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The response of *Cupriavidus metallidurans* CH34 to spaceflight in the international space station

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Abstract The survival and behavior of *Cupriavidus metallidurans* strain CH34 were tested in space. In three spaceflight experiments, during three separate visits to the 'International Space Station' (ISS), strain CH34 was grown for 10–12 days at ambient temperature on mineral agar medium. Space- and earth-grown cells were compared post-flight by flow cytometry and using 2D-gel protein analysis. Pre-, in- and post-flight incubation conditions and experiment design had a significant impact on the survival and growth of CH34 in space. In the CH34 cells returning from spaceflight, 16 proteins were identified which were present in higher concentration in cells developed in spaceflight conditions. These proteins were involved in a specific response of CH34 to carbon limitation and oxidative stress, and included an acetone carboxylase subunit, fructose biphosphate aldolase, a DNA protection during starvation protein, chaperone protein, universal stress protein, and alkyl hydroperoxide reductase. The reproducible observation of the over-expression of these same proteins in multiple flight experiments, indicated that the CH34 cells could experience a substrate limitation and oxidative stress in spaceflight where cells and substrates are exposed to lower levels of gravity and higher doses of ionizing radiation. Bacterium *C. metallidurans* CH34 was able to grow normally under spaceflight conditions with very minor to no effects on cell physiology, but nevertheless specifically altered the expression of a few proteins in response to the environmental changes.

Keywords *Cupriavidus metallidurans* CH34 · Spaceflight experiments · Flow cytometry · Proteome analysis

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Introduction

Microorganisms accompany humankind's journeys around the globe, and do so also in space. In closed manned spacecrafts, space stations and planetary bases microbes colonize the structures of the habitat and the systems to support human life, such as air revitalization systems, drinking, hygienic or cooling water loops, food storage, waste storage and recycling systems (Novikova 2004; Ott et al. 2004; Novikova et al. 2006; Van Houdt et al. 2009). These microbial consortia are a risk factor for biocorrosion or biodegradation of structural spacecraft components, and also a potential direct threat for crew health. The microbes might destabilize the beneficial

bacterial community in the human body (e.g., intestinal flora) and pathogenic bacteria might cause infection since the human immune system is known to be depressed in spaceflight conditions (Klaus and Howard 2006). Thus, the control, including prevention, monitoring, early detection, mitigation and remediation of the presence of the microbial population and its metabolic capacity in spacecraft, is vital for manned space missions (Castro et al. 2006). To enable the technological development of microbial monitoring tools it is essential to understand how bacteria survive and reproduce in spacecrafts and cope with a variety of spaceflight induced environmental changes including microgravity, ionizing radiation, electromagnetism, vibrations and hypervelocity during launch. Furthermore, with regards to biological life support systems it is necessary to survey the impact of spaceflight related environmental conditions on the interactions of microbes with human, animal or plant cells or other microbes and materials (Hendrickx and Mergeay 2007).

Numerous *Cupriavidus* and *Ralstonia* strains have been isolated from the former Mir space station water systems and free floating condensate water (Ott et al. 2004), the current international space station cooling water and Shuttle drinking water (Baker and Leff 2004; La Duc et al. 2004; Castro et al. 2004; Roman et al. 2006); or other spacecraft-related sites such as the surfaces of space robots and the floor, air and surfaces of spacecraft assembly rooms (La Duc et al. 2003; Moissl et al. 2007). The reasons for their successful resilience and flourishing in these highly controlled and oligotrophic space environments remain unclear. Besides soil and plant environments, harsh and oligotrophic man-made environments indeed seem to be a target of *Cupriavidus* and *Ralstonia* strains in general, as previously many were isolated from new emerging anthropogenic industrial environments (metal polluted soils and water) or clean environments (clean rooms in hospitals, technical assembly facilities, nuclear water basins) (Goris et al. 2001; Salanoubat et al. 2002; Sánchez and González 2007; Amadou et al. 2008; Satoshi et al. 2008). In addition, *Cupriavidus* and *Ralstonia* strains often possess mobile DNA fragments (such as large plasmids, genomic islands and transposons) that allow the strains to specifically adapt to their environment (Monchy et al. 2007; Mergeay et al. 2009). In a parallel study, *Cupriavidus* and *Ralstonia* strains isolated from spacecraft environments were collected and characterized in detail to identify their specific characteristics, including their plasmid content (Mergeay et al. 2009; Leys et al. unpublished data).

In this study, the *C. metallidurans* type strain CH34 (Mergeay et al. 1985) was cultured as a model organism in spaceflight conditions to study its characteristic responses. *C. metallidurans* CH34, was isolated from polluted soils and has been studied for over 30 years, with special emphasis on its response to metals (Mergeay et al. 1985, 2003; Monchy et al. 2007; Bersch et al. 2008, von Rozycki and Nies 2008). Also its full genome sequence was obtained. *C. metallidurans* CH34 is a robust and versatile bacterium which makes it a good test organism for spaceflight experiments which require often long duration, stand-alone and uncontrolled temperature experimental conditions. This report describes the results of three separate spaceflight experiments in the international space station with *C. metallidurans* CH34 grown on minimal agar medium in two different experimental designs for about 10–12 days, to investigate its overall fitness as well as its physiological and metabolic status in spaceflight conditions.

Materials and methods

Bacterial strains and culture conditions

Cupriavidus metallidurans type strain CH34 (LMG 1195, DSM 2839, ATCC 43123) (Mergeay et al. 1985) was cultivated in dark oxic heterotrophic conditions in a Tris buffered mineral medium (Mergeay et al. 1985) containing 2 g/l sodium gluconate (Merck) as sole C-source. For cultivation in spaceflight, the medium was supplemented with 20 g/l agar to solidify (Invitrogen) and 2 mg/l potassium nitrate (Merck) as alternative electron acceptor if oxygen would become depleted in the hermetically closed experiment package.

Spaceflight experimental setup

Three independent cultures of *C. metallidurans* CH34 cultured in liquid medium at 30°C in the dark in shaken aerobic conditions to stationary phase 8 or 10 days before launch were resuspended in isotonic

solution containing 8.5 g/l sodium chloride and transported at room temperature from the laboratory in SCK•CEN (Mol, Belgium) to the experiment preparation site. The MESSAGE-1 experiment package was fully prepared and assembled at the ESA technical facility (ESTEC) (Noordwijk, The Netherlands) 4 days prior to launch and was transported at 6 ± 5°C in a Polyfoam passive thermal insulator container with ice packs (Dolofriz Eutectic Gel, Sofrigam) to the launch site (Baikonour, Kazakhstan). For the MESSAGE-2 and BASE-A experiments, pre-launch preparation, assembly and safety control were performed 1 day prior to launch in a laboratory at the launch site (Baikonour, Kazakhstan).

For the MESSAGE-1 and -2 spaceflight experiments, cells were inoculated from the saline solution on the surface of 10 ml agar medium in polystyrene Petri dishes (cm-graduated bottom with marked letters and numerals, 55 mm diameter, 12 mm height, 24 cm^2 surface) (Surfair Plate, PBI International, Italy). For the MESSAGE-1 experiments, cells from 18 different independent CH34 cultures were spotted as 10 µl drops containing *ca.* 10^5 colony forming units (CFU) on the agar surface spread over 2 plates (9 drops per Petri dish) (Fig. 3a, b). For MESSAGE-2, 10 µl drops from of 3 independent CH34 cultures (*ca.* 5×10^7 CFU) (photograph not shown), as well as tenfold dilutions containing from *ca.* 5×10^6 CFU down to 5×10^1 CFU; Fig. 3c, d), were spotted on the agar surface spread over 2 Petri dishes (16 drops per Petri dish) for protein analysis and for viable count purposes. Also additional Petri dishes with cultures for generating zinc resistant mutants (Collard et al. 1993; Tibazarwa et al. 2000) in spaceflight were added to the experiment packages, but will not be discussed in this report. After inoculation, Petri plates were leak-tight hermetically sealed first with 1 layer of Parafilm and 1 layer of Kapton tape next, individually packed in polyethylene Ziplock bag (104 × 172 mm) and then jointly packed in a second polyethylene Ziplock bag (155 × 365 mm) and placed in a sealed polystyrene jar (Fig. 1a). This jar was wrapped in protective foam to protect the jar during intensive vibrations during launch and NOMEX fabric bags with Velcro strips for attachment in the international space station.

