiodurans* grown under simulated microgravity (Fig. 2B).

Additionally, an analysis with the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database[47] revealed a significant amount of protein-protein high confidence interactions of proteins which were higher abundant after growth in simulated microgravity (Fig. 3). Lower abundant proteins on the other hand, do not show a significant amount of interactions. Protein clusters with at least three proteins were further investigated.

Furthermore, protein clusters with high amount of protein-protein interactions identified by the STRING database were uploaded to the SMART (Simple Modular Architecture Research Tool) database. SMART is able to identify and annotate protein domains and analyse protein domain architectures[48]. Especially regulatory proteins (Fig. 4) showed a high level of conserved regions. Growth under simulated microgravity elevated the abundance of proteins with recognition domains, such as PAS (Per-Arnt-Sim) and PAC. Furthermore, these proteins contain signal transducer domains, for instance tetratrico peptide repeat (TPR) and the counterparts GGDEF and EAL which respond to certain environmental conditions to optimize gene expression. Ultimately, two of the regulatory proteins identified as higher abundant in cells, grown under simulated microgravity, harboured histidine kinase domains.

Metabolite profiling. In total, 24 metabolites were identified and quantified in the targeted approach (Table S2). Most metabolites identified by our targeted approach appeared more abundant in 1 g cells (Fig. 5).

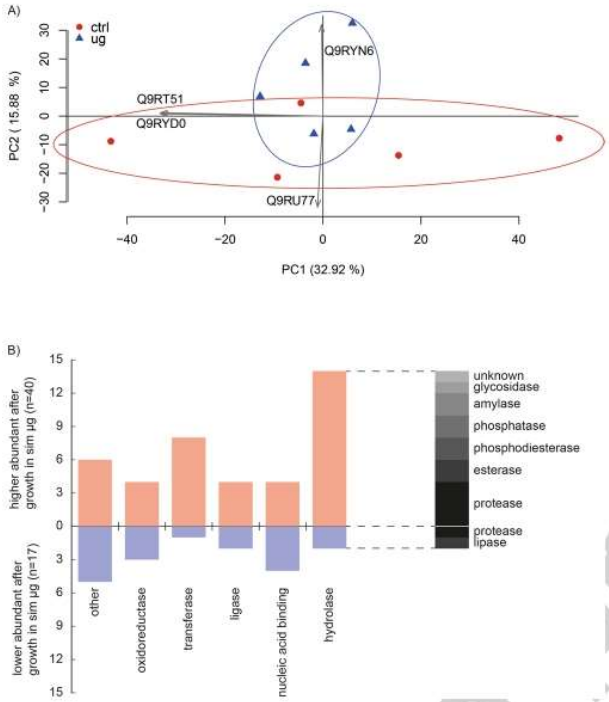

Figure 2. Proteomic response to simulated microgravity. (**A**) PCA of 1618 proteins identified in each of the five replicates in simulated microgravity (blue) and control condition (red). Explanation of the variances in percent of the most influential principal components in brackets. The two most influential loadings for both principal components are included as grey arrows (Uniprot IDs). (**B**) Gene Ontology clustering of protein classes according to the PANTHER classification tool. Out of the 73 proteins which were identified as more abundant under simulated microgravity, 40 were assigned to protein classes (red). 17 hits were annotated for the 46 less abundant proteins (blue).

However, some TCA cycle intermediates, which are malic acid, lactic acid and succinic acid are more abundant in colonies which were grown under simulated microgravity. The alteration of TCA cycle metabolites was already observed during our previous studies, where *D. radiodurans* cells were recovered after exposure to different extreme environmental conditions[49,50]. Furthermore, the amount of amino acids appears to be reduced whenever *D. radiodurans* cells experience environmental conditions that require a certain response from the cell. Apparently, growth under simulated microgravity causes an increased demand for amino acids. Amino acids are the preferred carbon source of *D. radiodurans*[51] and might serve as energy source during stress recovery[49].

Discussion

Although some studies were performed on gene expression changes after simulated microgravity in microorganisms, such as *Streptococcus* mutants[52], *E. coli*[53], *Bacillus cereus*[54], *Cupriavidus metallidurans*[55] and *Staphylococcus aureus*[56], this is the first study that presents alterations in the proteome of *D. radiodurans* induced by simulated microgravity. The overall changes in the proteome can be observed on the PCA level, as cells exposed to simulated microgravity show a reduced spreading compared to the control cells (Fig. 2A). When subjected to a specific parameter as reduced gravity, cells focus to adapt to this environmental change and therefore protein synthesis is aimed to tackle a specific task, which is visible as minimal spreading on the PCA (Fig. 2A). A similar result was shown after exposure of *D. radiodurans* cells to vacuum stress, which showed the same effects on the PCA[50] at the level of proteins.

General adaptation mechanisms induced by microgravity. The growth of *D. radiodurans* under simulated microgravity increased the abundance of several proteins associated with processes involving DNA, such as DR_2410 (DnaX), DR_1707 (PolA), DNA ligase DR_2069 (LigA) and the transcription repair coupling factor DR_1532 (Mfd) (Fig. 3). Among them, DnaX is the only differently abundant subunit of DNA polymerase III complex, a multichain enzyme responsible for most of the replicative synthesis in bacteria[57]. Another protein that was identified as higher abundant after microgravity exposure is PolA, a polymerase with 5′-3′ exonuclease activity, which is essential for an efficient DNA repair in *D. radiodurans*, for instance after heavy ionizing radiation exposure[58]. PolA primarily fills DNA gaps that arise during replication, excision repair and recombination[59]. Previous research data suggests a connection between simulated microgravity and the induction of DNA damage and stress response in human retinal pigment epithelial cells[60]. In accordance, an induced expression of stress response genes was observed in *E. coli*, grown under modelled reduced gravity conditions[61]. A

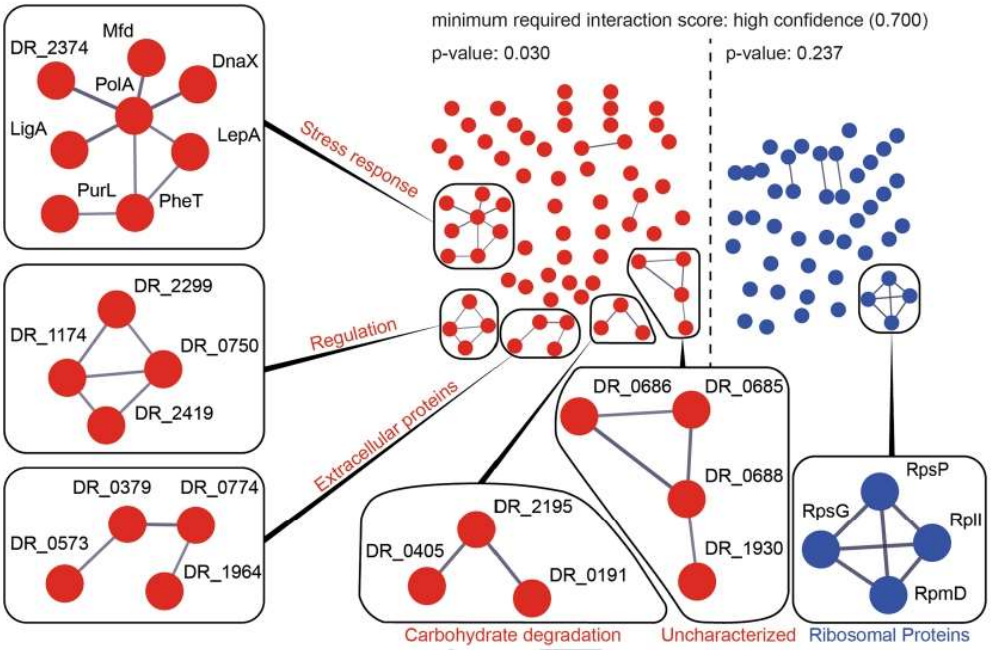

Figure 3. STRING database analysis of proteins higher abundant (red) and less abundant (blue) after growth in simulated microgravity. Protein-protein interactions with high confidence scores (0.700) are represented as nodes. A high p-value indicates that the number of nodes can be a result of coincidence. Protein-protein interactions with at least three proteins are emphasized.

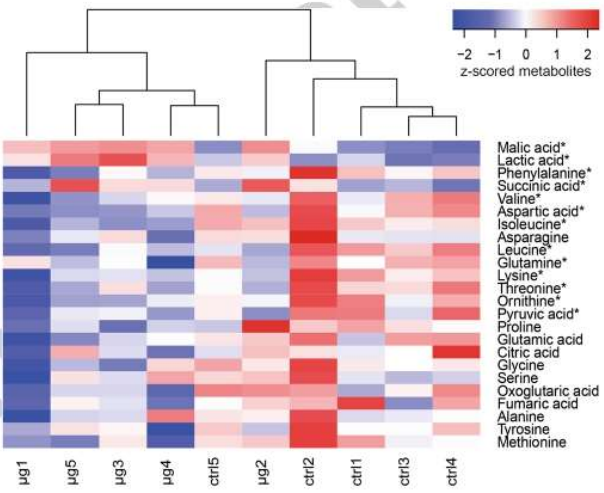

Figure 4. Heat map with the corresponding dendrogram of the targeted metabolomics approach. Metabolites were normalized to protein the amount of extracted proteins and z-scored. The plot was created in R with the heatmap.2 function included in the gplots package. Metabolites, which show a significantly different abundance between control and simulated microgravity samples are indicated with an asterisk (*).

separate transcriptomic study, performed on *E. coli*, subjected to simulated microgravity in a clinostat resulted in an increased expression of genes involved in stress response and DNA replication[53].

A proposed model, based on *E. coli* data from seven different shuttle flights describes an altered expression of genes directly and indirectly involved in glucose catabolism pathways[62]. In *Serratia marcescens*, spaceflight exposure induced alterations in genes and proteins associated with degradation and metabolism involving catabolic processes like glycolysis[63]. In our study, annotating catalytic activities of higher and lower abundant proteins after exposure to simulated microgravity revealed a distinctive peak for hydrolases (Fig. 2B). The upregulation of proteins with hydrolase activity was already observed in human mesenchymal stem cells under simulated

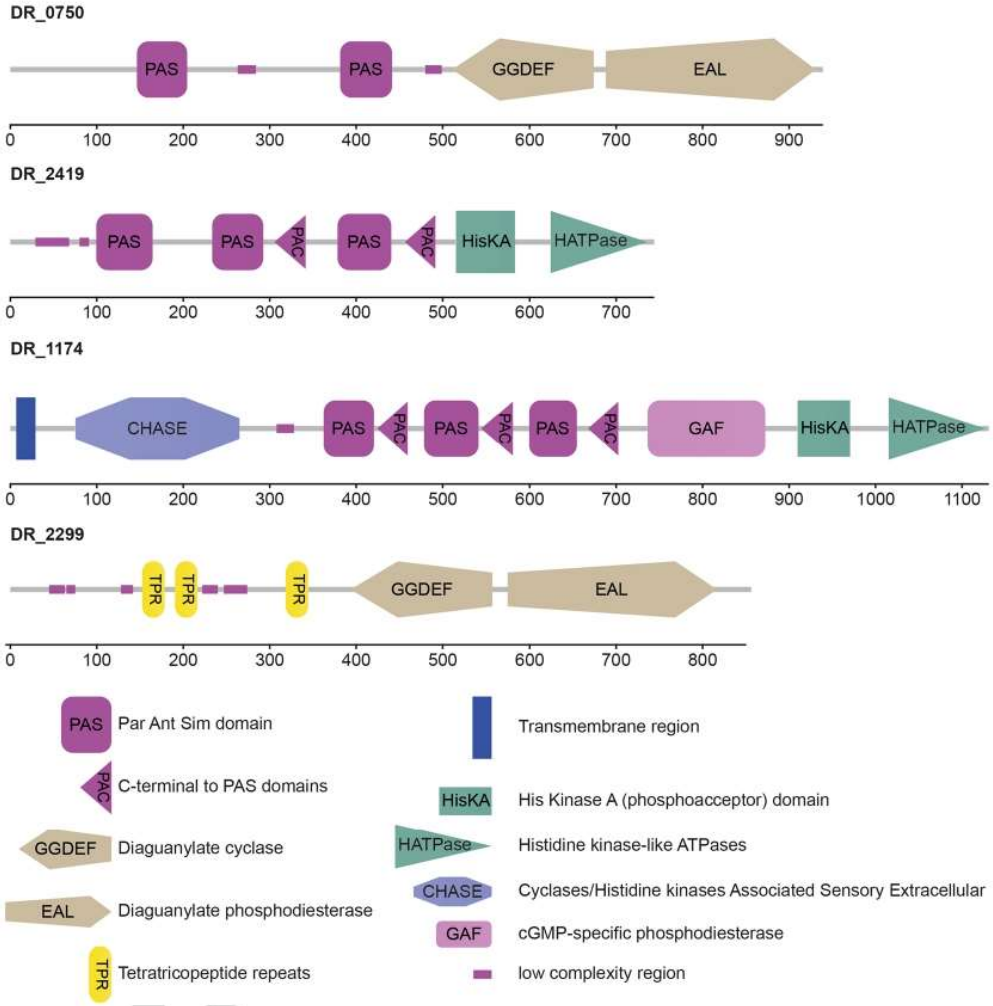

Figure 5. Group of signal transduction proteins that was uploaded to the SMART database to identify domains. Shows all identified domains and the corresponding length of the protein in base pairs. Identified domains in alphabetical order: CHASE (cyclase/histidine kinases associated sensory extracellular), EAL (diaguanylate phosphodiesterase), GAF (cGMP-specific phosphodiesterase), GGDEF (diaguanylate cyclase), HisKA (His Kinase A), HATPase (histidine kinase-like ATPase), PAC (C-terminal to PAS), PAS (Par Ant Sim domain), TPR (tetratricopeptide repeats)

microgravity[64]. In our work, proteins included in this group are annotated as glycogen debranching enzyme DR_0191, alpha-dextran endo-1, 6-alpha-glucosidase DR_0405, maltooligosyltrehalose synthase DR_0463 and the putative serine protease Acyl-peptide hydrolase DR_0165. Computationally derived database annotation of proteins often relies on orthologues and no experimental characterization of the proteins was performed for the used microorganism. There is a probability that the protein in the microorganism obtains the annotated or a similar function, however, it needs to be further experimentally investigated with using biochemical and molecular biology tools. The higher abundances of this group of proteins with putative hydrolase activity observed in *D. radiodurans* when grown in simulated microgravity may help with nutrient utilization to adapt to the stress caused by the extraordinary circumstances.

Cell envelope-associated events. Many microbial species, grown under low fluid shear environments, either in real spaceflight missions or in simulated conditions, showed an increase in extracellular polymeric substances (EPS), cell aggregation, cell-cell contacts and biofilm formation[16,65,66]. A microgravity simulation study with *R. rubrum*, cultivated in a rotating wall vessel identified several proteins belonging to cell envelope biogenesis/outer membrane as higher abundant[67]. In our study, the STRING database analysis identified a cluster of proteins associated with the extracellular milieu as higher abundant after growth in reduced gravity in *D. radiodurans* (Fig. 3). As part of the general secretion pathway, DR_1964 contribute to the secretion of unfolded proteins. In addition, the outer membrane protein DR_0379, which contains a PORTA (polypeptide transport associated)

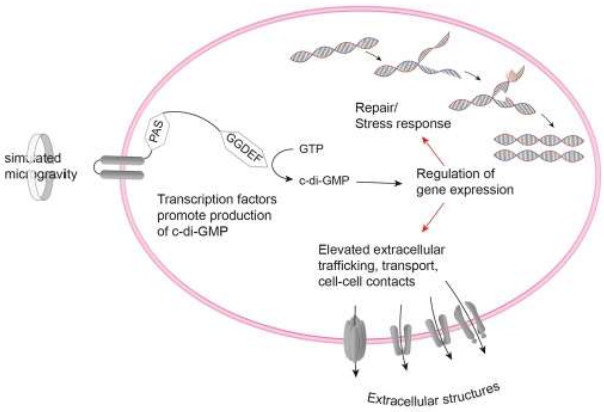

Figure 6. Model of the main molecular responses of *D. radiodurans* to growth under simulated microgravity. The reduced gravity is recognized by the cells through PAS/PAC regions of signal transduction proteins, which trigger GGDEF domains. Those increase the production of c-di-GMP, which affects gene expression to support DNA replication and production of extracellular structures.

domain was identified as higher abundant after growth in simulated microgravity[68]. DR_0573 as a protein specific to *Deinococcus-Thermus*[69] was identified as higher abundant when grown under simulated microgravity. According to BLAST (Basic Local Alignment Search Tool), DR_0573 is an orthologue to an autotransporter outer membrane protein from *Deinococcus actinosclerus*[70]. Finally, the type IV piliation system protein DR_0774 showed an increased abundance in *D. radiodurans* after growth in simulated microgravity. This protein was identified as a secretin like S-layer component of *D. radiodurans*[71]. DR_0774 and DR_2577 (SlpA), the most representative protein of the *D. radiodurans* cell wall, could contribute to a complex that could span both the inner and the outer membranes. DR_0774 is acting as a structural pillar that brings stability to the plane of the outer membrane[71] which is also the main channel through which trafficking is managed[72]. A high abundance of these channels may contribute to increased extracellular trafficking, cell-cell contacts and other cell envelop-associated events and in *D. radiodurans* as a consequence of growth under simulated microgravity (Fig. 6). Lastly, protein DR_2299, which was more abundant under simulated microgravity growth, contains TPR regions which basic function is to mediate protein-protein interactions and the assembly of multiprotein complexes[73]. Such complexes have been shown to fulfil important roles in biofilm formation in *Bacillus subtilis*[74] and may influence EPS production and/or membrane-associated events in organisms grown under reduced gravity.

Transcriptional regulation. Previous studies emphasized the importance of the Hfq regulon in various bacterial species as response to simulated or real microgravity conditions[20,36,75]. This regulon is missing in *D. radiodurans*[76], however comparing the obtained data (Table S1) to a previous spaceflight proteomics study performed on *P. aeruginosa* revealed in both cases a decreased abundance of ribosomal proteins[21]. Another microgravity simulation study, using a high-aspect rotating vessel, and a subsequent transcriptomic approach confirmed the lower abundance of ribosomal proteins in *Streptococcus mutans*, grown for 8 h under reduced gravity[52]. The same study showed an increase in expression of genes encoding transcriptional regulators for cells, grown under simulated microgravity. Furthermore, RNA seq of spaceflight exposed *S. aureus* and (to a lesser extent) *B. subtilis* showed in a transcriptomic approach a decreased expression of proteins involved in folding and associated processes[77]. Similar results were obtained by subjecting *Mycobacterium marinum* to simulated microgravity in a high aspect ratio vessel. A subsequent transcriptomic approach induced a reduction in the expression of proteins involved in translation[55]. In yeast, the transcriptional regulator Rap1p is responsible for the expression of many genes, including ribosomal proteins and those whose expression is altered in response to changes in growth rate[78]. Similar to Hfq or Rap1p, other regulatory proteins must be responsible for the response of *D. radiodurans* to reduced gravity conditions. A cluster of proteins which are related to signal transduction was identified throughout our analysis. In order to evaluate these proteins in more detail, the sequences were uploaded to the SMART database, which is able to identify and annotate genetically mobile domains (Fig. 4). Two of the identified proteins (DR_0750 and DR_2299) contain GGDEF domains, conserved regions which are detected in many prokaryotic proteins, often in various combinations with sensory regulatory components[79,80]. Its function is to act as diguanylate cyclase to catalyse cyclic (c)-di-GMP production, which is used as intracellular signalling molecule to control multicellular behaviour[81]. This includes biosynthesis of exopolysaccharides, formation of biofilms and regulation of gene expression[82]. In *P. aeruginosa* WspR, a GGDEF-type response regulator is correlated to auto aggregation[83]. Enhanced cell aggregation and clumping was observed for *S. enterica* Typhimurium cultured in space[75] as well as for *Candida albicans* during a short-term spaceflight[21]. After growth in simulated microgravity, assuming to be achieved by high-aspect-ratio vessels, several bacterial cultures tend to build self-aggregative biofilms[52,56,84]. It is assumed that differences in the EPS are responsible for cellular aggregation[85]. Although *D. radiodurans* R1 does not produce biofilms, it is likely that the extracellular trafficking and cell envelope-associated processing are altered during growth under reduced gravity (Fig. 6). Recent investigations report on biofilm formation of a minor genetically modified *D. radiodurans* strain[86], although these investigation requires further

critical assessment. To regulate the level of c-di-GMP, the EAL domain (can be activated if necessary) in the same protein is used, a diguanylate phosphodiesterase to break the phosphodiester bond, as high levels of c-di-GMP are toxic to the cells[87]. Together these messenger domains are assumed to be involved in modulating cell surface structures[88] and extracellular protein production[79].

