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Engineering *Escherichia coli* for survival in Outer Space conditions.

Enhancing bacterial resistance to extreme levels of radiation, temperature, and pressure

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Summary/Abstract

Some bacteria have shown the ability to naturally survive to extreme environments, even Outer Space. These bacteria have natural genetic mechanisms to protect and repair the DNA damage these severe conditions can cause, and this way can adapt and survive to these situations. A good example of this ability to survive can be found in *Deinococcus radiodurans*, which is known for its high radiation resistance and has proven its ability to survive under severe conditions such as the exposure unit of the International Space Station [1]. In this study, we translated some of these survival mechanisms to one of the most used experimental bacteria, *Escherichia coli*. Additionally, we also tested a protein from tardigrades, which is involved in DNA damage repair. The aim was to provide a certain strain of *E. coli* with the mechanisms it needs to survive harsh environments, which include extreme levels of ionizing and UV radiation, pressure, temperature, pH, and salinity. This research constitutes a first step in the creation of new strains and species engineered to survive severe conditions, opening the door to adapting existing species to survive on remote places and even other planets, such as Mars [2]. Furthermore, this study shed some light into the genetic mechanisms responsible for this survival, which could be beneficial for many scientific fields, such as medical sciences and life sciences engineering [3].

Keywords

Astrobiology, DNA Repair, Directed Molecular Evolution, UV Survival, Radiation Resistance, Low Pressure, Outer Space

Preface or prologue

Microorganisms and bacteria are essential for the ecosystem and our own survival, present almost everywhere on Earth and capable to survive to the most severe conditions. Besides this, bacterial strains have been detected and isolated aboard the International Space Station (ISS) [4], orbiting in the Low Earth Orbit, approximately 360km above Earth's surface. Some bacteria and microorganisms have even shown the ability to survive outside the ISS, in an environment similar to Outer Space. This is the case of some species of tardigrades [5], which have been found in some of the most extreme environments and are known for their extremophilic capabilities [6]. On the other hand, *Deinococcus radiodurans*, which has been widely studied due to its high radiation resistance [7], was exposed for three years in the ISS' Exposure Unit and exhibited a considerable survival [1]. In addition, Space Agencies such as the European Space Agency (ESA), have stated that microbes are the key to develop extra-terrestrial habitats [2], therefore studying the mechanisms by which the aforementioned organisms persevered in such severe conditions could impulse the possibilities behind Space exploration. Moreover, the knowledge obtained from these experiments could have many benefits and applications on science and technology on Earth.

The key to this bacterial survival resides, among other aspects, in the cellular mechanisms of protection and DNA repair [8]. With this objective, bacteria have developed many genetic and molecular systems that allow them to survive and adapt to their present conditions, including severe environments. Understanding and being able to improve these existent mechanisms is crucial for the development of bacteria capable of persevering to extreme environments, both on Earth and in an extra-terrestrial habitat. Additionally, studying bacterial resistance to high values of radiation, temperature and pressure conditions can be beneficial for the understanding of the molecular mechanisms involved in these resistances. This could benefit medical and life sciences engineering addressing treatments or diseases either directly related to any of these conditions, or that are nowadays treated using these conditions, as could be the case of some cancer treatments [3].

This project is focused on studying some of the natural mechanisms of protection and DNA repair, and on enhancing the resistance of *Escherichia coli* to extreme conditions, including a continuous spectrum of radiation, low and high temperatures, and low pressures and vacuum, while acquiring knowledge on the behaviour and survival of bacterial strains and different genes when exposed to adverse and extreme conditions. At a final stage, this should allow these strains to survive in an environment resembling Outer Space, but also in many other severe environments on Earth and beyond. The study and enhancement of these cellular resistance mechanisms required the use of systems and synthetic biology, directed evolution processes and genetic engineering to tackle the adverse effects extreme conditions can have on cellular metabolism and perseverance.

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1 Introduction

1.1 Life and its limiting parameters

Microorganisms and bacteria have been detected and isolated in a wide variety of environments, proving the ability of life to survive and adapt to a wide range of conditions, some of which can be considered as "extreme". However, the definition of "extreme conditions" has a strong anthropocentric criterion rather than microbial [9], as the conditions to which these microorganisms are highly adapted and we perceive as "extreme" are nominal for these species. Furthermore, our understanding of the environment is based on the current planetary surface conditions on Earth, which have only occurred for a short period of time compared to the existence of life [10], leading to the conclusion that extremophilic life has constituted a large part of the evolutionary history of life.

For life to exist, there are three main requirements: a liquid solvent, an energy source, and building blocks [11]. From these, the presence of a liquid solvent, which is usually water, appears to be the main factor controlling the presence of life on Earth, acting both as a solvent and a reactant in biochemical reactions. Moreover, numerous parameters responsible for limiting life, as could be temperature, pressure, or radiation, are in fact acting on the availability of water. The second main factor for life to exist is the access to an energy source to power chemical reactions inside the cells, and therefore, allowing the cells to survive. Depending on the environment, some of these parameters can more strongly influence microbial life over others, as could be the case of temperature in geothermal waters [12]. It is therefore important to consider the availability of each of these parameters to evaluate the survival feasibility of microorganisms in severe environmental conditions.

1.2 Enhancing Bacterial Survival

Deoxyribonucleic Acid, or DNA, is a molecule composed of two polynucleotide chains that coil forming a double helix carrying genetic information for the development, functioning, growth and reproduction of all known organisms. Asides from being an essential component of life, the role of these nucleotide chains has given rise to many metaphors, most of them using words like "instructions" and "determining". However, no metaphor or comparison can grasp all the meaning and functions of DNA. Due to its role and importance in cell functioning, the protection and repair of the DNA has proven essential for a cell to survive to adverse environments, specially in conditions with a high impact on genes and gene expression [1][8]. The different environmental factors a cell could be exposed to can cause several types of damage to the DNA, which could ultimately lead to cellular death. For instance, solar UV can induce, among other effects, pyrimidine dimerization [13][14], high vacuum exposure can lead to base deletion and/or insertion [15], and high-dose Ionizing Radiation (IR) and desiccation can cause Double and Single-Strand Breaks [16][17][18][19][20][21]. These are some of the effects each of these factors can have on bacterial DNA, but all of them can have multiple adverse consequences.

To avoid or minimize the possible effects of environmental factors, cells have developed a wide range of genetic and molecular mechanisms for the protection and repair of DNA. Understanding how these genetic mechanisms work when exposed to certain factors could help increase the cell's resistance and survival, making it able to persevere in new environments. In addition, expanding our knowledge on some of these genetic mechanisms

could be beneficial for the comprehension of several diseases and open the door to new diagnostic tools and treatments.

This study focused on enhancing the resistance of one of the most studied bacteria, *Escherichia coli*, to one of the most severe known environments, Outer Space, and the possibility of improving bacterial survival for the development of an extra-terrestrial habitat. For that, the values obtained during the three-year exposure of *Deinococcus radiodurans* outside the International Space Station were used to design, induce and analyse the different mechanisms to be implemented, as well as testing the survival of this potential new bacterial strain. The values obtained or estimated for the same conditions on Mars' equator [22][23] were also considered both when designing the experiments and discussing the results obtained and their outcome. Both sets of parameters can be found in Table 1.

Table 1: Environmental Factors considered for this study, extracted from the values registered during the Tanpopo mission in its three-year exposure experiment outside the ISS [1], and the values registered on the surface of Mars (at the equator) [22][23].

Environmental factor	Exposure Unit ISS [1]	Mars *(at equator)* [22][23]
UV fluence	$124 - 177$ MJ/m^2/year	~ 5 MJ/m^2
Ionizing Radiation	232 ± 5 mGy/year	~ 200 mGy/year
Temperature Range	$29 \pm 5 \sim -42 \pm 5$ ºC	$20 \sim -73$ ºC
Pressure Range	$10^{-4} \sim 10^{-7}$ Pa	$500 \sim 1000$ Pa

1.3 Ionizing and UV Radiation

Radiation in Space mainly consists of two types: Solar Cosmic Radiation (SCR) and Galactic Cosmic Radiation (GCR) [24]. The first one consists of low energy solar wind particles that constantly flow out of the Sun, and highly energetic solar particle events which originate from the magnetically disturbed regions of the Sun, which sporadically emits bursts of energetic charged particles [25]. This type of radiation varies depending on the distance from the emitter, in this case, the Sun. On the other hand, the Galactic Cosmic Radiation originates in Space beyond our Solar System. These ionizing radiation types can cause different kinds of DNA damage, including double and single strand breaks (DSBs and SSBs respectively) [16][18] and base and sugar modifications [26]. Additionally, ultraviolet radiation exposure can also induce DSBs and SSBs, as well as other effects like pyrimidine dimerization [13].

In the last decades there has been a notable interest in understanding the mechanisms by which some bacteria, like *D. radiodurans*, can decrease the effects of long-term exposure to ionizing or UV radiation [27]. Furthermore, many ionizing radiation-based treatments, common for example to treat some kinds of cancer, injure not only the targeted tissue, but also the surrounding area, limiting the effectiveness of these treatments and increasing their adverse effects. These issues highlight the urgent need for a better comprehension of the radiation protective mechanisms.

To study the effects of Ionizing Radiation on cellular survival, this project follows the guidelines established by Dennis R. Harris et al. (2009) [28]. In this article, the authors used evolved strains of *Escherichia coli* to perform a directed evolution process that led to the increase of the surviving fraction of the different strains. The dose range of the study was replicated, with levels from 0 to 3000 Grays (Gy), much more higher than the natural

environmental values on Earth, which varies depending on the specific location, being in the order of ∼200 milligrays per year [29]. To expose the cells to such levels of ionizing radiation, the Radiotherapy Department of *Hospital del Mar* allowed the use of a *Varian TrueBeam STX* ® accelerator, capable of generating high-energy X-rays in order to reach up to 3000 Gy. Before the Ionizing Radiation exposure, the cells were exposed to UV radiation, both inducing random mutations in the cells' genome and acting like a filter for the weaker bacteria, thus enhancing the overall survival rate to ionizing radiation. The radiation exposures were followed by a study of the survival rates and the growth rates in response to different doses.