For the BASE-A experiment, three biological independent culture suspensions were deposited as 4 spots of 10 µl on the *ca.* 10 cm^2 surface of a 5 ml layer of agar medium in 6 well culture plates (CellStar 6, Greiner Bio-One, Belgium) (Fig. 3e, f) and kept at ambient temperature. An oxygen indicator strip (Anaerotest, Merck) was added between the wells at the bottom of the multiwell plate, to indicate the presence of oxygen in the gas phase during the experiment. Culture plates were sealed with 1 layer of Parafilm and 1 layer of Scotch tape. Two culture plates were sealed hermetically in 1 polycarbonate Biocontainer (PedeoTechniek, Belgium) and vacuum sealed in a highly transparent Minigrip polyethylene bag (60 µm thick) (Fig. 1c). Two Biocontainers were placed together in 1 pouch of protective foam and NOMEX fabric (Fig. 1d, e).

Small programmable automatic miniature-sized temperature data loggers (CUBE from Meilhaus Electronic GmbH in Germany, or SmartButton from ACR Systems Inc. in US) were added inside each MESSAGE-2 jar and BASE-A biocontainer in the immediate vicinity of the culture plates. Inside the MESSAGE-2 jars and BASE-A biocontainers, also passive radiation data loggers (Track-Etch Detectors, Optically Stimulated Luminescence Detectors and ThermoLuminescent Detectors) (Goossens et al. 2006; Vanhavere et al. 2008) were added to monitor ionizing radiation exposure over the space mission. Finally, the exterior of the MESSAGE-1, MESSAGE-2 and BASE-A experiment packages was disinfected with 3% hydrogen peroxide wipes according to Russian flight procedures and stored at room temperature until integration into the Soyuz vehicle about 12–18 h before launch.

During the 2-days trip in the Soyuz vehicle to the international space station (Soyuz TMA-1 for MESSAGE-1; Soyuz TMA-3 for MESSAGE-2; Soyuz TMA-9 for BASE-A), the pouches were kept at ambient temperature (22 ± 1°C). Upon arrival in the international space station, the MESSAGE-1 jars were stored for a short period in the Russian Service Module 'Zvezda' of the international space station, and for most of the time in the return Soyuz vehicle, where the temperature profile recorded over the 10-days mission contained several periods of high temperature (max. 28°C) and low temperature (min. 12°C) (Fig. 2a). The MESSAGE-2 jars and the BASE-A Biocontainers were stored for 8–10 days in Zvezda behind structural bars (Fig. 1b), at a relative constant temperature of 20 ± 2°C (Fig. 2b, c). The experimental packages returned unopened from international space station

Fig. 1 Spaceflight experiments hardware. **a** The hardware used for the spaceflight experiments MESSAGE-1 and -2 was composed of basic commercially available standard sterile laboratory plastic components. **c**, **d**, **e** For the BASE-A spaceflight experiment a special transparent Biocontainer was constructed allowing manual in-flight photography of the colonies appearing in the hermetically closed containers (packed per 2 in a foam pouch) over time by the crew members. **b** The full passive (no power required) experimental packages MESSAGE-1, -2 and BASE were stored at ambient temperatures in the spacecraft Soyuz and behind the structural bars of the International space station

back to earth with the Soyuz vehicle (Soyuz TM-34 for MESSAGE-1; TMA-2 for MESSAGE-2; TMA-8 for BASE-A), after a total of 10–12 days flight (30 October–10 November 2002 for MESSAGE-1; 18–28 October 2003 for MESSAGE-2; 17–28 September 2006 for BASE-A). The total absorbed dose of ionizing radiation recorded during the MESSAGE-2 and BASE-A flight experiments, was about 157 μGy per day for the ionizing particles with low linear energy transfer and about 23 μGy per day for the ionizing particles with high linear energy transfer, meaning a total dose of about 180 μGy per day (Goossens et al. 2006; Vanhavere et al. 2008).

The experimental packages were transported at about 4°C (in a Polyfoam passive thermal insulator container with ice packs for MESSAGE-1 and -2, in a active controlled thermal container for BASE-A) without exposure to airport X-rays scanning from the landing area (the steppe around Arkalyk in Kazakhstan) to the laboratory in SCK•CEN (Mol, Belgium), within respectively 24, 36 and 40 h after landing. The analysis of the samples was started immediately.

Earth control experiments were prepared in parallel and returned immediately after preparation at ESTEC (Noordwijk, The Netherlands) or the launch site (Baikonur, Kazakhstan) to the laboratory in SCK•CEN (Mol, Belgium) (maintained at 22 ± 1°C during transport). Control experiments were cultured in dark conditions under comparable temperature (incubation at 22 ± 1°C) and time profile as the international space station samples, and were simultaneously cooled down (to 4°C) after landing of the space samples. The estimated total absorbed dose of background ionizing radiation for the control experiments on earth (Mol, Belgium) over the same period was about 2.5 μGy per day (Goossens et al. 2006; Vanhavere et al. 2008).

Cell physiology analysis by flow cytometry

Cells were harvested from the agar medium, suspended and diluted in an isotonic physiological solution to about 10^8 CFU/ml. An aliquot of bacterial suspension was transferred to a 5 ml polypropylene tube (Becton Dickinson), isotonic solution (no stain control) or one of the fluorescent stain solutions described below was added, and staining was allowed for 15 min at room temperature in the dark. The LIVE/DEAD BacLight Bacterial Viability Kit

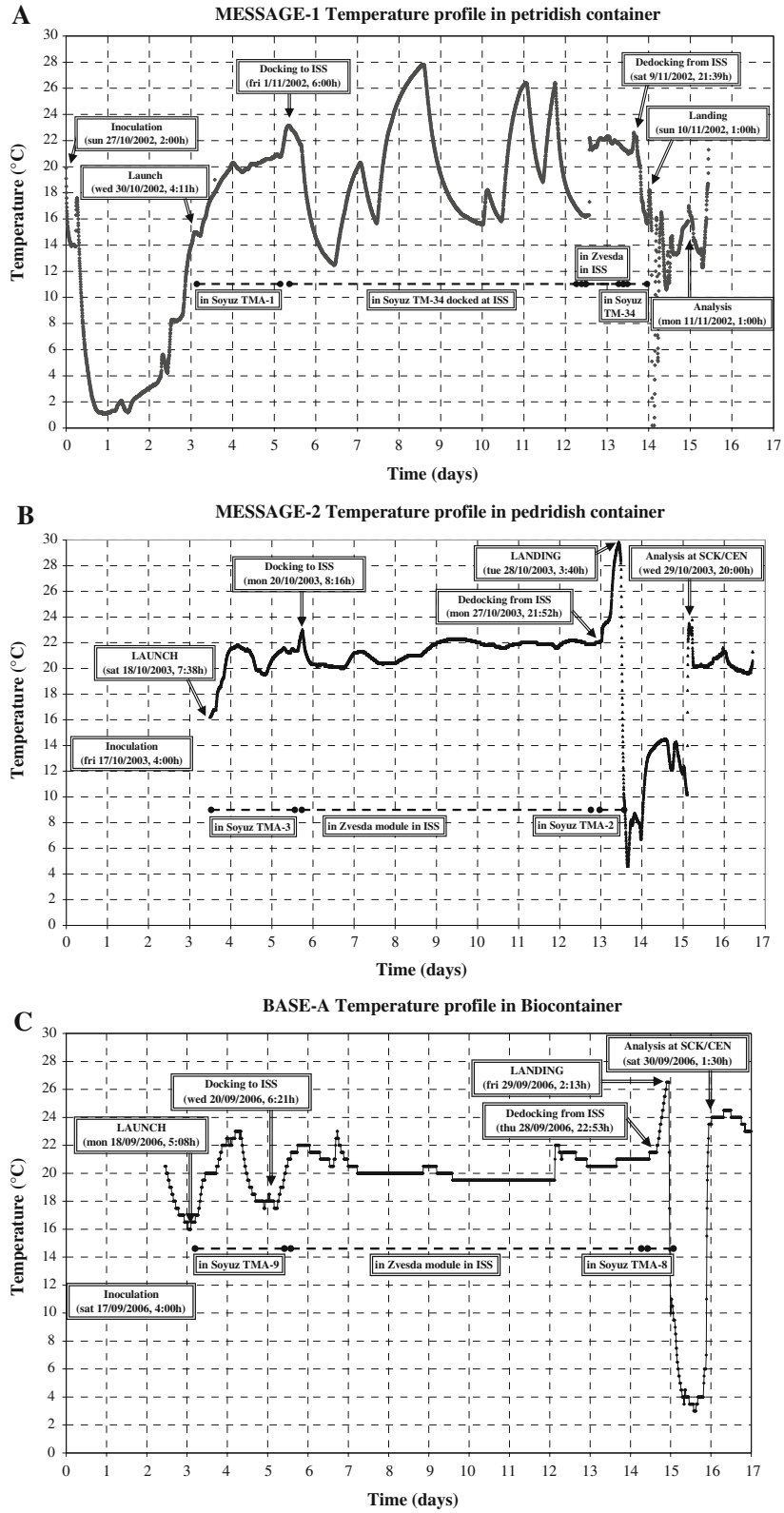

Fig. 2 Temperature conditions of the 3 passive spaceflight experiments. The temperature profile recorded over the flight duration showed high fluctuations for MESSAGE-1 **a**, but more stable temperatures for MESSAGE-2 **b** and BASE-A **c** space flight experiments. Dates and hours are given in Central European Standard Time, which is Universal Time Coordinate (UTC) + 1 h or Greenwich Mean Time (GMT) + 1 h