Another domain, which appear in three of the identified proteins (DR_0750, DR_2419 and DR_1174) is the PAS domain, a common signalling sensor in signalling proteins in all kingdoms of life[89]. Transduction of redox signals might be a common way to sensor by PAS domains, which are always located intracellularly. However, they are able to monitor the external milieu by detecting changes in the electron transport system[90]. PAS domains are often supported by PAC motifs, which occur C-terminal to many PAS domains and may contribute to PAS folding[91]. Certain environmental conditions, such as the availability of nutrients and oxygen can trigger biofilm dispersal[92]. In *P. aeruginosa*, this is enabled through a protein (RbdA), containing PAS and PAC regions as sensory domains as well as GGDEF and EAL domains. Under stressful conditions, the phosphodiesterase (EAL) domain of RbdA is active and catalyses the cleavage of the second messenger c-di-GMP, which ultimately leads to biofilm dispersal[93]. Although the enzymology behind c-di-GMP synthesis and degradation has been elucidated, the detailed mechanism through which it operates and how EPS and secretion processes are affected, remain obscure[94].

Additionally, cells grown under simulated microgravity showed elevated amounts of transferases (kinases, methyltransferases, glycosyltransferases and phosphorylases). These (Fig. 2B) might be responsible for transcriptional regulations and post-translational modifications as a response to the altered environmental condition. Apart from that, two high abundant proteins (DR_1174 and DR_2419) contain histidine kinase domains, key elements in two-component signal transduction systems which control complex cellular processes[95] and are involved in adapting to environmental changes[96].

Conclusion

This study under simulated microgravity conditions using 2D clinorotation was performed with respect to experimental exposure of *D. radiodurans* outside the ISS. Apart from radiation and vacuum, it is important to understand the molecular response to microgravity as one exceptional environmental factor present in outer space. However, considering that our study is performed under simulated conditions, further verification might be necessary in real microgravity in space. Most studies regarding microbial response to real microgravity focus on pathogens and biofilm forming bacteria. The growth of *D. radiodurans* under simulated microgravity obviously induce signal proteins responsible for the additional production of proteins connected to the extracellular milieu and cell envelope-associated events. The reduced gravity environment is recognized by PAS (and PAC) regions, which activate GGDEF to catalyse the production of c-di-GMP. As a result, *D. radiodurans* produces more proteins associated with the extracellular region, whereas in other EPS-forming microorganisms, biofilm production is increased. Other protein domains, e.g., TPR and GGDEF, convey the obtained signal to influence gene promotors. Therefore, the abundances of proteins which are responsible for DNA processing and extracellular membrane-associated events are increased and ultimately, those lead to a prolonged exponential phase and elevated extracellular trafficking (Fig. 6). Although results obtained from the applied approaches indicate essential components for the response of *D. radiodurans* to simulated microgravity, future studies are needed to validate the hypotheses. Computationally derived results in our study need to be further critically assessed by thorough biochemical analysis of targeted proteins. To verify enzymatic switches that respond to gravity stress, a comparison to cells grown under increased gravity is advised. Furthermore, the generation of mutant strains for identified signal transducer proteins combined with other methods such as a transcriptomic approach and various electron microscopy-based techniques can help to completely unravel the molecular mechanisms in *D. radiodurans* responsible to adapt to simulated microgravity conditions.

Methods

Strain, media and storage. A bacterial stock of *D. radiodurans* R1 (ATCC 13939, 1×10^8 CFU/ml) was stored at −80 °C in 1:1 glycerol and 2x TBG-broth (1% tryptone (w/v), 0.6% beef extract (w/v), 0.2% glucose (w/v). For recovery and pre-cultures, 1x TBG was inoculated with 10 µl of a 10^8 CFU/ml frozen stock solution and incubated at 37 °C for 15 h. For inoculating agar plates, 10 µl of a PBS-washed (0.7%, $Na_2HPO_4 \cdot 2\,H_2O$ (w/v), 0.4% NaCl (w/v), 0.3% KH_2PO_4 (w/v), pH 7.4) pre-culture were pipetted on 1x TBG (solidified with 1.5% (w/v) agar) and incubated at 37 °C for 2 days (Fig. 1).

2-D clinostat: simulation of microgravity. For the simulation of microgravity, a commercially available fast-rotating 2-D clinostat (UN-KTM2, Advanced Engineering Services, Japan) was used. The sample holder was slightly modified, to hold up to eleven petri-dishes. TBG-medium was poured in germ-counting petri-dishes (Greiner Bio-One GmbH, Germany), which were labelled with a grid on the backside of the plate. The centre of the grid represented the centre of the petri dish and therefore marked the inoculation position of the respective plate. Each plate was inoculated with 10 µl of a fresh PBS-washed overnight culture and air-dried for 2 min. Plates were wrapped with Parafilm to prevent contaminations and mounted in the 2-D clinostat, secured by a lid and four butterfly screws. The clinostat was placed in a standard laboratory incubator at 37 °C. The rotation axis was aligned parallel to the ground with a spirit level. The clinostat was set to 60 rpm, which correspond to ~0.0161 g residual acceleration assuming a final colony diameter of $\leq$0.8 cm (~r $\leq$ 0.4 cm). Control cells were placed at a similar position near the clinostat to mimic similar temperature and humidity conditions, however kept at static 1 g control.

Colony harvesting. After incubation for 48 h, colonies were immediately scratched off the agar by using a sterile 5 µl plastic loop and directly transferred into ice-cold PBS-buffer. For every biological replicate, five

individual colonies were pooled to one combined sample (n = 5). To secure reproducibility of the results, three clinostats were used at different time points. Combined samples were washed two times in ice-cold PBS-buffer at 4 °C. The supernatant was discarded, and the pellet was frozen in liquid nitrogen. Dry ice was used to transport the samples until the samples could be stored at −20 °C.

Extraction of proteins and metabolites. The integrative extraction of proteins and metabolites was performed as described before (27, 31). Approximately 0.5 g lysing matrix B (MP Biomedicals) and 750 µL ice-cold MCW (methanol: chloroform: water 2.5:1:0.5) were added to the frozen cell pellets. Homogenization was performed in a FastPrep 24 instrument (MP Biomedicals; 5 * 30 s, 6.5 ms^{-1}; cooled on ice between circles), followed by 15 min incubation on ice. Samples were centrifuged (21000 g/4 min/4 °C) and the supernatants were transferred in new tubes for subsequent metabolite purification. A second extraction of the pellets was performed with 250 µL MCW. Samples were vortexed, 5 min incubated at room temperature (RT), centrifuged (21000 g/4 min/4 °C) and the supernatant was transferred to the tubes for metabolite purification. H_2O (300 µL) was added to the supernatants to achieve a phase separation. After centrifugation (21000 g/4 min/4 °C), the upper polar phases were transferred to new tubes, carefully dried in a vacuum concentrator (ScanVac, Labogene) and stored at −20 °C until derivatization.

Protein purification and digestion. Protein pellets were washed with 1 mL methanol (MeOH), centrifuged (21000 g/5 min/4 °C) and air-dried within a laminar flow hood for 10 min. Pellets, containing nucleic acids and proteins were solubilized in 1 mL TRIzol. Together with the lysing matrix B, samples were homogenized using the bead beater one more time (30 s/6.5 ms^{-1}). Afterwards, samples were incubated 5 min on a turning wheel (20 rpm) at RT. To separate phases, 200 µL chloroform were added, samples were incubated for 3 min on a turning wheel (10 rpm) and centrifuged (21000 g/4 °C/15 min). The lower, apolar, protein containing phases were transferred to new tubes. They were washed with 550 µL H_2O, incubated 3 min on a turning wheel (20 rpm), centrifuged (21000 g/4 °C/5 min) and the lower, apolar phase was transferred to new tubes. For overnight precipitation at −20 °C, 1.5 mL 0.1 M NH_4Ac in MeOH (containing 0.5% β-mercaptoethanol) were added to each sample. Proteins were subsequently centrifuged (10000 g/15 min/4 °C) and the supernatants were discarded. The pellets were washed with 1 mL acetone, followed by disruption of the pellets in a ultrasonication bath for 5 min. Samples were centrifuged (10000 g/5 min/4 °C) and the supernatants were discarded. The washing procedure was repeated one time with 1 mL acetone and one time with 90% acetone. After the final washing step, samples were air dried for 15 min under a laminar flow hood.

Protein pellets were solubilized in 40 µL 8 M urea/4% SDS (sodium dodecyl sulphate) and the total protein concentration was estimated with a bicinchoninic acid assay (BCA) against bovine serum albumin (BSA) (Fig. S2). 80 µg proteins, mixed with Laemmli buffer (Bio-Rad) for each replicate were loaded on SDS-polyacrylamide gels (separation gel 12%, stacking gel 5%). Samples were run through the stacking gel with a voltage of 40 V, which was increased to 80 V once the samples reached the separating gel. After the bromophenol blue run approximately 1 cm into the separating gel, the electrophoresis was stopped. The gel was stained with 40% MeOH, 10% acetic acid, 0.1% Coomassie R-250 in milliQ-H_2O for 30 min. Destaining was performed 4 times (20 min) with 40% MeOH, 2% acetic acid, followed by washing the gel in H_2O for 30 min.

The protein bands for each sample were cut out of the gel and further cut into small pieces (of approximately 1 mm^3). To destain protein bands, 1 mL 25 mM ammonium bicarbonate (AmBic) in 50% acetonitrile (ACN) was added to each sample. Samples were incubated on a thermal shaker (650 rpm/15 min/37 °C) and the supernatants were discarded. This procedure was repeated two times until the blue colour disappeared from the pieces. To dry samples, 300 µL ACN were added, incubated 5 min at RT and the supernatants were discarded. Next, disulphide bonds were reduced with 20 mM dithiothreitol (DTT) in 100 mM AmBic (650 rpm/30 min/37 °C) and the supernatants were discarded. Gel pieces were washed with ACN and alkylation of reduced cysteine residues was performed with 55 mM iodoacetamide (IAA) in 100 mM AmBic (60 min/RT). Gel pieces from each sample were washed with 25 mM AmBic in H_2O, 25 mM AmBic in 50% ACN and in 100% ACN (650 rpm/15 min/37 °C). Proteins were digested by covering them with trypsin (12.5 ngµL^{-1}, in 25 mM AmBic, 10% ACN, 5 mM $CaCl_2$) for 16 h.

To extract peptides from each sample, 150 µL of 50% ACN including 1% formic acid (FA) were added. Samples were incubated for 5 min at RT, briefly sonicated and transferred into a new tube. The procedure was repeated once with 50% ACN (1% FA) and one time with 90% ACN (1% FA). Collected supernatants were dried down in a vacuum concentrator.

To desalt samples, peptides were suspended in 4% ACN (0.25% FA), incubated at RT and centrifuged (21000 g/2 min/4 °C). The desalting C18 spec plate (Agilent) membranes, connected to a water jet pump, were activated with 2 × 800 µL MeOH and washed with 2 × 800 µL H_2O. Samples were loaded on the membranes and incubated for 10 min at RT (only gravity). Peptides were first washed with 2 × 800 µL H_2O and finally eluted with 3 × 800 µL MeOH. Collected samples were dried in a vacuum concentrator.

Samples were resuspended in 100 µL 2% ACN (0.1% FA) and the total peptide concentration was estimated with a colorimetric peptide quantification assay (Pierce) (Fig. S2). The peptide concentrations were adjusted to 50 ng/µl for LC-MS/MS analysis.

HPLC nESI MS/MS. For shotgun proteomics measurements, 5 µL of each sample were injected into an nHPLC-Orbitrap QExactive (Thermo Fisher Scientific, Bremen, Germany), measurement settings were described before[49]. Data analysis was performed with Maxquant[97]. The minimum peptide length for identification was set to 7 amino acids and one unique peptide was required for protein identification (FDR 1%, based on target decoy database). For identification, measured spectra were compared to the *D. radiodurans* FASTA file from Uniprot (January 2018, 3085 sequences in the database). Further Maxquant settings: 20 ppm first search peptide tolerance,

4.5 ppm main search peptide tolerance, maximum of 2 missed cleavages, maximum number of 5 modifications per peptide (variable: oxidation (M) and acetylation of protein N-term, fixed: carbamidomethylation (C)), label free quantification of samples.

Derivatization and analysis of the metabolites with GC-TOF-MS. Polar metabolites were dissolved in 10 µL of 40 mg mL^{-1} methoxyamine-hydrochloride in pyridine through shaking at 650 rpm at 30 °C for 90 min. Subsequently, 40 µL of a silylation mix (1 mL N-methyl-N-trimethylsilyltrifluoroacetamid spiked with 30 µL of a mix of even-number alkanes (C10-C40)) was added and the mixture was incubated for 30 min at an agitation rate of 650 rpm at 37 °C. After centrifugation (14000 g, 2 min), the supernatant was transferred into a glass vial and 1 µL of it was injected into the GC (Agilent 6890 gas chromatograph) in splitless injection mode.

For separation of the metabolites, an Agilent HP-5MS column (30 m length, 0.25 mm diameter and 0.25 µm film) was used. Further parameters were set as following: flow rate 1 ml/min; injection temperature 230 °C; column temperature started at 70 °C for one minute, then heated up to 330 °C in 9 min, where it was hold for 8 min; recorded masses in the LECO Pegasus 4D GC × GC-TOF spectrometer were set between 40–700 m/z. Apart from the samples, a house intern standard mix of certain metabolites was measured to get level 1 identifications of common primary metabolites.

Identifications of the metabolites were based on matching the obtained MS-spectra and retention times with an in-house library (extended gmd database). Peak integration was performed with the LECO ChromaTOF software.

Statistical analysis. Data processing of proteomic and metabolomic measurements was performed similarly. The peptide content was normalized before measurement; therefore 250 ng peptides were injected for each replicate. Consequently, the LFQ (label free quantification) intensity results for each identified protein sequence were used for relative quantification without any further normalization steps. However, peaks derived from the metabolite measurements were normalized to the protein content measured by the BCA (Fig. S2) of each individual replicate. A *Welch's* t-test was performed to identify proteins and metabolites of interest (p-value below 0.05). For the STRING analysis, proteins of interest that were higher and lower abundant after simulated microgravity exposure were uploaded independently. The protein-protein interaction analysis was performed on high confidence level (0.700), to minimize false positives.

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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References

1. Anken, R. & Rahmann, H. *Gravitational Zoology: How Animals Use and Cope with Gravity*. (2002).
2. Grimm, D., Wise, P., Lebert, M., Richter, P. & Baatout, S. How and why does the proteome respond to microgravity? *Expert review of proteomics* **8**, 13–27, https://doi.org/10.1586/epr.10.105 (2011).
3. Strauch, S. M. *et al.* Current knowledge about the impact of microgravity on the proteome. *Expert review of proteomics* **16**, 5–16, https://doi.org/10.1080/14789450.2019.1550362 (2019).
4. Katkov, V. E. & Chestukhin, V. V. Blood pressure and oxygenation in different cardiovascular compartments of a normal man during postural exposures. *Aviation, space, and environmental medicine* **51**, 1234–1242 (1980).
5. Mader, T. H. *et al.* Optic disc edema, globe flattening, choroidal folds, and hyperopic shifts observed in astronauts after long-duration space flight. *Ophthalmology* **118**, 2058–2069, https://doi.org/10.1016/j.ophtha.2011.06.021 (2011).
6. Casaburi, G., Goncharenko-Foster, I., Duscher, A. A. & Foster, J. S. Transcriptomic changes in an animal-bacterial symbiosis under modeled microgravity conditions. *Scientific reports* **7**, 46318, https://doi.org/10.1038/srep46318 (2017).
7. Foster, J. S., Wheeler, R. M. & Pamphile, R. Host-microbe interactions in microgravity: assessment and implications. *Life (Basel)* **4**, 250–266, https://doi.org/10.3390/life4020250 (2014).
8. Foster, J. S., Khodadad, C. L., Ahrendt, S. R. & Parrish, M. L. Impact of simulated microgravity on the normal developmental time line of an animal-bacteria symbiosis. *Scientific reports* **3**, 1340, https://doi.org/10.1038/srep01340 (2013).
9. Ueda, J. *et al.* Growth and Development, and Auxin Polar Transport in Higher Plants under Microgravity Conditions in Space: BRIC-AUX on STS-95 Space Experiment. *Journal of Plant Research* **112**, 487–492, https://doi.org/10.1007/PL00013904 (1999).
10. Hemmersbach, R. & Braun, M. Gravity-sensing and gravity-related signaling pathways in unicellular model systems of protists and plants. *Signal Transduction* **6**, 432–442, https://doi.org/10.1002/sita.200600106 (2006).
11. Sievers, A. Gravity sensing mechanisms in plant cells. *ASGSB Bull* **4**, 43–50 (1991).
12. Buchen, B., Braun, M. & Sievers, A. *Statoliths, cytoskeletal elements and cytoplasmic streaming of Chara rhizoids under reduced gravity during TEXUS flights*. Vol. 1206 (1997).
13. Blancaflor, E. B. The cytoskeleton and gravitropism in higher plants. *Journal of plant growth regulation* **21**, 120–136, https://doi.org/10.1007/s003440010041 (2002).
14. Ingber, D. E. Tensegrity: The Architectural Basis of Cellular Mechanotransduction. *Annual Review of Physiology* **59**, 575–599, https://doi.org/10.1146/annurev.physiol.59.1.575 (1997).
15. Grimm, D. *et al.* The impact of microgravity-based proteomics research. *Expert review of proteomics* **11**, 465–476, https://doi.org/10.1586/14789450.2014.926221 (2014).
16. Kim, W. *et al.* Spaceflight Promotes Biofilm Formation by Pseudomonas aeruginosa. *PLOS ONE* **8**, e62437, https://doi.org/10.1371/journal.pone.0062437 (2013).
17. Chopra, V. *et al.* Alterations in the virulence potential of enteric pathogens and bacterial-host cell interactions under simulated microgravity conditions. *Journal of toxicology and environmental health. Part A* **69**, 1345–1370, https://doi.org/10.1080/15287390500361792 (2006).
18. Fajardo-Cavazos, P. & Nicholson, W. L. Cultivation of Staphylococcus epidermidis in the Human Spaceflight Environment Leads to Alterations in the Frequency and Spectrum of Spontaneous Rifampicin-Resistance Mutations in the rpoB Gene. *Frontiers in microbiology* **7**, 999–999, https://doi.org/10.3389/fmicb.2016.00999 (2016).