After the first exposure, two genes from *D. radiodurans*, and one from the tardigrade *R. varieornatus*, all known or suspected of enhancing radiation resistance mechanisms were transformed and a directed evolution process was performed, studying the effect these genes had in the survival to Ionizing and UV Radiation exposure and the different efficacies they showed, together with the impact this foreign genes had on *E. coli* cells. The selected genes were the RecA and uvrD genes from *D. radiodurans*, which are both known to have an important role in this bacteria's natural radiation resistance [30][27] and also have an orthologous gene in *E. coli*, and the Dsup gene from *R. varieornatus*, also known for having a big impact on this species' resistance to radiation [31]. During this directed evolution process, the cells were exposed to IR two more times, each of them studying their survival and selecting the surviving fraction. Once the directed evolution process was finished, single colonies were selected according to their growth and resistance, and their survival to Ionizing and UV Radiation was tested once more.

1.4 High and Low Temperature

The temperature of a body in Space, which is determined by the absorption and emission of energy, depends on multiple factors, including the position of the body with respect to the Sun or other orbiting bodies, size, surface, etc. [32]. Consequently, the temperature range varies a lot depending on the environment the cells are in. For instance, the temperature at the exposure unit of the ISS varies between 29 ± 5 and -42 ± 5 $^{\text{o}}$C [1], but at the equator of Mars, the range can go from 20 to -73 $^{\text{o}}$C [22]. Moreover, high and low temperatures can lead to molecular damage, but also to desiccation, which can induce DSBs or SSBs [19][21], ultimately causing severe DNA damage.

To induce and test the temperature resistance of the exposed *E. coli* cells, the guidelines described by Takahashi et al. (2011) [33] were followed. In this study, they described a temperature cycles experiment designed to simulate the temperature changes outside the ISS, with 90-minute cycles, which is the approximated duration of a "day" aboard the ISS, in which the temperature varies from 80 to -80 $^{\text{o}}$C. To analyse the cellular response to continuous exposure, multiple consecutive cycles can be performed. During this study, a similar approach was performed to test the resistance of the selected strains from the Ionizing and UV Radiation experiments.

1.5 Low Pressure, Vacuum and Microgravity

Low pressures and microgravity have negative effects in gene expression and cellular growth [34]. Several microgravity experiments have been done aboard the unique conditions of the ISS, and research on this topic is still being performed in order to delve into its effects at the macro and micro scales. In fact, in the words of David Brady, International Space Station (ISS) Assistant Program Scientist at NASA, *"Eliminating gravity from the equation facilitates understanding the rest"*, meaning that gravity itself and pressure have a very big impact on how life perseveres and evolves, but also on how we understand it. Moreover, vacuum can cause cell dehydration and desiccation, which can lead to severe damage to the DNA, but also on other cell components, such as the lipid membranes, proteins and nucleic acids [35][36].

Due to the wide range of DNA damage that vacuum and low pressures can cause, it has been suggested that the radiation resistance of the *Deinococcus* spp. might be related or consequence of an adaptation to prolonged desiccation [37]. However, this is not a proved fact, and some scientists suggest it may be the other way around.

Simulating vacuum on a laboratory is a very complex process. For this study, the pressure values used during the *D. radiodurans* test before its approval for the exposure outside the ISS [32] [1] and the values estimated for Mars' equator [22] were considered. Ultimately, the values used for this study were a little bit higher, around 7 kPa. However, this values correspond to a ∼93% vacuum and proved to have an important impact on cellular survival.

1.6 Salinity and pH

Although they are not known to have any major effect on DNA, salinity and pH can affect the availability of water, therefore limiting the survival of microorganisms.

Saline environments include a large fraction of the Earth, with a range that goes from a ∼3-4% salinity in marine environments, to up to 49.7% salinity in salt inclusions [38]. Salinity can highly influence water activities and availability, which at its turn affects microorganism proliferation and survival. The salinity range deemed optimum for isolated microorganisms ranges between 0 and 35% [39]. Further to this, currently, the most severe pH values in which extremophiles have been isolated are at pH 0 and pH 12.5, with an optimal pH between 0.7 and 11 for many species [39]. PH has a big impact on microorganisms, as all of them must maintain a near neutral cytoplasmic pH in order to enable cellular functions and metabolism [40][41], hence the importance of the pH of the environment and the protection mechanisms of the cells to keep a steady neutral pH.

2 Methods

2.1 Ionizing and UV Radiation Resistance

As mentioned earlier in this study, the main effects of Ionizing Radiation are DSBs and SSBs [16][18], and UV Radiation can mainly cause pyrimidine dimerization [13], among other effects. Any of these DNA damages or a combination of some of them could ultimately lead to cellular death. In the first part of this study, *Escherichia coli* K-12 MG1655 wild-type cells in exponential growth phase were exposed to 400 mJ of UV Fluence using a *Bio-Rad GS GENE LINKER™ UV Chamber* and to a continuous spectrum of Ionizing Radiation up to 3000 Gy using a *Varian TrueBeam STX* ® accelerator to study their survival rate, as well as the effects radiation could have on the growth-rate and metabolism of the cells. After this initial radiation exposure, three genes related with radiation resistance from *D. radiodurans* and *R. varieornatus* were transformed, and a directed evolution process was performed, enhancing the cell's survival rate. The chosen genes were RecA and uvrD, both from *D. radiodurans*, and Dsup, from *R. varieornatus*. Finally, 5 single colonies were selected according to their growth rates and survival, using a Plate-Reader analysis to measure their OD at 600 nm during their growth both when exposed to UV and when not exposed. Afterwards, a last test of the new survival rates to both UV and Ionizing Radiation for the selected colonies was performed.

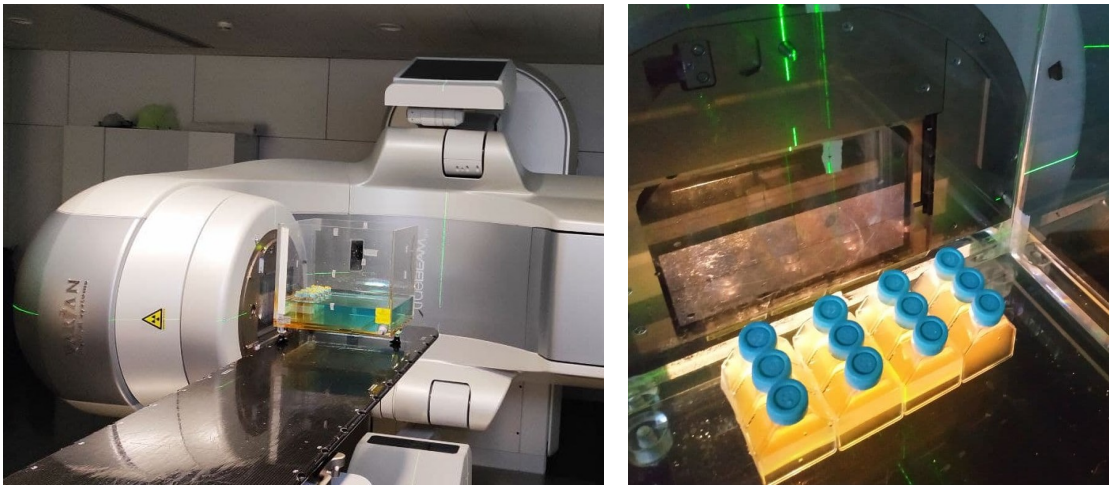
2.1.1 First UV and Ionizing Radiation Exposure

In the first stage of the radiation resistance experiments, wild-type *E. coli* K-12 MG1655 cell cultures were exposed to both UV and Ionizing Radiation, after which the surviving fraction of the cells to different doses of Ionizing Radiation was calculated, together with an initial characterization of the effects of these radiation exposures to the cell's growth rate.

For the UV Fluence exposure, a saturated wild-type *E. coli* K-12 culture was diluted to an Optical Density (OD) at 600nm of 0.0625, and let grow in a 37 ºC shaker for ∼1 hour until it reached an approximated OD of 0.5. This first dilution was done in order to get a cellular culture in an exponential growth phase [42], in which cells are more vulnerable to mutations and changes in the DNA, primarily due to their high replication rate. When the culture reached the exponential phase, 45 ml were distributed in three sterile Petri dishes in order to amplify the exposed surface and avoid UV shielding. The different samples were then exposed to UV Fluence in a *Bio-Rad GS GENE LINKER™ UV Chamber*. According to experimental data on cellular survival and mutagenesis previously obtained with the same device, the optimal UV Fluence was set to be 400 mJ, after which, although the mutagenesis increased, cellular survival dropped. The main objective of this first exposure was to generate random mutagenesis in the cell's genome, as well as enhancing the survival possibilities of the surviving culture to its exposure to IR [43]. However, exposing the cells to UV radiation also served as a filter for the weakest cells, which did not survive this irradiation. After the exposure, the culture was diluted with up to 200 ml of fresh LB medium and let grow overnight in a 37 ºC shaker until culture saturation.

After ∼18-20 hours, the 200 ml saturated culture was again diluted to an OD around 0.0625 and incubated in a 37 ºC shaker for an additional hour until it reached an exponential growth phase, at an OD of 0.494 (∼0.5). The culture was then distributed in 13 cell culture flasks of 66 ml, one per intended Ionizing Radiation dose, and moved to *Hospital del Mar*, where a *Varian TrueBeam STX* ® accelerator was used to irradiate the cells.

