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ORIGINAL ARTICLE

Experimental design and environmental parameters affect *Rhodospirillum rubrum* S1H response to space flight

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In view of long-haul space exploration missions, the European Space Agency initiated the Micro-Ecological Life Support System Alternative (MELiSSA) project targeting the total recycling of organic waste produced by the astronauts into oxygen, water and food using a loop of bacterial and higher plant bioreactors. In that purpose, the α-proteobacterium, *Rhodospirillum rubrum* S1H, was sent twice to the International Space Station and was analyzed post-flight using a newly developed *R. rubrum* whole genome oligonucleotide microarray and high throughput gel-free proteomics with Isotope-Coded Protein Label technology. Moreover, in an effort to identify a specific response of *R. rubrum* S1H to space flight, simulation of microgravity and space-ionizing radiation were performed on Earth under identical culture set-up and growth conditions as encountered during the actual space journeys. Transcriptomic and proteomic data were integrated and permitted to put forward the importance of medium composition and culture set-up on the response of the bacterium to space flight-related environmental conditions. In addition, we showed for the first time that a low dose of ionizing radiation (2 mGy) can induce a significant response at the transcriptomic level, although no change in cell viability and only a few significant differentially expressed proteins were observed. From the MELiSSA perspective, we could argue the effect of microgravity to be minimized, whereas *R. rubrum* S1H could be more sensitive to ionizing radiation during long-term space exploration mission.

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Introduction

In view of long-haul manned space exploration missions, the cargo weight to be uploaded is of major concern. Taking into account the crew's need for water and food and the corresponding wastes build-up during a several years trip, a biological recycling system seems to be essential (Farges *et al.*, 2008). For that purpose, the European Space Agency started the Micro-Ecological Life Support System Alternative (MELiSSA) project 20 years ago (Mergeay *et al.*, 1988). The MELiSSA concept is based on a lake ecosystem and consists of interconnected processes (that is, bioreactors, higher plant compartments, filtration units and so on) targeting the total recycling of the crew's organic waste into oxygen, water and food (Hendrickx *et al.*, 2006). Within the MELiSSA system, the purple non-sulfur α-proteobacterium, *Rhodospirillum rubrum* S1H, is used to convert volatile fatty acids released from the upstream raw waste-digesting reactor to carbon dioxide and biomass, and to complete the mineralization of amino acids into free ammonium that will be forwarded to the nitrifying compartment. The functional stability of the bioreactors in long-term use and under space flight conditions is of paramount importance for the efficiency of the life support system, and consequently, crew safety (Hendrickx *et al.*, 2006; Hendrickx and Mergeay, 2007).

The radiation on Earth comes from a combination of terrestrial (from the ^{40}K, ^{232}Th, ^{226}Ra, etc.) and cosmic

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radiation (photons, electrons and so on) and is fairly constant over the world, in order of 2–4 μGy day^{-1} (Idaho State University, 2008). Monitoring radiation on board the International Space Station (ISS) in low Earth orbit (*ca.* 400 km altitude) indicated an average dose of *ca.* 180 μGy day^{-1} (Goossens *et al.*, 2006; Vanhavere *et al.*, 2008). Thus, on board the ISS, organisms undergo radiation stress up to 80-fold higher than the background radiation levels on the Earth. In addition, gravity in low Earth orbit ranges between 10^{-6} and 10^{-3} g compared with 1 g on Earth (Nicholson *et al.*, 2000). As environmental changes induce and select for physiological, metabolic and/or genetic variations in microorganisms (Foster, 2007), it is envisioned that such adaptations will also likely occur under space flight conditions. Indeed, numerous in-flight studies have confirmed that space flight can have a pronounced effect on a variety of microbial parameters including changes in microbial proliferation rate, cell morphology, cell physiology, cell metabolism, genetic transfer among cells and viral reactivation within the cells (reviewed in Leys *et al.*, 2004; Nickerson *et al.*, 2004; Nicholson *et al.*, 2005; Klaus and Howard, 2006). However, previous studies have also shown that the results from space flight and space flight analog experiments can radically differ when using different bacteria or when using the same bacterium but different culture media (Baker and Leff, 2004, 2006; Benoit and Klaus, 2007; Wilson *et al.*, 2008; Leys *et al.*, in press).

In this study, *R. rubrum* S1H, inoculated on rich and minimal agar media, was sent for *ca.* 10 days to the ISS, respectively, in October 2003 (MESSAGE-part 2 experiment) and September 2006 (BASE-part A experiment). After flight, *R. rubrum* S1H cultures were subjected to both transcriptomic and proteomic analysis, respectively, by whole-genome oligonucleotide microarrays and high throughput proteomics to analyze the response of S1H to space flight. Moreover, ground simulation of space-ionizing radiation and microgravity were performed to identify radiation and gravity effects.

Materials and methods

Strain and media

R. rubrum strain S1H was obtained from the American Type Culture Collection (ATCC25903). Sistrom medium A containing 2 g l^{-1} Na-succinate (Sistrom, 1960) or Sistrom-peptone-yeast medium (Saegesser *et al.*, 1992) containing 30 g l^{-1} peptone and 10 g l^{-1} yeast extract (BD Franklin Lakes, NJ, USA), supplemented with 20 g l^{-1} agar for solid cultures, were used. For the MESSAGE 2-related experiments Sistrom-peptone-yeast was used, whereas Sistrom-succinate was used for the BASE-A-related experiments. The 869 medium (Mergeay *et al.*, 1985) supplemented with 2 mM H_6TeO_6 (Sigma-Aldrich, Bornem, Belgium) was used for post-flight analysis of tellurate resistance.

Space flight experiment set-up

The MESSAGE 2 experiment. Three independent cultures of *R. rubrum* S1H grown to stationary phase in Sistrom medium A in dark aerobic conditions were suspended in 0.85% NaCl (Saline tablets, Oxoid, Hampshire, United Kingdom) to a final OD_{680} of *ca.* 0.600 and transported at room temperature from the laboratory in SCK•CEN (Mol, Belgium) to the cosmodrome in Baikonour (Kazakhstan) 8 days before launch. Inoculation was performed 15 h before launch on Sistrom-peptone-yeast Petri dishes (Surfair Plate, PBI International, Milano, Italy). Drops of 10 μl containing 3×10^7 colony forming units of *R. rubrum* S1H, as well as dilution series (3×10^3 and 3×10^2 colony forming units) for viable count purpose, were spotted in biological triplicates spread over two agar plates (Supplementary Figure 1A). After inoculation, Petri plates were sealed first with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) and Kapton tape (3M, Cergy-Pontoise, France), next, individually packed in Ziplock bags, and then jointly packed in a second Ziplock bag and placed in a sealed jar. Radiation dosimeters (Track-Etch Detectors, Optically Stimulated Luminescence Detectors and ThermoLuminescent Detectors, described in Goossens *et al.*, 2006) and temperature sensors (CUBE, Meilhaus Electronic, Puchheim, Germany) were added in the jar. This jar was then wrapped in protective foam and NOMEX fabric bags (DuPont, Richmond, VA, USA) and stored at room temperature (Supplementary Figure 1). Finally, the exterior of the package was disinfected with 3% hydrogen peroxide wipes according to Russian flight procedures and was placed into the Soyuz (TMA-3) 8 h before launch. On arrival in the ISS (after 2 days at ambiant temperature in the Soyuz), the containers were incubated for 8 days in the Russian Service Module of the ISS behind structural bars (Supplementary Figure 1E) at a relative constant temperature of 21 ± 2 °C. After a total of 10 days flight, including *ca.* 2 days in the Soyuz and *ca.* 8 days in the ISS (18–28 October 2003), the samples returned to the Earth (Soyuz TMA-2). After landing in the Kazakhstan desert (near Arkalyk), the jar containing the cultures in Petri dishes was transported within 40 h at 4 °C without exposure to airport X-rays scanning, to SCK•CEN (Mol, Belgium). In contrast, the parallel-prepared ground control samples returned immediately after preparation in Kazakhstan to SCK•CEN and were cultured in dark conditions under comparable temperature and time profile as the ISS samples. From both ground control and ISS-exposed samples, colonies were harvested for both transcriptomic and proteomic analysis.

The BASE-A experiment. Cultures of *R. rubrum* S1H grown as mentioned above were transported at room temperature from SCK•CEN (Mol, Belgium) to Baikonour (Kazakhstan) 10 days before launch. Three biologically independent culture suspensions were deposited 24 h before launch as 4 spots of 10 μl for life count, as mentioned above, and as 1 mat of 150 μl

corresponding to 3×10^7 cells per *ca.* 10 cm^2 surface of a 5 ml layer Sistrom-succinate agar medium in six-well culture plates (CellStar 6, Greiner Bio-One, Frickenhausen, Germany) (Supplementary Figure 1F) and kept at ambient temperature. An oxygen indicator strip (Anaerotest, Merck, Darmstadt, Germany) was added at the bottom of the multiwell plate in between the wells, to visualize oxygen concentration in the gas phase in the multiwell plate during incubation pre-, in- and post-flight. Culture plates were sealed with one layer of Parafilm and one layer of Scotch tape (3M, Cergy-Pontoise, France). Radiation dosimeters (Track-Etch Detectors and Optically Stimulated Luminescence Detectors described in Vanhavere *et al.*, 2008) and temperature sensors (SmartButton, ACR Systems, Surrey, BC, Canada) were added in the container. Two culture plates were sealed hermetically in one polycarbonate 'Biocontainer' (PedeoTechniek, Oudenaarde, Belgium) (described in Vanhavere *et al.*, 2008) and vacuum sealed in a highly transparent Minigrip polyethylene bag (Minigrip, Brussels, Belgium) of 60-μm thickness. During the 2-day trip in the Soyuz (TMA-9) to the ISS, the temperature of the pouch was maintained at 22 ± 1 °C. After docking, all samples were stored at ambient temperature in the ISS Russian Zvezda service module (the exact location in the module is unknown) and a temperature of 21 ± 1 °C was recorded. The samples returned to the Earth after a total of 12 days in-flight including *ca.* 2 days in the Soyuz and *ca.* 10 days in the ISS (17–28 September 2006). Samples were handled as mentioned for the MESSAGE 2 experiment, and 24 h after landing of the Soyuz (TMA-8) the analysis was started at SCK•CEN. The parallel ground control experiment was maintained at 22 ± 1 °C during transport from Baikonur to Belgium in transportation box, followed by an incubation at 22 ± 1 °C, and simultaneously cooled down to 4 °C after landing of the space samples. From both ground control and ISS exposed samples, colonies and cell mats were harvested for transcriptomic and proteomic analysis.