19. Aunins, T. R. *et al.* Spaceflight Modifies Escherichia coli Gene Expression in Response to Antibiotic Exposure and Reveals Role of Oxidative Stress Response. *Frontiers in microbiology* **9**, 310–310, https://doi.org/10.3389/fmicb.2018.00310 (2018).
20. Crabbe, A. *et al.* Transcriptional and proteomic responses of Pseudomonas aeruginosa PAO1 to spaceflight conditions involve Hfq regulation and reveal a role for oxygen. *Appl Environ Microbiol* **77**, 1221–1230, https://doi.org/10.1128/AEM.01582-10 (2011).
21. Crabbe, A. *et al.* Spaceflight enhances cell aggregation and random budding in Candida albicans. *PLoS One* **8**, e80677, https://doi.org/10.1371/journal.pone.0080677 (2013).
22. Duscher, A. A. *et al.* Transcriptional profiling of the mutualistic bacterium Vibrio fischeri and an hfq mutant under modeled microgravity. *npj Microgravity* **4**, 25, https://doi.org/10.1038/s41526-018-0060-1 (2018).
23. Mastroleo, F. *et al.* Experimental design and environmental parameters affect Rhodospirillum rubrum S1H response to space flight. *The ISME journal* **3**, 1402–1419, https://doi.org/10.1038/ismej.2009.74 (2009).
24. Zea, L. *et al.* Phenotypic Changes Exhibited by E. coli Cultured in Space. *Front Microbiol* **8**, 1598, https://doi.org/10.3389/fmicb.2017.01598 (2017).
25. Mauclaire, L. & Egli, M. Effect of simulated microgravity on growth and production of exopolymeric substances of Micrococcus luteus space and earth isolates. *FEMS Immunol Med Microbiol* **59**, 350–356, https://doi.org/10.1111/j.1574-695X.2010.00683.x (2010).
26. Dainton, F. S. On the existence of free atoms and radicals in water and aqueous solutions subjected to ionizing radiation. *J Phys Colloid Chem* **52**, 490–517 (1948).
27. Jurkiewicz, B. A. & Buettner, G. R. Ultraviolet light-induced free radical formation in skin: an electron paramagnetic resonance study. *Photochem Photobiol* **59**, 1–4 (1994).
28. Potts, M. Desiccation tolerance of prokaryotes. *Microbiol Rev* **58**, 755–805 (1994).
29. Kawaguchi, Y. *et al.* Investigation of the Interplanetary Transfer of Microbes in the Tanpopo Mission at the Exposed Facility of the International Space Station. *Astrobiology* **16**, 363–376, https://doi.org/10.1089/ast.2015.1415 (2016).
30. Daly, M. J. *et al.* Small-Molecule Antioxidant Proteome-Shields in Deinococcus radiodurans. *PLOS ONE* **5**, e12570, https://doi.org/10.1371/journal.pone.0012570 (2010).
31. Battista, J. R. Against all odds: the survival strategies of Deinococcus radiodurans. *Annu Rev Microbiol* **51**, 203–224, https://doi.org/10.1146/annurev.micro.51.1.203 (1997).
32. Makarova, K. S. & Daly, M. J. *Comparative genomics of stress response systems in Deinococcus bacteria*. (2010).
33. Kobayashi, Y., Kikuchi, M., Nagaoka, S. & Watanabe, H. Recovery of Deinococcus radiodurans from radiation damage was enhanced under microgravity. *Biological Sciences in Space* **10**, 97–101 (1996).
34. Wang, H. *et al.* 2-D clinostat for simulated microgravity experiments with Arabidopsis seedlings. *Microgravity Science and Technology* **28**, 59–66 (2016).
35. Garschagen, L. S., Mancinelli, R. L. & Moeller, R. Introducing Vibrio natriegens as a Microbial Model Organism for Microgravity Research. *Astrobiology* **19**, 1211–1220 (2019).
36. Huang, B., Liu, N., Rong, X., Ruan, J. & Huang, Y. Effects of simulated microgravity and spaceflight on morphological differentiation and secondary metabolism of Streptomyces coelicolor A3(2). *Appl Microbiol Biotechnol* **99**, 4409–4422, https://doi.org/10.1007/s00253-015-6386-7 (2015).
37. Herranz, R. *et al.* Ground-based facilities for simulation of microgravity: organism-specific recommendations for their use, and recommended terminology. *Astrobiology* **13**, 1–17, https://doi.org/10.1089/ast.2012.0876 (2013).
38. Patel, M. J. *et al.* Identification of mechanosensitive genes in osteoblasts by comparative microarray studies using the rotating wall vessel and the random positioning machine. *Journal of cellular biochemistry* **101**, 587–599, https://doi.org/10.1002/jcb.21218 (2007).
39. Loon, J. Some history and use of the Random Positioning Machine, RPM, in gravity related research. *Advances in Space Research* **39**, 1161–1165, https://doi.org/10.1016/j.asr.2007.02.016 (2007).
40. Brungs, S. *et al.* Facilities for Simulation of Microgravity in the ESA Ground-Based Facility Programme. *Microgravity Science and Technology* **28**, 191–203, https://doi.org/10.1007/s12217-015-9471-8 (2016).
41. Hauslage, J., Cevik, V. & Hemmersbach, R. Pyrocystis noctiluca represents an excellent bioassay for shear forces induced in ground-based microgravity simulators (clinostat and random positioning machine). *NPJ Microgravity* **3**, 12, https://doi.org/10.1038/s41526-017-0016-x (2017).
42. Briegleb, W. Some qualitative and quantitative aspects of the fast-rotating clinostat as a research tool. *ASGSB Bull* **5**, 23–30 (1992).
43. Schwarzenberg, M. *et al.* Signal transduction in T lymphocytes — A comparison of the data from space, the free fall machine and the random positioning machine. *Advances in Space Research* **24**, 793–800, https://doi.org/10.1016/S0273-1177(99)00075-7 (1999).
44. Kawaguchi, Y. *et al.* The possible interplanetary transfer of microbes: assessing the viability of Deinococcus spp. under the ISS Environmental conditions for performing exposure experiments of microbes in the Tanpopo mission. *Orig Life Evol Biosph* **43**, 411–428, https://doi.org/10.1007/s11084-013-9346-1 (2013).
45. Yano, H. *et al.* In Lunar and Planetary Science Conference. 2934.
46. Muruganujan, A., Ebert, D., Mi, H., Thomas, P. D. & Huang, X. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Research* **47**, D419–D426, https://doi.org/10.1093/nar/gky1038 (2018).
47. Szklarczyk, D. *et al.* STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic acids research* **43**, D447–D452, https://doi.org/10.1093/nar/gku1003 (2015).
48. Letunic, I. & Bork, P. 20 years of the SMART protein domain annotation resource. *Nucleic Acids Research* **46**, D493–D496, https://doi.org/10.1093/nar/gkx922 (2017).
49. Ott, E. *et al.* Proteometabolomic response of Deinococcus radiodurans exposed to UVC and vacuum conditions: Initial studies prior to the Tanpopo space mission. *Plos One* **12**, e0189381, https://doi.org/10.1371/journal.pone.0189381 (2017).
50. Ott, E. *et al.* Proteomic and Metabolomic Profiling of Deinococcus radiodurans Recovering After Exposure to Simulated Low Earth Orbit Vacuum Conditions. *Frontiers in Microbiology* **10**, https://doi.org/10.3389/fmicb.2019.00909 (2019).
51. Venkateswaran, A. *et al.* Physiologic Determinants of Radiation Resistance in Deinococcus radiodurans. *Applied and Environmental Microbiology* **66**, 2620–2626 (2000).
52. Orsini, S. S., Lewis, A. M. & Rice, K. C. Investigation of simulated microgravity effects on Streptococcus mutans physiology and global gene expression. *NPJ Microgravity* **3**, 4, https://doi.org/10.1038/s41526-016-0006-4 (2017).
53. Arunasri, K. *et al.* Effect of simulated microgravity on E. coli K12 MG1655 growth and gene expression. *PLoS One* **8**, e57860, https://doi.org/10.1371/journal.pone.0057860 (2013).
54. Rea, G. *et al.* Microgravity-driven remodeling of the proteome reveals insights into molecular mechanisms and signal networks involved in response to the space flight environment. *Journal of proteomics* **137**, 3–18 (2016).
55. Abshire, C. F. *et al.* Exposure of Mycobacterium marinum to low-shear modeled microgravity: effect on growth, the transcriptome and survival under stress. *NPJ Microgravity* **2**, 16038, https://doi.org/10.1038/npjmgrav.2016.38 (2016).
56. Castro, S. L., Nelman-Gonzalez, M., Nickerson, C. A. & Ott, C. M. Induction of attachment-independent biofilm formation and repression of Hfq expression by low-fluid-shear culture of Staphylococcus aureus. *Appl Environ Microbiol* **77**, 6368–6378, https://doi.org/10.1128/AEM.00175-11 (2011).
57. Stukenberg, P. T., Studwell-Vaughan, P. S. & O'Donnell, M. Mechanism of the sliding beta-clamp of DNA polymerase III holoenzyme. *J Biol Chem* **266**, 11328–11334 (1991).

58. Slade, D., Lindner, A. B., Paul, G. & Radman, M. Recombination and replication in DNA repair of heavily irradiated Deinococcus radiodurans. *Cell* **136**, 1044–1055, https://doi.org/10.1016/j.cell.2009.01.018 (2009).
59. Kornberg, A. & Baker, T. A. *DNA replication*, 2nd ed. Vol. 17 (W. H. Freeman and Company, 1992).
60. Roberts, J. E. *et al*. Simulated microgravity induced damage in human retinal pigment epithelial cells. *Molecular vision* **12**, 633–638 (2006).
61. Vukanti, R., Model, M. A. & Leff, L. G. Effect of modeled reduced gravity conditions on bacterial morphology and physiology. *BMC microbiology* **12**, 4, https://doi.org/10.1186/1471-2180-12-4 (2012).
62. Zea, L. *et al*. A Molecular Genetic Basis Explaining Altered Bacterial Behavior in Space. *PLOS ONE* **11**, e0164359, https://doi.org/10.1371/journal.pone.0164359 (2016).
63. Wang, Y. *et al*. Transcriptomic and proteomic responses of Serratia marcescens to spaceflight conditions involve large-scale changes in metabolic pathways. *Advances in Space Research* **53**, 1108–1117 (2014).
64. Sheyn, D., Pelled, G., Netanely, D., Domany, E. & Gazit, D. The Effect of Simulated Microgravity on Human Mesenchymal Stem Cells Cultured in an Osteogenic Differentiation System: A Bioinformatics Study. *Tissue Engineering Part A* **16**, 3403–3412, https://doi.org/10.1089/ten.tea.2009.0834 (2010).
65. Wang, H. *et al*. Increased biofilm formation ability in Klebsiella pneumoniae after short-term exposure to a simulated microgravity environment. *Microbiology Open* **5**, 793–801, https://doi.org/10.1002/mbo3.370 (2016).
66. Searles, S. C., Woolley, C. M., Petersen, R. A., Hyman, L. E. & Nielsen-Preiss, S. M. Modeled microgravity increases filamentation, biofilm formation, phenotypic switching, and antimicrobial resistance in Candida albicans. *Astrobiology* **11**, 825–836, https://doi.org/10.1089/ast.2011.0664 (2011).
67. Mastroleo, F. *et al*. Modelled microgravity cultivation modulates N-acylhomoserine lactone production in Rhodospirillum rubrum S1H independently of cell density. *Microbiology (Reading, England)* **159**, 2456–2466 (2013).
68. Sanchez-Pulido, L., Devos, D., Genevrois, S., Vicente, M. & Valencia, A. POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. *Trends Biochem Sci* **28**, 523–526 (2003).
69. Griffiths, E. & Gupta, R. S. Identification of signature proteins that are distinctive of the Deinococcus-Thermus phylum. *International microbiology: the official journal of the Spanish Society for Microbiology* **10**, 201–208 (2007).
70. Sun Joo, E. *et al*. Deinococcus actinosclerus sp. nov., a novel bacterium isolated from soil of a rocky hillside. *International journal of systematic and evolutionary microbiology* **66**, 1003–1008, https://doi.org/10.1099/ijsem.0.000825 (2016).
71. Farci, D. *et al*. New features of the cell wall of the radio-resistant bacterium Deinococcus radiodurans. *Biochim Biophys Acta* **1838**, 1978–1984, https://doi.org/10.1016/j.bbamem.2014.02.014 (2014).
72. Korotkov, K. V., Gonen, T. & Hol, W. G. J. Secretins: dynamic channels for protein transport across membranes. *Trends in Biochemical Sciences* **36**, 433–443, https://doi.org/10.1016/j.tibs.2011.04.002 (2011).
73. D'Andrea, L. D. & Regan, L. TPR proteins: the versatile helix. *Trends Biochem Sci* **28**, 655–662, https://doi.org/10.1016/j.tibs.2003.10.007 (2003).
74. DeLoughery, A., Dengler, V., Chai, Y. & Losick, R. Biofilm formation by Bacillus subtilis requires an endoribonuclease-containing multisubunit complex that controls mRNA levels for the matrix gene repressor SinR. *Mol Microbiol* **99**, 425–437, https://doi.org/10.1111/mmi.13240 (2016).
75. Wilson, J. W. *et al*. Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. *Proc Natl Acad Sci USA* **104**, 16299–16304, https://doi.org/10.1073/pnas.0707155104 (2007).
76. Sun, X., Zhulin, I. & Wartell, R. M. Predicted structure and phyletic distribution of the RNA-binding protein Hfq. *Nucleic acids research* **30**, 3662–3671 (2002).
77. Morrison, M. D., Fajardo-Cavazos, P. & Nicholson, W. L. Comparison of Bacillus subtilis transcriptome profiles from two separate missions to the International Space Station. *npj Microgravity* **5**, 1, https://doi.org/10.1038/s41526-018-0061-0 (2019).
78. Pina, B., Fernandez-Larrea, J., Garcia-Reyero, N. & Idrissi, F. Z. The different (sur)faces of Rap1p. *Molecular genetics and genomics: MGG* **268**, 791–798, https://doi.org/10.1007/s00438-002-0801-3 (2003).
79. Galperin, M. Y., Nikolskaya, A. N. & Koonin, E. V. Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* **203**, 11–21, https://doi.org/10.1111/j.1574-6968.2001.tb10814.x (2001).
80. Ausmees, N. *et al*. Genetic data indicate that proteins containing the GGDEF domain possess diguanylate cyclase activity. *FEMS Microbiol Lett* **204**, 163–167, https://doi.org/10.1111/j.1574-6968.2001.tb10880.x (2001).
81. Chan, C. *et al*. Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci USA* **101**, 17084–17089, https://doi.org/10.1073/pnas.0406134101 (2004).
82. Ryjenkov, D. A., Tarutina, M., Moskvin, O. V. & Gomelsky, M. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J Bacteriol* **187**, 1792–1798, https://doi.org/10.1128/JB.187.5.1792-1798.2005 (2005).
83. D'Argenio, D. A., Calfee, M. W., Rainey, P. B. & Pesci, E. C. Autolysis and Autoaggregation in Pseudomonas aeruginosa Colony Morphology Mutants. *Journal of Bacteriology* **184**, 6481–6489, https://doi.org/10.1128/jb.184.23.6481-6489.2002 (2002).
84. Lynch, S. V., Mukundakrishnan, K., Benoit, M. R., Ayyaswamy, P. S. & Matin, A. Escherichia coli biofilms formed under low-shear modeled microgravity in a ground-based system. *Appl Environ Microbiol* **72**, 7701–7710, https://doi.org/10.1128/AEM.01294-06 (2006).
85. Kim, W. *et al*. Effect of spaceflight on Pseudomonas aeruginosa final cell density is modulated by nutrient and oxygen availability. *BMC microbiology* **13**, 241, https://doi.org/10.1186/1471-2180-13-241 (2013).
86. Shukla, S. K. & Subba Rao, T. The first recorded incidence of Deinococcus radiodurans R1 biofilm formation and its implications in heavy metals bioremediation. bioRxiv, 234781, https://doi.org/10.1101/234781 (2017).
87. Stock, A. M. Diguanylate cyclase activation: it takes two. *Structure* **15**, 887–888, https://doi.org/10.1016/j.str.2007.07.003 (2007).
88. Jenal, U. Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? *Current opinion in microbiology* **7**, 185–191, https://doi.org/10.1016/j.mib.2004.02.007 (2004).
89. Henry, J. T. & Crosson, S. Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annu Rev Microbiol* **65**, 261–286, https://doi.org/10.1146/annurev-micro-121809-151631 (2011).
90. Taylor, B. L. & Zhulin, I. B. PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiology and molecular biology reviews: MMBR* **63**, 479–506 (1999).
91. Ponting, C. P. & Aravind, L. PAS: a multifunctional domain family comes to light. *Curr Biol* **7**, R674–677, https://doi.org/10.1016/s0960-9822(06)00352-6 (1997).
92. Gjermansen, M., Ragas, P., Sternberg, C., Molin, S. & Tolker-Nielsen, T. Characterization of starvation-induced dispersion in Pseudomonas putida biofilms. *Environmental Microbiology* **7**, 894–904, https://doi.org/10.1111/j.1462-2920.2005.00775.x (2005).
93. Petrova, O. E. & Sauer, K. PAS domain residues and prosthetic group involved in BdlA-dependent dispersion response by Pseudomonas aeruginosa biofilms. *Journal of bacteriology* **194**, 5817–5828, https://doi.org/10.1128/JB.00780-12 (2012).
94. Valentini, M. & Filloux, A. Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from Pseudomonas aeruginosa and Other Bacteria. *J Biol Chem* **291**, 12547–12555, https://doi.org/10.1074/jbc.R115.711507 (2016).
95. Perego, M. & Hoch, J. A. Protein aspartate phosphatases control the output of two-component signal transduction systems. *Trends in genetics: TIG* **12**, 97–101 (1996).

96. Liu, Y. *et al.* A pH-gated conformational switch regulates the phosphatase activity of bifunctional HisKA-family histidine kinases. *Nature communications* **8**, 2104, https://doi.org/10.1038/s41467-017-02310-9 (2017).
97. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology* **26**, 1367, https://doi.org/10.1038/nbt.1511 https://www.nature.com/articles/nbt.1511#supplementary-information (2008).

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E.O. and F.M.F. performed experiments. All authors provided editorial input. All authors made substantial contributions to the acquisition, analysis, and interpretation of data described in this article. All authors critically reviewed the report and approved the final version.

Competing interests
The authors declare no competing interests.

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4.4 Molecular repertoire of *Deinococcus radiodurans* after 1 year of exposure to outer space

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Molecular repertoire of *Deinococcus radiodurans* after 1 year of exposure to outer space

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Abstract

The extraordinarily resistant bacterium *Deinococcus radiodurans* withstands harsh environmental conditions present in outer space. *D. radiodurans* was exposed for one year outside the International Space Station within Tanpopo orbital mission to investigate microbial survival and space travel. In addition, a ground-based simulation experiment with conditions that mirror those from the Low Earth orbit, was performed. We monitored *D. radiodurans* cells during early stage of recovery after outer space exposure using electron microscopy tools and integrative -omics approach. Cells with no morphological damage after space exposure accumulated numerous outer membrane-associated vesicles. This intensified trafficking/vesiculation was mirrored on proteotranscriptomic level. Detected decrease in primary metabolites indicates alterations in energy status of *D. radiodurans* needed for the repair processes during the early stage of stress response. Exceptionally abundant polyamine putrescine acts as reactive oxygen species scavenging molecule. The UvrABC endonuclease excision repair mechanism is triggered to cope with DNA damage. Ultimately, elevated vesiculation serves as a quick stress response, which augments cell survival as a part of a multifaceted response of *D. radiodurans* after space exposure.

Introduction

As humans continue to conquer the realms of the solar system, understanding the molecular mechanisms of survival in outer space becomes increasingly important. Outer space is a hostile environment, which constrains any form of life. Remarkably, a few extremophilic microbial species have been shown to withstand the drastic influence of the outer space factors [1-5]. Exposed to the outer space environment, microorganisms are challenged by several hostile parameters: galactic cosmic and solar UV radiation, extreme vacuum, temperature fluctuations, desiccation, freezing, and microgravity. The International Space Station (ISS) provides a suitable environment for astrobiological experiments in the low Earth orbit (LEO). Limited previous studies partially described microbial responses after exposure outside the ISS [6-10]. Significantly impacting the development of astrobiology, the EXPOSE experiments (2008-2015) concluded that not only spore-forming bacteria such as *Bacillus subtilis* can survive an interplanetary travel, but also seeds, lichens (e.g., *Stichococcus sp*, *Trichoderma sp* and *Acarospora sp*) [10-12], and non-spore forming thermophilic bacteria *Deinococcus geotermalis* [13]. However, we have been still missing an explicit knowledge of molecular mechanisms permitting survival and adaptation in the outer space environment. Space parameters affect microorganisms by altering a variety of physiological features, including proliferation rate, cell metabolism, cell division, cell motility, virulence, drug resistance, and biofilm production [9, 10, 14-16]. These physiological perturbations of space-exposed microorganisms are very poorly understood at the molecular level. In this context, in order to achieve a detailed understanding of the full functional molecular set up of microorganisms exposed to outer space, a comprehensive multi-omics analysis of their molecular responses is desirable.