The different flasks were exposed to a continuous spectrum of X-ray photons produced by a potential accelerator of 10 MV. In Figure 1 some photos of the IR exposure setup using the *TrueBeam* accelerator can be seen, together with an image of the water model the flasks were submerged in to reduce the absorbed dose estimated error and increase the homogeneity.



(a) *Varian TrueBeam STX* ® accelerator used for the cellular exposure to a continuous spectrum of X-ray photons at a dose rate of 1.35 Gy per minute.

(b) Positioning of the cell culture flasks previous and during the IR exposure, at a Source-Skin Distance of 65 cm.

Figure 1: First Ionizing Radiation exposure experiment setup, featuring the *Varian TrueBeam STX* ® accelerator used and the positioning of the flasks inside the water model.

Although the IR values registered during the exposure of *D. radiodurans* outside the ISS were around 232 mGy/year [1], the station is protected from cosmic radiation by the upper layers of the atmosphere, as well as Earth's magnetic field. Taking this into consideration, the dosage for this study was set between 0 and 3000 Gy in steps of 250 Gy (13 doses in total counting the control) at a dose rate of 1.35 Gy per minute. This dosage had an overall estimated error of the absorbed dose of 3.6%[1]. During the IR exposure, a flask was extracted per each dose, substituting it by a flask full of water to keep the homogeneity in the dose.

2.1.2 Gene Transformation and Directed Evolution Preparation

After the initial exposure to both UV and Ionizing Radiation, the surviving cells from the highest dose, 3000 Gy, were stored at -80 ºC to be used in a directed evolution process with the objective of enhancing their IR resistance. During this stage, the cells were exposed two times to IR, selecting each time the surviving population. Previous to the exposures, three different genes from *D. radiodurans* and *R. varieornatus* known and/or suspected of enhancing cellular resistance to radiation were transformed.

The first transformed gene was RecA, from *D. radiodurans*. This gene, which has an orthologous gene in *E. coli* with a similarity of ∼53.5% at an amino acid level, has been

[1]More information about the calculation of the absorbed dose estimated error can be found in the Additional Information Section (5.1).

characterized to be relevant to the *Deinococcus* spp. for their cellular damage repair and to achieve their natural radiation resistance [30] [27].

The second transformed gene, also from *D. radiodurans*, was uvrD. This gene, which also has an orthologous gene in *E. coli* with a similarity of ∼28% at an amino acid level, is one of the genes suspected of having a big impact on the radiation resistance of the *Deinococcus* spp., but its role is still not clear [27].

The last transformed gene was Dsup, from the tardigrades spp. *Ramazzottius varieornatus*. This gene is known for its role in DNA repair [31], and its effects on radiation resistance were tested in *E. coli* on this study.

The three genes were coupled with an arabinose inducible promoter, so before each new exposure, gene expression was induced with 1.32 μl/ml of arabinose, which has been characterized as the optimal concentration for the induction of this specific promoter [44]. The use of an inducible promoter was chosen to be able to control the expression of the genes and to study the cells behaviour with and without inducing the genes in further tests. A Chloramphenicol (50 µg/ml, Sigma, USA) resistance marker was used to select for colonies containing the plasmid.

First, the genes from *D. radiodurans* were extracted from the wild-type strain, performing a genomic DNA extraction using the *Norgen Biotek Corp.* Bacterial Genomic DNA Isolation Kit and a Kappa PCR to amplify and add the desired restriction sites to both the RecA and uvrD genes. The Dsup gene was obtained from a commercial plasmid, and a Kappa PCR was performed to amplify it and add the desired restriction sites.

Then, the inducible pBad/araC promoter[2] was cloned into the pSB1AC3 plasmid using the EcoRI and XbaI restriction sites. Both parts are available at the iGEM Registry of Standard Biological Parts[3]. After the pBad promoter was inserted and checked using a colony PCR and plasmid sequencing, the three different genes were inserted using the XbaI and SpeI restriction sites. The clonings were then checked again by colony PCR and plasmid sequencing. The resulting plasmid had a general structure as the one in Figure 2.

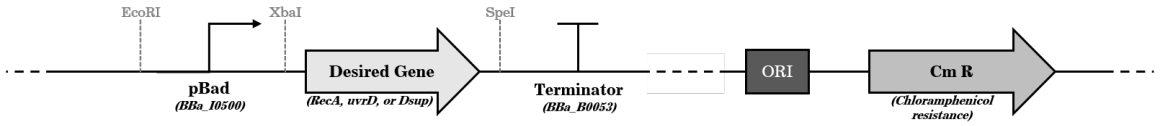


Figure 2: General scheme of the transformed circular plasmid, showing the position of the restriction sites with respect to the promoter, gene, and terminator, as well as the origin of replication and the Chloramphenicol resistance gene.

After the correct plasmids were obtained, the cells exposed during the first IR experiment were prepared for the transformation by electroporation. For that, the cells were grown overnight until culture saturation in a 37 ºC shaker and then diluted and grown until they reached an OD of approximately 0.4. Then the cells were centrifuged, pelleted, and washed with ice-cold sterile distilled water 3 times, after which they were resuspended with ice-cold sterile 10% glycerol. The electrocompetent cells were then used to transform the plasmids through a 2.5 kV pulse using a *Bio-Rad Gene Pulser Xcell™* and grown in LB and Chloramphenicol plates overnight at 37 ºC, after which all the colonies for each gene were picked to continue with the directed evolution process without losing popula-

[2]iGEM Registry of Standard Biological Parts code *BBa_I0500*.

[3]Link for the iGEM Registry of Standard Biological Parts: http://parts.igem.org/Main_Page

tion variability. These cells were then inoculated in 50 ml of fresh LB medium and let grow overnight until culture saturation in a 37 ºC shaker.

2.1.3 Second and Third Ionizing Radiation Exposures

The saturated 50 ml cultures were exposed to Ionizing Radiation up to 3000 Gy by following the same protocols as in the first IR exposure, by diluting the cultures up to 300 ml each and letting them grow until exponential phase before exposure. However, to be able to fit all the cell strains in the accelerator without losing dose homogeneity, the studied doses were reduced to 0, 500, 1500 and 3000 Gy, with the same dose rate as in the previous exposure. Besides this, due to technical constrains, between the 500 and 1500 doses, a stop of ∼20-30 minutes was done. In both exposures, the surviving population was selected, and the survival curves were calculated to study the impact and efficacy of the transformed genes, as well as testing the response of the cell strains to a continuous exposure to high levels of Ionizing Radiation.

For the second Ionizing Radiation exposure, the exposed cultures included a control from the first IR exposure, and the three new strains, named after the transformed genes: RecA, uvrD and Dsup.

For the third and last exposure, new cultures were added to test different properties and effects of Ionizing Radiation exposure, as well as being able to compare different survival rates depending on the cellular conditions. The exposed cultures were: a wild-type *E. coli* K-12 MG1655, a wild-type *E. coli* K-12 previously exposed to 400 mJ of UV Fluence (only doses 0 and 500 Gy), the four selected clones from the second exposure and a wild-type *D. radiodurans* culture, which was grown on p53 medium in a 30 ºC shaker.

In both exposures, the procedure was very similar to the first IR exposure, inoculating the cultures for each sample, growing them overnight until culture saturation in a 37 ºC shaker and diluting them to an OD of 0.0625 to grow until they reached an exponential growth phase (OD ∼0.5). In the case of the transformed strains, the cultures were induced with arabinose when diluted in order to activate the expression of the transformed genes [44]. Once the cultures reached the exponential growth phase, they were distributed in flasks and exposed to the different Ionizing Radiation doses.

2.1.4 Data Acquisition and Analysis

After each radiation exposure, the survival rates were computed and compared to analyse the effects of Ionizing Radiation in cell survival, but also the relevance of the different transformed genes for radiation resistance. The survival rate was calculated by the Colony-Forming Units of each exposed culture. To do so, the cultures were diluted from 10^{-1} to 10^{-4} for the first exposure and from 10^{-1} to 10^{-6} for the second and third exposure, as by selecting the surviving population, higher CFU values were expected for these exposures. For each of these dilutions, nine drops of 5μl were plated in three separate LB plates. The surviving fraction of each strain was then calculated from the titer of the surviving population, which was calculated from the CFU of the culture one day after the exposure, divided by the titer of the non-exposed culture, the control dose. The errors were calculated using Equation 1, where n is the number of drops counted, and SD is the standard deviation of the colonies counted per drop.

$$e = \frac{SD}{\sqrt{n}}; \quad where: \; SD = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2} \tag{1}$$

In the first exposure, a Hill Function (Equation 2) was used to fit the experimental data and check if the survival curve followed the tendency from this model, as many natural molecular and genetic mechanisms can be modelled using a modified version of this function [45].

$$H = \frac{b}{1 + (\frac{x}{k})^n} \tag{2}$$

In addition to the survival rates, in the first IR exposure, a *TECAN Infinite M NANO*$^+$ Plate-Reader was used to measure the OD at 600 nm throughout the growth of five colonies per each dose to compare the growth-rates of the different exposed cultures and analyse the possible effects of Ionizing and UV radiation in cellular growth. After the third IR exposure, a new Plate-reader analysis was performed measuring the OD at 600 nm through the growth of 120 colonies from the 3000 Gy dose of the different exposed cultures, in addition to 12 wild-type *E. coli* control colonies (more details on the number of colonies per strain can be found in Table 2). The number of colonies was chosen in proportionality with their overall surviving fraction to the third IR exposure. Each colony's growth rate was studied twice, once diluting the colony, growing it until exponential phase and exposing the culture to a 400 mJ UV Fluence right before the analysis, and once without this UV exposure. The different strains tested were not induced with arabinose for this characterization, as the aim was to detect and analyse the possible beneficial mutations each colony had. This characterization of the colonies allowed the study of the growth-rates and the effects of radiation on the selected cells, as well as selecting the colonies with a better metabolic performance with and without UV exposure. From this individual growth-rate study, the colonies with best performance were selected to continue with the study. For the RecA and the uvrD strains, one single colony was selected, whereas for the Dsup strain 3 different colonies were selected.