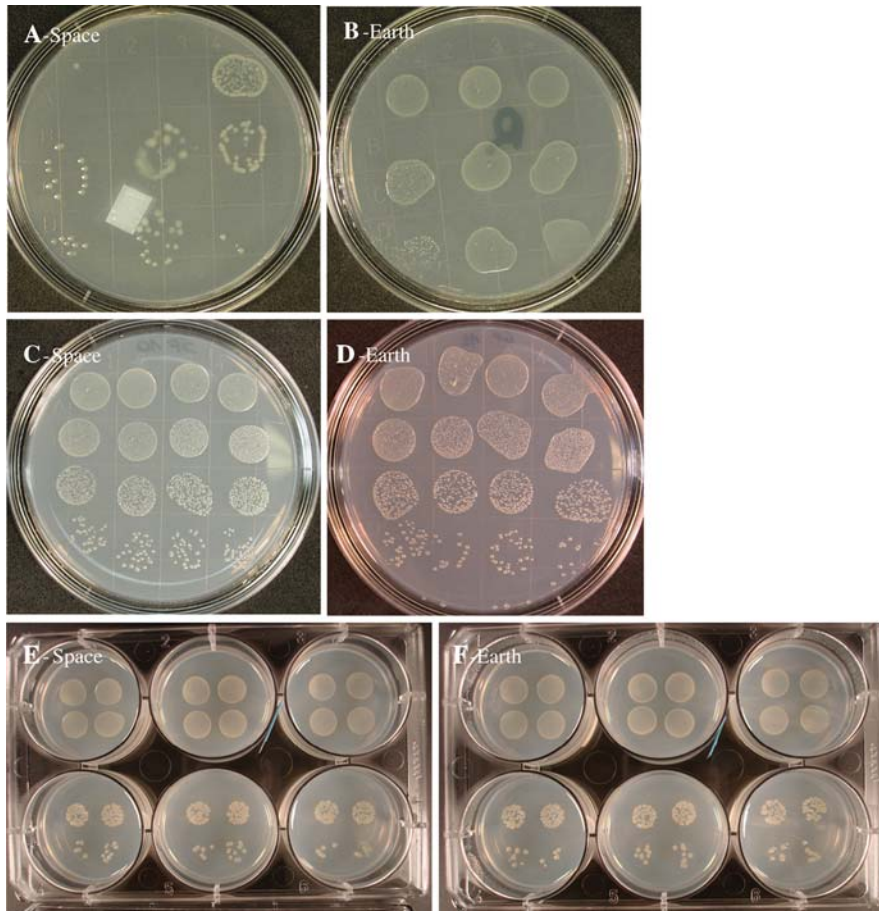

Fig. 3 Photographs of *C. metallidurans* CH34 growth on minimal agar medium after *ca*. 10–12 days in space or on earth. The MESSAGE-1 cultures for space flight (**a**) and earth control (**b**) were inoculated as 9 spots of 10 µl (3 spots for each of the 3 biological cultures) per 68 mm diameter Petri dish with ca. 10^5 CFU per spot. The MESSAGE-2 cultures for spaceflight (**c**) and earth control (**d**) were inoculated as 16 spots of 10 µl (4 dilution spots for each of the 3 biological cultures) per 68 mm diameter Petri dish with 3 times ca. 5×10^4 (row 1), ca. 5×10^3 (row 2), ca. 5×10^2 (row 3), ca. 5×10^1 (row 4) CFU per spot. The BASE-A cultures for spaceflight (**e**) and earth control (**f**) were inoculated in 6-welll plates (2 wells for each of the 3 biological cultures) as 4 spot of 10 µl per well. The wells 1, 2 and 3 contained 4 spots of ca. 5×10^6 CFU per spot, the wells 5, 6, and 7 contained 2 spots of ca. 5×10^2 CFU per spot, and 2 spots of ca. 5×10^1 CFU CH34 per spot

(Molecular Probes, Invitrogen), providing stock solutions of the green fluorescent SYTO 9 (334 mM) and the red fluorescent propidium iodide (PI) (20 mM) nucleic acid dyes, was used according to manufacturer instructions to assess the ratio of live cells with intact plasma membrane (containing SYTO 9) over dead cells with compromised membrane (containing SYTO 9 and PI) in the culture (Table 1). The SYTO 9 and propidium iodide dyes were mixed and used in a final concentration of 10 and 60 µM, respectively, in contact with the cells. The red fluorescent propidium iodide (PI) dye (Molecular Probes, Invitrogen) was used individually to assess the cell membrane permeability of the cells in the culture (Baatout et al. 2006, 2007) (Table 1). Propidium iodide is a relative small hydrophilic dye molecule that is unable to penetrate a bacterial cell with an intact cell membrane (no fluorescence) but penetrates in cells that have a disrupted membrane and where it intercalates in dsDNA (red fluorescence). Propidium iodide staining solution was prepared at 20 mM in deionized water, conserved at 4°C in the dark, and used in a final concentrations 60 µM in contact with the cells for the staining. A lipophilic voltage-sensitive cyanine dye,

Table 1 Conclusions from the physiological parameters analysed in CH34 cells postflight by flow cytometry

Parameter assessed	MESSAGE-1 (11 days in space)	MESSAGE-2 (10 days in space)	BASE-A (12 days in space)
Cell exterior			
Size[a]	Space < Earth*	Space = Earth	Space = Earth
Shape[b]	Space < Earth*	Space = Earth	Space = Earth
Cell membrane			
Live/dead ratio[c]	ND	Space = Earth	Space < Earth**
Membrane permeability[d]	Space < Earth*	Space = Earth	Space = Earth
Membrane Potential[e,f,g]	Space > Earth*[,e,f]	Space = Earth[f]	Space > Earth*[,g]
Intracellular pH[h]	Space = Earth	Space = Earth	ND
Electron transport chain function[i]	ND	ND	Space = Earth
Cell interior			
$O_2^{\cdot-}$ concentration[j]	Space = Earth	Space = Earth	ND
H_2O_2 concentration[k]	ND	Space = Earth	ND
Thiol concentration[l]	ND	Space = Earth	ND
Ca^{2+} concentration[m]	ND	Space = Earth	ND
Esterase activity[n]	Space = Earth	Space = Earth	ND
DNA/RNA ratio[o]	ND	Space = Earth	ND

Signal or fluorescent dye used : [a] *FS* forward scatter, [b] *SS* side scatter, [c] fluorescence ratio of Syto-9 over PI, [d] *PI* Propidium iodide, [e] Rhodamine-123, [f] $DioC_6(3)$ = 3,3′-dihexyloxacarbocyanine iodide, [g] $DioC_2(3)$ = 3,3′-diethyloxacarbocyanine iodide, [h] CFDASE = 5-(6) carboxy-fluorescein diacetate succinimidyl ester, [i] fluorescence ratio of RSG = redox sensor green over PI, [j] HE = Hydroethidine, [k] *DHR* dihydrorodamine-123, [l] *MO* mercury orange, [m] Fluo 3 AM, [n] *FDA* fluorescein diacetate, [o] *AO* acridine orange