The gram-positive bacteria *Deinococcus radiodurans* possess numerous remarkable properties [17-19], which made it a suitable candidate for long-term space exposure experiments in the frame of the Tanpopo orbital project [20-22]. Preliminary experiments indicated that cells of *D. radiodurans* were able to withstand solar UV radiation on the ISS for one year as multilayers of more than 200 µm thickness [21]. Additionally, several initial investigations prior to the Tanpopo space mission resolved molecular response of *D. radiodurans* to selected simulated parameters of the outer space environment [23, 24]. Exposure to extreme conditions such as ionizing radiation, UV radiation and desiccation cause severe damage to nucleic acids, as the level of reactive oxygen species is rising. With a dose of ionizing radiation that is already lethal to *E. coli*, within 3-4 hours the nucleic acid fragments in *D. radiodurans* are reassembled into complete chromosomes and the cells return to normal growth [25]. Although the nucleic acid repair mechanisms show no clear genetic difference from *E.*

coli [26], *D. radiodurans* is 50 times more resistant to ionizing radiation and 33 times more resistant to UV radiation [17]. The mechanisms which allow *D. radiodurans* to survive under such conditions are not completely unraveled yet. Possible explanations are the condensed nature of the *D. radiodurans* genome [27], an increased ROS-scavenging and ROS-detoxifying activity via orthophosphate-manganese-small molecule complexes [28], and higher manganese to iron ratio than in radiation sensitive microorganisms [29]. Though, these physiological and metabolic adaptions are unique for *D. radiodurans*, it is hard to explain extraordinary genome reconstitution without considering DNA repair pathways [25]. Generally, in prokaryotes, there are several DNA repair systems, such as photoreactivation, nucleotide excision repair, base excision repair, mismatch repair, double strand break repair, homology directed repair and ultraviolet damage endonuclease repair, each specialized on a certain type of damage. Nucleotide excision repair stands out from the other repair mechanisms, since it is able to recognize a broad range of structurally unrelated DNA damages [30]. It consists of four crucial proteins, UvrA, UvrB, UvrC and UvrD, which in conjunction with each other orchestrate effective DNA repair performance. The Uvr cluster proteins are triggered by the exposure of *D. radiodurans* to several space-related conditions (UVC and vacuum), albeit the response to these factors is a multilayer process, in which many other molecular components, in addition to DNA damage repair, are involved [23, 24].

In this study, dehydrated cells of *D. radiodurans* were exposed to LEO conditions for one year outside the ISS in the frame of the Tanpopo space mission [31]. After exposure and subsequent recovery in complex medium, metabolites, proteins and mRNAs were extracted from space-exposed cells, analyzed with integrative –omics techniques and compared to ground controls. The results show the early molecular response of *D. radiodurans* after LEO exposure, help to understand which molecular tools are used to cope with the damage induced by outer space conditions and highlight the power of combining different –omics techniques to unravel molecular stress response mechanisms.

Results

Survival and post-exposure analysis

Dehydrated cells of *D. radiodurans* survived an exposure to low Earth orbit for one year (Fig. 1a), however, with decreased survival rates compared to the ground controls (Fig. 1a). Survival rates were calculated by colony forming units as N/N_0, where N is the number of CFU's/mL after 1 year and N_0 is the number of CFU's/mL of the stock before dehydration. After recovery for 2 h in complex medium, an insignificant decrease in OD_{600} (Fig. 1b) was observed for both LEO exposed and ground control cells. In order to investigate cellular integrity after long-term LEO exposure, the surface of dehydrated clustered cell layers of *D. radiodurans* deposited on aluminum plates was examined with scanning electron microscopy (SEM). The surface of dehydrated *D. radiodurans* cells showed no detectable damage and preserved its integrity after LEO exposure. However, SEM observations revealed the accumulation of multiple nano-sized particles over the surface of LEO-returned cells (Fig. 1b-f and Supplementary Fig. 1 and 2). These spherical-like morphologies were not represented on the surface of ground control cells and could be attributed to the results of direct influence of outer space parameters on biological material, *e.g.*, Maillard reactions [24, 32].

As survival of space-returned cells was confirmed (Fig. 1a) successfully, a recovery time of 2 h was chosen to see the early response after exposure to harsh outer space conditions. Subsequently, the morphology of space-returned dehydrated and ground control cells of *D. radiodurans* was inspected upon recovery in a liquid complex medium (Fig. 2, Supplementary Fig. 3). The observed typical morphology of diplococci and tetracocci of *D. radiodurans* is shown in Fig. 2 and Supplementary Fig. 3. Compared to the ground control, cells in early stages of recovery after space exposure were characterized by cell surface-associated vesicular structures (Fig. 1c, e and Supplementary Fig. 3a). Investigations with transmission electron microscopy (TEM) confirmed this observation. TEM qualitative observations of space-returned cells of *D. radiodurans* revealed pronounced outer membrane-associated events with numerous vesicles accumulated around the cell surface (Fig. 2d and Supplementary Fig. 3b). To obtain a comprehensive perspective of molecular changes, induced by LEO exposure we further examined the transcriptome, exoproteome, intracellular proteome, and metabolome responses of space-returned *D. radiodurans*.

Outer space driven proteomic alterations

Data processing with Maxquant identified 325 proteins in the extracellular compartment in each replicate. According to p-values (below 0.05), 8 proteins were higher abundant in the extracellular milieu of space exposed cells and 3 proteins were less abundant (Supplementary Fig. 4a). Out of the 8 proteins which were present in all replicates and identified as more abundant in the space exposed cells, one S-layer array related protein (p-value 0.0487) and one hemin transporter (p-value 0.0332) were

identified. The Maxquant data processing resulted in 1828 protein hits for the intracellular compartment throughout all replicates (59 % of the whole *D. radiodurans* proteome). First, a quantitative comparison between controls and space-exposed cells was performed. For that purpose, only proteins which were identified in all replicates of both conditions were compared. The intensities for these 1170 remaining proteins were scaled and used as loadings for a principle component analysis. Supplementary Fig. 4c shows a clear separation for the two conditions on PC1 level, which explained 40.82 % of the variance in the dataset. The five most influential loadings for PC levels are indicated as black arrows. Welch's t-tests were performed to identify proteins with a higher abundance in the control and the space-exposed cells. These resulted in 226 proteins (19.3 %) that were identified in all replicates and differentially expressed (p-value below 0.05) between the conditions, with 153 being more abundant after LEO exposure and 73 more abundant in the control cells (Supplementary Fig. 4b). To cluster these proteins in molecular function categories, the online tool of the Gene Ontology Consortium, Panther (V 13.1), was used (Supplementary Fig. 5a). 79 hits were found for proteins, which were higher abundant after the outer space exposure, whereas 48 hits were found for the proteins which were less abundant. The relative distribution between the categories differed between the two groups. Proteins with catalytic activity and transporter activity were more abundant after exposure, while proteins with binding abilities became less abundant (Supplementary Fig. 5a).

D. radiodurans **transcriptional response**

We subsequently compared the transcriptome changes in response to outer space. Out of 3099 identified mRNAs, 146 were more abundant in cells which were exposed to outer space and 142 were less abundant after the outer space exposure (Supplementary Fig. 4d). $\log_2$ fold changes $\frac{\mathrm{ISS}}{\mathrm{Ctrl}}$ reached from $-4<x<2$. For further interpretations, all mRNAs with a p-value below 0.05 were manually categorized (Supplementary Fig. 5b). The number of mRNAs which code for proteins connected to resistance/repair and metabolic processes was considerably higher in the outer space exposed cells. However, mRNAs which code for proteins that are associated with replication were only identified as higher abundant in ground control cells. Most of these proteins were transposases, which are important for gene reshuffling.

D. radiodurans **metabolomic response**

Primary metabolites were measured after recovery of LEO exposed cells and control cells. Results of the targeted analysis are presented in Fig. 4a, 4b and Supplementary Table 4. In general, most metabolites were higher abundant in controls compared to cells after LEO exposure (Fig. 4a). TCA cycle related metabolites, *e.g.*, pyruvic acid, succinic acid, and fumaric acid were also higher abundant in control cells. Amino acids showed a similar pattern throughout the samples. Stress molecule polyamine putrescine is the only metabolite more represented after LEO exposure compared to the control

(Fig. 4b). In case of the untargeted GC metabolomics approach, 68 peaks were annotated by one of the three databases used (Supplementary Table 5). Untargeted analysis revealed, that sugars identified via databases were less presented in LEO exposed cells upon recovery (Fig. 4c).

Discussion

The current findings based on integrated -omics with EM-assisted analysis provide a better understanding of molecular mechanisms of the complex rewiring which cells experience on early stages of recovery from the outer space environment. In line with the extreme multiple resistance of *D. radiodurans*, there was no detectable damage of cell surface and morphology of *D. radiodurans* observed after LEO exposure (Fig. 1). On a molecular scale, effects of the LEO environment are mirrored in several layers. Transcriptomic, proteomic, and metabolomic networking of space exposed cells focuses on repair mechanisms and metabolizing exogenous resources on early stages of recovery after LEO exposure. Although responses on different -omics levels intertwine, stress induced changes in the transcriptome and metabolome might appear quicker compared to the proteome. Due to limited, precious space returned material, provided results give a snapshot impression after 2 h of recovery in complex medium.

Proteometabolic rearrangements as response to the outer space environment

The overall amount of free amino acids, organic acids (TCA cycle intermediates) and sugars is decreased in exposed cells. Although limited under space-simulated conditions, previous studies have shown a similar tendency after exposure of *D. radiodurans* to UVC radiation combined with vacuum and solely vacuum [23, 24]. Our untargeted metabolomics approach indicated a reduced level of sugars during repair after LEO exposure (Fig. 4c). Sugars can be used as carbon and energy source, which can be utilized by various repair mechanisms, for instance repair of damaged nucleic acids. The organotrophic bacteria *D. radiodurans* possess proteolytic properties for protein degradation and amino acid catabolism. In 2010, Daly et al. [28] showed an induction of proteolytic activity following ionizing radiation. The proteomics dataset after LEO exposure supports this assumption. Based on proteins, identified in all replicates, nine proteases were found higher abundant after LEO exposure, two of them significantly increased (Supplementary Fig. 4e). Amino acids are used as nitrogen and carbon source, which can further be utilized for any metabolic processes, *e.g.*, growth and nucleic acid repair. As *D. radiodurans* is not able to utilize ammonia as nitrogen source [33], it completely relies on exogenous amino acids, and this elevated pull of proteases presumably aims to degrade damaged proteins in order to deliver more amino acids during recovery from LEO exposure. The relative amount of amino acids (Fig. 4a) shows most difference between ground control and space exposed cells for glutamine and glutamic acid, as more of these amino acids are needed for nucleic acid repair mechanisms after space exposure [33]. Glutamine and glutamate certainly play

an important role in growth, but in case of alleviation from stress, which they received from outer space exposure, results indicate that they might be utilized as intermediates for repair processes. Similar results were observed after exposure to simulated LEO conditions (Supplementary Fig. 6). Simulated LEO conditions likewise reduced the abundance of most amino acids and organic acids throughout the early repair stage, with glutamine and glutamate showing a strong response.

How to cope with space induced DNA damage?

D. radiodurans does not possess any special mechanisms that prevents it from nucleic acid damage, such as double strand breaks after ionizing gamma irradiation and the amount of radiation induced double strand breaks is fairly similar between *E. coli* and *D. radiodurans* [34]. Preliminary studies [21, 35] showed that dehydrated *D. radiodurans* cells are damaged by vacuum and temperature cycles in the LEO. However, UV radiation (100 to 280 nm) is most problematic for survival of *D. radiodurans* cells. On Earth, harmful solar UVC radiation is not dangerous to biota, because of an oxygen and ozone shield in the Earth's atmosphere. LEO, however, does not provide such defense possibilities. Therefore, dried cells outside the ISS were protected from most deleterious UVC radiation below 200 nm by a SiO_2 glass window. Apart from breaks in the DNA, UV radiation can cause bipyrimidine photoproducts, which later lead to mutations as they interfere with DNA replication [36]. Usually, UV induced lesions can be repaired by photoreactivation, which uses energy from visible light to enzymatically remove pyrimidine dimers [37]. However, as the necessary enzyme is not present in *D. radiodurans* [38], damages, caused by solar UV radiation on dehydrated *D. radiodurans* cells are repaired by nucleotide excision repair. Truglio et al [30] already recognized the flexibility of the UvrABC excision repair mechanisms in prokaryotes, which is very well suited to repair damage caused by UV radiation. Our proteomics data (Supplementary Table 2) shows that all key proteins involved in the UvrABC mechanism, which are responsible for detecting lesions and cutting DNA are more abundant after exposure (Fig. 3b). Apparently, there are two UvrA proteins in *D. radiodurans*, which transport UvrB to the damaged DNA. Usually, the intracellular concentration of UvrB is much higher than UvrA, as one UvrA dimer can transport multiple UvrB proteins onto different damage sites [30]. The second UvrA protein might accelerate transport it increases the specificity to identify target regions. Apart from both UvrA proteins (both p-value 0.0065), also UvrB (p-value 0.0100) and UvrC (p-value 0.0141), which are responsible to cut out a twelve or thirteen-mer with the DNA lesion were higher abundant after space exposure (Fig. 3b). Finally, the transcription repair coupling factor Mfd (p-value 0.0075) showed an increase in abundance after LEO exposure. In bacteria, Mfd scans DNA to find RNA polymerases which are blocked by strand lesions. Upon detection, it mediates the release of the stalled RNA polymerase and recruits the nucleotide excision repair machinery to the damaged site [39]. Furthermore, both simulated LEO exposure and real LEO exposure showed an increased abundance of proteins involved in pyrimidine biosynthesis, which most certainly plays a role in response to these stresses (Fig. 5).

The stress molecular reactions after outer space exposure

Exposure to extreme environmental conditions, as those present in LEO, is very deleterious to nucleic acids due to surplus ROS which are produced upon oxidative stress damage. If the UvrABC mechanism proteins are still intact, space induced DNA damage can be repaired while cells seem to be still in the metabolically not very active state. On transcripts and proteins level, (Fig. 3a) we observed an increase of transporter proteins after space exposure. Obviously, LEO exposed cells experience needs in external nutrients (*e.g.*, amino acids, sugars, and metal cofactors), which are necessary for repair processes. At the same time, EM-based observations of *D. radiodurans* recovering after outer space exposure revealed intensive vesicle trafficking associated with the outer membranes (Fig. 2). According to our proteotranscriptomic analysis, such intensified membrane-associated trafficking reflects elevated levels of membrane-bound molecular machinery of LEO exposed *D. radiodurans* (Fig. 3a). Intensified vesiculation after recovery from LEO exposure can serve as a quick stress response, which augments cell survival by withdrawing stress products (*e.g.*, damaged or misfolded proteins). Additionally, outer membrane vesicles may contain proteins important for nutrient acquisition, DNA transfer, transport of toxins and quorum sensing molecules, eliciting the activation of resistance mechanisms after space exposure.

The ground control cells, which did not suffer exposure damage, showed an increase of transcripts, which code for replication proteins and transposases (Supplementary Fig. 5b). Control cells are already closer to the exponential phase than the space exposed cells. Proteomics data supports this assumption, as ribosomal proteins and proteins involved in folding of new proteins were higher abundant in the control cells, to enable upcoming cell replication. Three ribosomal proteins (RlmN, RpmC and RtcB) were exclusively found in all three control replicates (Supplementary Table 1).

One cause of the extreme resistance of *D. radiodurans* against radiation and oxidative damage is based on the high levels of constitutively expressed catalase activity and superoxide dismutase activity [40]. These enzymatic systems are devoted to the protection of cells against toxic reactive oxygen species. Our transcriptomic analysis revealed that genes coding oxidative resistance proteins, such as the catalases *katA* (p-value <0.0001) and catalase *DR_A0259* (p-value <0.0001), are more abundant in LEO exposed cells (Supplementary Table 3). Previous proteomics studies showed an overrepresentation of proteins involved in the oxidative defense system after ionizing radiation was applied [41, 42]. Obviously, upon recovery from LEO exposure, *D. radiodurans* realized a potential oxidative threat and prioritized transcribing oxidation response proteins. The gene *PdxT* and its protein product “redox active pyridoxal 5’-phosphate synthase” are both overrepresented after LEO exposure in our proteotranscriptomic analysis (Fig. 4d). We previously reported the elevated expression of the pyridoxine biosynthesis proteins in *D. radiodurans* in response to space-related stress stimulus (UVC and vacuum) [23]. The enzymes of pyridoxal 5'-phosphate

317 biosynthesis are singlet oxygen resistance proteins involved in the synthesis of vitamin
318 B_6, an efficient singlet oxygen quencher and a potential antioxidant [43].
319 Apart from that, polyamines (*e.g.*, putrescine) as primordial form of stress molecules
320 [44] have been represented in metabolomic response of LEO exposed cells (Fig. 4b).
321 Additionally, the proteomic and transcriptomic analysis revealed several genes and
322 their products of putrescine biosynthesis are more abundantly represented in LEO
323 exposed cells (Fig. 4d). Furthermore, several proteins involved in this pathway were
324 also identified as higher abundant after simulated LEO conditions (Fig. 5). This
325 indicates that polyamines are used as general stress response molecules during recovery
326 of *D. radiodurans* from space exposure.
327 In summary, these data provide molecular evidence for a multifaceted response of
328 *D. radiodurans* after LEO exposure (Fig. 6) by combining proteomic, transcriptomic
329 and metabolomic analysis with electron microscopy tools. Ultimately, survival of *D.*
330 *radiodurans* in outer space for a longer period is possible due to its efficient molecular
331 response system. This certainly spreads hope that even longer, farther journeys are
332 possible for organisms with such capabilities.
333

334 **Experimental Design**

335 **Tanpopo Mission exposure**

336 For this space exposure experiment, *Deinococcus radiodurans* cells were placed inside
337 wells of aluminium plates until 1 µm of cell layers was reached, desiccated and
338 positioned inside an exposure panel designed by the Tanpopo space mission team [31].
339 SiO_2 filters were put on top of the plates to cut off harmful UV radiation shorter than
340 200 nm. The exposure panels were on board the SpaceX Dragon commercial cargo
341 spaceship, which launched on April 15th, 2015 from Cape Canaveral (USA) by the
342 Space-X Falcon-9 rocket. They were manually attached to the exposed experiment
343 handrail attachment mechanism (ExHAM) on the Japanese exposure facility of the
344 international space station, which was transferred to its final position on May 26th,
345 2015. For one year, samples were exposed to a total UV fluence of $3.1*10^3$ kJ/m^2 (200-
346 315 nm), total cosmic radiation of 250-298 mGy, temperature fluctuations between –
347 21.0 ± 5 °C and 23.9 ± 5 °C, pressure between 10^{-7} Pa and 10^{-4} Pa and 0 % humidity.
348 Space exposed (LEO) cells returned after one year on August 26th, 2016 on board the
349 SpaceX Dragon C11, which landed in the Pacific Ocean. Ground control cells (Ground
350 Control) were prepared simultaneously and stored in a desiccator during all exposure
351 time.

352 **Exposure to simulated LEO environmental factors**

353 *D. radiodurans* cells deposited in dried form on Tanpopo exposure plates as for the
354 mission were exposed in PSI 5 of the Astrobiology Space Simulation facilities at DLR
355 Cologne equipped with an UV-transparent SiO_2 window and a temperature control
356 plate to a final pressure of 8.7 s 10-5 Pa for 90 days (Sim_exp). Samples were in parallel
357 exposed to UV radiation of 3.4 x 103 kJm^{-2} with wavelengths > 200 nm from a solar
358 simulator SOL2 (Dr. Hönle GmbH) and temperature cycles from -21 °C to +24 °C by
359 an attached cryostat (Lauda). Unexposed simulation controls (Sim_ctrl) were stored
360 inside a desiccator with silica gel at 21 °C.

361 **Survival Assays**

362 To evaluate survivability of the LEO returned cells, colony forming units were counted
363 on plates and compared to the growth of ground control cells. After exposure,
364 dehydrated cells were recovered from the aluminium plate wells by resuspending the
365 cell pellet in sterile phosphate buffer. Suspensions were serially diluted in phosphate
366 buffer and dropped onto TGB (1 % tryptone, 0.2 % glucose, 0.6 % beef extract) plates.
367 Colonies were counted after incubation at 30 °C for 36 h. Surviving cell fractions were
368 determined as N/N_0, where N was the number of colony-forming units remaining after
369 space exposure or one year in the desiccator (ground control) and N_0 was that at the
370 time of cell preparation one year before.