Table 2: Analysed single colonies per each gene after the radiation exposure experiments. These colonies were studied during their growth to analyse and compare their metabolic activity.

	Wild-Type *E. coli*	UV Exposed *E. coli*	Control strain	RecA strain	uvrD strain	Dsup strain	Total
Colonies	12	6	12	26	30	52	**138**

2.1.5 Final Ionizing Radiation Exposure

The three selected colonies of the strain which had a bigger survival rate, the Dsup gene, were exposed again to Ionizing Radiation to characterize the final radiation resistance of these colonies. The exposed samples were the three selected Dsup colonies and the Dsup strain population from the last IR exposure. As before, the cultures were inoculated overnight and grown until saturation in a 37 ^{o}C shaker, after which they were diluted and induced, grown until exponential growth phase, and exposed. For this final test, and due to some constraints, only the 0 and the 500 Gy dose were tested (at the same dose rate as previous exposures). Taking into account the Ionizing Radiation values both in the equator of Mars [23], and outside the ISS [1], which can be seen in Table 1, this values give a very good estimation of the survival possibilities of the selected strains in harsh environments beyond Earth.

2.1.6 Final UV Fluence Exposure

To test the UV Fluence resistance of the new cellular strains, a culture for each strain, including the selected colonies, the full populations and the wild-types *E. coli* and *D. radiodurans* were inoculated and grown overnight until culture saturation in a 37 ^{o}C shaker. The cultures were then diluted to an OD of 0.0625, induced with arabinose (except for the wild-type strains) and grown until exponential growth phase ($\sim$0.5). The cultures were then placed in sterile Petri dishes and exposed to different doses of UV Fluence using a *Bio-Rad GS GENE LINKER™ UV Chamber*. The doses for this exposure reached from 0 to 785 mJ, as can be seen in Figure **??**. After this, the results were obtained in the same way as the Ionizing Radiation exposures, by calculating the CFU and obtaining the surviving fraction of each strain, which were then compared and analysed.

2.1.7 Whole Genome Sequencing Analysis

From the selected colonies for the three strains containing the selected genes RecA, uvrD and Dsup, a whole genome extraction was done using a *Norgen Biotek Corp.* Bacterial Genomic DNA Isolation Kit. Whole Genome Sequencing (WGS) of the selected colonies was then conducted and the sequences were compared with the wild-type genome of *E. coli* K12 MG1655. The WGS results were analyzed by mapping against the wild-type genome assembly using the Burrows-Wheeler Aligner [46]. To differentiate the sequencing errors from the potentially mutated genome sequence a variant calling algorithm was applied and the position of the base variation with respect to the wild-type genome analyzed. This analysis is still in progress.

On the other hand, plasmid DNA was extracted from the selected clones and the genes (UvrD, RecA, Dsup) were sequenced to identify mutations which could enhance their radiation resistance capabilities.

2.2 Temperature and Low Pressure Resistance

As mentioned earlier, both vacuum and high and low temperatures can lead to desiccation, which can cause severe damage to the DNA and other cell components [34]. The main effects these conditions have on the DNA are DSBs and SSBs [19][21]. After having exposed the *E. coli* cells to Ionizing and UV Radiation, the selected strains, together with

wild-type strains of *E. coli* K-12 and *D. radiodurans*, were tested by exposing to low-pressures of 90-93% vacuum and a temperature cycles experiment designed by Takahashi et al. [33]. This experiment was designed to simulate the temperature conditions outside the ISS, making it very useful for testing the possible survival of the cells to extreme ranges of temperature in short periods of time.

To test both conditions, the cells were exposed for 0, 3 and 7 days to low pressures around 90% vacuum ($\sim$ 7 kPa). Right after the exposure, the same tubes went through the temperature cycles experiment, completing 0, 1, 3 or 5 cycles. After each cycle finished, the cultures were diluted and plated as done for the IR exposures to obtain the CFUs and study their survival. This process was done with the 5 selected colonies from the radiation experiments, as well as a wild-type strain of *E. coli* and *D. radiodurans*. Each strain was tested with and without pelleting the culture, and the three strains containing the selected genes were tested with and without gene induction. The functioning of the experiments is detailed below.

2.2.1 Low Pressure and Vacuum Simulation

The vacuum simulation process was performed using a desiccator coupled with a vacuum pump, placing the cells in 1 ml tubes inside the desiccator and applying -700 mmHg of negative pressure to expose the cells to a positive pressure of $\sim$7 kPa, which can be translated to a $\sim$90% of vacuum.

Cell cultures for all the strains were grown overnight until culture saturation in a 37 ^{o}C shaker. For each strain, two cell cultures were prepared, one in which the inserted genes were induced with arabinose, and a second culture without induction. The saturated cultures were taken out after $\sim$18-20h of growth, the OD was measured to check the different cultures had similar values and therefore were on similar growth phases, and the cultures were distributed in 1 ml tubes. Each culture was divided into two tubes per dose. One of these tubes was centrifuged and pelleted and the supernatant removed, drying the cell pellet, and the other one was left as a liquid culture.

2.2.2 Temperature Cycles Experiment

The temperature cycles experiment, designed by Takahashi et al. [33], was performed to test the cell's survival to a wide range of temperatures. In this experiment, the cells are exposed to temperatures that go from -80 to 80 ^{o}C in 90 minutes cycles, simulating the length of a day aboard the ISS. Each cycle consists of cooling the cells to -80 ^{o}C for 20 minutes, leaving them at room temperature for 25 minutes, heating them up to 80 ^{o}C for 20 minutes and finally leaving them at room temperature for an additional 25 minutes. This 90-minute cycle is then repeated multiple times to simulate several "days".

In this study, the first approach was to test the cells using the original temperature range, but after exposure to one cycle, an almost zero survival was observed, and the temperature range was altered to reduce the highest temperature, which was deemed to be responsible for the low survival. The temperature range used to test the cells was changed to go from 50 to -80 ^{o}C, simulating each temperature with a thermal bath and a laboratory freezer, respectively. For each low-pressure exposure (0, 3 and 7 days), the cells were tested during 0, 1 and 3 temperature cycles, plus an additional 2 cycles in the 7 days low-pressure exposure.

To calculate the temperature variation throughout the experiment, the Ordinary Differential Equation 3 representing Newton's law of cooling was used, calculating the proportionality constant k experimentally. The resulting plot showing the temperature variations can be seen in the figure below.

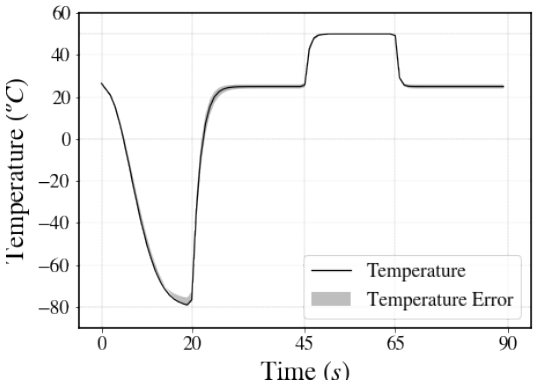


Figure 3: Temperature variation in one cycle, ranging from 50 to -80 ºC. This cycle was then repeated multiple consecutive times.

$$\frac{dT}{dt} = k(T + T_m)$$
$$T(t) = T_m + (T_0 - T_m)\ e^{-k(t_0 - t)} \tag{3}$$

2.3 Salinity and pH Resistance

Salinity and pH can have a big impact on the growth and survival of cells, mainly affecting the availability of water, and therefore, the metabolism of the microorganisms. To study these effects, an analysis of the growth rates of the different selected colonies, and the wildtypes for *E. coli* and *D. radiodurans* was performed using a Plate-Reader to measure the OD at 600 nm during their growth for approximately 20 hours. Different concentrations of salt and different pH were tested in order to establish the current life limitations of the different strains, both with the genes induced and non-induced.

For the salinity study, LB media was prepared with different concentrations of salt to study the variations this could induce to the cellular growth rate. The concentrations went from a 0% of salt to a 57% with respect to the total quantity of each ingredient of the medium, covering a wide range of concentrations that are now known to be the limits.

For the pH study, LB media with different pH from 3 to 11 were prepared using HCl and NaOH to compensate the acidity and alkalinity of the media.

In both cases, overnight saturated cultures were used to inoculate 200 μl of fresh LB media in a 96-well plate, which was then introduced to the Plate-Reader and the analysis started. First the temperature was set to 37 ºC, and then the plate was shaken for 10 minutes before taking a first measure, continuing like this for up to 20 hours. Afterwards, the results were analysed and plotted to see which effects the different LB media had to the growth of the different cell strains.

3 Results

In this study, the boundaries of *Escherichia coli* survival have been tested, trying to enhance its resistance to life-limiting factors. More specifically, its resistance to Ionizing and UV Radiation, vacuum, and high and low temperatures have been analysed, focusing on ways to expand those limits. Its growth in different salinity and pH conditions, both conditions known to affect the water availability and activity, was also analysed, trying to set its current boundaries. With the main aim of outer space survival, the cells were tested under severe conditions for all the aforementioned environmental factors.