ND not determined

* Significant with $P < 0.05$, ** highly significant with $P < 0.01$

Rhodamine-123 (Molecular Probes, Invitrogen), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Molecular Probes, Invitrogen) or 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$) (MitoProbe™ $DiOC_2(3)$ Assay Kit for Flow Cytometry, Molecular Probes, Invitrogen), was used to assess the cell membrane potential (Baatout et al. 2006, 2007) (Table 1). A high transmembrane potential (high positive charge outside and high negative charge inside) of active live bacterial cells, stimulate the positively charged rhodamine or cyanine dye to enter the negatively charged cell rapidly and to accumulate in cells. The dye will bind with the nucleic acids and the fluorescence of the cell is increased. In contrast, dead bacteria with depolarized membranes (low negative charge inside) will show slow and low dye uptake and thus minimal fluorescence. When the green fluorescent rhodamine or cyanine dye accumulates more in cells, red emission increases due to dye stacking. Red and green signals from intact cells increase proportionally, but using the red over green intensity ratio corrects for size differences when staining bacteria. The rhodamine-123 and 3,3′-dihexyloxacarbocyanine iodide staining solutions were prepared at 400 µM and 3 mM in dimethyl sulfoxide, stored at 4°C in the dark, and used in a final concentration of 26 and 30 µM for staining the cells. The 3,3′-diethyloxacarbocyanine iodide was provided in the MitoProbe™ $DiOC_2(3)$ Assay Kit as a 10 µM stock solution in dimethyl sulfoxide, stored at 4°C in the dark, and used in a final concentrations of 50 nM for staining the cells. To measure intracellular pH, the dye 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDASE) (Sigma) was used (Baatout et al. 2006) (Table 1). The relative intracellular pH of the cells was determined from the fluorescence signal emitted by carboxyfluorescein diacetate succinimidyl ester at wavelength 525 nm when exposed to the pH-sensitive excitation wavelength 488 nm. Carboxyfluorescein diacetate succinimidyl ester was dissolved at 7 mM dimethyl sulfoxide as stock solution, and used in a final staining concentration of 1 µM. The BacLight

RedoxSensor Green Vitality Kit (Molecular Probes, Invitrogen), was used according to manufacturer instructions to assess the electron transport chain function in the cell membrane (Table 1). The kit provides stock solutions of the RedoxSensor Green (1 mM in dimethyl sulfoxide) dye and the propidium iodide (20 mM in dimethyl sulfoxide) nucleic acid dye to rapidly distinguish live cells with reductase activity (active electron transport chain) (fluoresce green) from dead bacteria with compromised membranes (fluoresce red). RedoxSensor Green reagent penetrates passively the bacterial cell and produces a stable green-fluorescent signal upon reduction inside the cell. The RedoxSensor Green and propidium iodide dyes were stored at −20°C in the dark and mixed together in a final concentration of 1 and 20 μM, respectively for staining. The intracellular concentration of superoxide anion ($O_2^{\cdot-}$) and peroxide (H_2O_2) were measured by their reactions with the fluorescent dyes Hydroethidine (HE) (Molecular Probes) and Dihydrorodamine-123 (DHR-123) (Sigma), respectively (Baatout et al. 2006) (Table 1). Hydroethidine and dihydrorhodamine diffuse passively into the cell and upon oxidation by intracellular $O_2^{\cdot-}$ and H_2O_2 they are converted to ethidium respectively rhodamine-123, that bind to the nucleic acids and emit red fluorescence. The higher the concentration of $O_2^{\cdot-}$ and H_2O_2 in the cells is, the more red fluorescence is emitted. Hydroethidine and dihydrorhodamine-123 were prepared at 5 mM in dimethyl sulfoxide, stored at −20°C protect from air and light, and used in a final concentration of 5 μM. The intracellular concentration of glutathione was determined by the dye mercury orange (MO) (Molecular Probes) that forms an insoluble red fluorescent product with non-protein thiols (Table 1). Mercury orange was prepared in a stock solution of 1 mM, and used in a final concentration of 5 μM for staining. The fluorescent calcium indicator dye Fluo-3 AM (Molecular Probes) was used to determine the intracellular concentration of cytosolic free ionic calcium (Ca^{2+}), the most common signal transduction element in bacterial cells (Table 1). The measured Fluo-3 AM fluorescence intensity is directly correlated with the Ca^{2+} concentration in the cell. Fluo-3 AM was prepared in a stock solution of 5 mM and used at a final concentration of 5 μM. The dye acridine orange (AO) (Sigma) was used to estimate the DNA versus RNA concentration ratio of the cells, to assess whether the cells were quiescent or activated (Table 1). Intracellular esterase activity was assessed using fluorescein diacetate (FDA) (Sigma) (Baatout et al. 2006) (Table 1). Fluorescein diacetate is a non fluorescent esterase substrate, that is only taken up and hydrolysed by intracellular esterases in live cells. The product of the hydrolysis, fluorescein, is highly fluorescent and is retained in cells with intact membranes. Fluorescein diacetate was dissolved to 5 mM in acetone, maintained at −20°C, and used at a final concentration of 24 μM. Acridine orange interacts with DNA by intercalation, causing it to fluoresce green (at 525 nm), and interacts with RNA by electrostatic attraction respectively, causing it to fluoresce red (at >630 nm). Acridine orange was prepared at 6.6 mM stock solution in water, and used in a final concentration of 33 nM. The concentrations of dimethyl sulfoxide and acetone used to prepare the stain stock concentrations were tested and were shown to have no effect on the physiology of the bacterial cells (data not shown). Flow cytometry was carried out using a Coulter Epics XL flow cytometer equipped with an air-cooled argon ion laser of 15 mW output and a fixed wavelength excitation of 488 nm. Before each experiment, the instrument was calibrated with fluorescent beads (Flow-Check, Beckman-Coulter) until measurement were uniform, with coefficients of variation always <2 for size and fluorescence. Software discriminators were set on forward side scatter (FS) signals to eliminate electronic and small particle (originating from the media, buffers or sheath fluid) noise. A total of 10,000 bacteria was recorded for each sample and each sample was analyzed in triplicate. The relative volume or size of individual bacterial cells was assessed by measuring the scattering of the light at a forward angle of the laser beam on the flow cytometer (the forward scatter). The relative cell shape, i.e., granularity or the presence of inclusion bodies, was assessed using the changes in refractory index of the laser beam (the side scatter). The fluorescence detectors were used to detect appropriately filtered light at green wavelength (FL1, 525 nm) emitted by SYTO-9, rhodamine-123, 3,3′-dihexyloxacarbocyanine iodide, 3,3′-diethyloxacarbocyanine iodide, acridine orange, 5(6)-carboxyfluorescein diacetate succinimidyl ester, Fluo-3 AM, dihydrorhodamine, redox sensor green and fluorescein diacetate; and orange wavelength (FL3, 620 nm) emitted by propidium iodide, acridine orange, hydroethidine and

mercury orange. Data in list mode files were analyzed off-line using the System II software (Beckman-Coulter), followed by statistical analysis using the *t*-test of the Microsoft Excel 2002 package. Statistical significance levels were expressed as highly significant if $P \leq 0.01$ (**), significant if $P \leq 0.05$ (*) or non significant if $P > 0.05$.

Proteomic analysis

Sample preparation

The bacterial pellets were suspended in lysis buffer (8 M urea, 4% w/v 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris, 0.2% v/v Pharmalytes 3–10, 2 mM tributyl phosphine (Bio-Rad), 0.25 tablet/ml Complete mini EDTA Free Protease Inhibitor Cocktail (Roche)) and incubated for 5 min in an Elma Transonic 450/H sonicator at 4°C. The samples were centrifuged at 13,200 rpm at 4°C for 15 min. Protein concentration of the supernatant fluids was measured with the Bio-Rad Protein Assay kit, with bovine gamma-globuline as a protein standard. Supernatants were stored at −20°C.

Proteome profiling by 2-dimensional gel electrophoresis

In first dimension, each sample (100 µg) was subjected to isoelectric focusing in Immobiline Dry strips of 18 cm at pH 4–7 (Amersham Pharmacia Biotech). The strips were rehydrated overnight in rehydration solution (2% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 8 M urea, 0.5% v/v pharmalyte 3–10, 13 mM dithioerythritol). Isoelectric focusing was performed on a Pharmacia Biotech Multiphor II system equipped with a Pharmacia Biotech EPS3500 XL power supply using a 3 phases program. The first phase was set at 500 V for 1 min, the second set was a linear gradient spanning from 500 to 3,500 V over 1.5 h and the final phase was set at 3,500 V for 16.3 h, according to the manufacturer's recommendations. After isoelectric focusing, the gels were equilibrated two times for 20 min each, first in equilibration solution (6 M urea, 30% v/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), 50 mM Tris–HCl pH 6.8) containing 65 mM dithioerythritol, and second in equilibration solution containing 135 mM iodoacetamide. The strips were then placed on top of 12.5% SDS-polyacrylamide gels (PAGE) in 0.4% w/v agarose gel made with SDS–PAGE running buffer (192 mM glycine, 0.1% w/v SDS, 25 mM Tris–HCl, pH 8.3). The second dimension was run for approximately 4 h at 500 V, 40 mA per gel, in a Bio-Rad Protean II Multicell gel system. Visualization of protein spots in the gels was obtained by silver staining (Mortz et al. 2001). Protein patterns within the gels were analyzed as digitalized images using a high-resolution scanner in combination with the molecular analysis software PDQuest (Bio-Rad) for the quantification.