371 **Recovery and Growth Conditions**

372 In total, three biological replicates of LEO exposed cells and ground control cells were
373 recovered and used for –omics analysis. In case of the simulation experiment, four
374 replicates from the exposed (Sim_exp) and control cells (Sim_ctrl) were used for

375 analysis. For each biological replicate, the content of two wells with *D. radiodurans*
376 cells (each 1500 µm thickness of cell layer) was resuspended in 30 mL of TGB
377 medium. OD_{600} was measured for each replicate before incubating the cells at 30 °C,
378 150 rpm for 2 h. After 2 h of incubation, OD_{600} was measured again (Fig. 1) and cells
379 were harvested by centrifugation at 3000 g/2 min/4 °C. The supernatant was kept for a
380 subsequent analysis of proteins, present in the extracellular compartment. For washing,
381 the pellets were resuspended in 20 mL sterile, ice cold PBS and centrifuged at
382 3000 g/2 min/4 °C. The supernatant was discarded, pellets were resuspended in 1.5 mL
383 ice cold PBS and centrifuged at 1500 g/2 min/4 °C. For the final washing step, 1.5 mL
384 PBS was added and the cells suspension was separated in a 500 µL aliquot for
385 metabolite extraction and a 1000 µL aliquot for protein and RNA extraction. After
386 centrifugation at 1500 g/2 min/4 °C, the supernatant was discarded, cells were snap
387 frozen in liquid nitrogen and stored at -80 °C overnight until further extraction.

388 **Integrative Extraction and Measurements of biological Molecules**

389 **Polar Metabolite Extraction**

390 Cells from each well were resuspended in 1 mL methanol/chloroform/water (2.5:1:0.5)
391 and ceramic beads were added to the samples. Homogenization was performed with a
392 MagNA-Lyser (Roche) five times at 7000 rpm/30 s with cooling in between the cycles.
393 Samples were incubated 15 min on ice, centrifuged at 21000 g/6 min/RT and the
394 supernatant was transferred into new tubes. 200 µL of water were added to the
395 supernatant to achieve a phase separation. Samples were centrifuged at
396 10000 g/5 min/RT and the upper, polar phase was transferred into a new tube. The polar
397 phase was put under constant nitrogen steam at 35 °C until total dryness. Samples were
398 frozen at -20 °C until further analysis.
399

400 **Analysis of polar metabolites with GC-TOF**

401 Before injection to the GC-TOF, samples were derivatized. Derivatization, further
402 sample preparation steps and instruments for measurements were described previously
403 [24].
404 Identifications of metabolites were based on comparing the obtained spectra to those of
405 a standard mix, containing several important primary metabolites with ChromaTOF.
406 For quantification, a high abundant, preferably unique mass for each metabolite was
407 selected and the peak on the chromatogram was integrated. Areas were normalized to
408 OD_{600} values which were measured before the extraction.
409

410 **RNA Extraction**

411 For extraction of RNA, cells were resuspended in 1000µl QIAzol together with ceramic
412 beads (Roche, Basel, Switzerland; MagNA Lyser Green Beads, CatNo 03358941001).
413 Homogenization was performed as described above. After cell disruption, samples
414 were incubated for 5 min at RT on a rotating wheel and afterwards 200 µL chloroform
415 were added to each sample. After 3 min incubation at RT on the rotating wheel, samples
416 were centrifuged at 21000 g/15 min/4 °C to separate phases. The upper, polar phase
417 with the RNA was transferred into a new tube for purification with the RNeasy Lipid
418 Tissue kit according to the manufacturer´s manual including DNAse treatment.

Samples were eluted with 20 µL TE buffer (pH 8) and stored at -80 °C until further analysis.

Analysis of mRNA with Illumina HiSeq

The purified, total RNA was quantified on a NanoDrop (Supplementary Fig. 4) and subsequently measured on a Bioanalyzer BA2100 (Agilent; Foster City, CA, USA) to calculate RNA integrity numbers. rRNA was depleted with the MICROBExpress™ Bacterial mRNA Enrichment Kit using 100 ng total RNA as input, followed by library construction with the NEB® Ultra™ RNA Library Prep Kit for Illumina according to the manufacturers' manuals. The samples were measured on an Illumina HiSeq instrument at the Vienna BioCenter Core Facilities (VBCF). Resulting bam-files were converted to fastq with bedtools2 [45] and mapped via bowtie2 [46] on the updated genome of *D. radiodurans* type strain R1 [47]. Annotation according to the reference genome and differential gene expression calculations were done with CUFFDIFF [48].

Intracellular Protein Extraction

After QIAzol extraction and phase separation, the lower, phenolic phase was transferred into a fresh tube for protein purification. To further wash the samples, 550 µL of H_2O were added to the samples, centrifuged at 10000 g/5 min/RT and the lower phase was transferred into a new tube. To precipitate the proteins, 1.5 mL of 0.1 M NH_4Ac in MeOH (with 0.5 % 2-mercaptoethanol) were added and the samples were put at -20 °C overnight. On the following day, proteins were centrifuged at 10000 g/15 min/4 °C. The pellets were subsequently washed three times with acetone and centrifuged at 10000 g/5 min/4 °C. After the final washing step, pellets were air dried and stored at -20 °C until further analysis.

Extracellular Protein Extraction

To identify proteins represented in the extracellular milieu, the extracellular medium/supernatant of each replicate was analyzed. To remove remaining bacteria first, the supernatant samples were filtered through a 0.22 µm membrane (Watson). To each sample, trichloroacetic acid was added to a final concentration of 10 % (V/V) and samples were incubated on ice for 2 h. To pellet the proteins, samples were centrifuged at 38000 g/30 min/4 °C and the supernatant was discarded. Pellets were washed three times with acetone and centrifuged at 10000 g/5 min/4 °C. After the final washing step, pellets were air dried and stored at -20 °C until further analysis.

Analysis of Proteins with LC-Orbitrap

Further sample preparation steps as digestion, peptide purification and peptide desalting were performed as described previously [23]. For the intracellular compartment, peptides were normalized with a fluorometric peptide assay (Pierce). LCMS analyses were performed using a single-shot LCMS approach with 120 min gradient using a Dionex Ultimate 3000 system (Thermo Fisher Scientific) coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Germany) with LCMS parameters as described previously [49].
Data analysis was performed with Maxquant [50] with following settings: min 7 amino acid mass for peptide identification, min 1 unique peptide for protein identification,

label free quantification (LFQ), max missed cleavages 2, max number of modifications per peptide 5, cuts after proline are allowed.

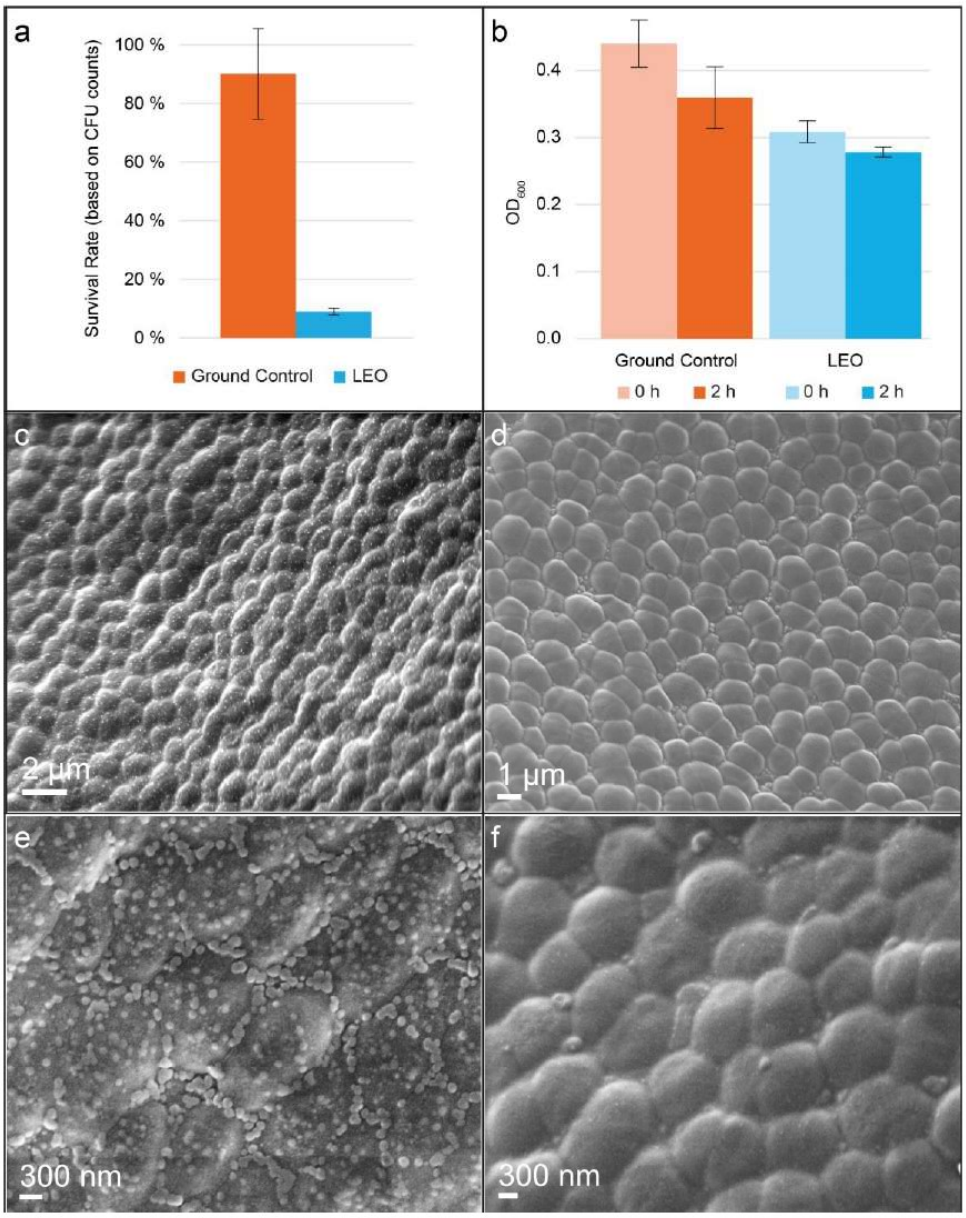
SEM/TEM Analysis

The morphology and cellular integrity of the dehydrated cells of *D. radiodurans* deposited on aluminum plates were examined with a Zeiss Supra 55 VP scanning electron microscope. The dehydrated cells were coated with a thin Au/Pd layer (Laurell WS-650-23 spin coater). The imaging of dehydrated clustered cell layers was performed with an acceleration voltage of 5 kV. For electron microscopy analyses of recovered cells after LEO exposure, *D. radiodurans* cells were sampled at 0 and 120 minutes after recovery, washed three times in PHEM-buffer (360mM PIPES, 150mM HEPES, 60mM EGTA, 12mM MgCl) and subsequently fixed in the same buffer containing 2.5% glutaraldehyde. After the second wash cycle post-fixation was carried out using 1% OsO_4 in H_2O and pellets were dehydrated in a graded series of ethanol. For transmission electron microscopy (TEM), pellets were transferred to dried acetone for subsequent infiltration and embedding in epoxy resin (Agar Scientific, Low Viscosity Resin Kit). Ultrathin sections (50-70 nm) were mounted on TEM support grids (Agar Scientific, copper, 200 mesh hex) coated with formvar film, stained with gadolinium triacetate [51] and lead citrate [52], and finally examined in a Zeiss Libra 120. For scanning electron analysis (SEM), samples were treated equally (washed, fixed, EtOH dehydrated) until TEM samples were transferred into dried acetone. Pellets for SEM examination were spread on filters (Whatman, 13mm, 0.2µm pore size), dried via critical point drying (Leica EM CPD 300), mounted on stubs and coated with gold (JEOL JFC 2300 HR). Samples were viewed with a JEOL IT 300.

Quantification and Statistical Analysis

Statistical evaluation of transcriptomics data was based on fold changes and t test results calculated by CUFFDIFF. Relative comparison of proteomics data was based on the LFQ intensities calculated by Maxquant. To compare exposed and control samples, fold changes were calculated ($mean_{exp}/mean_{ctrl}$) and Welch`s t tests were performed. To compare metabolomics data, the peak areas calculated by ChromaTOF were normalized to $OD_{600}$ values of the corresponding sample. Afterwards, fold changes and p-values (Welch`s t test) for all identified metabolites were calculated.

496



497

498 Fig. 1. **Survival and post-exposure analysis.**
499 (a) Survival rate of LEO exposed and ground control cells based on colony forming units counting. (b)
500 OD_{600} measurements of exposed and control cells at t_0 and after two hours of recovery in complex
501 medium. (c-f) Scanning electron microscopy (SEM) images showing upper surface of multilayers of
502 dehydrated cells of *D. radiodurans* deposited on aluminum plates. (c, e) SEM images of *D. radiodurans*
503 cells exposed to LEO in Tanpopo mission. (d, f) SEM images of ground control cells of *D. radiodurans*.
504 (e, f) Higher magnification SEM images displaying upper surface of multilayers of dehydrated cells of
505 *D. radiodurans*.

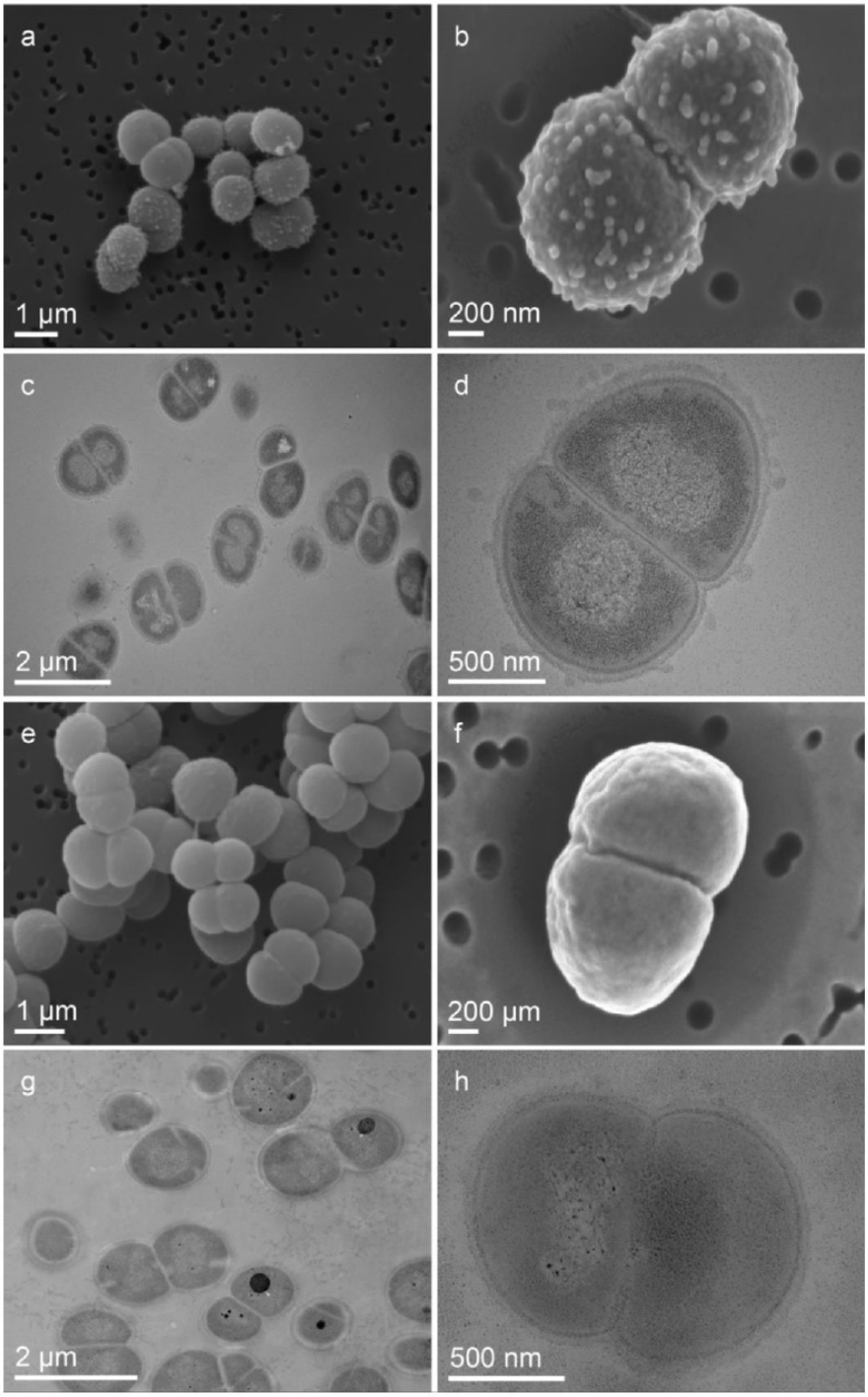


Fig. 2. **Scanning and transmission electron microscopy (SEM and TEM) images of *D. radiodurans* cells recovered after LEO exposure in complex medium.**
(a, b) SEM images of recovered *D. radiodurans* cells after LEO exposure. (c, d) TEM images of recovered *D. radiodurans* cells after LEO exposure. (e, f) SEM images of ground control *D. radiodurans* cells. (g, h) TEM images of ground control *D. radiodurans* cells.

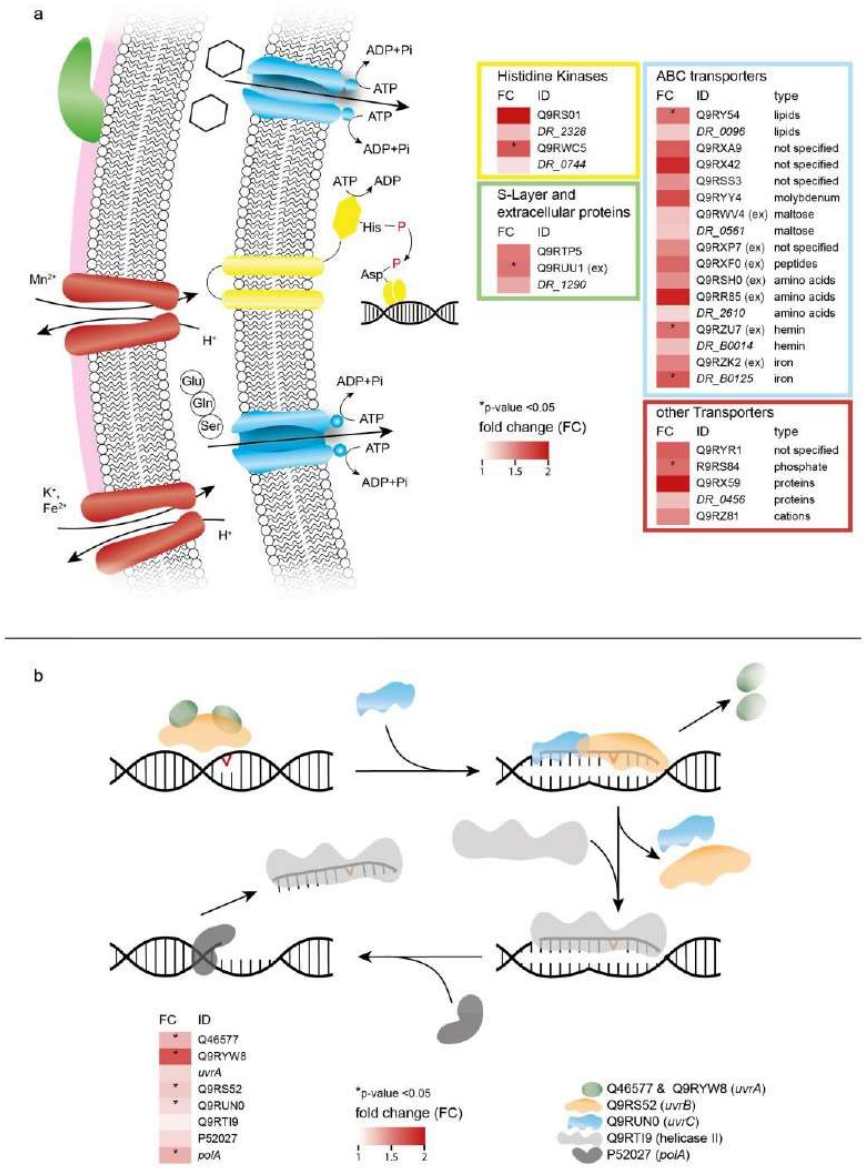


Fig. 3. **Proteotranscriptomic rearrangements as response to the outer space environment**
(a) Membrane associated proteins/Transporters/Extracellular proteins which were identified higher abundant after LEO exposure in *D. radiodurans*. Proteins identified through intracellular and extracellular (ex) proteomics measurements are indicated as Uniprot IDs, transcriptomics data is shown as *Locus tags*. (b) Quantitative UvrABC nucleotide excision repair mechanism data in *D. radiodurans* after exposure to LEO. Intracellular proteomics data is shown as Uniprot IDs, transcriptomics data as *gene names*.