3.1 Ionizing and UV Radiation Resistance

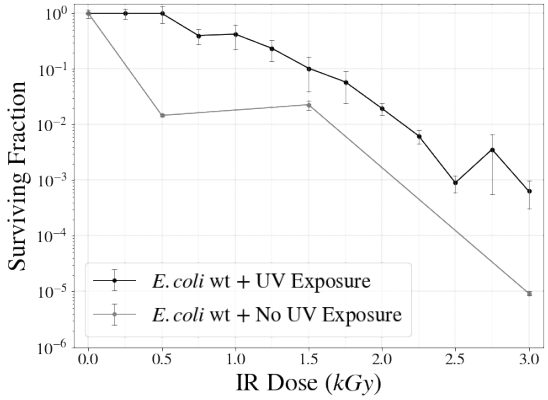
3.1.1 First Ionizing Radiation Exposure

After exposing the *E. coli* cells to 400 mJ of UV Fluence using a UV Chamber to generate a mutagenic process, an exposure to up to 3000 Gy of Ionizing Radiation was perfomred in a *Varian TrueBeam STX* ® accelerator, testing the survival of these cells to both types of radiation. After this double exposure, the CFU of the different doses was calculated and normalized to obtain the corresponding survival curve. The graph in Figure 4a shows the curves obtained for both, the wild-type *E. coli* previously exposed to UV Fluence, and a non-exposed control. As the IR dose increases, the surviving fraction of both cell populations decreases. However, the comparison between the UV exposed cells and the wild-type control shows an increase in the surviving fraction of the UV exposed cells and, consequently, an enhanced resistance to Ionizing Radiation. The exposed cells show a very good survival to the first doses, with the surviving fraction starting to decrease after the 500 Gy dose was reached. The surviving fraction after being exposed to 3000 Gy of IR is also significant, being one order of magnitude higher than the wild-type, which has a similar surviving fraction to the obtained by Dennis R. Harris et al. (2009) [28]. The reason of this increase may be related to the mutagenetic process induced by the UV exposure, which could have introduced beneficial mutations, as well as killed the cells with less radiation resistance.

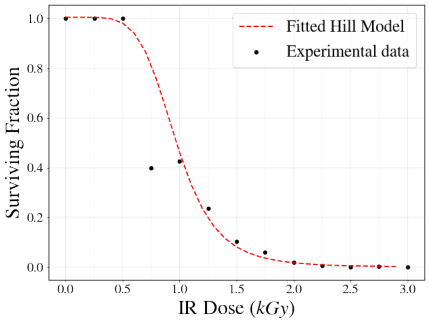
This data was then used to fit a Hill function, which can be seen in Equation 4. In Figure 4b, the fitted Hill function is represented, together with the data obtained during the IR exposure experiment, showing there is a correlation between both plots. The use of a Hill function was determined from the shape of the curve, but also due to its utility in modeling molecular and genetic systems [45].

$$H = \frac{b}{1 + (\frac{x}{k})^n}; \quad where: \ b = 1.00587; \ k = 0.97062; \ n = 5.6 \tag{4}$$

Asides from the surviving curve, the growth rate of the exposed cells was also studied to analyse the possible effects of the Ionizing and UV Radiation exposure to their metabolism. After a 22 hours continued analysis of the OD at 600 nm of 5 different colonies per dose, the results obtained showed very similar values and slopes between the doses, as can be seen in Figure 5. Although there were not any major differences, it is noticeable that the control's curve (0 Gy) had a bigger slope, which was translated to a steeper growth curve, therefore reaching the saturation faster than the rest of the exposed colonies. However, no other significant effects on the metabolism of the exposed *E. coli* cells could be observed.



(a) Survival curve of wild-type *E. coli* K-12 cells after being exposed to Ionizing Radiation, both previously exposed to UV Fluence and wild-type.



(b) Trend line of the survival curve of wild-type *E. coli* after UV and IR exposure.

Figure 4: First UV and Ionizing Radiation exposure survival results showing and increase in the overall survival of the *E. coli* wild-type strain exposed to UV Fluence with respect to the non-exposed wild-type *E. coli*. A trend line showing the tendency of the survival fraction as the radiation dose increases was also plotted, following a Hill model function (Equation 4).

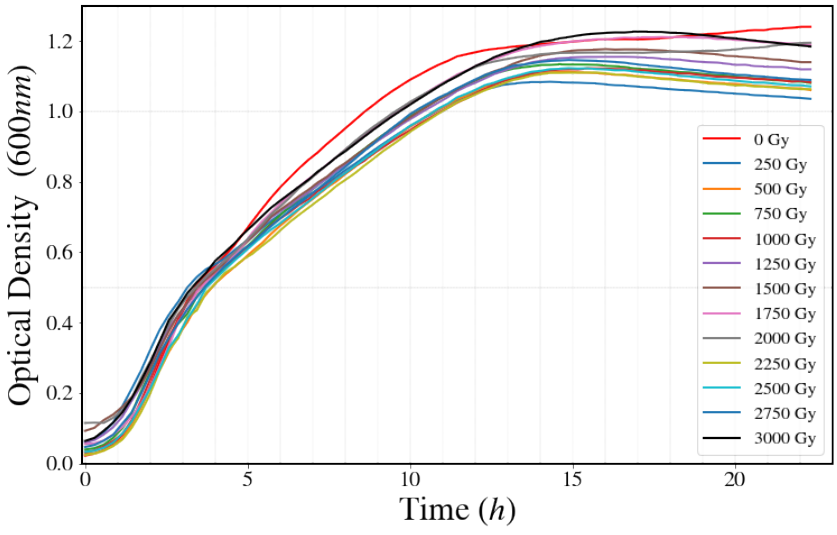
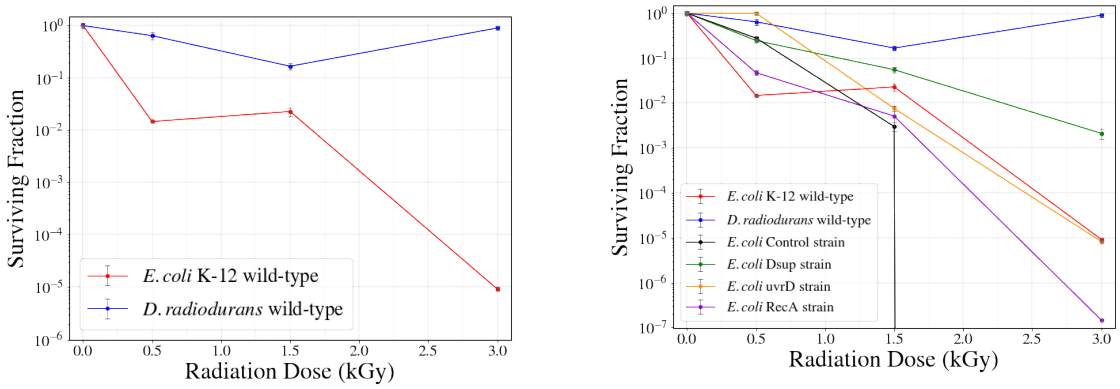


Figure 5: Growth rate curves comparison after the first Ionizing Radiation exposure of *E. coli*. Each curve corresponds to the average values of 5 surviving colonies per each dose.

3.1.2 Second and Third Ionizing Radiation Exposures

After the directed evolution process consisting on two new Ionizing Radiation exposures of the surviving fraction of the cells, the survival curve was again computed and analysed.

In Figure 6a, a comparison between the wild-types of *E. coli* K-12 and *D. radiodurans* can be observed, showing a big difference between their natural survivals and highlighting the radiation resistance of *D. radiodurans*.



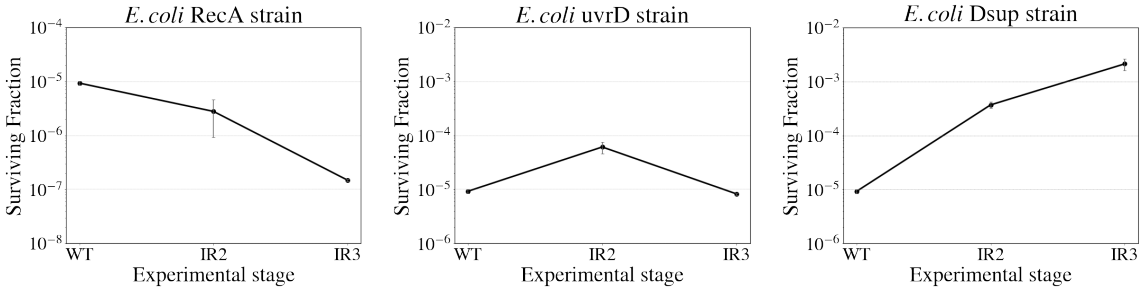
(a) Survival curve comparison between the wild-type *E. coli* K-12 and the wild-type *D. radiodurans* cells after being exposed to Ionizing Radiation.

(b) Survival curve comparison for the different *E. coli* evolved strains and the wild-types of *E. coli* and *D. radiodurans*.

Figure 6: Survival curves of the different cell strains after the third exposure to Ionizing Radiation and a comparison to the wild-types of *E. coli* K-12 and *D. radiodurans*.

Furthermore, in Figure 6b, the different *E. coli* evolved strains can be seen and compared with both wild-types, showing a different behaviour depending on the gene transformed. The Dsup gene from *R. varieornatus* was the one showing a bigger impact in the cell's surviving fraction, resulting in an increase of up to two orders of magnitude with respect to the wild-type. The uvrD gene showed very good results for the lowest doses, between 0 and 500 Gy, but then decreased and ended with an overall survival similar to the wild-type. The RecA gene showed a survival even lower than the wild-type, proving to be more harmful than beneficial for the cell's survival. This could be due to the similarity with *E. coli*'s orthologous gene, as some interaction could be affecting their effectiveness. Finally, the control strain showed zero survival to the 3000 Gy dose. However, when a culture was grown from the exposed flask, the culture grew until saturation. Therefore, although it may have been to low as to be properly detected in the CFU values, some cells from the control strain did survive the exposure.

A further analysis on the variation of the overall survival at 3000 Gy for the three evolved strains with the selected genes RecA, uvrD, and Dsup, shows the impact each of these genes had on *Escherichia coli*'s radiation resistance. Figure 7 shows the progress of the surviving fraction from the three strains at 3000 Gy throughout the different IR exposures. It is clear that the Dsup gene did have a big impact in radiation resistance, increasing the surviving fraction by more than two orders of magnitude. For the other two strains, corresponding to the genes from *D. radiodurans*, the survival was either maintained, as is the case of the uvrD gene, or dropped, as in RecA gene's case. It is however of note, that for the uvrD gene, an increase in the survival to 500 Gy was



(a) Surviving fraction variation for the RecA strain, showing a decrease in the overall final radiation resistance for this particular strain.