Simulated microgravity experiment set-up

R. rubrum S1H cultures, prepared according to the space flight experimental set-ups described above, were mounted on the European Space Agency random-positioning machine (RPM) facility located at the University of Sassari (Italy) in a room with an ambient temperature of *ca.* 22 °C (Figure 1a). The RPM was built by Dutch Space in Leiden (The Netherlands) and is similar to an earlier Japanese model, which was extensively reviewed in Hoson *et al.* (1997). The total sample package ($20 \times 20 \times 10$ cm^3) was mounted at the center of the two independent frames for which rotation is driven by two separate motors. The RPM was operated as a random walk three-dimensional clinostat (basic mode) with an angular velocity of rotation of 60 deg s^{-1} (Walther *et al.*, 1998) for 10 days (maximum cultivation time allowance for that facility). Random rotation at 60 deg s^{-1}, 1–10 cm away from the center of rotation, yields gravity contours from 1.12×10^{-3} to 1.12×10^{-2} g as calculated by $g' = (\omega^2 R)/g_0$, where $\omega = 1.05$ radian s^{-1}, $R = 0.01$–0.10 m and $g_0 = 9.81$ m s^{-2} (Hoson *et al.*, 1997; Boonyaratanakornkit *et al.*, 2005).

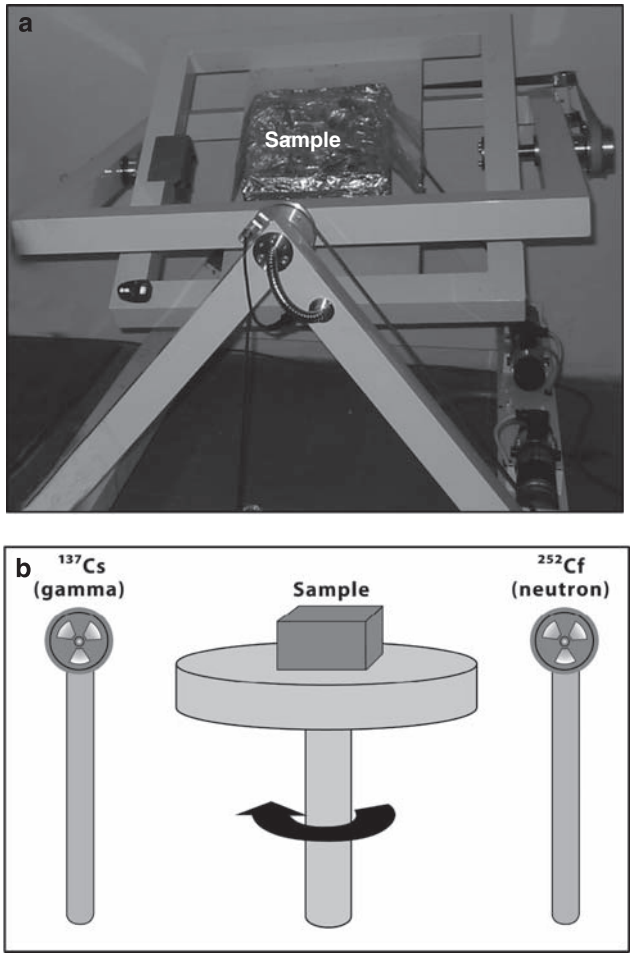

Figure 1 Random positioning machine (RPM) (**a**) and ionizing radiation (**b**) experimental set-up.

Simulated space-ionizing radiation experiment set-up

To mimic the ISS-ionizing radiation environment, ^{137}Cs γ and ^{252}Cf neutron sources were used as representative of low-linear energy transfer (LET) and high-LET particles, respectively. ^{137}Cs is a common mono-energetic source of 0.662 MeV γ rays (LET up to 10 keV μm^{-1}). The neutron source ^{252}Cf has been studied in the SCK•CEN calibration room facility (Vanhavere *et al.*, 2001) and is expelling a neutron spectrum with an average energy of 2.1 MeV (LET up to 250 keV μm^{-1}). The source reference values were traceable to primary standards (PTB for γ and NPL for neutrons). *R. rubrum* S1H agar cultures, prepared according to the space flight experimental set-ups, were exposed to ionizing radiation in the calibration room facility at SCK•CEN (Mol, Belgium).

During irradiation, samples were kept rotating at 2 r.p.m. in between the two sources for 10 days (maximum irradiation time allowance for the facility) at 21 ± 2 °C (Figure 1b), being exposed to *ca.* 0.16 mGy day^{-1} of γ rays and 0.02 mGy day^{-1} of neutron rays, totaling *ca.* 1.8 mGy per 10 days, which is comparable with the actual space flights experiments (Goossens *et al.*, 2006; Vanhavere *et al.* 2008). The total cumulative absorbed dose was measured *in situ* by using ThermoLuminescent Detectors (LiF:Mg, Ti 'TLD-100' from Harshaw Bicron, Solon, OH, USA and LiF:Mg,Cu,P 'MCP-N' from TLD-Poland, Kraków, Poland) and Optically Stimulated Luminescence Detectors (Al2O3:C 'TLD-500' from Harshaw Bicron) for γ dosimetry and bubble detectors (BD-PND/BDT from Bubble Technology Industry, Chalk River, ON, Canada) for neutron dosimetry. Non-irradiated control samples were kept outside the irradiation bunker at the same temperature for the same period of time.

Transcriptomic analysis

Microarray platform. The *R. rubrum* S1 genome comprising a chromosome (4.35 Mb; GenBank ID: CP000230) and a plasmid (53.7 kb; GenBank ID: CP000231) was used to design 60-mer aminosilane-modified oligonucleotide probes corresponding to the 3829 candidate protein-encoding genes. The oligonucleotides were spotted in triplicate onto glass slides by Eurogentec SA (Liège, Belgium), thus providing three technical replicates for each sample. The full description of the array analysis platform has been deposited at the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/) under accession number GPL7256. The full experimental details on the RNA extraction, labeling, hybridization and microarray analysis can be found in Supplementary Data. All microarray data have been deposited at the Gene Expression Omnibus under accession number GSE14265.

Data analysis. Only genes with a *P*-value below 0.05 and showing a fold change below 0.5 or above 2.0 were kept for data interpretation. Genes of interest were explored and some were re-annotated using the Magnifying Genomes (MaGe) platform (Genoscope, Evry, France), a microbial genome annotation system (Vallenet *et al.*, 2006). The parent strain *Rhodospirillum rubrum* S1 is part of the MaGe 'MagnetoScope' project available at https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/MageHome/index.php?webpage = mage.

Proteomic analysis

Protein extraction and quantification. Bacterial cells were collected by centrifugation at 7000 r.p.m. for 10 min. Protein samples were obtained by high-power sonication (U50 control, IKA Labortechnik, Staufen, Germany) of the bacterial pellet suspended in 6 M (v/v) guanidine chloride. Sonication was realized by three cycles of 10 s (40% amplitude, cycle 1) followed by 1 min cooling on ice. Samples were cleared by centrifugation at 13 500 r.p.m. for 15 min at 4 °C. Protein concentration of the supernatants was measured by the Bradford method (Bradford, 1976), according to the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA), with bovine γ-globulin as a protein standard. Protein concentration of each sample was adjusted to 5 μg μl^{-1} and equal amounts of the three biological replicates were pooled before labeling.

Isotope-coded protein labeling. Isotope-coded protein labeling (ICPL) was performed using the ICPL-kit (SERVA Electrophoresis, Heildberg, Germany) as described previously (Schmidt *et al.*, 2005). Briefly, two protein mixtures of 100 μg each, obtained from two different culture conditions, were first individually reduced and alkylated to denature proteins and to ensure easier access to the free amino acid groups that are subsequently derivatized with the ^{12}C (light) or ^{13}C (heavy) form of the ICPL reagent (N-nicotinoyloxy-succinimide). The heavy (H) form of the IPCL reagent was used to label the experimental sample, whereas the light (L) form was used to label the control sample. Labeled samples were then combined in a H/L ratio of 1:1. After subsequent overnight digestions by trypsin (protein/enzyme ratio of 1/50 and incubation at 37 °C) and by endoproteinase Glu-C (protein/enzyme ratio of 1/30 and incubation at 25 °C), peptides were desalted using HyperSep SpinTip C18 (Thermo Electron, Columbia, MD, USA) and analyzed by MudPIT (Multi-Dimensional Protein Identification Technology). Each ICPL sample was run twice to increase the peptides detection number and the protein identification and quantification accuracy. As differentially labeled protein samples derived from identical peptides differ in mass, they appeared as doublets in the acquired mass spectra. From the ratio of the ion intensity of these sister peptide pairs, the relative abundance of their parent protein in the original samples was determined. H/L ratios greater than 1 and lower than 1 indicate, respectively, the overexpression and downexpression of the protein of interest in the experimental sample. Experimental details on the MudPIT approach including the chromatographic separation and the mass spectrometry analysis can be found in Supplementary data.