Identification of proteins by mass spectrometry

Spots on the gel were excised using a 1 mm sample corer (Fine Science Tools Inc.). Excised gel pieces were placed in a Protein LoBind tube (Eppendorf) and washed 2 times 15 min in 40 µl of 25 mM ammonium bicarbonate (NH_4HCO_3). The gel pieces were then destained for few seconds in 250 µl of 30 mM potassium hexacyanoferrate ($C_6FeK_3N_6$) and 0.1 M sodium thiosulphate ($Na_2S_2O_3$) and three washes of 15 min in water. After two additional washes of 15 min in 40 µl of 25 mM ammonium bicarbonate and two washes of 15 min in 25 mM ammonium bicarbonate with 50% v/v acetonitrile (CH_3CN), gel pieces were dehydrated in a centrifugal evaporator (Heto, Drywinner, Denmark). The proteins from each dried gel piece were enzymatically digested by 10 µl of 0.02 µg/µl trypsin (Promega) in 25 mM ammonium bicarbonate, by overnight incubation at 37°C. The reaction was stopped with 1 µl of 5% v/v formic acid (CH_2O_2). Next, 1 µl of the digestion supernatant fluid was mixed 1:1 v/v ratio with a saturated solution of α-cyano-4-hydroxycinnamic acid ($C_{10}H_7NO_3$) in 50% v/v acetonitrile and 0.1% v/v trifluoroacetic acid (CF_3CO_2H). This mix was applied onto the 96 target wells and allowed to air-dry. Peptide mass fingerprints were obtained with a MALDI mass spectrometer (Micromass, Manchester, UK) working in reflectron mode with 15 kV of source voltage, 2.5 kV of pulse voltage, and 2 kV of reflecting voltage. Mass accuracy for peptide mass fingerprint analysis was 0.1 Da with external calibration, and internal calibration was carried out using enzyme autolysis peaks; resolution was 11,000. The resulting peptide masses were automatically searched

for in a local copy of the Swiss-Prot and TrEMBL databases (Boeckmann et al. 2003) using the ProteinLynx global server and the Protein Probe (Micromass Ltd., Manchester, UK) and Mascot (Matrix Science) search engines. One missed cleavage per peptide was allowed, a mass tolerance of 50 ppm was used, and the following variable modifications were taken into account: carbamidomethylation of cysteine and oxidation of methionine. Protein identification results were manually evaluated. Only identification results with a confidence level above 95% were confirmed as positive hits.

Gene annotation

Some genes of interest were further explored and re-annotated using the Magnifying Genomes (MaGe) platform (Vallenet et al. 2006). The *C. metallidurans* CH34 genome is part of the MaGe Cupriavidus2-Scope' project publically available at https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/MageHome/index.php?webpage=mage.

Results and discussion

Survival and growth of CH34 during spaceflight

The bacterial survival and reproduction in spacecraft during flight were assessed by visual quantification of colony growth on the mineral agar medium surface post-flight. The cultures retrieved from the MESSAGE-1 flight experiment showed a significant lower number of colonies than in the parallel earth based control experiment (Fig. 3). These differences in bacterial survival and growth observed in the first MESSAGE-1 experiment were, however, not observed anymore in the following space experiments MESSAGE-2 and BASE-A. It should be noted that a more stable temperature control during pre-, in- and post-flight in the space experiment MESSAGE-2 and BASE-A was possible, compared to the first MESSAGE-1 experiment (Fig. 2). Therefore, it is concluded that the reduced survival observed in the MESSAGE-1 experiment was possibly due to a synergetic effect of the lower inoculation cell concentrations, the pre-flight prolonged cold storage and in-flight fluctuating temperature profile, and the spaceflight. For the MESSAGE-2 and BASE-A experiments, no significant difference in number, size or morphology of spots or colonies was observed when comparing the cultures grown in spaceflight and earth conditions (Fig. 3). And no significant difference in total biomass harvested from the agar cultures from space or earth was observed (data not shown). For the motile α-proteobacterium *Rhodospirillum rubrum* S1H, which tested together with CH34 during the same MESSAGE-2 and BASE-A flight experiments, similar results were obtained, i.e., no significant difference in cell survival counts on agar medium or total biomass harvested between space and earth grown cultures (Mastroleo et al. 2009).

CH34 derivates have also successfully been cultured in liquid medium in spaceflight (De Boever et al. 2007). Most bacterial spaceflight experiments have been performed in liquid cultures, and the few performed on agar media showed survival and growth of bacteria under spaceflight conditions (reviewed in Leys et al. 2004). Most studies report a significant increase in bacterial growth (shorter lag time, higher growth rate, higher final cell concentrations) in liquid cultures in spaceflight (Leys et al. 2004). However, others did not observe changes for liquid or agar cultures in spaceflight or the same results for 1 g flight controls and the 1 g earth controls (Leys et al. 2004). It has been suggested that not direct cellular dynamics but mainly indirect fluid dynamics and extracellular transport phenomena cause the increases in growth of non-motile bacteria in liquid cultures in microgravity (Benoit et al. 2008). Based on theoretical calculations it is unlikely that bacteria can sense gravity directly due to the small mass of the internal cellular components and the negligible gravitational force compared to Brownian motion. However, mathematical calculations suggest that fluid quiescence under microgravity could lead to lack of cell sedimentation and to more efficient transfer of nutrients to and waste products from the cells (Benoit et al. 2008).

Spaceflight effect on cell size and cell shape

The flow cytometer side versus forward scatters dot-plots of *C. metallidurans* CH34 cells grown in spaceflight and earth control, indicated a significant difference in the MESSAGE-1 flight experiment: a more homogeneous cell size (a lower variation in the

value of the forward scatter) and a more spherical cell shape (a lower value of the side scatter) was observed in cultures returning from space (Table 1). However, no clear changes in cell shape and size were observed for the MESSAGE-2 or BASE-A flight experiments. These differences may have been due to the less optimal experimental conditions and flight effects in the MESSAGE-1 flight as mentioned above.

To our knowledge, this is the first study using flow cytometry to evaluate cell size and shape after spaceflight. Scanning electron microscopic analysis of *Salmonella typhimurium* cultured aerobically in rich liquid medium in spaceflight and on earth similarly did not show any apparent differences in the size and shape of individual cells (Wilson et al. 2007). Previous flow cytometry studies have indicated similarly that environmental stresses (such as exposure to low or high pH and hydroperoxide) have little or no effect on cell size and cell shape parameters of *C. metallidurans* CH34 (Baatout et al. 2006, 2007). This was in contrast with for example *Escherichia coli* which showed significant changes in cell size and shape upon exposure to environmental stresses (Baatout et al. 2006, 2007). *C. metallidurans* CH34 is a robust and versatile bacterium originating from metal polluted soil or sediments where it can survive dry and wet seasons, high and low temperatures, oxic and anoxic conditions, long periods of oligotrophic conditions, and toxic pollutants (Diels and Mergeay 1990; Mergeay 2000; Mergeay et al. 2003).