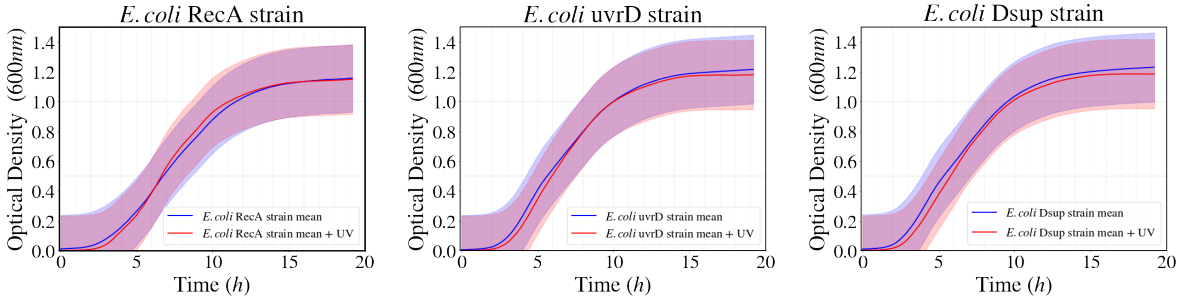
(b) Surviving fraction variation for the uvrD strain, showing similar radiation resistance values between the wild-type *E. coli* and the final IR exposure of this particular strain.

(c) Surviving fraction variation for the Dsup strain, showing a significant increase in the overall final radiation resistance for this particular strain.

Figure 7: Overall Ionizing Radiation survival variation for the evolved gene strains of *E. coli*. The progress between the wild-type *E. coli* K-12 (WT), and the values obtained during the second (IR2) and third (IR3) Ionizing Radiation exposures can be seen, showing how the strains evolved towards IR resistance.

perceived, resulting in an almost 100% of the population surviving to low doses of IR, values that were not obtained with any other of the selected colonies and are higher than the wild-type *D. radiodurans*.

Asides from the survival rates of the selected strains, different colonies were picked to study their growth rates, measuring their OD at 600 nm for ∼20 hours. Figure 8 shows the growth rate graphs for the RecA, uvrD, and Dsup strains. Using this plots, one colony from the RecA and uvrD strains, and three colonies from the Dsup strain were selected to continue the study. The selection was done taking into account the slopes, maximum, time until saturation, and comparison between the UV-exposed and the non-exposed culture of each colony, selecting the ones with a best overall performance. The selected colonies were: colony 7 from the RecA strain (C7), colony 1 from the uvrD strain (C1), and colonies 15, 16, and 17 from the Dsup strain (C15, C16, C17).



(a) Growth rate of the E. coli RecA strain.

(b) Growth rate of the E. coli uvrD strain.

(c) Growth rate of the E. coli Dsup strain.

Figure 8: Growth rates of different colonies for RecA, uvrD, and Dsup strains, measured using their OD at 600 nm for ∼20 hours. Each colony was studied with and without a previous exposure to UV Fluence, and the means were calculated.

3.1.3 Final Ionizing Radiation and UV Exposures

After exposing the selected colonies from the Dsup strain (C15, C16 and C17), together with the strain's population, to up to 500 Gy of Ionizing radiation, the results obtained showed a big variety of results, but all over the 10^{-1} magnitude order. As can be seen in Figure 9, the colony with a better survival was C16, with a 100% survival rate. After this colony, the full population had the next best result, with a survival over 90%. Finally, C15 had a survival rate over 50%, while C17 had a survival rate ∼30%, all of them having better results than the same strain in the third IR exposure. When compared with the wild-type *E. coli* K-12 survival rate, which is around the 10^{-2} magnitude order, this results clearly show the progress the cells have gone through during the directed evolution process, as well as the benefiting effects of the Dsup gene for their resistance to high and low levels of radiation.

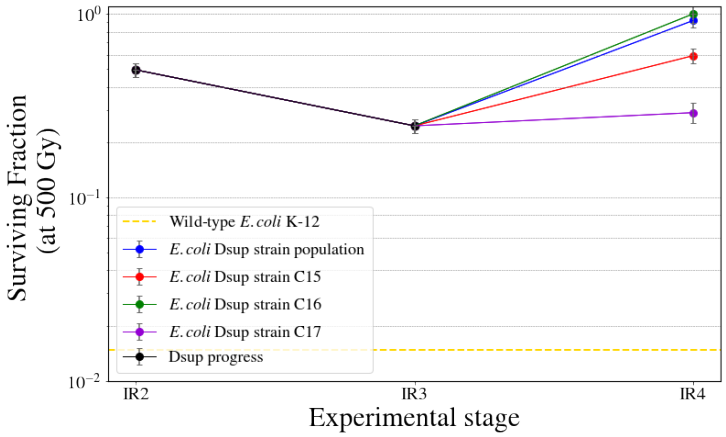


Figure 9: Surviving fraction at 500 Gy of Ionizing Radiation exposure for the selected Dsup colonies compared to the population.

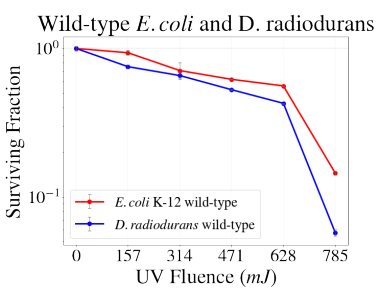
As well as exposing the selected colonies from the Dsup strain to Ionizing Radiation, all the selected colonies including RecA, uvrD, and Dsup, together with the wild-type strains of *E. coli* K-12 and *D. radiodurans* were exposed to UV Fluence to study their survival to different doses of radiation. The results from this exposure can be seen in Figure 10. The overall survival of all the samples was very high compared to the Ionizing Radiation survival. Moreover, from the experimental data, the wild-type *E. coli* K-12 strain had better results than *D. radiodurans* at 785 mJ, as can be seen in Figure 10a.

The RecA strain, both for the selected colony and the full population, had very similar results to the wild-type *E. coli*, showing no relevant effect of the gene's induction to the UV Fluence survival.

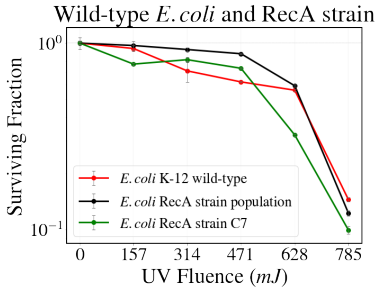
The uvrD strain, both for the selected colony and the full population, had lower survival results than the wild-type *E. coli*. The full population had a better survival to the first UV doses, but then decreased down to a survival rate under 10^{-1}.

Finally, the Dsup strain, both for the selected colonies and the full population, had a high variability in the results, with the full population and the colony C17 obtaining better results than the wild-type. This behaviour was expected, as colony 17 was the first selected colony for the Dsup strain due to the similarity between the growth rates with and without UV exposure, but contrasts with the results obtained by the same colony when exposed to Ionizing Radiation. This emphasizes the differences between both radiation types and the effects they have on cell metabolism and perseverance. This highlights

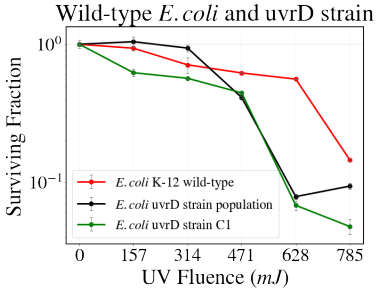
the relevance of the methods used to choose the colonies that had the best possibilities of survival. The other two colonies, C15 and C16, had similar results, both below the wild-type and near to 10^{-1}.



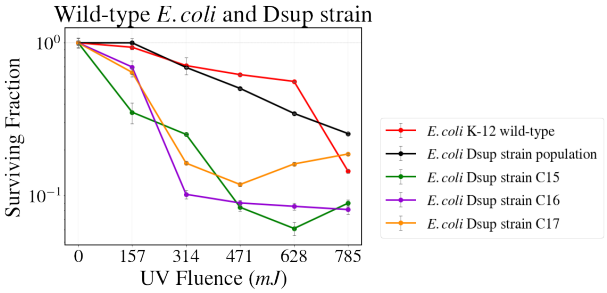
(a) Surviving fraction for the wild-type *E. coli* and *D. radiodurans* strains to different doses of UV Fluence.



(b) Surviving fraction for the wild-type *E. coli* and RecA strains to different doses of UV Fluence.



(c) Surviving fraction for the wild-type *E. coli* and uvrD strains to different doses of UV Fluence.



(d) Surviving fraction for the wild-type *E. coli* and Dsup strains to different doses of UV Fluence.

Figure 10: Surviving fractions for the different strains after the UV Fluence exposure up to 785 mJ using a *Bio-Rad GS GENE LINKER™ UV Chamber*.