Data extraction and database search. Data acquired from both runs were combined in a single file using AnalysisCombiner (Bruker Daltonics, Bremen, Germany) and using DataAnalysis 3.4 (Bruker Daltonics) a peak list in Mascot Generic File format was generated. The peak list was searched against a local copy of the NCBInr database released in June 2008 (taxonomy = *Rhodospirillum rubrum* S1) using an in-house Mascot 2.2 server (Matrix Science, Boston, MA, USA) for protein identification ($P < 0.05$). Protein identification was achieved using the Mascot search engine including the following parameters: database = NCBInr,

organism = *Rhodospirillum rubrum* S1, fixed modification = carbamidomethyl cystein, variable modification = oxidation of methionine, ICPL light and heavy (on lysine-K- and N-terminal), mass tolerance in MS = 1.3 Da and mass tolerance in MS/MS = 0.5 Da. Correct mixing of the experimental (H-labeled) and the control (L-labeled) samples was checked by plotting the protein fold change (that is, H/L ratio) distribution. Accepted value for the median value of the H/L ratio distribution was 1.0 ± 0.1 (Supplementary Figure 2). The false discovery rate (FDR) was estimated using the Mascot 'decoy' option. If TP was true-positive matches and FP was false-positive matches, the number of matches in the target database was TP + FP and the number of matches in the decoy database was FP. The quantity that was reported was the FDR = FP/(FP + TP) (Elias *et al.*, 2005). Only proteins identified with a Mascot score above 50 and quantified by at least two different peptides or by the same peptide detected at different times during the analysis were considered. The Mascot score is given as $S = -10 \times \log_{10}(P)$, where P is the probability that the observed match is a random event. Protein quantification was completed using WARP-LC (Bruker Daltonics) that detects isotopic pairs resulting from differentially labeled proteins and calculates intensity ratio of each peptide pair based on peak intensity. Proteins quantified with a mean calculated peptide ratio showing a standard deviation higher than 20% were manually checked. Proteins were considered as significant when fold change was higher than 1.5 or lower than 0.7.

Results

MESSAGE 2 and related experiments: sistrom-peptone-yeast medium

Transcriptomic analysis. For the MESSAGE 2 space flight experiment, the ground control and ground-based simulations of microgravity and ionizing radiation, the same hardware configurations were used consisting of 10-day incubation time on rich medium in Petri dishes under dark aerobic conditions. Using the whole-genome DNA chip, 4.94% and 0.78% of the genes (out of 3826 genes retained) were identified as, respectively, significantly up- and downregulated after space flight compared with the ground control. Concerning the modeled microgravity experiment, 3.88% and 0.26% of the genes (out of the 3814 genes retained) were identified as, respectively, significantly up- and downregulated when cultured on the RPM compared with culturing in normal gravity. Finally, for the ionizing radiation simulation, 0.13% and 0.10% of the genes (out of 3794 genes retained) were identified, respectively, as significantly up- and downregulated compared with non-irradiated samples.

When sorting the differentially expressed genes according to their COG functional category (Table 1 and http://www.ncbi.nlm.nih.gov/COG/), the 'unclassified' (Un.) class was the most numerically abundant in the ionizing radiation and RPM experiments, and the second most in the MESSAGE 2 experiment (Figure 2). Moreover, of the 373 significant genes found in at least one of the tests, 30% coded for hypothetical proteins (Supplementary Table 1).

No genes met the threshold criteria for differential expression in all three culture conditions tested, that is, space flight, RPM and ionizing radiation experiments (Figure 2c). However, one hypothetical gene (Rru_A0197) was downregulated in the irradiation and the RPM experiment and showed a 0.59-fold change ($P < 0.001$) in the MESSAGE 2 experiment. Moreover, Rru_A0196 and Rru_A0198, both encoding for hypothetical proteins, were significantly downregulated (0.51-fold change) in the irradiation experiment and were slightly, but significantly, downregulated in the RPM (only Rru_A0196) and MESSAGE 2 (both Rru_A0196 and Rru_A0198) experiments. For the 11 significant genes that were jointly induced in the MESSAGE 2 flight and RPM experiments, four coded for hy-

Table 1 Representation of genes grouped by COG functional categories within the *R. rubrum* S1 genome

		%	No.
Information storage and processing			
A	RNA processing and modification	0.05	2
K	Transcription	5.79	222
L	DNA replication, recombination and repair	3.55	136
B	Chromatin structure and dynamics	0.05	2
Cellular processes			
D	Cell division and chromosome partitioning	0.97	37
V	Defense mechanisms	1.51	58
T	Signal transduction mechanisms	4.83	185
M	Cell envelope biogenesis, outer membrane	5.37	206
N	Cell motility and secretion	3.36	129
O	Posttranslational modification, protein turnover, chaperones	3.26	125
Z	Cytoskeleton	0.00	0
U	Intracellular trafficking and secretion	0.97	37
J	Translation, ribosomal structure and biogenesis	4.02	154
Metabolism			
C	Energy production and conversion	5.84	224
G	Carbohydrate transport and metabolism	4.20	161
E	Amino acid transport and metabolism	8.58	329
F	Nucleotide transport and metabolism	1.77	68
H	Coenzyme metabolism	3.63	139
I	Lipid metabolism	2.87	110
P	Inorganic ion transport and metabolism	4.85	186
Q	Secondary metabolites biosynthesis, transport and catabolism	1.56	60
Poorly characterized			
R	General function prediction only	9.21	353
S	Function unknown	7.51	288
Un.	Unclassified	16.25	623

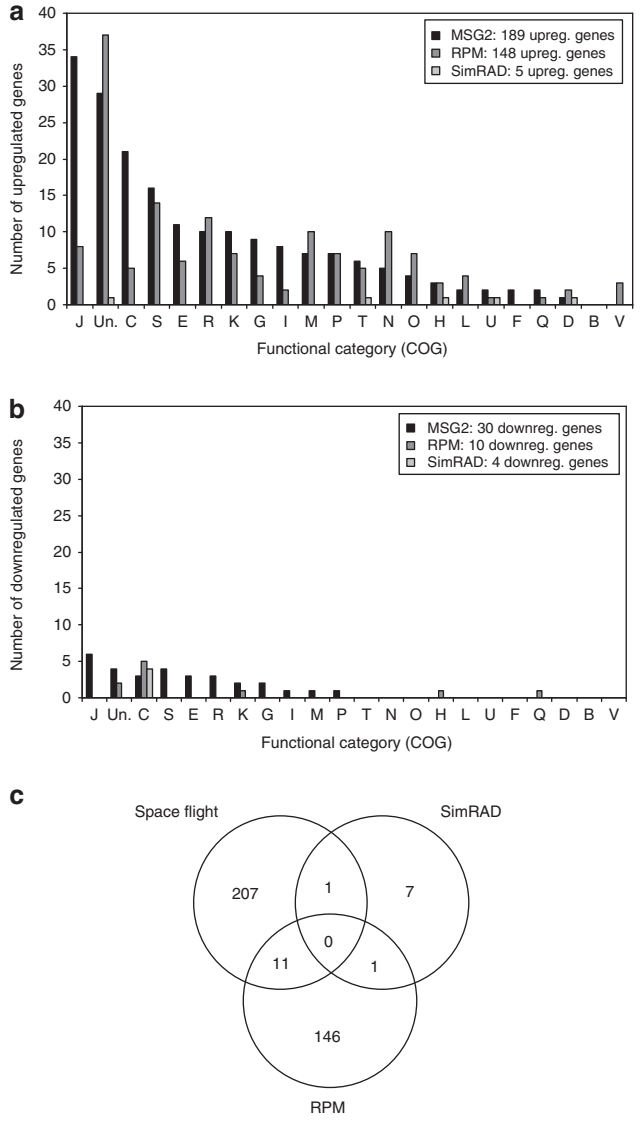

Figure 2 Functional classification of the significant genes upregulated (**a**) and downregulated (**b**) during the MESSAGE 2 space flight experiment (MSG2), the modeled microgravity experiment (RPM) and the simulation of ISS-ionizing radiation (SimRAD). (**c**) Venn diagrams showing the relation between the 373 significant genes categorized from the MSG2, the RPM and the SimRAD experiments ($0.5 >$ fold change > 2 and P-value < 0.05). See Table 1 for the COG nomenclature.

pothetical proteins (Table 2), with the Rru_A3369 gene being induced the most during the flight experiment (13.99-fold). One single hypothetical gene (Rru_A1244) was downregulated both in the MESSAGE 2 and the ionizing radiation experiment. The data specific to each experiment are discussed in detail below.

Transcriptomic analysis of the MESSAGE 2 space flight experiment. As mentioned above, genes from the 'unclassified' (Un.) and the 'function unknown' (S) classes were numerically very abundant in all the differentially expressed genes. The top three of most induced genes encoded for hypothetical proteins (Rru_A3369, Rru_A2713 and Rru_A0160). The 'Un.' category contained 28 genes coding for hypothetical proteins that were differentially expressed only in the space samples. On the other hand, the S class contained 10 space-specific upregulated hypothetical protein-encoding genes (Supplementary Table 1).

Another clear observation was the high number of upregulated genes related to the functional category 'translation, ribosomal and biogenesis' (J) (Figure 2a). Indeed, 29 ribosomal protein-encoding genes, as well as 1 translation initiation- and 2 translation elongation-related genes were found only in the MESSAGE 2 flight experiment and not in the two simulations experiments (Supplementary Table 1). Furthermore, class J was significantly enriched, as indicated by a Fisher's exact test ($P < 0.05$).

Multiple clusters, which classified within the 'energy production and conversion' (C) class, were only significantly upregulated in the space samples like the operon *sdhCDAB* (Rru_A1202 to Rru_A1205) coding for a succinate dehydrogenase, with *sdhA* and *sdhB* being significantly overexpressed, respectively, 1.73- and 1.92-fold, the operon coding for a ubiquinone oxidoreductase *nuoABCDEFG* (Rru_A1555 to Rru_A1561), with *nuoA*, *nuoD* and *nuoE* showing fold changes between 1.86 and 1.96 ($P < 0.05$), five F_0F_1 ATP synthase subunits (Rru_A1223, Rru_A1225 and Rru_A3244 to Rru_A3246), and *hppA* (Rru_A1818) coding for membrane-bound proton-translocating pyrophosphatase. Related to transcription (class K), an alternative sigma factor of extracytoplasmic function (Rru_A3287) and six transcriptional regulators were specifically upregulated in the space samples (Supplementary Table 1).