Viability and cultivability of CH34 after spaceflight

It is known that environmental stresses can affect the cell membrane integrity and thus the cell viability of *C. metallidurans* (Baatout et al. 2006, 2007). Therefore, the fraction of viable and dead cells in the cultures returning from space was assessed more in detail, by measuring via flow cytometry the ratio of live of dead cells in the culture (using the LIVE/DEAD BacLight Bacterial Viability Kit), cell membrane permeability (using propidium iodide), cell membrane potential (using rhodamine-123, 3,3′-dihexyloxacarbocyanine iodide or 3,3′-diethyloxacarbocyanine iodide) and the cell electron transport chain function (using Redox sensor green). The fluorescence histograms for both space-exposed and control cultures, indicated that in the MESSAGE-1 flight experiment the space cultures contained fewer cells with damaged membrane and more cells with a higher membrane potential than the control cultures (Table 1). These data indicated that, despite the lower initial survival, the cultures that did grow in spaceflight in the MESSAGE-1 experiment contained a larger portion of viable cells. In the MESSAGE-2 experiment, however, no significant difference between space and earth cultures was observed (Table 1). Cultures from the BASE-A flight experiment returning from space contained live cells with intact cell membranes, but with an overall significant higher membrane potential (Table 1). Thus, the overall data indicated that after 10–12 days growth either in space or on earth, the major fraction of the CH34 cells had intact cytoplasmic membranes, with respiratory chain function leading to a normal membrane potential and intra/extra-cellular pH gradient. Thus these cells were presumed to be metabolically active and to be able to reproduce. Indeed, consistent with these flow cytometry data, all cultures provided normally proliferating daughter cultures post-flight (data not shown). Also the post-flight swimming motility (using the proton gradient over the membrane) of *C. metallidurans* CH34 in semi-solid LB agar (0.6% agar) medium showed no significant differences between earth and space-grown cultures (data not shown).

Spaceflight effects on the intracellular concentration of reactive oxygen species

Exposure to ionizing radiation can generate inside bacterial cells additional reactive oxygen species (ROS), a group of strong oxidant molecules, including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (Cabiscol et al. 2000). ROS can cause irreversible damage to cellular components and thus are normally rapidly detoxified by antioxidant defense systems, including enzymes such as catalases, superoxide dismutases, small proteins like thioredoxin and glutaredoxin, and antioxidant molecules such as the glutathione (Cabiscol et al. 2000). Therefore, the ionizing radiation damage after spaceflight was assessed by measuring the intracellular concentration of $O_2^{\cdot -}$, H_2O_2 and glutathione, using fluorescent dyes hydroethidine, dihydrorhodamine and mercury orange in flow cytometry.

These flow cytometry data indicated no significant changes in intracellular $O_2^{\cdot-}$, H_2O_2 or glutathione concentrations for cultures from space in the MESSAGE-1 and -2 flight experiments (Table 1). As such, these observations provide evidence that *C. metallidurans* CH34 did not experience additional oxidative stress or was able to deal with the exposure to a total dose of about 180 μGy of ionizing radiation per day during the 10–12 days spaceflight inside the international space station. This is consistent with the results mentioned above as the cultures returning from space indeed contained only very few dead cells, and not more than earth-grown cultures. It has been reported that in space flight bacterial cells are able to deal with radiation stress and for example repair radiation-induced DNA damage close to normality (Horneck et al. 1996).

Spaceflight effect on intracellular and membrane protein content (proteome)

Proteomic profiles based on two-dimensional gel electrophoresis analyses showed minor differential cellular protein expression between space- and earth-grown cultures of *C. metallidurans* CH34 in the MESSAGE-1, MESSAGE-2 and BASE-A flight experiments. Although there was no significant difference in the concentrations of total protein in the space and earth extracts, a few proteins were detected in significantly higher concentrations in space-grown cells in comparison to earth grown cells (Fig. 4; Table 2). In contrast, no protein was detected as significantly over expressed in earth condition compared to the spaceflight conditions. Moreover, the protein profiles were very similarly changed in cultures from the MESSAGE-1 and MESSAGE-2 experiments (Fig. 4; Table 2), indicating the overproduction of these proteins was not a random effect but had a physiological function in the bacterial response to spaceflight in the given experimental set-up. Similar overall protein profiles were also observed in the BASE-A experiment but there were no significantly differences in protein concentrations in space versus earth cultures, excepted for one protein (AcxC) for which the earth cultures contained this time higher concentrations than the spaceflight samples (Fig. 4; Table 2). *R. rubrum* S1H also showed significant differentially expressed markers in its proteome and transcriptome profiles after 10 days cultivation in rich medium in the MESSAGE-2 experiment, but did not significantly do so when cultivated in minimal medium in the BASE-A flight experiment in the international space station (Mastroleo et al. 2009).

The most differentially expressed proteins under space conditions in MESSAGE-1 and -2, were AcxC (Rmet_4107) and AtoA (Rmet_1154) (Fig. 4; Table 2), subunits of the enzymes which were later discovered to be involved in the degradation of acetone and isopropanol in *C. metallidurans* CH34 (Rosier et al. unpublished data). Acetone carboxylase (AcxABC) catalyzes the carboxylation of acetone to acetoacetate. Acetoacetate is transformed via acetyl-CoA:acetoacetate CoA transferase (AtoDA) (Rmet_1153 and Rmet_1154) in acetoacetyl-CoA, which is further processed by a 3-ketoacyl-CoA thiolase to 2 acetyl-CoA molecules that are oxidised in the tricarboxylic acid (TCA) cycle (Rosier et al. unpublished data). The expression of these acetone degradation enzymes in spaceflight conditions was an unexpected observation as the acetone degradation capacity was not observed or suspected before in CH34 and as only gluconate was provided in the medium. In CH34, gluconate is predicted to be metabolized in the presence of oxygen through the Entner-Doudoroff pathway to 2 pyruvate molecules, which are transformed to acetyl-CoA. The acetyl group of acetyl-CoA is in most organisms fully oxidized to CO_2 via the TCA cycle followed by complete conversion of its chemical energy via NADH, $FADH_2$, and GTP to ATP in oxidative phosphorylation. CH34 has in addition the necessary enzymes to bypass some steps in the TCA cycle where carbon is lost in the form of CO_2 via the glyoxylate cycle (glyoxylate shunt), and can as such use acetyl-CoA for biosynthesis of cell constituents via gluconeogenesis. In the glyoxylate cycle, acetyl-CoA is converted to oxaloacetate. During gluconeogenesis, oxaloacetate is decarboxylated, phosphorylated and finally transformed to fructose-1,6-biphosphate by fructose bisphosphate aldolase. In fact, a fructose bisphosphate aldolase class II (CbbA3) (Rmet_0503) was found in both space and earth samples, but in higher concentrations in space samples (Table 2). The fructose bisphosphate aldolase is sometimes also involved at the end of the Calvin–Benson–Bassham cycle using CO_2 as carbon source. Also a putative subunit of soluble [NiFe]-hydrogenase HoxI (Rmet_1527), allowing growth at the expense of