3.1.4 Whole Genome Sequencing Analysis

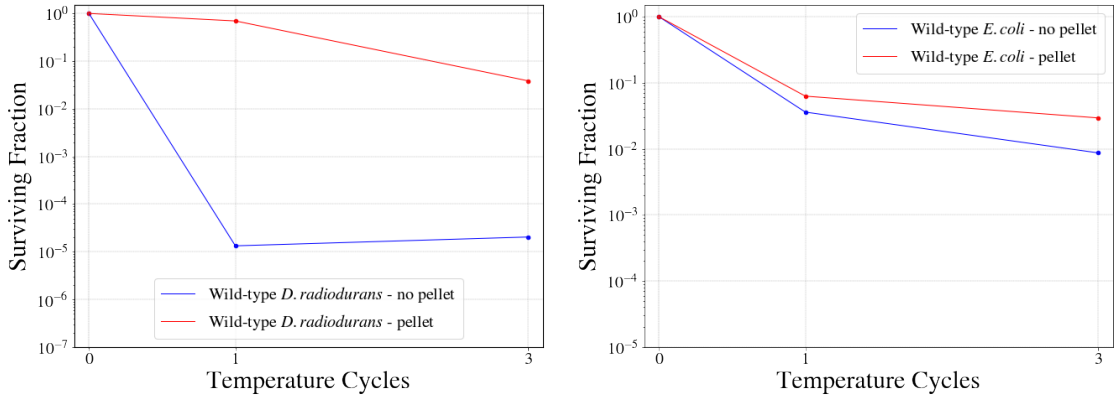
Although the Whole Genome Sequencing analysis is still in progress, the results for the Sanger sequencing of the RecA, uvrD, and Dsup genes were analysed and compared to the original sequences to identify possible mutations that could have benefited the radiation resistance of their respective strains. However, no mutations were perceived for any of the genes in the extracted plasmids, which were the colonies selected after the third Ionizing Radiation Exposure. This could mean that these genes were not affected by radiation, and so their protective and repair effects were conserved after the exposures.

3.2 Temperature and Low Pressure Resistance

The selected colonies from the RecA, uvrD, and Dsup strains, as well as the wild-types of *E. coli* and *D. radiodurans*, were tested against high and low temperatures and vacuum, both pelleted and non-pelleted to study the effects of desiccation and cell aggregation for the survival under low pressures in the order of ∼7 kPa and temperatures in a range

between 50 and -80 ºC. These experiments gave several survival results to the different variables, so only the most relevant results are shown in this section.

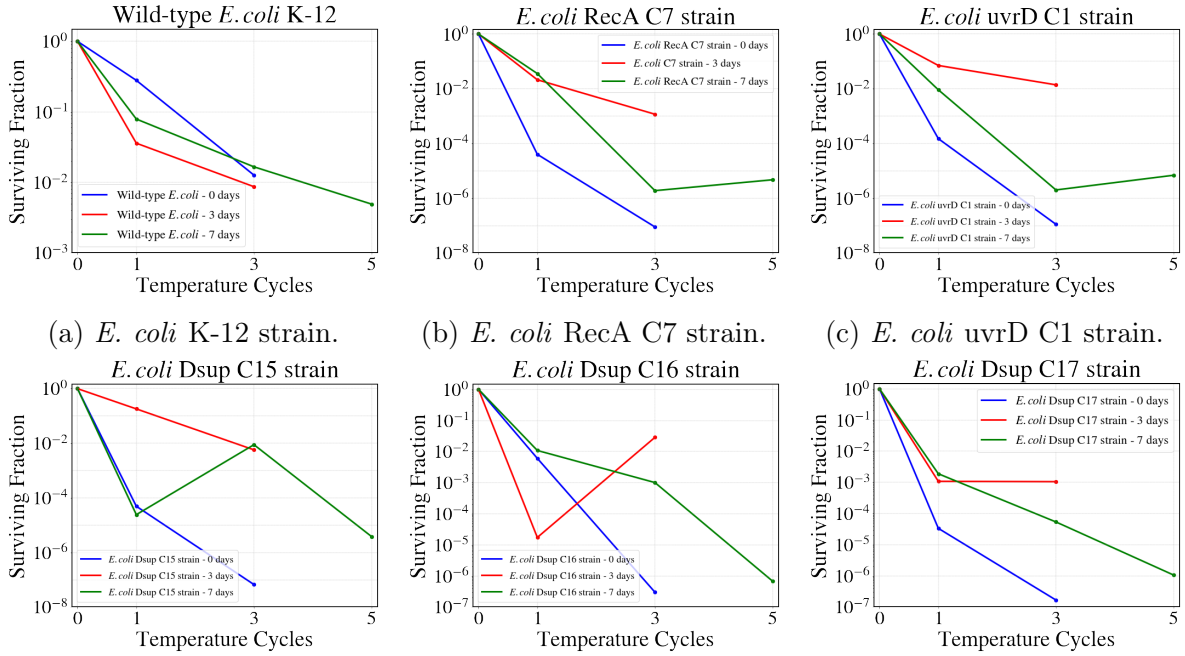
In Figure 11, a comparison between the wild-type *E. coli* K-12 and the wild-type *D. radiodurans* after a three-day exposure to low pressures is done, with both strains studied when exposed as a cell aggregation (pellet) and as a liquid culture. In the case of the wild-type *D. radiodurans* strain, cell aggregation clearly has a very big impact on survival, increasing its surviving fraction in approximately three orders of magnitude. This corresponds with the results obtained in previous by Y. Kawaguchi et al. (2013 and 2020) when testing this strain's resistance to radiation [1][32], so it would seem that the increase of survival due to aggregation is also observable when exposed to vacuum and temperature cycles. For the wild-type *E. coli* strain, this difference is not appreciated, with very similar survival results for both studies.



(a) *D. radiodurans* strain. (b) *E. coli* K-12 strain.

Figure 11: Cell aggregation importance for the survival of *E. coli* and *D. radiodurans* after 3 days of ∼93% vacuum exposure and 3 cycles of high and low temperatures.

As cell aggregation didn't seem to have a big impact in the survival of *E. coli* when exposed to vacuum and the cell cycles experiments, the graphs on Figure 12 show the values obtained during the different temperature cycles experiments after the cell's exposure to 0, 3 and 7 days of ∼93% vacuum in a saturated liquid culture. These results show that the different genes transformed, together with the mutations from the Ionizing and UV Radiation exposures did not have a big impact on the cell's survival to neither the vacuum exposure, nor the temperature cycles. In fact, the different evolved strains had a lower survival than the wild-type, showing that either the mutations on this colonies' genome are not beneficial for their survival under these conditions, or the expression of the genes was a metabolic load that negatively affected the cells' performance. However, the non-induced colonies were also studied and presented similar survival rates.



(a) *E. coli* K-12 strain. (b) *E. coli* RecA C7 strain. (c) *E. coli* uvrD C1 strain.

(d) *E. coli* Dsup C15 strain. (e) *E. coli* Dsup C16 strain. (f) *E. coli* Dsup C17 strain.

Figure 12: Temperature cycles survival after 0, 3, 7 days of vacuum exposure for the wild-type *E. coli* K-12 strain and the different RecA, uvrD and Dsup selected colonies.

3.3 Salinity and pH Resistance

The salinity and pH tests were performed in order to study the limitations the evolved strains had with respect to the effects this factors can have on water availability and metabolic activity.

The salinity growth rate analysis revealed that all the different strains tested could persevere and survive for all the conditions, therefore the known limits for these strains would be from 0 to 20 grams per liter of media. Further tests could be done to expand this range with higher concentrations. In Figure 13, a representation of the time each strain needed to get over the mid-exponential phase, which was estimated to be at an OD $\sim$0.6. The results are very similar for all the strains, with a faster slope at concentrations around 5 g/l, which is usually the standard value for commercially sold LB powder media. With lower and higher concentrations, the cultures grew similarly, but reached the mid-exponential growth phase later.

The pH analysis was studied in a similar way, growing the cells in LB adjusted to different pH values and studying how fast the culture reached the mid-exponential growth phase. For pH 3 and 11, none of the cultures grew neither with the gene induced or non-induced, ergo the current known limits for these evolved strains survival are between pH 5 and 9. However, further tests could be done to try and amplify this range by testing the margin between 3 and 5, and 9 and 11. As for the values studied, represented in Figure 14, the different strains had similar results for some pH values, as for example pH 7, in which all the strains grew faster than the other pH studied. However, the RecA strain seemed to need more time to reach the mid-exponential phase compared to the rest, specially for the non-induced strain and pH = 5. This indicates that either the gene's presence is not beneficial for the cell, or that the rest of the genes balance out the harmful effect of this metabolic load. It is noticeable that, in general, pH 7 or neutral is the most beneficial for

the cell's growth, but alkaline pH seem to affect less to the growth rates than acid, which is a general trend through all the strains studied.

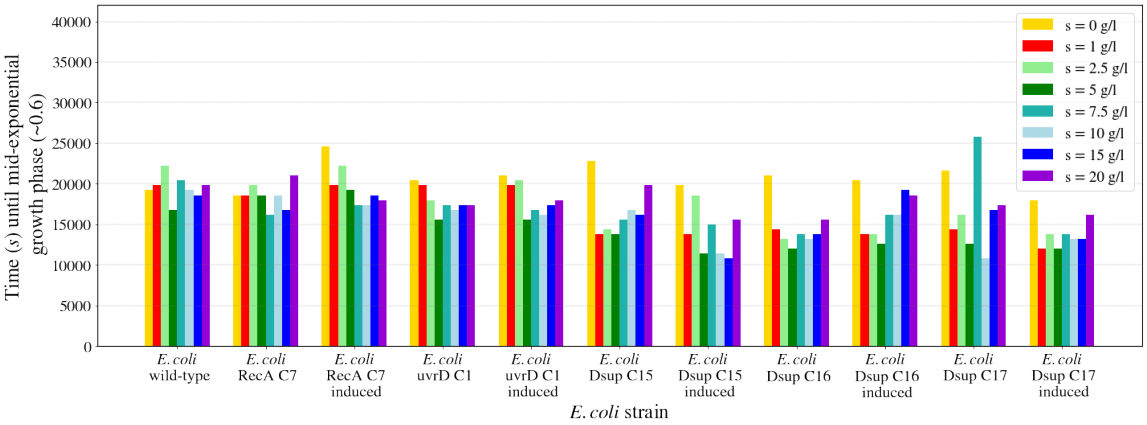


Figure 13: Analysis of the time each strain needed to grow up to mid-exponential growth phase (∼0.6) for different LB salinity levels.