Genes related to putative oxidative and osmotic stress were induced, including genes involved in the redox balance of the cell, such as a ferredoxin (Rru_A0077), a Fe-S cluster-related gene (Rru_A1069), a *dps*-related gene (Rru_A1499), a superoxide dismutase (Rru_A1760) and a bacterioferritin *bfr* (Rru_A2195). In addition, genes involved in tellurium resistance like *terB* (Rru_A0891) and *terD* (Rru_A0894), and to a lesser extent *terC* (Rru_A0890), Rru_A0892 and *terZ* (Rru_A0893) were specifically upregulated in space samples. This enhanced expression for the space samples was consistent with post-flight phenotypic observations. Plating space and control colonies on 869 solid medium supplemented with 2 mM tellurate showed full growth for the space samples contrary to the ground samples (Supplementary Figure 3). Genes involved in solute transport and thus osmotic regulation that were upregulated included the porins *ompC* (Rru_A2211) and *ompA* (Rru_A3328), the mechanosensitive ion channel gene *mscS2* (Rru_A3072), a potassium efflux protein-encoding gene (Rru_A3670), and Rru_A2485 and the gene cluster Rru_A1604–Rru_A1605 involved in the biosynthesis of trehalose, whereas, the Kdp K^+-uptake system, including the three ATPase subunits

Table 2 Selected significant genes list ($P<0.05$) from the MESSAGE 2 space experiment and the related simulation of microgravity (RPM) and ionizing radiation (SimRAD) experiments

Gene number	*Gene name*	*Product name*	*COG*	*FC MSG2*	*FC RPM*	*FC SimRAD*
Rru_A0197	—	Hypothetical protein Rru_A0197	Un.	0.59	**0.49**	**0.46**
Rru_A0198	—	Hypothetical protein Rru_A0198	Un.	0.68	NS	**0.50**
Rru_A0637	—	Hypothetical protein Rru_A0637	S	**3.18**	**3.51**	NS
Rru_A1244	—	Hypothetical protein Rru_A1244	Un.	**0.48**	NS	**0.21**
Rru_A1537	—	Hypothetical protein Rru_A1537	Un.	**2.70**	**2.88**	NS
Rru_A1771	—	Sec-independent protein translocase TatC	U	NS	NS	**2.20**
Rru_A2091	—	Hypothetical protein Rru_A2091	Un.	**3.97**	**2.75**	NS
Rru_A2367	—	Response regulator receiver domain-containing protein	K	NS	NS	**2.24**
Rru_A2535	—	Flagellar hook-associated protein 2 (FliD, filament cap protein)	N	**2.81**	**2.61**	NS
Rru_A2666	*rpsM*	30S ribosomal protein S13	J	**3.08**	**2.35**	0.89
Rru_A2721	—	2-oxoglutarate synthase, alpha subunit	C	**2.50**	**2.24**	NS
Rru_A2933	—	Hypothetical protein Rru_A2933	Un.	0.71	1.43	**0.47**
Rru_A2994	—	Hypothetical protein Rru_A2994	D	NS	NS	**2.57**
Rru_A3205	—	50S ribosomal protein L33P	J	**3.08**	**2.58**	0.57
Rru_A3369	—	Hypothetical protein Rru_A3369	Un.	**13.99**	**2.35**	NS
Rru_A3636	—	Hypothetical protein Rru_A3636	Un.	NS	NS	**2.22**
Rru_A3681	—	YceI	S	**2.27**	**2.31**	1.30
Rru_A3719	—	Methyltransferase FkbM	H	NS	NS	**2.26**
Rru_A3746	—	50S ribosomal protein L25P	J	**2.77**	**2.05**	NS
Rru_A3775	—	XRE family transcriptional regulator	K	**2.08**	**2.23**	NS

Abbreviations: FC, fold change; NS, not significant.
Genes with fold change above 2 or below 0.5 are shown in bold. See Table 1 for the COG nomenclature.

kdpABC, the sensor kinase *kdpD* and the cytosolic response regulator *kdpE* (Rru_A1155 to Rru_A1159), were slightly but significantly ($P<0.05$) downregulated (fold changes between 0.84 and 0.54).

Finally, the gene cluster Rru_A3283–Rru_A3288 was significantly upregulated. By using the MaGe annotation platform (Vallenet *et al.*, 2006), we found that this region contains a divergent putative two-component signal transduction system separated by a putative transcriptional regulator (Rru_A3286) and an alternative sigma factor (Rru_A3287). This sensing and regulatory module is flanked by the upregulated genes Rru_A3284 and Rru_A3283 encoding for, respectively, a putative molecular chaperone for exported proteins and a GTPase. The latter is similar to the small GTPases involved in the formation of intracellular vesicles in magnetic bacteria (Okamura *et al.* 2001) and could also stabilize the protein transport-related SecA protein (Rru_A0235) (Müller *et al.* 1992), which was upregulated 1.8-fold ($P<0.05$).

Transcriptomic analysis of the modeled microgravity (RPM) experiment using the MESSAGE 2 set-up. The ground simulation experiment of microgravity showed 158 significant differentially expressed genes, for which 37% seemed to code for hypothetical proteins (Figure 2, Supplementary Table 1). Genes from the 'unclassified' (Un.) and 'function unknown' (S) functional categories were numerically most abundant (Figure 2). The top 20 of the most induced genes included genes involved in transport (for example, Rru_A0249, Rru_A2387, Rru_A2893 and Rru_A3767), in transcriptional regulation (for example, Rru_A2396 and Rru_A2896), cell envelope biosynthesis (for example, Rru_A0938, Rru_A2938 and Rru_B0046) and many coding for hypothetical proteins. Most of the downregulated genes were coding for hypothetical proteins (Supplementary Table 1).

Transcriptomic analysis of the simulation of ISS ionizing radiation using the MESSAGE 2 set-up. The 2 mGy irradiation experiment mimicking the dose received during flight elicited only a moderate response with five up- and four downregulated genes, for which two of the downregulated genes were also downregulated either in the space flight or in the microgravity simulation experiments. All down- and two upregulated genes coded for hypothetical proteins (Table 2).

Proteomic analysis

Proteomic analysis of the MESSAGE 2 space flight experiment. Two-dimensional protein maps were made for the three independent cultures from MESSAGE 2 space flight and the corresponding ground controls. These two-dimensional maps indicated several changes in protein expression when comparing space versus ground samples: both up- (13 spots) and downregulation (8 spots) were detected in the space samples compared with that in the ground control samples (data not shown). Unfortunately, it was not possible to identify the spots of interest due the low amount of starting material (about 40 μg of proteins).

Proteomic analysis of the modeled microgravity (RPM) experiment using the MESSAGE 2 set-up. In

Table 3 Transcriptomics versus differential proteomics approach for the random positioning machine (RPM) experiment using the MESSAGE 2 set-up

Gene number	*Gene name*	*Product name*	*COG*	*FC RPM mRNA*	*FC RPM protein*
Rru_A0414	—	SSU ribosomal protein S6P	J	2.22	1.29 ± 0.13
Rru_A1072	*rpmE*	50S ribosomal protein L31	J	2.68	0.95 ± 0.05
Rru_A1527	—	Putative ABC transporter ATP-binding protein	R	2.89	IBNQ
Rru_A1537	—	Hypothetical protein Rru_A1537	Un.	2.88	IBNQ
Rru_A1799	—	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	O	2.38	0.79 ± 0.04
Rru_A2302	—	Periplasmic-binding protein/LacI transcriptional regulator	G	2.23	0.87 ± 0.15
Rru_A2666	*rpsM*	30S ribosomal protein S13	J	2.35	1.27 ± 0.17
Rru_A2721	—	2-oxoglutarate synthase, alpha subunit	C	2.24	IBNQ
Rru_A3205	—	50S ribosomal protein L33P	J	2.58	0.83 ± 0.07
Rru_A3249	—	DSBA oxidoreductase	O	2.33	IBNQ
Rru_A3282	—	Hypothetical protein Rru_A3282	S	2.01	0.90 ± 0.02
Rru_A3643	—	GrpE protein	O	2.74	1.34 ± 0.05
Rru_A3700	—	Secretion protein HlyD	M	2.08	IBNQ
Rru_A3744	—	Signal transduction protein	T	3.00	IBNQ
Rru_A3746	—	50S ribosomal protein L25P	J	2.05	IBNQ
Rru_B0025	—	Hypothetical protein Rru_B0025	Un.	2.78	IBNQ

Abbreviations: FC, fold change; IBNQ, identified protein but not quantified by at least two H/L occurrences (see Materials and methods).
'Rru_A' and 'Rru_B' refer to gene located, respectively, on the chromosome and on the plasmid. Proteins are quantified by mean ± standard deviation. See Table 1 for the COG nomenclature.

Table 4 Significant proteins from the random positioning machine (RPM) experiment using the MESSAGE 2 set-up

Gene number	*Product name*	*COG*	*FC RPM*	*No. (H/L)*	*Mascot score*	*MW (kDa)*
Rru_A0738	Glutaredoxin GrxC	O	1.55 ± 0.13	5	271.50	9.63
Rru_A0893	Stress protein	T	1.57 ± 0.05	19	1185.06	20.46
Rru_A0894	Stress protein	T	1.56 ± 0.08	6	851.47	20.29
Rru_A2083	CreA	S	1.60 ± 0.11	5	189.51	17.12
Rru_A2161	Polyphosphate kinase	P	2.04 ± 0.25	3	113.83	84.55

Abbreviations: FC, fold change; MW, molecular weight; No. (H/L), number of H/L occurrences used for protein identification and quantification (See Materials and methods).
'Rru_A' refers to gene located on the chromosome, whereas 'Rru_B' refers to gene located on the plasmid. Proteins are quantified by mean ± standard deviation. See Table 1 for the COG nomenclature.

the modeled microgravity experiment, 406 proteins were identified for which 224 were quantified with at least two peptides (Supplementary Table 2). This constituted, respectively, 10.6% and 5.9% of the total candidate protein-encoding genes with a computed FDR of 0.59% estimated at the peptide level. The median value for the protein fold change distribution plot was 1.04, indicating a correct mixing of the control and the experimental samples during the differential proteomic protocol (Supplementary Figure 2A).