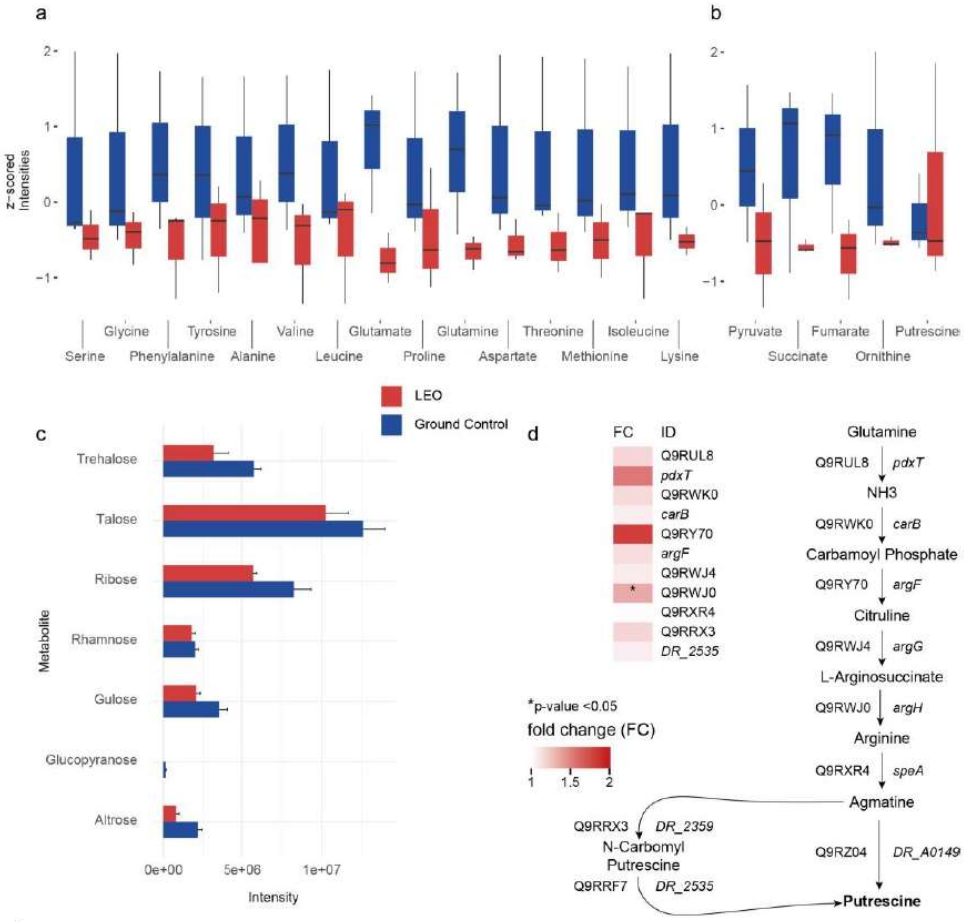


Fig. 4. **Metabolic response after LEO exposure**
(a-b) Results of the targeted metabolomics approach, illustrating amino acids (a) and organic acids and polyamine (precursors) (b). (c) Abundances of sugars identified via untargeted metabolomics approach. (d) Proteomics and transcriptomics data for the biosynthesis pathway from glutamine to putrescine. Proteins are indicated as Uniprot IDs and mRNAs are shown as *gene names* or *Locus tags*.

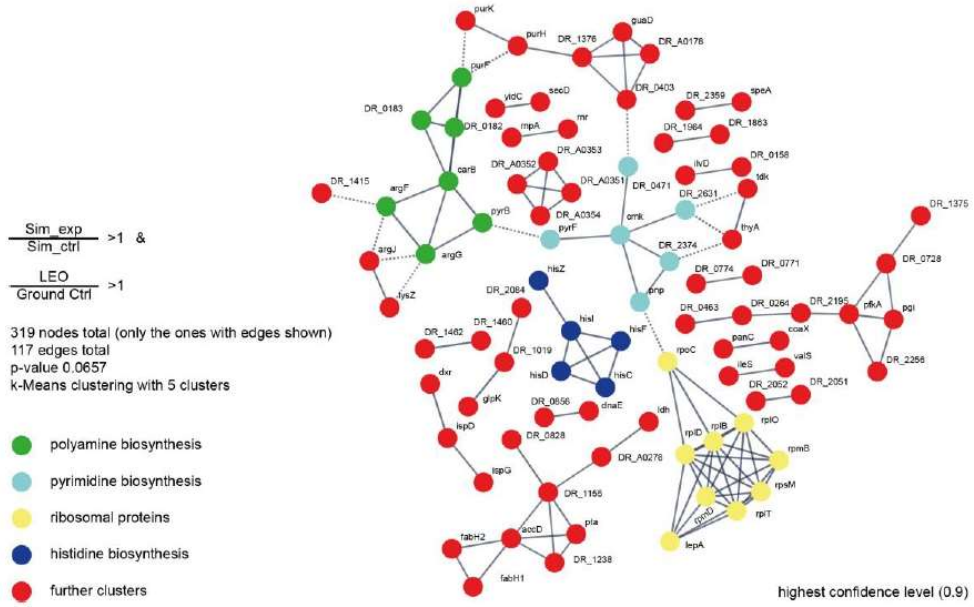


Fig. 5. **Comparative protein-protein network analysis.**
Proteins of *D. radiodurans* which were higher abundant after LEO exposure compared to ground control and proteins which were higher abundant after exposure to simulated LEO conditions compared to corresponding non-exposed control chosen for the network. This list was uploaded to the STRING database. Network construction was performed at highest confidence level (0.9) with k-Means clustering containing five clusters. Annotation was performed according to proteins present in the clusters.

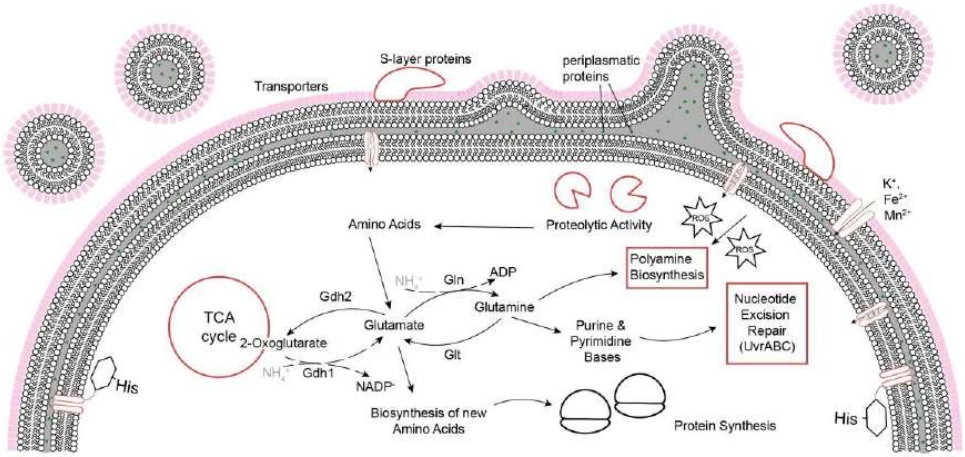


Fig. 6. **Multifaceted response of *D. radiodurans* after LEO exposure**
Molecular response of *D. radiodurans* after exposure to LEO conditions. Recovered after LEO, *D. radiodurans* cells accumulate transporters, show increased abundance of proteases, nucleotide excision repair proteins and increased polyamine biosynthesis.

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Author contributions

E.O., Y.K., D.K., E.R., and M.M. performed experiments. All authors provided editorial input. All authors made substantial contributions to the acquisition, analysis, and interpretation of data described in this article. All authors critically reviewed the report and approved the final version.

Competing financial interests

The authors declare no competing interests.

1. Wassmann M, Moeller R, Rabbow E, Panitz C, Horneck G, Reitz G, et al. Survival of spores of the UV-resistant Bacillus subtilis strain MW01 after exposure to low-earth orbit and simulated martian conditions: data from the space experiment ADAPT on EXPOSE-E. Astrobiology. 2012;12(5):498-507.
2. Cockell CS, Rettberg P, Rabbow E, Olsson-Francis K. Exposure of phototrophs to 548 days in low Earth orbit: microbial selection pressures in outer space and on early earth. The ISME journal. 2011;5(10):1671-82.
3. Sancho LG, de la Torre R, Horneck G, Ascaso C, de Los Rios A, Pintado A, et al. Lichens survive in space: results from the 2005 LICHENS experiment. Astrobiology. 2007;7(3):443-54.
4. Selbmann L, Zucconi L, Isola D, Onofri S. Rock black fungi: excellence in the extremes, from the Antarctic to space. Current genetics. 2015;61(3):335-45.
5. Saffary R, Nandakumar R, Spencer D, Robb FT, Davila JM, Swartz M, et al. Microbial survival of space vacuum and extreme ultraviolet irradiation: strain isolation and analysis during a rocket flight. FEMS Microbiology Letters. 2002;215(1):163–8.
6. Panitz C, Horneck G, Rabbow E, Rettberg P, Moeller R, Cadet J, et al. The SPORES experiment of the EXPOSE-R mission: Bacillus subtilis spores in artificial meteorites. International Journal of Astrobiology. 2015;14(1):105-14.
7. Rabbow E, Rettberg P, Parpart A, Panitz C, Schulte W, Molter F, et al. EXPOSE-R2: The Astrobiological ESA Mission on Board of the International Space Station. Frontiers in microbiology. 2017;8:1533-.
8. Chiang AJ, Malli Mohan GB, Singh NK, Vaishampayan PA, Kalkum M, Venkateswaran K. Alteration of Proteomes in First-Generation Cultures of Bacillus pumilus Spores Exposed to Outer Space. mSystems. 2019;4(4).
9. Nicholson WL, Moeller R, Horneck G. Transcriptomic responses of germinating Bacillus subtilis spores exposed to 1.5 years of space and simulated martian conditions on the EXPOSE-E experiment PROTECT. Astrobiology. 2012;12(5):469–86.
10. Vaishampayan PA, Rabbow E, Horneck G, Venkateswaran KJ. Survival of Bacillus pumilus spores for a prolonged period of time in real space conditions. Astrobiology. 2012;12(5):487-97.
11. Onofri S, de la Torre R, de Vera JP, Ott S, Zucconi L, Selbmann L, et al. Survival of rock-colonizing organisms after 1.5 years in outer space. Astrobiology. 2012;12(5):508–16.
12. Neuberger K, Lux-Endrich A, Panitz C, Horneck G. Survival of Spores of Trichoderma longibrachiatum in Space: data from the Space Experiment SPORES on EXPOSE-R. International Journal of Astrobiology. 2014;14(01):129–35.
13. Panitz C, Frosler J, Wingender J, Flemming HC, Rettberg P. Tolerances of Deinococcus geothermalis Biofilms and Planktonic Cells Exposed to Space and Simulated Martian Conditions in Low Earth Orbit for Almost Two Years. Astrobiology. 2019;19(8):979-94.
14. Li J, Liu F, Wang Q, Ge P, Woo PC, Yan J, et al. Genomic and transcriptomic analysis of NDM-1 Klebsiella pneumoniae in spaceflight reveal mechanisms underlying environmental adaptability. Scientific reports. 2014;4:6216.
15. Mastroleo F, Van Houdt R, Leroy B, Benotmane MA, Janssen A, Mergeay M, et al. Experimental design and environmental parameters affect Rhodospirillum rubrum S1H response to space flight. The ISME journal. 2009;3(12):1402-19.

16. Wilson JW, Ott CM, Honer zu Bentrup K, Ramamurthy R, Quick L, Porwollik S, et al. Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. Proc Natl Acad Sci U S A. 2007;104(41):16299-304.
17. Slade D, Radman M. Oxidative stress resistance in Deinococcus radiodurans. Microbiol Mol Biol Rev. 2011;75(1):133–91.
18. Potts M. Desiccation tolerance of prokaryotes. Microbiol Rev. 1994;58(4):755-805.
19. Krisko A, Radman M. Biology of extreme radiation resistance: the way of Deinococcus radiodurans. Cold Spring Harbor perspectives in biology. 2013;5(7).
20. Leuko S, Bohmeier M, Hanke F, Böettger U, Rabbow E, Parpart A, et al. On the Stability of Deinoxanthin Exposed to Mars Conditions during a Long-Term Space Mission and Implications for Biomarker Detection on Other Planets. Frontiers in microbiology. 2017;8:1680-.
21. Kawaguchi Y, Yang Y, Kawashiri N, Shiraishi K, Takasu M, Narumi I, et al. The possible interplanetary transfer of microbes: assessing the viability of Deinococcus spp. under the ISS Environmental conditions for performing exposure experiments of microbes in the Tanpopo mission. Orig Life Evol Biosph. 2013;43(4-5):411–28.
22. Kawaguchi Y, Yokobori S, Hashimoto H, Yano H, Tabata M, Kawai H, et al. Investigation of the Interplanetary Transfer of Microbes in the Tanpopo Mission at the Exposed Facility of the International Space Station. Astrobiology. 2016;16(5):363–76.
23. Ott E, Kawaguchi Y, Kölbl D, Chaturvedi P, Nakagawa K, Yamagishi A, et al. Proteometabolomic response of Deinococcus radiodurans exposed to UVC and vacuum conditions: Initial studies prior to the Tanpopo space mission. PLOS ONE. 2017;12(12):e0189381.
24. Ott E, Kawaguchi Y, Özgen N, Yamagishi A, Rabbow E, Rettberg P, et al. Proteomic and Metabolomic Profiling of Deinococcus radiodurans Recovering After Exposure to Simulated Low Earth Orbit Vacuum Conditions. Frontiers in Microbiology. 2019;10(909).
25. Cox MM, Keck JL, Battista JR. Rising from the Ashes: DNA Repair in Deinococcus radiodurans. PLoS Genet. 2010;6(1):e1000815.
26. Krisko A, Radman M. Protein damage and death by radiation in Escherichia coli and Deinococcus radiodurans. Proc Natl Acad Sci U S A. 2010;107(32):14373–7.
27. Levin-Zaidman S, Englander J, Shimoni E, Sharma AK, Minton KW, Minsky A. Ringlike structure of the Deinococcus radiodurans genome: a key to radioresistance? Science. 2003;299(5604):254–6.
28. Daly MJ, Gaidamakova EK, Matrosova VY, Kiang JG, Fukumoto R, Lee D-Y, et al. Small-Molecule Antioxidant Proteome-Shields in Deinococcus radiodurans. PLOS ONE. 2010;5(9):e12570.
29. Ghosal D, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Venkateswaran A, et al. How radiation kills cells: survival of Deinococcus radiodurans and Shewanella oneidensis under oxidative stress. FEMS Microbiol Rev. 2005;29(2):361–75.
30. Truglio JJ, Croteau DL, Van Houten B, Kisker C. Prokaryotic Nucleotide Excision Repair: The UvrABC System. Chemical Reviews. 2006;106(2):233–52.
31. Yano H, Yamagishi A, Hashimoto H, Yokobori S, Kobayashi K, Yabuta H, et al., editors. Tanpopo Experiment for Astrobiology Exposure and Micrometeoroid

662 Capture Onboard the ISS-JEM Exposed Facility. Lunar and Planetary Science
663 Conference; 2014.
664 32. Horneck G, Klaus DM, Mancinelli RL. Space microbiology. Microbiol Mol
665 Biol Rev. 2010;74(1):121–56.
666 33. Venkateswaran A, McFarlan SC, Ghosal D, Minton KW, Vasilenko A,
667 Makarova K, et al. Physiologic Determinants of Radiation Resistance in
668 Deinococcus radiodurans. Applied and Environmental Microbiology.
669 2000;66(6):2620–6.
670 34. Daly MJ. A new perspective on radiation resistance based on Deinococcus
671 radiodurans. Nature reviews Microbiology. 2009;7(3):237-45.
672 35. Pogoda de la Vega U, Rettberg P, Reitz G. Simulation of the environmental
673 climate conditions on martian surface and its effect on Deinococcus radiodurans.
674 Advances in space research : the official journal of the Committee on Space
675 Research (COSPAR). 2007;40(11):1672–7.
676 36. Rastogi RP, Richa, Kumar A, Tyagi MB, Sinha RP. Molecular mechanisms of
677 ultraviolet radiation-induced DNA damage and repair. J Nucleic Acids.
678 2010;2010:592980.
679 37. Sancar A, Smith FW, Sancar GB. Purification of Escherichia coli DNA
680 photolyase. J Biol Chem. 1984;259(9):6028–32.
681 38. Moseley BE, Evans DM. Isolation and properties of strains of Micrococcus
682 (Deinococcus) radiodurans unable to excise ultraviolet light-induced pyrimidine
683 dimers from DNA: evidence for two excision pathways. Journal of general
684 microbiology. 1983;129(8):2437–45.
685 39. Park J-S, Marr MT, Roberts JW. E. coli Transcription Repair Coupling Factor
686 (Mfd Protein) Rescues Arrested Complexes by Promoting Forward Translocation.
687 Cell. 2002;109(6):757–67.
688 40. Chou FI, Tan ST. Manganese(II) induces cell division and increases in
689 superoxide dismutase and catalase activities in an aging deinococcal culture. J
690 Bacteriol. 1990;172(4):2029-35.
691 41. Zhang C, Wei J, Zheng Z, Ying N, Sheng D, Hua Y. Proteomic analysis of
692 Deinococcus radiodurans recovering from γ-irradiation. PROTEOMICS.
693 2005;5(1):138–43.
694 42. Basu B, Apte SK. Gamma Radiation-induced Proteome of Deinococcus
695 radiodurans Primarily Targets DNA Repair and Oxidative Stress Alleviation.
696 Molecular & Cellular Proteomics : MCP. 2012;11(1):M111.011734.
697 43. Mooney S, Leuendorf J-E, Hendrickson C, Hellmann H. Vitamin B6: A Long
698 Known Compound of Surprising Complexity. Molecules. 2009;14(1):329-51.
699 44. Rhee HJ, Kim EJ, Lee JK. Physiological polyamines: simple primordial stress
700 molecules. J Cell Mol Med. 2007;11(4):685–703.
701 45. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing
702 genomic features. Bioinformatics (Oxford, England). 2010;26(6):841-2.
703 46. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat
704 Methods. 2012;9(4):357-9.
705 47. White O, Eisen JA, Heidelberg JF, Hickey EK, Peterson JD, Dodson RJ, et al.
706 Genome sequence of the radioresistant bacterium Deinococcus radiodurans R1.
707 Science. 1999;286(5444):1571-7.
708 48. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L.
709 Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat
710 Biotechnol. 2013;31(1):46-53.

711 49. Stojanovic T, Orlova M, Sialana FJ, Hoger H, Stuchlik S, Milenkovic I, et al.
712 Validation of dopamine receptor DRD1 and DRD2 antibodies using receptor
713 deficient mice. Amino acids. 2017;49(6):1101–9.
714 50. Cox J, Mann M. MaxQuant enables high peptide identification rates,
715 individualized p.p.b.-range mass accuracies and proteome-wide protein
716 quantification. Nature Biotechnology. 2008;26:1367.
717 51. Nakakoshi M, Nishioka H, Katayama E. New versatile staining reagents for
718 biological transmission electron microscopy that substitute for uranyl acetate.
719 Journal of electron microscopy. 2011;60(6):401–7.
720 52. Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain
721 in electron microscopy. The Journal of cell biology. 1963;17:208–12.
722
723

5 References

Anderson, A.O., Nordon, Hc, Cain, Ronald B., Parrish, Gillian, Duggan, Deirdre E., Nordan, Harold Cecil, Parish, Geoffrey B., Cullum-Dugan, D (1956). Studies on a radio-resistant micrococcus. I, Isolation, morphology, cultural characteristics, and resistance to gamma radiation. *Food Technology*, 575-578.

Anken, R., and Rahmann, H. (2002). Gravitational Zoology: How Animals Use and Cope with Gravity. *Astrobiology*, 315-333. doi: 10.1007/978-3-642-59381-9_21.

Arrhenius, S. (1903). Die Verbreitung des Lebens im Weltenraum. *Die Umschau* 7, 481-486.