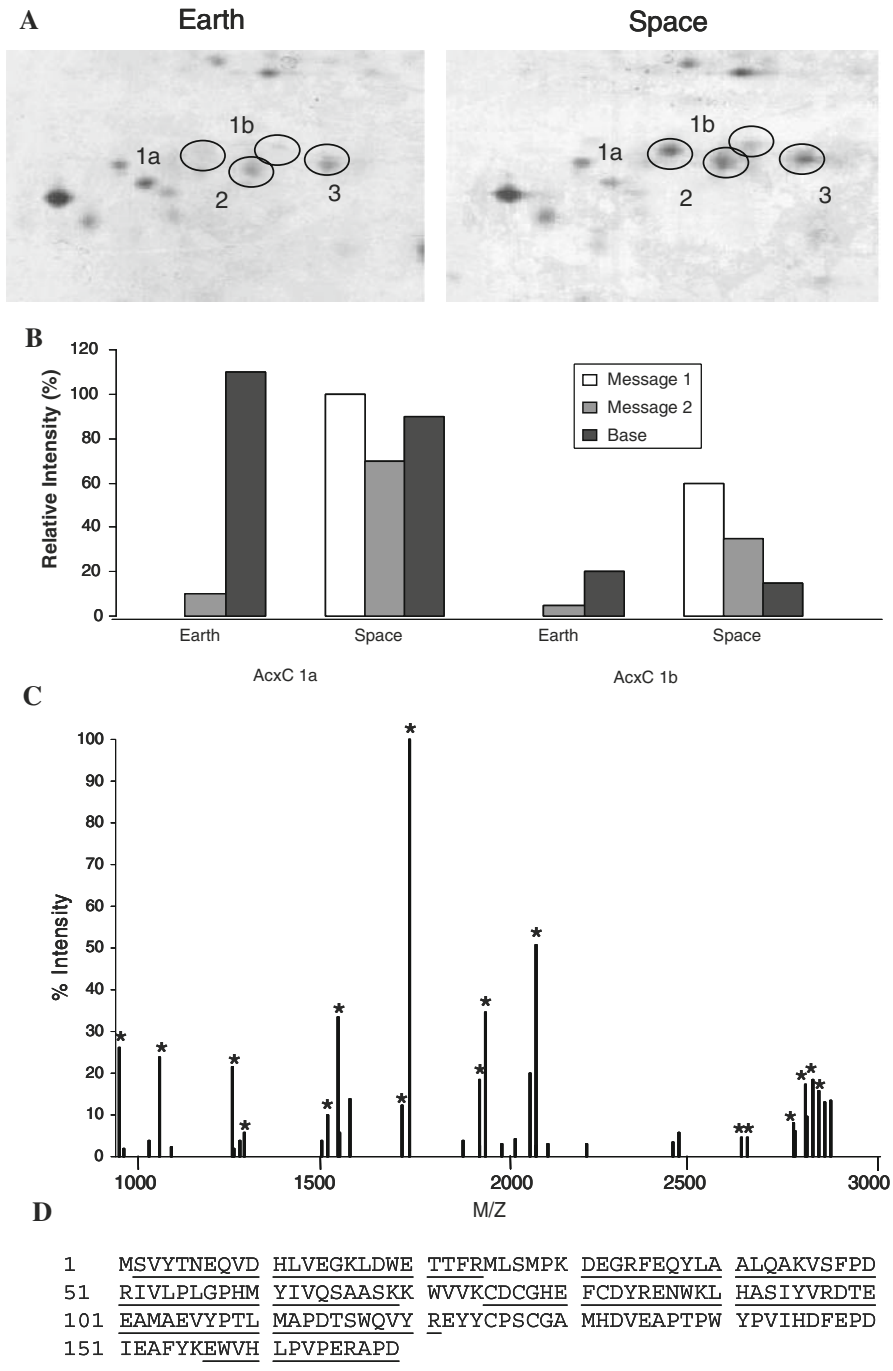

Fig. 4 Protein extracts from *C. metallidurans* CH34 grown in space or earth conditions in the MESSAGE 1, MESSAGE 2 and BASE experiments. **a** Area of two dimensional gel electrophoresis including the spots corresponding to AcxC (*spots 1a* and *1b*), AhpC1 (*spot 2*) and GrpE (*spot 3*). **b** Relative intensity of AcxC isoforms in earth and space conditions measured with PDQuest software. **c** MALDI-TOF mass spectrum of AcxC (*spot 1a*). **d** Amino acid sequence of AcxC: the *underlined* parts of the sequence correspond to the peptides identified in the MALDI-TOF mass spectrum (*) (Sequence recovery = 72%)

hydrogen as electron donor when fixating CO_2 (chemolithoautotrophic growth), was detected in CH34 cells from space and earth (Table 2). It is known that gluconeogenesis occurs during periods of starvation, is highly energy absorbing, and is often associated with ketogenesis. It is possible that, when oxygen or carbohydrates became scarce in the space and earth cultures after 10 days, energy was obtained from breaking down short-chain fatty acids or poly-hydroxybutyrate to acetyl-CoA molecules, which were forwarded to the TCA cycle. The MESSAGE-1 and -2 space samples indeed contained high

Table 2 Proteins present in higher concentrations in CH34 cells retrieved from space in the MESSAGE-1, MESSAGE-2 and BASE flight experiments

Protein name	Protein function	Protein pI/MW[a]	Protein Tag (NCBI No)	Gene Tag (NCBI No)	Gene location[b]	Protein conc. ratio space/earth[c]		
						MSS-1	MSS-2	BAS-A
AcxC	Acetone carboxylase, gamma subunit	4.85/19799.4	GI:94313034	Rmet_4107	CHROM 2	>10	5.0 ± 0.4	0.8 ± 0.2
AtoA	Acetyl-CoA:acetoacetate CoA transferase, subunit B	4.92/22474.0	GI:94310099	Rmet_1154	CHROM 1	1.8 ± 0.2	2.0 ± 0.5	1.2 ± 0.4
AldB	Acetaldehyde dehydrogenase	5.81/55158.1	GI:94314047	Rmet_5128	CHROM 2	5.6 ± 0.4	3.0 ± 0.8	1.3 ± 0.2
AcdA	Acyl CoA dehydrogenase like (fragment)	8.47/23558.2	GI:94152505	Rmet_6103	pMOL30	2.0 ± 0.4	1.8 ± 0.2	0.9 ± 0.3
CbbA3	Fructose bisphosphate aldolase, class II	5.67/38510.9	GI:94309448	Rmet_0503	CHROM 1	2.5 ± 0.3	1.9 ± 0.5	1.1 ± 0.2
GlnB	Nitrogen response transcriptional regulator	5.70/12337.3	GI:94309626	Rmet_0681	CHROM 1	2.1 ± 0.3	1.6 ± 0.2	1.1 ± 0.3
DpsA	DNA binding and protection protein	5.83/18046.7	GI:94311872	Rmet_2940	CHROM 1	3.5 ± 1.1	2.5 ± 0.4	1.09 ± 0.4
GrpE	Chaperone protein of DnaK (Hsp70)-type heat shock ATPases	5.08/19755.1	GI:94309949	Rmet_1004	CHROM 1	2.4 ± 0.2	2.6 ± 0.1	1.3 ± 0.2
UspA3	Universal stress protein	5.16/15811.2	GI:94310329	Rmet_1387	CHROM 1	1.9 ± 0.4	1.8 ± 0.3	0.8 ± 0.3
AhpC1	Thioredoxin-dependent alkyl hydroperoxide reductase	4.98/20226.7	GI:94310888	Rmet_1950	CHROM 1	2.5 ± 0.4	1.8 ± 0.3	0.9 ± 0.2
TrxA	Thioredoxin	4.98/11858.7	GI:94311072	Rmet_2134	CHROM 1	2.2 ± 0.1	2.3 ± 0.4	1.1 ± 0.1
HoxI	Putative subunit of soluble [NiFe]-hydrogenase	5.15/18534.1	GI:94310469	Rmet_1527	CHROM 1	1.5 ± 0.2	1.8 ± 0.1	0.9 ± 0.3
Tuf	Protein translation elongation factor Tu	5.41/43088.3	GI:94312255	Rmet_3324	CHROM 1	3.2 ± 1.1	3.2 ± 0.7	1.1 ± 0.1
Tsf	Protein translation elongation factor Ts	5.36/30948.5	GI:94310378	Rmet_1436	CHROM 1	3.0 ± 0.5	2.5 ± 1.0	1.3 ± 0.2
RpsA	30S ribosomal subunit S1	5.15/61648.9	GI:94309667	Rmet_0722	CHROM 1	3.1 ± 1.1	2.5 ± 0.5	0.9 ± 0.3
RplL	50S ribosomal subunit L7/L12	4.83/12610.5	GI:94312266	Rmet_3335	CHROM 1	2.1 ± 0.4	1.8 ± 0.3	1.0 ± 0.3

[a] The MW and pI values given in this table correspond to theoretical values evaluated with the Mascot system, and may differ from the values in the NCBI data base

[b] NCBI Accession No of CHROM 1 = CP000352, CHROM2 = CP000353, pMOL30 = CP000354, pMOL28 = CP000355

[c] Mean and standard deviation values of three biological and two technical replicates ($n = 6$)

concentrations of a Acyl-CoA dehydrogenase like protein (AcdA, Rmet_6103) (Table 2), possibly catalyzing the conversion of butyryl-CoA to acetoacetyl-CoA, which than further can be cleaved to 2 acetyl-CoA molecules. Acetyl-CoA could however, probably not be fully recycled through the TCA cycle because the cycle intermediates (mainly oxaloacetate, needed to initiate the TCA cycle) had been depleted to feed the gluconeogenesis pathway. The resulting accumulation of acetyl-CoA may have activated ketogenesis, i.e., the production of ketone bodies such as acetoacetate and acetone. Two acetyl-CoA molecules were condensed to acetoacetyl-CoA which was transformed by acetyl-CoA:acetoacetate CoA transferase (AtoDA) to acetoacetate (Table 2). Acetoacetate decarboxylation to acetone and CO_2, occurs spontaneously in aqueous solution (Boyd et al. 2004). In some bacteria the spontaneous decomposition of acetoacetate to acetone is further accelerated by acetoacetate decarboxylase (Boyd et al. 2004), but the CH34 genome did not seem to contain the genes coding for such enzymes. However, in CH34 the acetone produced by the spontaneous decomposition of acetoacetate may have induced the expression of acetone carboxylase (AcxABC) (Fig. 4; Table 2). In addition, an secondary aldehyde dehydrogenase (AldB) (Rmet_5128), a possible candidate for the further conversion of acetone to less toxic isopropanol, was found in the CH34 space culture proteomes (Table 2).