Of the 158 significant differentially expressed genes in modeled microgravity, 16 were confirmed to be present as protein in the same sample (Table 3). However, none of the latter quantified proteins passed the threshold limit set to define differential expression. Proteins that did pass the threshold limit were the electron carrier GrxC, the tellurium resistance-related TerZ and TerD, CreA (function is unknown) and the polyphosphate kinase (Table 4).

Proteomic analysis of the simulation of ISS-ionizing radiation experiment using the MESSAGE 2 set-up. For the simulation of ISS-ionizing radiation, 341 proteins were identified including 192 quantified with at least two peptides (Supplementary Table 3). This represents 6.9% and 5% of the total candidate protein-encoding genes with a computed FDR of 0.34% estimated at the peptide level. The median value for the protein fold change distribution plot was 1.04, indicating again a correct mixing of the control and the experimental samples during the differential proteomic protocol (Supplementary Figure 2B). None of the nine differentially expressed genes from the simulation of the ISS-ionizing radiation using the MESSAGE 2 set-up were identified or quantified using the differential proteomic approach. Proteomic analysis revealed only three proteins showing a significant upregulation considering the threshold mentioned in the Materials and methods part (Table 5). However, the slight upregulation of the osmoprotectant glycine betaine transporter periplasmic subunit ProX (Rru_A2477) could be pointed out.

BASE-A and related experiments: sistrom-succinate medium

Transcriptomic analysis. The methylene oxygen indicator strip placed inside the six-well plate

showed that at the end of all the BASE-A-related experiments the culture conditions were still aerobic.

Using the whole-genome DNA chip, respectively, 1.7% and 0.4% of the genes (out of 3143 genes retained) were identified as significantly up- and downregulated after a 12-day journey in space compared with that in the ground control. For the modeled microgravity experiment, 0.6% and only one of the genes (out of the 3797 genes retained) were identified as, respectively, significantly up- and downregulated after 10 days of culturing on the RPM compared with that on the normal gravity control. For the ionizing radiation simulation, 4.5% and 1.2% of the genes (out of 3723 genes retained) were identified as, respectively, significantly up- and downregulated after a 10-day ionizing radiation experiment compared with non-irradiated samples.

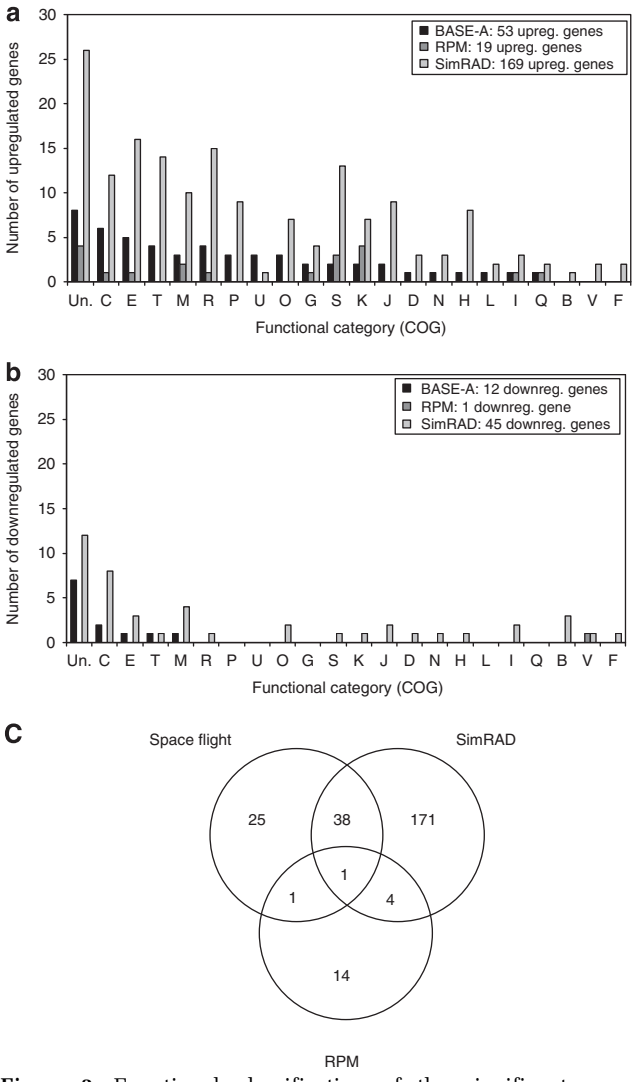

Figure 3 Functional classification of the significant genes upregulated (**a**) and downregulated (**b**) during the BASE-A experiment, the modeled microgravity experiment (RPM) and the simulation of ISS-ionizing radiation (SimRAD). (**c**) Venn diagrams showing the relation between the 254 significant genes categorized from the BASE-A, the RPM and the SimRAD experiments ($0.5 >$ fold change > 2 and P-value < 0.05). See Table 1 for the COG nomenclature.

Considering the 254 significant genes differentially expressed found in at least one of the three cultures conditions tested, 31% seemed to code for hypothetical proteins (Supplementary Table 4). In addition, the 'unclassified' (Un.) functional category (that included 44 hypothetical proteins) seemed to be the most numerically abundant category whatever the culture conditions (Figure 3).

Only three genes (Rru_A0119, Rru_A0269 and Rru_A3286) were differentially expressed in all the three culture conditions. These genes were downregulated in BASE-A and in the simulation of ionizing radiation, whereas they were upregulated during simulation of microgravity (Table 6). Rru_A0119 encoded for a hypothetical protein, Rru_A0269 for a putative outer membrane protein (only a fold change of 0.53 for simulation of radiation but with $P<0.05$) and Rru_A3286 for a putative transcriptional regulator (a fold change of 3.21 for simulation of microgravity but with $P=0.08$). However, BASE-A space flight and the simulation of ISS-ionizing radiation shared 38 differentially expressed genes (same trend) (Figure 3c). These include, for the upregulated genes, the chaperones (Rru_A3332 and Rru_A3433), as well as the cell envelope related genes *mscS1* (Rru_A1608), *wcaA* (Rru_A2737) and Rru_A3715. For the downregulated genes, we found *hfq* encoding for a RNA-binding regulatory protein. Finally, the simulation of ionizing radiation and of microgravity shared five differentially expressed genes with opposite trend (Table 6, Supplementary Table 4). The data specific to all three conditions are described separately below.

Table 5 Significant differentially expressed proteins from the simulation of ISS-ionizing radiation (SimRAD) using the MESSAGE 2 set-up

Gene number	*Gene name*	*Product name*	*COG*	*FC SimRAD*	*No. (H/L)*	*Mascot score*	*MW (kDa)*
Rru_A0930	—	Iron-containing alcohol dehydrogenase	C	2.08 ± 0.02	2	85.92	39.91
Rru_A0931	—	Aldehyde dehydrogenase	C	1.54 ± 0.07	8	534.37	55.73
Rru_A2477	*proX*	Glycine betaine transporter periplasmic subunit	E	1.58 ± 0.10	2	162.49	37.23

Abbreviations: FC, fold change; MW, molecular weight; No. (H/L), number of H/L occurrences used for protein identification and quantification (See Materials and methods).
'Rru_A' refers to gene located on the chromosome. Proteins are quantified by mean ± standard deviation. See Table 1 for the COG nomenclature.

Table 6 List of the 44 significant genes ($P<0.05$) shared by at least two culture conditions within the BASE-A, the random positioning machine (RPM) and the simulation of ISS-ionizing radiation (SimRAD) experiments