Arvidson, R.E., Squyres, S.W., Bell, J.F., 3rd, Catalano, J.G., Clark, B.C., Crumpler, L.S., et al. (2014). Ancient aqueous environments at Endeavour crater, Mars. *Science* 343(6169), 1248097. doi: 10.1126/science.1248097.

Bailey, C.H. (1938). The Origin of Life (Oparin, A. I.). *Journal of Chemical Education* 15(8), 399. doi: 10.1021/ed015p399.1.

Baqué, M., de Vera, J.-P., Rettberg, P., and Billi, D. (2013). The BOSS and BIOMEX space experiments on the EXPOSE-R2 mission: Endurance of the desert cyanobacterium Chroococcidiopsis under simulated space vacuum, Martian atmosphere, UVC radiation and temperature extremes. *Acta Astronautica* 91, 180-186. doi: 10.1016/j.actaastro.2013.05.015.

Basu, B., and Apte, S.K. (2012). Gamma Radiation-induced Proteome of Deinococcus radiodurans Primarily Targets DNA Repair and Oxidative Stress Alleviation. *Molecular & Cellular Proteomics : MCP* 11(1), M111.011734. doi: 10.1074/mcp.M111.011734.

Battista, J.R. (1997). Against all odds: the survival strategies of Deinococcus radiodurans. *Annual Review of Microbiology* 51, 203-224. doi: 10.1146/annurev.micro.51.1.203.

Bauche, C., and Laval, J. (1999). Repair of oxidized bases in the extremely radiation-resistant bacterium Deinococcus radiodurans. *Journal of Bacteriology* 181(1), 262-269.

Benzi, G., and Moretti, A. (1995). Are reactive oxygen species involved in Alzheimer's disease? *Neurobiology of Aging* 16(4), 661-674. doi: https://doi.org/10.1016/0197-4580(95)00066-N.

Billi, D., Verseux, C., Rabbow, E., and Rettberg, P. (2017). "Endurance of desert-cyanobacteria biofilms to space and simulated Mars conditions during the EXPOSE-R2 space mission", in: *EANA 2017.* (Aarhus, Denmark).

Blasius, M., Sommer, S., and Hubscher, U. (2008). Deinococcus radiodurans: what belongs to the survival kit? *Critical Reviews in Biochemistry and Molecular Biology* 43(3), 221-238. doi: 10.1080/10409230802122274.

Briegleb, W. (1992). Some qualitative and quantitative aspects of the fast-rotating clinostat as a research tool. *ASGSB bulletin: publication of the American Society for Gravitational and Space Biology* 5(2), 23-30.

Brim, H., McFarlan, S.C., Fredrickson, J.K., Minton, K.W., Zhai, M., Wackett, L.P., et al. (2000). Engineering Deinococcus radiodurans for metal remediation in radioactive mixed waste environments. *Nature Biotechnology* 18(1), 85–90. doi: 10.1038/71986.

Bruggeman, F.J., Hornberg, J.J., Boogerd, F.C., and Westerhoff, H.V. (2007). Introduction to systems biology. *EXS* 97, 1-19.

Brungs, S., Egli, M., Wuest, S.L., Christianen, P.C., Van Loon, J.J., Anh, T.J.N., et al. (2016). Facilities for simulation of microgravity in the ESA ground-based facility programme. *Microgravity Science and Technology* 28(3), 191-203.

Choi, M.H., Jeong, S.W., Shim, H.E., Yun, S.J., Mushtaq, S., Choi, D.S., et al. (2017). Efficient bioremediation of radioactive iodine using biogenic gold nanomaterial-containing radiation-resistant bacterium, Deinococcus radiodurans R1. *Chemical Communications* 53(28), 3937-3940. doi: 10.1039/c7cc00720e.

Cockell, C.S., Brack, A., Wynn-Williams, D.D., Baglioni, P., Brandstätter, F., Demets, R., et al. (2007). Interplanetary Transfer of Photosynthesis: An Experimental Demonstration of A Selective

Dispersal Filter in Planetary Island Biogeography. *Astrobiology* 7(1), 1–9. doi: 10.1089/ast.2006.0038.

Conselice, C.J., Wilkinson, A., Duncan, K., and Mortlock, A. (2016). The evolution of galaxy number density at z< 8 and its implications. *The Astrophysical Journal* 830(2), 83.

Cox, C.S. (1993). Roles of water molecules in bacteria and viruses. *Origins of Life and Evolution of Biospheres* 23(1), 29–36.

Cox, M.M. (2003). The bacterial RecA protein as a motor protein. *Annual Review of Microbiology* 57, 551-577. doi: 10.1146/annurev.micro.57.030502.090953.

Cox, M.M., and Battista, J.R. (2005). Deinococcus radiodurans - the consummate survivor. *Nature Reviews Microbiology* 3(11), 882-892. doi: 10.1038/nrmicro1264.

Crabbe, A., Schurr, M.J., Monsieurs, P., Morici, L., Schurr, J., Wilson, J.W., et al. (2011). Transcriptional and proteomic responses of Pseudomonas aeruginosa PAO1 to spaceflight conditions involve Hfq regulation and reveal a role for oxygen. *Applied and Environmental Microbiology* 77(4), 1221-1230. doi: 10.1128/AEM.01582-10.

Dai, X., and Guerras, E. (2018). Probing Extragalactic Planets Using Quasar Microlensing. *The Astrophysical Journal* 853(2). doi: 10.3847/2041-8213/aaa5fb.

Daly, M.J. (2009). A new perspective on radiation resistance based on Deinococcus radiodurans. *Nature Reviews Microbiology* 7(3), 237-245. doi: 10.1038/nrmicro2073.

Daly, M.J., Gaidamakova, E.K., Matrosova, V.Y., Kiang, J.G., Fukumoto, R., Lee, D.-Y., et al. (2010). Small-Molecule Antioxidant Proteome-Shields in Deinococcus radiodurans. *PLOS ONE* 5(9), e12570. doi: 10.1371/journal.pone.0012570.

Daly, M.J., Gaidamakova, E.K., Matrosova, V.Y., Vasilenko, A., Zhai, M., Leapman, R.D., et al. (2007). Protein oxidation implicated as the primary determinant of bacterial radioresistance. *PLOS Biology* 5(4), e92. doi: 10.1371/journal.pbio.0050092.

de Groot, A., Dulermo, R., Ortet, P., Blanchard, L., Guerin, P., Fernandez, B., et al. (2009). Alliance of proteomics and genomics to unravel the specificities of Sahara bacterium Deinococcus deserti. *PLOS Genetics* 5(3), e1000434. doi: 10.1371/journal.pgen.1000434.

de Vera, J.-P.P., Alawi, M., Backhaus, T., Baqué, M., Billi, D., Böttger, U., et al. (2019). Limits of Life and the Habitability of Mars: The ESA Space Experiment BIOMEX on the ISS. *Astrobiology* 19(2).

del Carmen Huesca Espitia, L., Caley, C., Bagyan, I., and Setlow, P. (2002). Base-change mutations induced by various treatments of Bacillus subtilis spores with and without DNA protective small, acid-soluble spore proteins. *Mutation Research* 503(1-2), 77-84.

Donato, M.M., Seleiro, E.A., and Da costa, M.S. (1990). Polar Lipid and Fatty Acid Composition of Strains of the Genus Thermus. *Systematic and Applied Microbiology* 13(3), 234-239. doi: https://doi.org/10.1016/S0723-2020(11)80191-8.

Dose, K., Bieger-Dose, A., Dillmann, R., Gill, M., Kerz, O., Klein, A., et al. (1995). ERA-experiment "Space Biochemistry". *Advances in Space Research* 16(8), 119–129.

Dose, K., Bieger-Dose, A., Labusch, M., and Gill, M. (1992). Survival in extreme dryness and DNA-single-strand breaks. *Advances in Space Research* 12(4), 221–229.

Drechsel, D.A., and Patel, M. (2008). Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. *Free Radical Biology and Medicine* 44(11), 1873-1886. doi: https://doi.org/10.1016/j.freeradbiomed.2008.02.008.

Fajardo-Cavazos, P., Link, L., Melosh, H.J., and Nicholson, W.L. (2005). Bacillus subtilis Spores on Artificial Meteorites Survive Hypervelocity Atmospheric Entry: Implications for Lithopanspermia. *Astrobiology* 5(6), 726–736. doi: 10.1089/ast.2005.5.726.

Farci, D., Bowler, M.W., Kirkpatrick, J., McSweeney, S., Tramontano, E., and Piano, D. (2014). New features of the cell wall of the radio-resistant bacterium Deinococcus radiodurans. *Biochimica et Biophysica Acta* 1838(7), 1978–1984. doi: 10.1016/j.bbamem.2014.02.014.

Farci, D., Slavov, C., Tramontano, E., and Piano, D. (2016). The S-layer Protein DR_2577 Binds Deinoxanthin and under Desiccation Conditions Protects against UV-Radiation in Deinococcus radiodurans. *Frontiers in Microbiology* 7, 155. doi: 10.3389/fmicb.2016.00155.

Filkin, D. (1997). *Stephen Hawking's Universe: The Cosmos Explained.* BasicBooks.

Furtauer, L., Pschenitschnigg, A., Scharkosi, H., Weckwerth, W., and Nagele, T. (2018). Combined multivariate analysis and machine learning reveals a predictive module of metabolic stress response in Arabidopsis thaliana. *Molecular Omics* 14(6)**,** 437-449. doi: 10.1039/c8mo00095f.

Galperin, M.Y., Nikolskaya, A.N., and Koonin, E.V. (2001). Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiology Letters* 203(1)**,** 11-21. doi: 10.1111/j.1574-6968.2001.tb10814.x.

Ghosal, D., Omelchenko, M.V., Gaidamakova, E.K., Matrosova, V.Y., Vasilenko, A., Venkateswaran, A., et al. (2005). How radiation kills cells: survival of Deinococcus radiodurans and Shewanella oneidensis under oxidative stress. *FEMS Microbiology Reviews* 29(2)**,** 361–375. doi: 10.1016/j.femsre.2004.12.007.

Gladyshev, E., and Meselson, M. (2008). Extreme resistance of bdelloid rotifers to ionizing radiation. *Proceedings of the National Academy of Sciences* 105(13)**,** 5139. doi: 10.1073/pnas.0800966105.

Goosen, N., Moolenaar, G.F., Visse, R., and van de Putte, P. (1998). "Functional Domains of the E. coli UvrABC Proteins in Nucleotide Excision Repair," in *DNA Repair,* eds. F. Eckstein & D.M.J. Lilley. (Berlin, Heidelberg: Springer Berlin Heidelberg), 103-123.

Green, J., Stapleton, M.R., Smith, L.J., Artymiuk, P.J., Kahramanoglou, C., Hunt, D.M., et al. (2014). Cyclic-AMP and bacterial cyclic-AMP receptor proteins revisited: adaptation for different ecological niches. *Current Opinion in Microbiology* 18**,** 1-7. doi: 10.1016/j.mib.2014.01.003.

Grotzinger, J.P. (2014). Habitability, Taphonomy, and the Search for Organic Carbon on Mars. *Science* 343(6169)**,** 386.

Grotzinger, J.P., Sumner, D.Y., Kah, L.C., Stack, K., Gupta, S., Edgar, L., et al. (2014). A Habitable Fluvio-Lacustrine Environment at Yellowknife Bay, Gale Crater, Mars. *Science* 343(6169).

Gupta, P., Gayen, M., Smith, J.T., Gaidamakova, E.K., Matrosova, V.Y., Grichenko, O., et al. (2016). MDP: A Deinococcus Mn2+-Decapeptide Complex Protects Mice from Ionizing Radiation. *PLOS ONE* 11(8)**,** e0160575. doi: 10.1371/journal.pone.0160575.

Gutman, P.D., Fuchs, P., Ouyang, L., and Minton, K.W. (1993). Identification, sequencing, and targeted mutagenesis of a DNA polymerase gene required for the extreme radioresistance of Deinococcus radiodurans. *Journal of Bacteriology* 175(11)**,** 3581-3590. doi: 10.1128/jb.175.11.3581-3590.1993.

Harris, D.R., Pollock, S.V., Wood, E.A., Goiffon, R.J., Klingele, A.J., Cabot, E.L., et al. (2009). Directed Evolution of Ionizing Radiation Resistance in <em>Escherichia coli</em&gt. *Journal of Bacteriology* 191(16)**,** 5240. doi: 10.1128/JB.00502-09.

Hassler, D.M., Zeitlin, C., Wimmer-Schweingruber, R.F., Ehresmann, B., Rafkin, S., Eigenbrode, J.L., et al. (2014). Mars' Surface Radiation Environment Measured with the Mars Science Laboratory's Curiosity Rover. *Science* 343(6169).

Hauslage, J., Cevik, V., and Hemmersbach, R. (2017). Pyrocystis noctiluca represents an excellent bioassay for shear forces induced in ground-based microgravity simulators (clinostat and random positioning machine). *npj Microgravity* 3(1)**,** 12.

Henne, A., Bruggemann, H., Raasch, C., Wiezer, A., Hartsch, T., Liesegang, H., et al. (2004). The genome sequence of the extreme thermophile Thermus thermophilus. *Nature Biotechnology* 22(5)**,** 547-553. doi: 10.1038/nbt956.

Horneck, G., Moeller, R., Cadet, J., Douki, T., Mancinelli, R.L., Nicholson, W.L., et al. (2012). Resistance of bacterial endospores to outer space for planetary protection purposes--experiment PROTECT of the EXPOSE-E mission. *Astrobiology* 12(5)**,** 445-456. doi: 10.1089/ast.2011.0737.

Horneck, G., Stöffler, D., Ott, S., Hornemann, U., Cockell, C.S., Moeller, R., et al. (2008). Microbial Rock Inhabitants Survive Hypervelocity Impacts on Mars-Like Host Planets: First Phase of Lithopanspermia Experimentally Tested. *Astrobiology* 8(1)**,** 17–44. doi: 10.1089/ast.2007.0134.

Huang, B., Liu, N., Rong, X., Ruan, J., and Huang, Y. (2015). Effects of simulated microgravity and spaceflight on morphological differentiation and secondary metabolism of Streptomyces coelicolor A3(2). *Applied Microbiology and Biotechnology* 99(10)**,** 4409-4422. doi: 10.1007/s00253-015-6386-7.

Im, S., Song, D., Joe, M., Kim, D., Park, D.H., and Lim, S. (2013). Comparative survival analysis of 12 histidine kinase mutants of Deinococcus radiodurans after exposure to DNA-damaging agents. *Bioprocess and Biosystems Engineering* 36(6)**,** 781–789. doi: 10.1007/s00449-013-0904-8.

Jenal, U. (2004). Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? *Current Opinion in Microbiology* 7(2)**,** 185-191. doi: 10.1016/j.mib.2004.02.007.

Joshi, B., Schmid, R., Altendorf, K., and Apte, S.K. (2004). Protein recycling is a major component of post-irradiation recovery in Deinococcus radiodurans strain R1. *Biochemical and Biophysical Research Communications* 320(4)**,** 1112-1117. doi: 10.1016/j.bbrc.2004.06.062.

Jung, S., Joe, M., Im, S., Kim, D., and Lim, S. (2010). Comparison of the genomes of deinococcal species using oligonucleotide microarrays. *Journal of Microbiology and Biotechnology* 20(12)**,** 1637-1646.

Kawaguchi, Y., Yang, Y., Kawashiri, N., Shiraishi, K., Takasu, M., Narumi, I., et al. (2013). The possible interplanetary transfer of microbes: assessing the viability of Deinococcus spp. under the ISS Environmental conditions for performing exposure experiments of microbes in the Tanpopo mission. *Origins of Life and Evolution of Biospheres* 43(4-5)**,** 411–428. doi: 10.1007/s11084-013-9346-1.

Kawaguchi, Y., Yokobori, S., Hashimoto, H., Yano, H., Tabata, M., Kawai, H., et al. (2016). Investigation of the Interplanetary Transfer of Microbes in the Tanpopo Mission at the Exposed Facility of the International Space Station. *Astrobiology* 16(5)**,** 363–376. doi: 10.1089/ast.2015.1415.

Kennan, R.M., Lovitt, C.J., Han, X., Parker, D., Turnbull, L., Whitchurch, C.B., et al. (2015). A two-component regulatory system modulates twitching motility in Dichelobacter nodosus. *Veterinary Microbiology* 179(1-2)**,** 34–41. doi: 10.1016/j.vetmic.2015.03.025.

Kerr, R.A. (1997). An Icy World Looks Livelier. *Science* 275(5299)**,** 478.

Kim, J.I., Sharma, A.K., Abbott, S.N., Wood, E.A., Dwyer, D.W., Jambura, A., et al. (2002). RecA Protein from the extremely radioresistant bacterium Deinococcus radiodurans: expression, purification, and characterization. *Journal of Bacteriology* 184(6)**,** 1649-1660. doi: 10.1128/jb.184.6.1649-1660.2002.

Kim, W., Tengra, F.K., Young, Z., Shong, J., Marchand, N., Chan, H.K., et al. (2013). Spaceflight Promotes Biofilm Formation by Pseudomonas aeruginosa. *PLOS ONE* 8(4)**,** e62437. doi: 10.1371/journal.pone.0062437.

Klyce, B. (Year). "Panspermia asks new questions", in: *Photonics West 2001 - LASE*: SPIE), 4.

Kobayashi, Y., Kikuchi, M., Nagaoka, S., and Watanabe, H. (1996). Recovery of Deinococcus radiodurans from radiation damage was enhanced under microgravity. *Biological Sciences in Space* 10(2)**,** 97-101.

Krisko, A., and Radman, M. (2010). Protein damage and death by radiation in Escherichia coli and Deinococcus radiodurans. *Proceedings of the National Academy of Sciences of the United States of America* 107(32)**,** 14373–14377. doi: 10.1073/pnas.1009312107.

Krisko, A., and Radman, M. (2013). Biology of extreme radiation resistance: the way of Deinococcus radiodurans. *Cold Spring Harbor Perspectives in Biology* 5(7). doi: 10.1101/cshperspect.a012765.

Krisko, A., and Radman, M. (2019). Protein damage, ageing and age-related diseases. *Open Biology* 9(3)**,** 180249. doi: 10.1098/rsob.180249.

Leuko, S., Bohmeier, M., Hanke, F., Böettger, U., Rabbow, E., Parpart, A., et al. (2017). On the Stability of Deinoxanthin Exposed to Mars Conditions during a Long-Term Space Mission and Implications for Biomarker Detection on Other Planets. *Frontiers in Microbiology* 8**,** 1680-1680. doi: 10.3389/fmicb.2017.01680.

Leys, N., Baatout, S., Rosier, C., Dams, A., s'Heeren, C., Wattiez, R., et al. (2009). The response of Cupriavidus metallidurans CH34 to spaceflight in the international space station. *Antonie Van Leeuwenhoek* 96(2)**,** 227-245. doi: 10.1007/s10482-009-9360-5.

Li, T., Chang, D., Xu, H., Chen, J., Su, L., Guo, Y., et al. (2015). Impact of a short-term exposure to spaceflight on the phenotype, genome, transcriptome and proteome of Escherichia coli. *International Journal of Astrobiology* 14(3)**,** 435-444. doi: 10.1017/S1473550415000038.

Limaye, S.S., Mogul, R., Smith, D.J., Ansari, A.H., Słowik, G.P., and Vaishampayan, P. (2018). Venus' Spectral Signatures and the Potential for Life in the Clouds. *Astrobiology* 18(9)**,** 1181-1198. doi: 10.1089/ast.2017.1783.

Liochev, S.I. (2013). Reactive oxygen species and the free radical theory of aging. *Free Radical Biology and Medicine* 60**,** 1-4. doi: https://doi.org/10.1016/j.freeradbiomed.2013.02.011.

Lipton, M.S., Paša-Tolić, L., Anderson, G.A., Anderson, D.J., Auberry, D.L., Battista, J.R., et al. (2002). Global analysis of the Deinococcus radiodurans proteome by using accurate mass tags. *Proceedings of the National Academy of Sciences* 99(17)**,** 11049-11054. doi: 10.1073/pnas.172170199.

Liu, Y., Rose, J., Huang, S., Hu, Y., Wu, Q., Wang, D., et al. (2017). A pH-gated conformational switch regulates the phosphatase activity of bifunctional HisKA-family histidine kinases. *Nature Communications* 8(1)**,** 2104. doi: 10.1038/s41467-017-02310-9.