The genes coding for these metabolic proteins detected as differentially expressed in the CH34 space and earth cultures were spread over chromosome 1 and chromosome 2. Only 1 pMOL30 plasmid and no pMOL28 plasmid encoded proteins were found differentially expressed. Further genomic sequence analysis revealed that for most of these metabolic proteins, the gene transcription is dependent of a RNA polymerase containing the σ^{54} (RpoN) subunit. The transcription of the *acxABC* genes is regulated by a transcriptional activator (AcxR) (Rmet_4107), which contains a σ^{54} binding site. This operon structure is similar to what has been found for *acxRABC* in *Xanthobacter autrophicus* Py2 and *Rhodobacter capsulatus* B10 (Sluis et al. 2002). Also the transcription of the *aldB* (Rmet_5128) gene is putatively regulated by a σ^{54} dependent transcriptional regulator (Rmet_5127) in CH34. In *E. coli*, the transcription of *atoDA* is also regulated by a σ^{54} dependent activator, but so far no regulator with a clear σ^{54} signature was found close to the *atoDA* genes in the genome of CH34. Nevertheless, also the σ^{54} dependent nitrogen metabolism P-II transcription regulator GlnB (Rmet_0681) (Mouz et al. 2001), was found to be overproduced in CH34 in space in the MESSAGE-1 and -2 experiments.

Some other proteins that were produced in higher concentrations in space grown cells, are known to be implicated in general stress response, and included the DNA protection during starvation protein DpsA (Rmet_2940), the chaperone protein GrpE (Rmet_1004), and the universal stress protein UspA3 (Rmet_1387). Dps proteins are mini-ferritins that catalyze reactions with $Fe^{2+}/H_2O_2/O_2$ and trap minerals inside protein nanocages to minimize radical oxygen-chemistry (Liu et al. 2006). The expression of Dps was also found differentially regulated by spaceflight and possibly regulated by the Hfq chaperone for small non-coding RNAs in *Salmonella* cultured in mineral or rich liquid medium (Wilson et al. 2007, 2008). The expression of the stress protein genes *uspA* and *grpE* can be induced by starvation and increases thermal resistance in *E. coli* (Zhang and Griffiths 2003; Siegele 2005). Next to DpsA, UspA3 and GrpE, also the production of enzymes involved in thiol specific oxidoreduction reactions, such as the thioredoxin-dependent alkyl hydroperoxide dehydrogenase (AhpC1) (Rmet_1950) and a thioredoxin (TrxA) (Rmet_2134), was upregulated in CH34 under space flight conditions. The expression of TrxA was also differentially regulated by spaceflight in *Salmonella* cultured in both mineral and rich liquid medium (Wilson et al. 2007). The expression of these redox-active proteins AhpC1 and TrxA has been reported to be induced upon stationary phase in *E. coli*. In *E. coli*, the transcription of genes encoding the NADPH-dependent alkyl hydroperoxide reductase (AhpC) and the protective DNA-protection protein during starvation (Dps) are controlled by the *oxyR* gene and the activation of these responses greatly increases cellular resistance to oxidative agents (Cabiscol et al. 2000). Thus all these proteins, DpsA, GprE, UspA3, AhpC1 and TrxA, were possibly produced in response to carbon limitation, while protecting the cells additionally against environmental stresses such as oxidative or heat stress.

Several ribosomal structural proteins (RpsA, RplL) (Rmet_0722 and Rmet_3335) and enzymes involved in ribosomal protein translation (TufA, Tsf)

(Rmet_3324 and Rmet_1436) were found in higher concentrations in cells exposed to spaceflight in the MESSAGE-1 and -2 experiments. These translation-related proteins were possibly needed to support the higher expression of the metabolic and stress response proteins described above.

Most of these proteins (CbbA3, DpsA, GrpE, AhpC, TufA, Tsf) observed at higher levels in the colonies from CH34 in the spaceflight experiments are coded by genes that are predicted to be highly expressed in prokaryotic genomes (Karlin and Mrázek 2000). Ribosomal proteins, translation and transcription processing factors, chaperone proteins and proteins of principal energy metabolism such as glycolysis and TCA cycles are predicted to be highly expressed in most prokaryotic genomes (Karlin and Mrázek 2000). Moreover, these groups of highly expressed proteins are suggested to play a role in the survival and stress resistance of the cells (Karlin and Mrázek 2000).

The differential expression of groups of proteins involved in carbon limitation and stress response have also been reported for other bacteria after spaceflight. As an example, CbbA3, Dps, UspA, TrxA, RplL, RpsA, TufA and Tsf, together with a large number of TCA and carbon cycle, ATP synthesis, chaperone and ribosomal proteins, and some proteins related to the RpoN and PstN nutrient limitation responding regulators, were detected in *S. typhimurium* grown in minimal medium in spaceflight (Wilson et al. 2008). And the *dps*, *trxA* and *rplL* genes were found differentially expressed on the RNA level in *S. typhimurium* grown in rich medium in the international space station (Wilson et al. 2007). The upregulation of genes involved in the starvation and stress response have also been observed for *E. coli* cultures grown in simulated microgravity in liquid medium (Vukanti et al. 2008). In fact it has been demonstrated for *Pseudomonas* sp., *Stenotrophomonas* sp., *Sphingobacterium* and *Ralstonia picketti* cultured in liquid medium in slow turning lateral vessels that the response to simulated reduced gravity was less apparent under starvation conditions than under rich nutrient conditions (Baker and Leff 2004, 2006).

Our data do not demonstrate any involvement of RpoS (σ^{38}), the primary sigma factor required for the expression of genes for survival during stationary phase, in a spaceflight response. This is similar to what has been reported before for *Salmonella* cultured in liquid medium in simulated microgravity in high aspect ratio vessels (HARV system) (Wilson et al. 2002). Despite the many results indicating the importance of *rpoS* gene in environmental stress responses, it was concluded that instead of the RpoS regulon, mainly new genes are expressed to accommodate the organisms to the new environment (Saint-Ruf et al. 2004). Some data also indicated that when nutrients are limiting, bacteria reduce their metabolic rate and activate a variety of genes to enable them to survive nutrient limitation and cope with stresses such as heat and oxidation that they might encounter before the return of nutrients. The differential expression of such proteins in response to a differential carbon limitation in spaceflight could thus trigger a physiology change and provide additional resistance of the cells post-flight against a variety of environmental stresses such as oxidative, heat, acid, metal stress or antibiotics, as has been reported for several bacteria (Wilson et al. 2007; Mastroleo et al. 2009). Also increased production of secondary metabolites or extracellular matrix and biofilm formation (Wilson et al. 2007; Crabbé et al. 2008) has been reported in cells returning from spaceflight or after growth in simulated microgravity, and it is known that extracellular matrix/biofilm formation is a means of bacteria to increase survival of under various conditions.

Conclusions

This is the first study on the spaceflight response of environmental *Cupriavidus* bacteria. The inevitable constraints of spaceflight experiments, such as hardware design as well as pre-, in- and post-flight storage, have a significant impact on the experimental observations, and therefore play an important role when comparing several spaceflight experiments or spaceflight versus earth-grown cells. The experimental design of the BASE-A experiment, possibly leading to faster oxygen and nutrient limitation, indeed gave somewhat different results when compared to those of the earlier MESSAGE-1 and -2 experiments. It appears that under stable temperature conditions, spaceflight did not affect survival or proliferation of *C. metallidurans* type strain CH34 on mineral agar medium. Flow cytometry analysis showed no large changes in cell size and shape, cell envelope and cell interior physiology in CH34

cultures returning from space. To our knowledge this was the first study using flow cytometry to monitor the cell physiology of bacterial cells returning from space. Flow cytometry provides individual cell information in a homogeneous or heterogeneous population that is otherwise not possible to obtain. The proteomic data indicated that the about 10–12 days old CH34 colonies on mineral agar medium were responding, probably via RpoN regulated pathway, to carbohydrate or oxygen limitation and that this response was stronger in space than on earth as the key proteins were present in higher concentrations in space cultures. Overall, CH34 displayed only a weak response to spaceflight as only a few proteins were differentially expressed with only minor concentration changes. Nevertheless, they revealed new metabolic functions of CH34 such as the acetone degradation pathway. As many attributes of bacteria are only expressed under stress, it is likely that more novel and environment-specific genes and proteins will be discovered by the study of the bacterial response to spaceflight conditions.

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