Gene number	*Gene name*	*Product name*	*COG*	*FC BASE-A*	*FC RPM*	*FC SimRAD*
Rru_A0119	—	Hypothetical protein Rru_A0119	Un.	**0.42**	**2.02**	**0.37**
Rru_A0235	—	Preprotein translocase subunit SecA	U	**2.12**	NS	**9.21**
Rru_A0269	—	Hypothetical protein Rru_A0269	M	**0.50**	**2.20**	0.53
Rru_A0306	—	Hydrogenase formation HypD protein	O	**2.58**	NS	**5.05**
Rru_A0663	—	Cold-shock DNA-binding protein family protein	K	0.53	**2.37**	**0.41**
Rru_A0672	—	Hypothetical protein Rru_A0672	Un.	**0.50**	1.85	**0.37**
Rru_A0780	—	Binding protein-dependent transport system inner membrane protein	E	**2.12**	NS	**8.76**
Rru_A0858	—	EmrB/QacA family drug resistance transporter	G	**2.65**	NS	**3.15**
Rru_A0920	—	Acetate kinase	C	**2.20**	NS	**4.83**
Rru_A0927	—	Luciferase-like	C	**2.15**	NS	**2.35**
Rru_A0974	—	Linocin_M18 bacteriocin protein	S	0.59	**3.93**	**0.37**
Rru_A1107	—	Hypothetical protein Rru_A1107	K	**2.27**	NS	**2.39**
Rru_A1172	—	AsnC family transcriptional regulator	K	**2.64**	NS	**6.61**
Rru_A1236	—	Iojap-related protein	S	**0.39**	1.73	**0.50**
Rru_A1315	—	Sterol-binding	I	NS	**2.09**	**0.46**
Rru_A1382	—	Butyryl-CoA:acetate CoA transferase	I	**2.26**	NS	**3.04**
Rru_A1411	—	Hypothetical protein Rru_A1411	S	**2.26**	NS	**3.13**
Rru_A1608	—	MscS mechanosensitive ion channel	M	**2.21**	NS	**5.70**
Rru_A1684	*hfq*	RNA-binding protein Hfq	R	**0.45**	NS	**0.41**
Rru_A1881	—	Pyruvate dehydrogenase (lipoamide)	C	**2.03**	NS	**3.26**
Rru_A1934	—	TetR family transcriptional regulator	K	**2.89**	NS	**5.21**
Rru_A1995	—	Divalent cation transporter	P	**2.55**	NS	**2.58**
Rru_A2016	—	Hypothetical protein Rru_A2016	Un.	**2.06**	NS	**2.11**
Rru_A2286	—	Nitrogenase MoFe cofactor biosynthesis protein NifE	C	**2.24**	NS	**5.34**
Rru_A2289	—	Sulfate adenylyltransferase subunit 2	E	**2.67**	NS	**14.65**
Rru_A2389	—	ABC transporter component	P	**2.35**	NS	**4.85**
Rru_A2561	—	Hypothetical protein Rru_A2561	Un.	**2.71**	NS	**4.41**
Rru_A2737	—	Glycosyl transferase family protein	M	**2.27**	NS	**2.29**
Rru_A2837	—	Response regulator receiver domain-containing protein	K	**2.16**	NS	**2.41**
Rru_A2850	—	Hypothetical protein Rru_A2850	M	0.72	**2.02**	**0.33**
Rru_A2895	—	Twin-arginine translocation pathway signal	P	**2.03**	NS	**8.30**
Rru_A3034	—	Hypothetical protein Rru_A3034	Un.	**2.02**	NS	**3.76**
Rru_A3181	—	Hypothetical protein Rru_A3181	S	**0.48**	NS	**0.47**
Rru_A3277	—	Acetylornithine aminotransferase	E	**2.70**	NS	**2.33**
Rru_A3286	—	Hypothetical protein Rru_A3286	Un.	**0.40**	NS	**0.30**
Rru_A3317	—	Hypothetical protein Rru_A3317	Un.	**2.01**	NS	**28.69**
Rru_A3320	—	Pantothenate synthetase	H	**3.55**	NS	**4.05**
Rru_A3332	—	Cytochrome c oxidase cbb3-type, subunit I	O	**2.18**	NS	**6.02**
Rru_A3394	—	Hypothetical protein Rru_A3394	Un.	**0.47**	NS	**0.35**
Rru_A3405	—	Formaldehyde dehydrogenase (glutathione)	C	**2.09**	NS	**5.08**
Rru_A3433	—	Thioredoxin	O	**2.52**	NS	**3.81**
Rru_A3715	—	Lipopolysaccharide biosynthesis	M	**2.41**	NS	**4.88**
Rru_A3733	—	HNH endonuclease	V	**0.48**	NS	**0.41**
Rru_A3746	—	50S ribosomal protein L25P	J	**2.57**	NS	**4.43**

Abbreviations: FC, fold change; NS, not significant.
Genes with fold change above 2 or below 0.5 are shown in bold. See Table 1 for the COG nomenclature.

Transcriptomic analysis of the BASE-A space flight experiment. For the BASE-A flight experiment, the highest induction (3.83-fold) was found for Rru_A3097 encoding for a hypothetical protein. The 52 upregulated genes encode for numerous functions related to translation (for example, Rru_A3746), transport (for example, Rru_A0235 (*secA*)), metabolism, transcriptional regulation, transposition (Rru_A2588, Rru_A2590 and Rru_A3310) and stress, including a DNA mismatch repair gene *mutL* (Rru_A2946) and the mechanosensitive channel *mscS1* (Rru_A1608). Most downregulated genes encoded for hypothetical proteins (Supplementary Table 4).

Transcriptomic analysis of the modeled microgravity (RPM) experiment using the BASE-A setup. With one down- and eight upregulated genes, the simulation of microgravity induced less differential gene expression than the BASE-A space flight experiment or the simulation of ISS-ionizing radiation (Supplementary Table 4). The downregulated gene Rru_A1311 encoded for a MerR-family transcriptional regulator. Among the upregulated genes, we could identify genes encoding for transcriptional regulators (for example, Rru_A1116 and Rru_A2049), transport functions and hypothetical proteins.

Transcriptomic analysis of the simulation of ISS-ionizing radiation using the BASE-A set-up. With 169 up- and 41 downregulated genes, this condition gave the highest number of significant differentially expressed genes (Supplementary Table 4). Furthermore, whereas the fold change neither in the space flight nor in the RPM experiments exceeded 4, the ionizing radiation rendered 20 genes with a fold change above 5. Genes that were upregulated coded for functions involved in transport, cell envelope biosynthesis or maintenance, redox balance, transcriptional regulation, amino acid metabolism and stress response. These included *secA* (Rru_A0235) and *mcsS1* (Rru_A1608), which were also upregulated in the BASE-A flight experiment; a ferric uptake regulator *fur* (Rru_A3788), which was also slightly (1.83-fold), but significantly, upregulated in the MESSAGE 2 flight experiment; the DNA mismatch repair genes *mutS* (Rru_A3541) and *mutL* (Rru_A2946), the latter only slightly upregulated and also upregulated in the BASE-A flight experiment. The downregulated genes not only included a lot of genes encoding for hypothetical proteins but also Rru_A2723, which codes for rubrerythrin and *hfq* (Rru_A1684), also downregulated in the BASE-A space experiment.

Proteomic analysis

Proteomic analysis of the BASE-A space flight experiment. For the BASE-A flight experiment, 366 proteins were identified (Supplementary Table 5) including 248 quantified with at least two peptides. This represents 9.5% and 6.5% of the total candidate protein-encoding genes with a computed FDR of 0.32% estimated at the peptide level. The median value for the protein fold change distribution plot was 0.91 (Supplementary Figure 2C). Of the 65 significant differentially expressed genes, 11 could be confirmed at the proteomic level (Table 7). None of these proteins satisfied the threshold for significance. However, nine downregulated proteins, three hypothetical ones, showed significant differences in concentration in the cell extract from space and control samples (Supplementary Table 5).

Proteomic analysis of the modeled microgravity (RPM) experiment using the BASE-A set-up. With a median value of 1.04, the protein fold change distribution showed again a correct mixing of the control and the experimental samples (Supplementary Figure 2D). Although 429 proteins were identified (Supplementary Table 6) and 282 quantified with at least two peptides (computed FDR was 0.30% estimated at the peptide level) for the ground simulation of microgravity using the BASE-A set-up, no proteins showed a fold change above 1.5 or below 0.7. Of the 29 significant differentially expressed genes, 3 were identified at the proteomic level (Table 8) but without differential expression.

Proteomic analysis of the simulation of ISS-ionizing radiation using the BASE-A set-up. For the ground simulation of ionizing radiation, 387 proteins were

Table 7 Transcriptomics versus differential proteomics approach for the BASE-A experiment

Gene number	*Product name*	*COG*	*FC BASE-A mRNA*	*FC BASE-A protein*
Rru_A0235	Preprotein translocase subunit SecA	U	2.12	0.94 ± 0.06
Rru_A1566	NADH dehydrogenase subunit L	C	2.99	1.01 ± 0.00
Rru_A1881	Pyruvate dehydrogenase (lipoamide)	C	2.03	1.39 ± 0.22
Rru_A2692	30S ribosomal protein S7	J	2.70	0.82 ± 0.03
Rru_A2837	Response regulator receiver domain-containing protein	K	2.16	0.85 ± 0.12
Rru_A3317	Hypothetical protein Rru_A3317	Un.	2.01	IBNQ
Rru_A3320	Pantothenate synthetase	H	3.55	1.10 ± 0.09
Rru_A3332	Cytochrome c oxidase cbb3-type, subunit I	O	2.18	IBNQ
Rru_A3405	Formaldehyde dehydrogenase (glutathione)	C	2.09	IBNQ
Rru_A3433	Thioredoxin	O	2.52	0.96 ± 0.05
Rru_A3746	50S ribosomal protein L25P	J	2.57	IBNQ

Abbreviations: FC, fold change; IBNQ, identified protein but not quantified by at least two H/L occurrences (see Materials and methods). 'Rru_A' refers to gene located on the chromosome. Proteins are quantified by mean ± standard deviation. See Table 1 for the COG nomenclature.

Table 8 Transcriptomics versus differential proteomics approach for the random positioning machine (RPM) experiment using the BASE-A set-up

Gene number	*Gene name*	*Product name*	*COG*	*FC RPM mRNA*	*FC RPM protein*
Rru_A0974	—	Linocin_M18 bacteriocin protein	S	3.93	IBNQ
Rru_A1072	*rpmE*	50S ribosomal protein L31	J	2.09	1.04 ± 0.03
Rru_A1311	—	MerR family transcriptional regulator	K	0.33	1.12 ± 0.06

Abbreviations: FC, fold change; IBNQ, identified protein but not quantified by at least two H/L occurrences (see Materials and methods). 'Rru_A' refers to gene located on the chromosome. Proteins are quantified by mean ± standard deviation. See Table 1 for the COG nomenclature.