Liu, Y., Zhou, J., Omelchenko, M.V., Beliaev, A.S., Venkateswaran, A., Stair, J., et al. (2003). Transcriptome dynamics of Deinococcus radiodurans recovering from ionizing radiation. *Proceedings of the National Academy of Sciences of the United States of America* 100(7)**,** 4191-4196. doi: 10.1073/pnas.0630387100.

Loeb, A. (2014). The habitable epoch of the early Universe. *International Journal of Astrobiology* 13(04)**,** 337–339. doi: 10.1017/s1473550414000196.

Luan, H., Meng, N., Fu, J., Chen, X., Xu, X., Feng, Q., et al. (2014). Genome-Wide Transcriptome and Antioxidant Analyses on Gamma-Irradiated Phases of Deinococcus radiodurans R1. *PLOS ONE* 9(1)**,** e85649. doi: 10.1371/journal.pone.0085649.

Makarova, K.S., Aravind, L., Daly, M.J., and Koonin, E.V. (2000). Specific expansion of protein families in the radioresistant bacterium Deinococcus radiodurans. *Genetica* 108(1)**,** 25-34.

Makarova, K.S., Aravind, L., Wolf, Y.I., Tatusov, R.L., Minton, K.W., Koonin, E.V., et al. (2001). Genome of the Extremely Radiation-Resistant Bacterium Deinococcus radiodurans Viewed from the Perspective of Comparative Genomics. *Microbiology and Molecular Biology Reviews* 65(1)**,** 44–79. doi: 10.1128/MMBR.65.1.44-79.2001.

Markillie, L.M., Varnum, S.M., Hradecky, P., and Wong, K.K. (1999). Targeted mutagenesis by duplication insertion in the radioresistant bacterium Deinococcus radiodurans: radiation sensitivities of catalase (katA) and superoxide dismutase (sodA) mutants. *Journal of bacteriology* 181(2)**,** 666-669.

Mastrapa, R., Glanzberg, H., Head, J., Melosh, H., and Nicholson, W. (Year). "Survival of Bacillus subtilis spores and Deinococcus radiodurans cells exposed to the extreme acceleration and shock predicted during planetary ejection", in: *Lunar and Planetary Science XXXI*).

Mastroleo, F., Van Houdt, R., Leroy, B., Benotmane, M.A., Janssen, A., Mergeay, M., et al. (2009). Experimental design and environmental parameters affect Rhodospirillum rubrum S1H response to space flight. *The ISME Journal* 3(12)**,** 1402-1419. doi: 10.1038/ismej.2009.74.

Mattimore, V., and Battista, J.R. (1996). Radioresistance of Deinococcus radiodurans: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. *Journal of Bacteriology* 178(3)**,** 633–637. doi: 10.1128/jb.178.3.633-637.1996.

Mauclaire, L., and Egli, M. (2010). Effect of simulated microgravity on growth and production of exopolymeric substances of Micrococcus luteus space and earth isolates. *FEMS Immunology and Medical Microbiology* 59(3)**,** 350-356. doi: 10.1111/j.1574-695X.2010.00683.x.

McKay, C.P. (2016). Titan as the Abode of Life. *Life* 6(1)**,** 8. doi: 10.3390/life6010008.

Mishra, S., Chaudhary, R., Singh, S., Kota, S., and Misra, H.S. (2019). Guanine Quadruplex DNA Regulates Gamma Radiation Response of Genome Functions in the Radioresistant Bacterium Deinococcus radiodurans. *Journal of Bacteriology* 201(17). doi: 10.1128/JB.00154-19.

Mooney, S., Leuendorf, J.-E., Hendrickson, C., and Hellmann, H. (2009). Vitamin B6: A Long Known Compound of Surprising Complexity. *Molecules* 14(1)**,** 329-351.

Munakata, N., Saitou, M., Takahashi, N., Hieda, K., and Morohoshi, F. (1997). Induction of unique tandem-base change mutations in bacterial spores exposed to extreme dryness. *Mutation Research* 390(1-2)**,** 189–195.

Narumi, I., Satoh, K., Cui, S., Funayama, T., Kitayama, S., and Watanabe, H. (2004). PprA: a novel protein from Deinococcus radiodurans that stimulates DNA ligation. *Molecular Microbiology* 54(1)**,** 278-285. doi: 10.1111/j.1365-2958.2004.04272.x.

nasa.gov (2019). *Exoplanet Exploration - Planets Beyond Our Solar System* [Online].[Available: https://exoplanets.nasa.gov/ Accessed 2019].

Neuberger, K., Lux-Endrich, A., Panitz, C., and Horneck, G. (2014). Survival of Spores of Trichoderma longibrachiatum in Space: data from the Space Experiment SPORES on EXPOSE-R. *International Journal of Astrobiology* 14(01)**,** 129–135. doi: 10.1017/s1473550414000408.

Ngo, K.V., Molzberger, E.T., Chitteni-Pattu, S., and Cox, M.M. (2013). Regulation of Deinococcus radiodurans RecA protein function via modulation of active and inactive nucleoprotein filament states. *Journal of Biological Chemistry* 288(29)**,** 21351-21366. doi: 10.1074/jbc.M113.459230.

Nicholson, W.L. (2009). Ancient micronauts: interplanetary transport of microbes by cosmic impacts. *Trends Microbiol* 17(6)**,** 243-250. doi: 10.1016/j.tim.2009.03.004.

Nicholson, W.L., Moeller, R., Team, P., and Horneck, G. (2012). Transcriptomic responses of germinating Bacillus subtilis spores exposed to 1.5 years of space and simulated martian conditions on the EXPOSE-E experiment PROTECT. *Astrobiology* 12(5)**,** 469-486. doi: 10.1089/ast.2011.0748.

Novikova, N., Deshevaya, E., Levinskikh, M., Polikarpov, N., Poddubko, S., Gusev, O., et al. (2015). Study of the effects of the outer space environment on dormant forms of microorganisms, fungi and plants in the 'Expose-R' experiment. *International Journal of Astrobiology* 14(01)**,** 137-142. doi: 10.1017/s1473550414000731.

Olsson-Francis, K., and Cockell, C.S. (2010). Experimental methods for studying microbial survival in extraterrestrial environments. *Journal of Microbiological Methods* 80(1)**,** 1–13. doi: 10.1016/j.mimet.2009.10.004.

Omelchenko, M.V., Wolf, Y.I., Gaidamakova, E.K., Matrosova, V.Y., Vasilenko, A., Zhai, M., et al. (2005). Comparative genomics of Thermus thermophilus and Deinococcus radiodurans: divergent routes of adaptation to thermophily and radiation resistance. *BMC evolutionary biology* 5**,** 57-57. doi: 10.1186/1471-2148-5-57.

Onofri, S., de la Torre, R., de Vera, J.P., Ott, S., Zucconi, L., Selbmann, L., et al. (2012). Survival of rock-colonizing organisms after 1.5 years in outer space. *Astrobiology* 12(5)**,** 508–516. doi: 10.1089/ast.2011.0736.

Ott, E., Kawaguchi, Y., Özgen, N., Yamagishi, A., Rabbow, E., Rettberg, P., et al. (2019). Proteomic and Metabolomic Profiling of Deinococcus radiodurans Recovering After Exposure to Simulated Low Earth Orbit Vacuum Conditions. *Frontiers in Microbiology* 10(909). doi: 10.3389/fmicb.2019.00909.

Pask-Hughes, R., and Williams, R. (1978). Cell envelope components of strains belonging to the genus Thermus. *Microbiology* 107(1)**,** 65-72.

Pavlov, A.K., Kalinin, V.L., Konstantinov, A.N., Shelegedin, V.N., and Pavlov, A.A. (2006). Was Earth Ever Infected by Martian Biota? Clues from Radioresistant Bacteria. *Astrobiology* 6(6)**,** 911-918. doi: 10.1089/ast.2006.6.911.

Peana, M., Chasapis, C.T., Simula, G., Medici, S., and Zoroddu, M.A. (2018). A Model for Manganese interaction with Deinococcus radiodurans proteome network involved in ROS response and

defense. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS)* 50**,** 465-473. doi: 10.1016/j.jtemb.2018.02.001.

Pearson, K.A., Palafox, L., and Griffith, C.A. (2017). Searching for exoplanets using artificial intelligence. *Monthly Notices of the Royal Astronomical Society* 474(1)**,** 478–491.

Pollmann, K., Raff, J., Merroun, M., Fahmy, K., and Selenska-Pobell, S. (2006). Metal binding by bacteria from uranium mining waste piles and its technological applications. *Biotechnology Advances* 24(1)**,** 58-68. doi: 10.1016/j.biotechadv.2005.06.002.

Potts, M. (1994). Desiccation tolerance of prokaryotes. *Microbiological Reviews* 58(4)**,** 755-805.

Querec, T.D., Akondy, R.S., Lee, E.K., Cao, W., Nakaya, H.I., Teuwen, D., et al. (2009). Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. *Nature immunology* 10(1)**,** 116-125. doi: 10.1038/ni.1688.

Quintela, J.C., Zöllner, P., García-del Portillo, F., Allmaier, G., and de Pedro, M.A. (1999). Cell wall structural divergence among Thermus spp. *FEMS Microbiology Letters* 172(2)**,** 223-229. doi: 10.1111/j.1574-6968.1999.tb13472.x.

Rabbow, E., Parpart, A., and Reitz, G. (2016). The planetary and space simulation facilities at DLR cologne. *Microgravity Science and Technology* 28(3)**,** 215–229.

Rhee, H.J., Kim, E.J., and Lee, J.K. (2007). Physiological polyamines: simple primordial stress molecules. *Journal of Cellular and Molecular Medicine* 11(4)**,** 685–703. doi: 10.1111/j.1582-4934.2007.00077.x.

Ricardo, A., and Szostak, J.W. (2009). Origin of life on earth. *Scientific American* 301(3)**,** 54–61.

Roth, L., Saur, J., Retherford, K.D., Strobel, D.F., Feldman, P.D., McGrath, M.A., et al. (2014). Transient Water Vapor at Europa's South Pole. *Science* 343(6167)**,** 171.

Rothfuss, H., Lara, J.C., Schmid, A.K., and Lidstrom, M.E. (2006). Involvement of the S-layer proteins Hpi and SlpA in the maintenance of cell envelope integrity in Deinococcus radiodurans R1. *Microbiology* 152(Pt 9)**,** 2779-2787. doi: 10.1099/mic.0.28971-0.

Saarimaa, C., Peltola, M., Raulio, M., Neu, T.R., Salkinoja-Salonen, M.S., and Neubauer, P. (2006). Characterization of Adhesion Threads of Deinococcus geothermalis as Type IV Pili. *Journal of Bacteriology* 188(19)**,** 7016-7021. doi: 10.1128/jb.00608-06.

Scott, D. (1983). Ionizing Radiation: Sources and Biological Effects. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine* 43(5)**,** 585-586. doi: 10.1080/09553008314550691.

Searles, S.C., Woolley, C.M., Petersen, R.A., Hyman, L.E., and Nielsen-Preiss, S.M. (2011). Modeled microgravity increases filamentation, biofilm formation, phenotypic switching, and antimicrobial resistance in Candida albicans. *Astrobiology* 11(8)**,** 825-836. doi: 10.1089/ast.2011.0664.

Sghaier, H., Narumi, I., Satoh, K., Ohba, H., and Mitomo, H. (2007). Problems with the current deinococcal hypothesis: an alternative theory. *Theory in Biosciences* 126(1)**,** 43-45. doi: 10.1007/s12064-007-0004-x.

Shukla, M., Chaturvedi, R., Tamhane, D., Vyas, P., Archana, G., Apte, S., et al. (2007). Multiple-Stress Tolerance of Ionizing Radiation-Resistant Bacterial Isolates Obtained from Various Habitats: Correlation Between Stresses. *Current Microbiology* 54(2)**,** 142-148. doi: 10.1007/s00284-006-0311-3.

Slade, D., Lindner, A.B., Paul, G., and Radman, M. (2009). Recombination and replication in DNA repair of heavily irradiated Deinococcus radiodurans. *Cell* 136(6)**,** 1044–1055. doi: 10.1016/j.cell.2009.01.018.

Slade, D., and Radman, M. (2011). Oxidative stress resistance in Deinococcus radiodurans. *Microbiology and Molecular Biology Reviews* 75(1)**,** 133–191. doi: 10.1128/mmbr.00015-10.

Soberon-Chavez, G., Alcaraz, L.D., Morales, E., Ponce-Soto, G.Y., and Servin-Gonzalez, L. (2017). The Transcriptional Regulators of the CRP Family Regulate Different Essential Bacterial Functions and Can Be Inherited Vertically and Horizontally. *Frontiers in Microbiology* 8**,** 959. doi: 10.3389/fmicb.2017.00959.

Su, L., Zhou, L., Liu, J., Cen, Z., Wu, C., Wang, T., et al. (2014). Phenotypic, genomic, transcriptomic and proteomic changes in Bacillus cereus after a short-term space flight. *Advances in Space Research* 53(1)**,** 18-29. doi: 10.1016/j.asr.2013.08.001.

Sun, X., Zhulin, I., and Wartell, R.M. (2002). Predicted structure and phyletic distribution of the RNA-binding protein Hfq. *Nucleic acids research* 30(17)**,** 3662-3671.

Suzuki, Y.J., Carini, M., and Butterfield, D.A. (2010). Protein carbonylation. *Antioxidants & redox signaling* 12(3)**,** 323-325. doi: 10.1089/ars.2009.2887.

Sweet, D.M., and Moseley, B.E.B. (1974). Accurate repair of ultraviolet-induced damage in Micrococcus radiodurans. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 23(3)**,** 311–318. doi: https://doi.org/10.1016/0027-5107(74)90104-3.

Sweet, D.M., and Moseley, B.E.B. (1976). The resistances of Micrococcus radiodurans to killing and mutation by agents which damage DNA. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 34(2)**,** 175-186. doi: https://doi.org/10.1016/0027-5107(76)90122-6.

Tian, B., Xu, Z., Sun, Z., Lin, J., and Hua, Y. (2007). Evaluation of the antioxidant effects of carotenoids from Deinococcus radiodurans through targeted mutagenesis, chemiluminescence, and DNA damage analyses. *Biochimica et Biophysica Acta* 1770(6)**,** 902-911. doi: 10.1016/j.bbagen.2007.01.016.

Truglio, J.J., Croteau, D.L., Van Houten, B., and Kisker, C. (2006). Prokaryotic Nucleotide Excision Repair: The UvrABC System. *Chemical Reviews* 106(2)**,** 233–252. doi: 10.1021/cr040471u.

Vaishampayan, P.A., Rabbow, E., Horneck, G., and Venkateswaran, K.J. (2012). Survival of Bacillus pumilus spores for a prolonged period of time in real space conditions. *Astrobiology* 12(5)**,** 487-497. doi: 10.1089/ast.2011.0738.

Van Houten, B., and Snowden, A. (1993). Mechanism of action of the Escherichia coli UvrABC nuclease: clues to the damage recognition problem. *Bioessays* 15(1)**,** 51-59. doi: 10.1002/bies.950150108.

Venkateswaran, A., McFarlan, S.C., Ghosal, D., Minton, K.W., Vasilenko, A., Makarova, K., et al. (2000). Physiologic Determinants of Radiation Resistance in Deinococcus radiodurans. *Applied and Environmental Microbiology* 66(6)**,** 2620–2626.

Waite, J.H., Glein, C.R., Perryman, R.S., Teolis, B.D., Magee, B.A., Miller, G., et al. (2017). Cassini finds molecular hydrogen in the Enceladus plume: Evidence for hydrothermal processes. *Science* 356(6334)**,** 155.

Wang, H., Yan, Y., Rong, D., Wang, J., Wang, H., Liu, Z., et al. (2016). Increased biofilm formation ability in Klebsiella pneumoniae after short-term exposure to a simulated microgravity environment. *Microbiology* 5(5)**,** 793-801. doi: 10.1002/mbo3.370.

Wang, Y., Xu, Q., Lu, H., Lin, L., Wang, L., Xu, H., et al. (2015). Protease Activity of PprI Facilitates DNA Damage Response: Mn(2+)-Dependence and Substrate Sequence-Specificity of the Proteolytic Reaction. *PLOS ONE* 10(3)**,** e0122071. doi: 10.1371/journal.pone.0122071.

Waris, G., and Ahsan, H. (2006). Reactive oxygen species: Role in the development of cancer and various chronic conditions. *Journal of Carcinogenesis* 5(1)**,** 14-14. doi: 10.1186/1477-3163-5-14.

Webber, W.R., and McDonald, F.B. (2013). Recent Voyager 1 data indicate that on 25 August 2012 at a distance of 121.7 AU from the Sun, sudden and unprecedented intensity changes were observed in anomalous and galactic cosmic rays. *Geophysical Research Letters* 40(9)**,** 1665–1668. doi: 10.1002/grl.50383.

Weckwerth, W. (2011). Green systems biology - From single genomes, proteomes and metabolomes to ecosystems research and biotechnology. *J Proteomics* 75(1)**,** 284-305. doi: 10.1016/j.jprot.2011.07.010.

Weckwerth, W., Wenzel, K., and Fiehn, O. (2004). Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. *Proteomics* 4(1)**,** 78–83. doi: 10.1002/pmic.200200500.

White, O., Eisen, J.A., Heidelberg, J.F., Hickey, E.K., Peterson, J.D., Dodson, R.J., et al. (1999). Genome sequence of the radioresistant bacterium Deinococcus radiodurans R1. *Science* 286(5444)**,** 1571-1577.

Wilson, J.W., Ott, C.M., Honer zu Bentrup, K., Ramamurthy, R., Quick, L., Porwollik, S., et al. (2007). Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. *Proceedings of the National Academy of Sciences of the United States of America* 104(41)**,** 16299-16304. doi: 10.1073/pnas.0707155104.

Yamagishi, A., Yano, H., Okudaira, K., Kobayashi, K., Yokobori, S.-i., Tabata, M., et al. (2007). TANPOPO: Astrobiology exposure and micrometeoroid capture experiments. *Biological Sciences in Space* 21(3)**,** 67-75.

Yang, S., Xu, H., Wang, J., Liu, C., Lu, H., Liu, M., et al. (2016). Cyclic AMP Receptor Protein Acts as a Transcription Regulator in Response to Stresses in Deinococcus radiodurans. *PLOS ONE* 11(5)**,** e0155010. doi: 10.1371/journal.pone.0155010.

Yano, H., Yamagishi, A., Hashimoto, H., Yokobori, S., Kobayashi, K., Yabuta, H., et al. (Year). "Tanpopo Experiment for Astrobiology Exposure and Micrometeoroid Capture Onboard the ISS-JEM Exposed Facility", in: *Lunar and Planetary Science Conference*), 2934.

Zahradka, K., Slade, D., Bailone, A., Sommer, S., Averbeck, D., Petranovic, M., et al. (2006). Reassembly of shattered chromosomes in Deinococcus radiodurans. *Nature* 443**,** 569. doi: 10.1038/nature05160.

Zea, L., Larsen, M., Estante, F., Qvortrup, K., Moeller, R., Dias de Oliveira, S., et al. (2017). Phenotypic Changes Exhibited by E. coli Cultured in Space. *Frontiers in Microbiology* 8**,** 1598. doi: 10.3389/fmicb.2017.01598.

Zhang, C., Wei, J., Zheng, Z., Ying, N., Sheng, D., and Hua, Y. (2005). Proteomic analysis of Deinococcus radiodurans recovering from γ-irradiation. *Proteomics* 5(1)**,** 138–143. doi: 10.1002/pmic.200300875.

Zhang, L., Yang, Q., Luo, X., Fang, C., Zhang, Q., and Tang, Y. (2007). Knockout of crtB or crtI gene blocks the carotenoid biosynthetic pathway in Deinococcus radiodurans R1 and influences its resistance to oxidative DNA-damaging agents due to change of free radicals scavenging ability. *Archives of Microbiology* 188(4)**,** 411-419. doi: 10.1007/s00203-007-0262-5.

Zhang, Y.M., Liu, J.K., and Wong, T.Y. (2003). The DNA excision repair system of the highly radioresistant bacterium Deinococcus radiodurans is facilitated by the pentose phosphate pathway. *Molecular Microbiology* 48(5)**,** 1317-1323. doi: 10.1046/j.1365-2958.2003.03486.x.