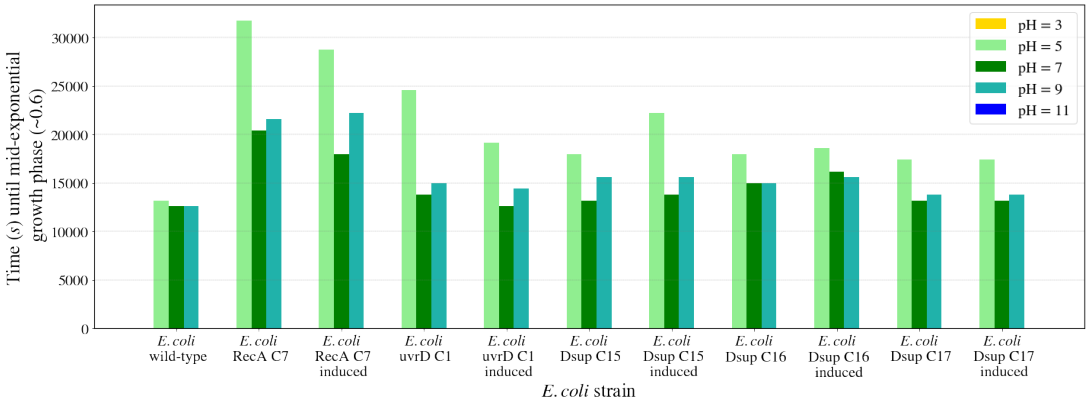


Figure 14: Analysis of the time each strain needed to grow up to mid-exponential growth phase (∼0.6) for different LB pH values. PH values 3 and 11 showed no cell growth for any of the strains.

4 Discussion

A lot of research has been done trying to determine the habitability of different planetary bodies [47][48], and some studies have evidenced the survival and growth of microorganisms under lab-simulated planetary conditions, as is the case of Mars [49][50] or Saturn's moon Enceladus [51]. Moreover, as shown in this project, studies aboard the ISS have proven that some microorganisms and bacteria have many genetic and molecular mechanisms to survive high levels of radiation, vacuum and temperature ranges, and defining the limits of life on Earth is crucial for analysing these experiments and understanding, not only the possible development of extra-terrestrial habitats, but also the possible contamination due to human exploration, the potential of panspermia and microbe transfer, and the exploration of extinct and extant life.

The exposure of *E. coli* to high levels of Ionizing and UV Radiation proved to have a big impact on survival. However, some microorganisms can persevere under similar radiation conditions. These microorganisms have genetic mechanisms to protect and repair the DNA to avoid the damage caused by radiation. Some of these genes have been widely studied and tested, and for this study, three of these genes were selected to try to enhance the natural radiation resistance of *E. coli*. In particular, the Dsup gene of the tardigrade spp. *Ramazzottius varieornatus*, which has been previously studied due to its DNA protection role in this species, was the gene which better enhanced the resistance to both radiation types, having a big impact on Ionizing Radiation survival after the directed evolution process, obtaining a 100% survival after being exposed to 500 Gy of radiation, and increasing the survival at 3000 Gy in over two orders of magnitude. When tested under UV Fluence, the Dsup gene also obtained the best results, although these were very similar to the values obtained by the wild-type and the other two genes studied, which were the RecA and uvrD genes, both from *Deinococcus radiodurans*. From these, uvrD was the gene with better results, with a very high survival when exposed to 500 Gy of radiation. However, when the radiation levels reached 3000 Gy, its survival was similar to the wild-type. The RecA gene did not enhance the resistance of *E. coli*, but decreased it, probably because of its similarity with the orthologous *E. coli* RecA gene, which could have interacted with the transformed gene. This underlines the current limitations of interspecies gene editing and transformation. Still, the overall results obtained by the three genes highlight the possibility of enhancing the radiation resistance of cells, which could be very useful for life sciences engineering, but also for medical tools for the diagnosis or treatment of some diseases.

Vacuum and a wide range of temperatures can have big effects on cellular metabolism, which can result in major impairments in the survival of exposed cells. For the *E. coli* strains tested in this study, the best results were obtained by the wild-type, while the strains containing one of the genes mentioned above showed a lower survival. Besides this, the survival for all the strains was very low, especially when compared to other species like *D. radiodurans*,where cell aggregation helped enhance its survival. This effect was not observed in *E. coli*, in which no significant change on the survival between aggregated and non-aggregated cultures has been observed. To further study the limitations of the studied strains, a directed evolution process could be applied to try to enhance the resistance to temperatures and low pressures by exposing and selecting the survival cells. Moreover, some genes related to thermal resistance [52][53][54] could also be transformed and characterized to study their possible use in enhancing cellular resistance to high and low temperatures.

Finally, the study of the growth of the selected strains in media with different salinity and pH values revealed the current limits for these strains, showing a closed range of pH in which the cells can proliferate, and a much wider range of salt concentrations in which the cells can grow. Still, further testing should be done to study the cells metabolic response to higher salinity, and a more specific range of pH in which the cells could persevere.

This study has evidenced that an enhancement of cellular resistance to harsh environments is possible but is also limited by the current knowledge on the natural genetic and molecular mechanisms of protection and DNA repair existing in extremophiles. Consequently, increasing the knowledge on these mechanisms is still key to develop resistant bacterial strains and extra-terrestrial habitats. In this study, the cellular resistance of E. coli was enhanced to the point that it should allow the survival of some of the selected strains in environments like the equator of Mars, but also outside of the International Space Station. New tests and procedures should be performed to further study and characterize the surviving possibilities and limitations of the evolved strains. For instance, the shielding properties of cell aggregation could increase the survival to UV radiation, but also to corpuscular Ionizing Radiation, such as beta or gamma rays. Also, a combination between vacuum and continuous wide temperature cycles exposure should be done to further characterize the limits of the selected strains, together with the directed evolution processes and genetic editions mentioned earlier.

The development of environments hospitable to life and the possibilities of building extra-terrestrial habitats are tied to the knowledge and capacity of enhancing bacterial, fungi and cellular resistance to the existent conditions. For this, many environmental factors must be considered, but also some ethical and moral concerns. The selection of the most adequate species for the development of the desired ecosystem has a major role, and the capacity of controlling the survival of the introduced species is key for the preservation of the natural conditions. The preservation of environments and the avoidance of microbe transfer has led to many serviceable spacecrafts and satellites being terminated to minimize the risks of microbe transfer, as was the case of NASA's orbiter Cassini [55], which was burned in Saturn's atmosphere before an eventual crash into Enceladus, which has been studied as an eminently habitable environment. Similar reasoning led to the termination of NASA's Jupiter orbiter Galileo [56]. These procedures comply with the planetary protection protocols, established to minimize the risks of depositing Earth microbes into possibly habitable environments. However, it has proven impossible to construct a microbe-free spacecraft, and experiments such as the ones done with D. radiodurans prove that some species can remain viable in outer space. These studies have led to very strict policies on space exploration, but still, the risks are not eliminated. In addition, the idea of human exploration adds more concerns to this field, and any human mission in space would almost certainly have to break those policies to survive. On the other hand, space exploration is not the only source of microbe transfer, as debris caused by asteroid impacts could also carry potentially viable microbes. All this leads to the conclusion that eventual microbe transfer is inevitable unless space exploration is abandoned, and no exchange between other planets or organic matter is happening.

Space exploration is essential for survival, expanding not only our knowledge and resources, but also opening the door to many new possibilities. However, as this study has revealed, there is still plenty of knowledge and resources to be gained on Earth that could help in the exploration and development of extra-terrestrial habitats. Additionally, many life science engineering strategies could be potentially used in this field, and the search of Earth's life limits and their expansion could provide some insight into the generation of habitable environments. Space is believed to be the last frontier, but the truth is, we are still a frontier to ourselves.

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Supporting information

S.1 Ionizing Radiation Absorbed Dose Estimated Error

In this section, the estimated error of the IR dose exposure is explained based on the method and the parameters used to irradiate the cells using the *Varian TrueBeam STX* ® linear accelerator from *Hospital del Mar*.

- **Experiment Conditions**

  The cells were presented in different flasks of liquid culture, one for each dose. The useful dimensions for the irradiation were 4.5x2.5x6.2 cm^3. The irradiated doses were from 250 Gy to 3000 Gy in steps of 250 Gy (12 doses in total) at a dose rate of 1.35 Gy per minute. The 12 flasks were compacted in packs of 3. The irradiation was done using the linear accelerator *Varian TrueBeam STX* ® from *Hospital del Mar*'s Radiotherapy Department. Due to the dosage and the conditions of the study, the irradiation was done with 10 MV photons. Using the Planning System *Eclipse* the geometry and the irradiation times were calculated. The packs were rotated $180^{\underline{o}}$ every 125 Gy to homogenize the dose. To homogenize the dose, the flasks were submerged in water using the model *WP1D phantom* from *IBA Dosimetry*, with 1 cm PMMA walls and internal dimensions 34x40x35 cm^3. Each time a flask is extracted, it was replaced by a flask full of water (inactive flask).

- **Estimated Error Calculations**

  From tomographic images, the liquid culture's electronic density was 1.015 and water's electronic density was 1.009. The difference is <0.6%, which means that the error on the dosage due to the use of the model is limited to 0.6%.

  To adjust the irradiation, the Source-Skin Distance was set to 65 cm. This distance to the source adds an additional error to the dosage. This error was estimated to be 0.57%.

  Taking into account the homogeneity of the dose and considering a lineal decrease of the dose along the flask packs, there's an expected error or 3%

  Finally, the verification test of the administered dose by the linear accelerators showed a difference of -0.57%.

- **Final Absorbed Dose Estimated Error**

  Taking into consideration the different errors described above, the error in the dosage is estimated at 3.6%.

S.2 Data Availability Statement

The original contributions and data presented in this study are available from the corresponding author, JP, upon reasonable request at jaume.p98@gmail.com