Table 9 Transcriptomics versus differential proteomics approach for the simulation of ISS-ionizing radiation (SimRAD) using the BASE-A set-up

Gene number	*Gene name*	*Product name*	*COG*	*SimRAD FC mRNA*	*SimRAD FC protein*
Rru_A0146	—	Inorganic diphosphatase	C	7.17	1.04 ± 0.14
Rru_A0210	*rpmB*	50S ribosomal protein L28	J	0.34	1.07 ± 0.07
Rru_A0521	—	CheA Signal transduction histidine Kinases (STHK)	N	0.40	1.09 ± 0.10
Rru_A0586	—	Chaperonin Cpn10	O	0.47	1.10 ± 0.04
Rru_A0665	—	Response regulator receiver domain-containing protein	K	2.36	1.18 ± 0.18
Rru_A0892	—	Stress protein	R	0.49	IBNQ
Rru_A0972	—	Prephenate dehydrogenase	E	2.15	IBNQ
Rru_A0974	—	Linocin_M18 bacteriocin protein	S	0.37	IBNQ
Rru_A1043	*rpsU*	30S ribosomal protein S21	J	2.01	1.09 ± 0.09
Rru_A1234	—	Phosphoglyceromutase	G	10.94	1.08 ± 0.05
Rru_A1551	*clpX*	ATP-dependent protease ATP-binding subunit	O	0.46	1.05 ± 0.06
Rru_A1881	—	Pyruvate dehydrogenase (lipoamide)	C	3.26	1.24 ± 0.09
Rru_A1917	—	Extracellular solute-binding protein	E	2.40	1.10 ± 0.05
Rru_A2167	—	Phosphoribosylformylglycinamidine cyclo-ligase	F	2.49	IBNQ
Rru_A2687	—	50S ribosomal protein L4P	J	14.25	0.97 ± 0.07
Rru_A2837	—	Response regulator receiver domain-containing protein	K	2.41	1.41 ± 0.09
Rru_A3177	—	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C	J	2.14	1.08 ± 0.09
Rru_A3279	—	Hypothetical protein Rru_A3279	S	2.12	IBNQ
Rru_A3320	—	Pantothenate synthetase	H	4.05	0.78 ± 0.11
Rru_A3405	—	Formaldehyde dehydrogenase (glutathione)	C	5.08	IBNQ
Rru_A3433	—	Thioredoxin	O	3.81	1.05 ± 0.05
Rru_A3631	—	Leucyl-tRNA synthetase	J	2.56	1.01 ± 0.08
Rru_A3711	—	Thioredoxin-related	O	2.10	IBNQ
Rru_A3746	—	50S ribosomal protein L25P	J	4.43	IBNQ
Rru_A3785	—	Polynucleotide phosphorylase/polyadenylase	J	2.22	1.12 ± 0.07

Abbreviations: FC, fold change; IBNQ, identified protein but not quantified by at least two H/L occurrences (see Materials and methods).
'Rru_A' refers to gene located on the chromosome. Proteins are quantified by mean ± standard deviation. See Table 1 for the COG nomenclature.

Table 10 Significant proteins from the space (BASE-A) and the simulation of ISS-ionizing radiation (SimRAD) experiments using the BASE-A set-up

Gene number	*Gene name*	*Product name*	*COG*	*FC BASE-A*	*No. (H/L)*	*Mascot score*	*MW (kDa)*
Rru_A0148	—	GTP-binding protein TypA	T	0.63 ± 0.05	7	156.56	67.32
Rru_A0221	*pgk*	Phosphoglycerate kinase	G	0.69 ± 0.09	3	437.72	41.69
Rru_A0684	—	Hypothetical protein Rru_A0684	H	0.64 ± 0.05	2	124.32	45.36
Rru_A1262	—	Extracellular solute-binding protein	G	0.63 ± 0.05	3	84.47	46.98
Rru_A1656	—	Hypothetical protein Rru_A1656	S	0.67 ± 0.02	2	116.27	13.57
Rru_A1760	—	Superoxide dismutase	P	0.63 ± 0.02	2	168.71	24.93
Rru_A2557	—	Hypothetical protein Rru_A2557	S	0.70 ± 0.06	13	596.75	35.38
Rru_A3572	—	YciI-like protein	S	0.66 ± 0.03	2	184.69	10.24
Rru_B0037	—	Hemolysin-type calcium-binding region	Un.	0.70 ± 0.06	2	157.86	81.39
Gene number	*Gene name*	*Product name*	*COG*	*FC SimRAD*	*No. (H/L)*	*Mascot Score*	*MW (kDa)*
Rru_A0148	—	GTP-binding protein TypA	T	0.63 ± 0.05	6	158.04	67.32
Rru_A0936	—	Hypothetical protein Rru_A0936	S	0.69 ± 0.03	2	67.95	41.66

Abbreviations: FC, fold change; MW, molecular weight; No. (H/L), number of H/L occurrences used for protein identification and quantification (See Materials and methods).
'Rru_A' refers to gene located on the chromosome, whereas 'Rru_B' refers to gene located on the plasmid. Proteins are quantified by mean ± standard deviation. See Table 1 for the COG nomenclature.

identified (Supplementary Table 7), including 282 quantified with at least two peptides. Plotting the fold change to the protein number also showed a correct median value (1.05) (Supplementary Figure 2E). In total, 25 of these proteins also were shown to be differentially transcribed. However, none of these proteins had a fold change above 1.5 or below 0.7 (Table 9). In this analysis, only two proteins were shown to be significantly downregulated including one protein also found in the BASE-A flight sample (Rru_A0148) and a hypothetical protein (Table 10).

Comparison of the MESSAGE 2- and BASE-A-related experiments

The two space flight experiments shared only five genes that were commonly differentially expressed within the threshold limits including two genes

encoding for hypothetical proteins (Rru_A0119, Rru_A0269), the NADH dehydrogenase subunit L (Rru_A1566), a ribosomal protein (Rru_A3746) and the putative transcriptional regulator Rru_A3286. The latter was downregulated in the BASE-A flight experiment and its simulation of ionizing radiation and upregulated in the BASE-A simulation of microgravity. Furthermore, Rru_A3286 is part of the cluster Rru_A3283–Rru_A3288 upregulated in the MESSAGE 2 flight experiment.

However, as mentioned above, about 600 genes from the BASE-A space experiment were not kept for statistical analysis after the microarray spot filtering quality control. As a direct consequence, of the 219 significant differentially expressed genes from the MESSAGE 2 experiment, 49 have no corresponding fold induction value in the BASE-A experiment.

One hypothetical protein-encoding gene (Rru_A2994) was jointly overexpressed in the ISS-ionizing radiation simulations of MESSAGE 2 and BASE-A, whereas two genes (Rru_A1537 and Rru_A2850 coding for hypothetical proteins) were common to the microgravity simulation experiments. Noteworthy is that the cluster Rru_A0196–Rru_A0198, which was slightly but significantly ($P<0.05$) downregulated in the MESSAGE 2 and related simulations (except for Rru_A0198 in microgravity simulation), was also slightly but significantly downregulated in BASE-A and its simulation of ionizing radiation. Within the differential proteomic approach, no overlap was found between the MESSAGE 2- and BASE-A-related experiments (that is, flight, ionizing radiation and RPM).

In the MESSAGE 2 experimental set-up, conducted on rich medium, space flight and simulation of microgravity elicited the highest number of differentially expressed genes and showed the largest overlap (Figure 2c), whereas for the BASE-A experimental set-up, performed on minimal medium, space flight and simulation of ionizing radiation showed the highest number of significant differentially expressed genes and the largest overlap (Figure 3c). A general comparison of the differentially expressed genes from the two space flight and their related simulation experiments on the different culture media indicated an overall low overlap (Figure 4).

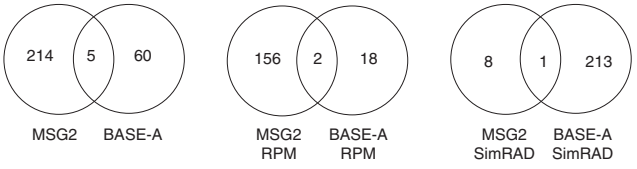

Figure 4 Comparison of significant genes expressed in the MESSAGE 2 (MSG2) and BASE-A related experiments. RPM, modeled microgravity experiment using the random-positioning machine; SimRAD, simulation of ISS-ionizing radiation.

Discussion

Experiment set-up and bacterial response

A different response of *R. rubrum* S1H cultivated in space-related environmental conditions was observed for rich compared with that for minimal medium, and overall there was limited overlap observed in differential gene expression for the conditions tested. In addition, when interpreting differential gene expression of large sets of genes, it should be considered that the observed overlap could be expected just by chance. Indeed, considering the length of the compared genes lists and the total number of genes surveyed, an overlap could be expected of, respectively, seven, two and three genes ($P<0.05$) for the space, the ionizing radiation and the modeled microgravity experiments taken 2 by 2.

The BASE-A set-up involving minimal medium showed a more pronounced effect for the simulation of ionizing irradiation than for microgravity simulation, whereas the opposite was observed for the MESSAGE 2 set-up with rich medium. Several studies already reported a more apparent bacterial response when cultivation in space flight, and space flight analogs was performed on rich medium (Baker and Leff, 2004, 2006; Benoit and Klaus, 2007; Wilson *et al.*, 2008). However, the difference in growth medium composition is probably not the only factor responsible for the difference in expression pattern for MESSAGE 2 and BASE-A. Particularly, the experimental set-up is an important additional difference. The use of different hardware dimensions like Petri dishes (MESSAGE 2) compared with six-well plates (BASE-A), and different inoculation load affect amongst others the surface area and oxygen availability per cell. In combination with (simulated) microgravity that will affect gas and fluid transport processes (Klaus *et al.*, 1997) and ionizing radiation, the hardware properties probably influenced the effect of space flight and consequently influenced the bacterium's response to it.

Osmotic stress in MESSAGE 2 rich medium cultivation. The MESSAGE 2 transcriptomic results suggest a response to what is felt by the cell as possibly an osmotic stress. Indeed, together with the gene coding for the biosynthesis of the osmoprotectant trehalose synthesis, two membrane-based osmosensors, the mechanosensitive channel MscS and, to a lesser extent, the two-component sensor kinase KdpD as well as a potassium efflux protein were differentially expressed in the MESSAGE 2 space samples (Wood, 1999; Ballal *et al.*, 2007; Gunasekera *et al.*, 2008; Hurst *et al.*, 2008). During a hypo-osmotic shock, that is, sudden reduction of the external osmolarity, induction of a mechanosensitive channel could act as an emergency safety valve (reviewed in Booth *et al.*, 2007) and decreasing potassium influx (down-regulation Kdp-system) and increasing potassium efflux would help to adapt to this hypo-osmotic conditions. A disruption of the

cellular homeostasis may partially explain the observed increase in expression of genes related to energy production and conversion, to restore the homeostasis. Another evidence for a response to stress could be the accumulation of trehalose. Indeed, studying *Saccharomyces cerevisiae*, Benaroudj *et al*. (2001) showed that in addition to its structural function in desiccation tolerance, trehalose accumulates during cellular stress (exposure to mild heat shock at 38 °C or to a proteasome inhibitor) and markedly protects cells and proteins from damage generated from an oxygen radical-generating system (that is, H_2O_2 and $FeCl_3$ through the Fenton reaction). A similar role was suggested by Cytryn *et al*. (2007) who studied the desiccation-induced stress in the α-proteobacterium *Bradyrhizobium japonicum*.

Oxidative stress in BASE-A minimal medium cultivation. Stress elicited by ionizing radiation solely has apparently a more pronounced effect in a minimal medium compared with that in a rich medium where less reactive oxygen species are generated (Lee *et al*., 2006) or where the produced reactive oxygen species are more rapidly scavenged by the anti-oxidant compounds present in the medium. Differential gene expression that could be linked to oxidative stress putatively included the upregulation of the ferric uptake repressor *fur* (Rru_A3788), which links cellular iron status to oxidative stress by scavenging iron (Hantke, 2001; Imlay, 2003), and the downregulation of *hfq*, which negatively regulates the expression of Fur in *E. coli* (Vecerek *et al*., 2003). However, rubrerythrin involved in oxidative stress defense in anaerobic bacteria (Lehmann *et al*., 1996; Lumppio *et al*., 2001) was downregulated. Previous reports on the effect of low doses (in the range of 0.1 Gy of X-rays) on prokaryotes only focused on the induction of the adaptive response by means of SOS repair-related genes (Huang and Claycamp, 1993; Ewing, 1995; Basak, 1996) and Ewing (1995) predicted the threshold dose that just activates a physiological response (that is, SOS repair mechanism) in *E. coli* to be as low as 1 mGy. Our study was the first to analyze the global transcriptomic and proteomic response to such low doses of ionizing radiation (*ca*. 2 mGy) and indicated a pronounced transcriptomic response of *R. rubrum* S1H to low-dose radiation when grown in minimal medium.

Space flight versus simulations of microgravity and ionizing radiation on Earth

Simulation of microgravity with the RPM. Except for the preliminary work of de Vet and Rutgers (2007), who tested bacterial fuel cells in both real space and RPM-simulated microgravity conditions, this study is unique in cultivating bacteria using the RPM and comparing it with real space flight data at both the transcriptomic and proteomic levels. The low overlap between space and ground simulation experiments, however, raises the question of the proper simulation of microgravity using the RPM. The principle of the RPM is that the direction of the gravity vector experienced by an organism continuously changes in three-dimensional space (Hoson *et al*., 1997). Although the RPM device is currently the only facility allowing to study agar culture under microgravity simulation, it is clear that there is a difference in gravity values present in space and the ones obtained using microgravity simulators. In addition, particular aspects of the space flight experiment including launch and landing effects (high vibrations, hyper-g) and the ISS on-board environment regarding vibrations are not included in such simulations and might have had an impact on the final results. Future studies involving activation, cultivation and fixation of the bacterial culture in-flight, starting the complete experiment after launch and stopping it before return to the Earth, could minimize the effects mentioned above. Moreover, the possible use of an in-flight centrifuge, such as the KUBIK incubator (COMAT Aerospace, Flourens, France) from the European Space Agency, could bring an additional control, eliminating the effect of microgravity. But still, some questions would remain, as launch acceleration, vibration and so on, may cause damage to the 'inactive' cell that will be processed when the bacterium is activated in-flight and the elicited response could still be more than space flight alone.

Simulation of ISS-ionizing radiation. In our experimental set-up, a combination of single beams of low-LET γ rays and high-LET neutron rays was chosen. The high-LET neutrons are important because they are among the most important secondary radiation particles found in the space craft in terms of radiation protection (that is, biological effectiveness) (Armstrong and Colborn, 2001; Benton and Benton, 2001; Mitaroff and Silari, 2002). When passing through the skin and structure of the ISS, primary ionizing particles can undergo interactions with the nuclei that constitute the spaceship's mass, producing a wide variety of secondary particles, such as neutrons, protons, recoil nuclei, projectile fragments, γ-particles and so on. These particles occupy a quite broad energy spectrum and range from low-LET to high-LET including LET values from approximately 10^{-1} to $10^{3}\,\mathrm{keV\,\mu m^{-1}}$ (Armstrong and Colborn, 2001; Benton and Benton, 2001). Therefore, the difference between single beam ground-based irradiation (γ and neutron with a maximum LET of $250\,\mathrm{keV\,\mu m^{-1}}$) and the complex mixture of particles over a wide range of energy inside the ISS could account for discrepancies that need to be taken into account when comparing ground simulation with space flight data. Rea *et al*. (2008) recently underlined the difficulty to measure and above all, to reproduce different radiation

components at the same time over a wide energy range.

In a few words, no simulations set-up on the Earth would be able to reproduce all the stress factors related to a space flight mission. Still, they will always be more accessible than flight opportunities (but also in terms of experiment size, weight, electric power requirements and so on) and can give an indication of which particular physiological aspect to monitor during cultivation in actual space flight conditions.

Integrating transcriptomics and quantitative proteomics results

The specific search for differentially expressed proteins in the MudPIT approach obviously limited the number of detected proteins. Nevertheless, the relative poor correlation between transcriptomic and proteomic data has been reported before (Cox *et al.*, 2007; Giotis *et al.*, 2008). Moreover, Julka and Regnier (2004) concluded their research by mentioning that one must be cautious in concluding that the presence or absence of significant mRNA abundance change of a gene detected by transcriptomics necessarily corresponds to the presence or absence of significant protein abundance change detected by proteomics. The discrepancy between the mRNA log-ratio and the protein log-ratio of a gene can stem from sustained protein presence from transient transcriptional induction, post-transcriptional regulation or possible measurement errors or any combinations of these causes.

Therefore, although not differentially expressed at the transcriptomic level, the upregulation of the osmoprotectant transporter ProX (among the very few upregulated proteins) in the simulation of ionizing radiation using the MESSAGE 2 set-up remains an interesting result that could be related to a probable oxidative stress too. Indeed, exposure of the cells to one type of stress can also condition them against other, seemingly unrelated, stresses. In this respect, it has been shown that when bacteria are challenged with high osmolarity, they acquired increased resistance to high temperature and oxidative stress through an RpoS-dependent mechanism (Hengge-Aronis *et al.*, 1993; Smirnova *et al.*, 2000; Cánovas *et al.*, 2001). However, based on the genome annotation, *R. rubrum* does not seem to have a specific sigma factor involved in general stress or entry into stationary phase such as RpoS. This has also been observed for the closely related α-proteobacterium, *Rhodobacter sphaeroides* 2.4.1 (Mackenzie *et al.*, 2007). Thus, we can suspect these functions to be ascribed to another sigma factor or to non-sigma-factor-type regulator(s). Palma *et al.* (2004) reported the response of *Pseudomonas aeruginosa* to hydrogen peroxide to induce an upregulation of extracellular protein such as extracytoplasmic function. Interestingly, we found the upregulation of an alternative sigma factor of extracytoplasmic function in the MESSAGE 2 experiment that could be involved in the oxidative stress response of *R. rubrum*.

Genes coding for hypothetical proteins

This group seemed to constitute an important part (30%) of the differentially expressed genes detected in the three studied culture conditions related to MESSAGE 2 and even occupied the top three upregulated genes in the MESSAGE 2 space flight experiment (Rru_A3369, Rru_A2713 and Rru_A0160). The amount of genes annotated as hypothetical proteins (*ca.* 25%) in the genome of *R. rubrum* indicates that a substantial part of the genetics of this organism is not well known. The expression of these genes in response to environmental parameters not encountered on the Earth raises the question of their earthly niche origin and how these genes have been kept during evolution. Therefore, exploring new environmental and stress conditions, such as space or RPM cultivation, could assign novel functions to these hypothetical proteins. After manual annotation using the MaGe platform, four genes coding for hypothetical proteins were further explored: (i) Rru_A2588, Rru_A2590 and Rru_A3310, induced in the BASE-A space samples, seemed to belong to mobile genetic elements, that is, insertion sequences. The mobility of insertion sequences has been linked to various bacterial stress responses (reviewed in Foster, 2007); (ii) Rru_A2016, induced in the space and the ionizing radiation experiments using the BASE-A set-up, is a conserved protein frequently associated with DnaJ-like chaperones, and therefore gave another argument in favor of a stress response.

Conclusion and perspectives

Transcriptomic and proteomic data showed that *R. rubrum* S1H is able to sense and react to environmental changes such as microgravity and ionizing radiation that are linked to space flight. Subtle effects were already seen after 10 days in space featuring an ionizing radiation dose as low as 2 mGy. Thus, it is not excluded that similar responses will occur in a continuous culture bioreactor during extended space exploration missions of several years and may eventually affect the bioreactor performance. Within the MELiSSA system, it is, however, foreseen that the *R. rubrum* S1H bioreactor is fully mixed to allow homogeneous culture feeding and illumination. Therefore, we could argue that the effect of microgravity would be minimized due to intensive mixing. Although the composition of the *R. rubrum* reactor input flow coming from the upstream fermentation reactor is presently not fully characterized, the medium could potentially also provide specific antioxidants (such as present in a rich culture medium), which may

protect the bacterium also against ionizing radiation. Moreover, also the selection of *R. rubrum* radio-resistant mutants could be considered. On the other hand, the bioreactor conditions may elucidate additional stress (medium composition, light intensity, long-term culturing and so on), which may stress the cells and in combination with radiation be nevertheless a risk for good reactor performance. Therefore, additional space experiments and ground simulations (for example, space microgravity and ionizing radiation) in continuous bioreactors are crucial to draw final conclusions concerning the space flight impact for MELiSSA. New space experiments need to be done in conditions mimicking the future MELiSSA loop conditions (with light and in anaerobic conditions), and should include a detailed metabolic analysis in addition to the proteomic and transcriptomic profiling. As shown in this study, conducting scientific space flight experiments remains a technical and logistical challenge but space research is truly a rich source of new findings.

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