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Hypobaric bacteriology: growth, cytoplasmic membrane polarization and total cellular fatty acids in *Escherichia coli* and *Bacillus subtilis*

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Abstract: *Escherichia coli* JM109 (Gram-negative) and *Bacillus subtilis* (Gram-positive) were grown under hypobaric conditions for 19 days at 25 °C to study the effects of 33 and 67 kPa low pressures on selected physiological responses; growth, cytoplasmic membrane polarization (measure of cytoplasmic membrane fluidity) and total cellular fatty acids. In the first experiment, cytoplasmic membrane polarization in *B. subtilis* increased under both hypobaric conditions, indicating the membrane became more rigid or less fluid. This experiment was repeated and the effect of the hypobaric conditions was not evident as in the first experiment with *B. subtilis*. In addition, total cellular fatty acids analysis for *B. subtilis* showed that hypobaric conditions did not alter the ratio of saturated to unsaturated fatty acids. The cytoplasmic membrane remained in the same fluid state in hypobaric grown *E. coli* cell cultures as in the 101 kPa ambient control cells in both experiments. However, the saturated to unsaturated ratios were altered in *E. coli* under hypobaric conditions. It is important to note the ratios for *E. coli* were less than 1, while the ratios for *Bacillus* were in the 28–50 range. Growth of both species was also measured by colony forming units at the termination of the 19 day experiment. Both bacterial species were capable of growth under hypobaric conditions and no distinct trend emerged as to the effect of hypobaric pressure on bacterial growth and cytoplasmic membrane fluidity.

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Introduction

Mars is a possible future long-term space mission, yet a paucity of knowledge exists in astrobiology research on diverse living organisms in space under reduced gravity and hypobaric conditions. An engineering advantage is gained in space if some structural facilities can be operated at reduced pressures. These include decreased pressures that allow structurally simpler and lighter facilities to be transported and assembled. This is significant, as fewer construction materials need to be transported to Mars. Secondly, there will be less leakage to the ambient Martian atmosphere from the constructed facility. However, research needs to be completed on the effects of low pressure on microbial and plant growth and human activities. Microbes will be present on space missions as contaminants on seeds, plants, in the hydroponic plant food systems, in and on the human body, on facility surfaces and in waste treatment facilities (Peterson *et al*. 2004) such as bioreactors and water purification systems.

The current space missions are generally short-term and do not employ complex, living, life support systems such as plants and microbial bioreactors. In the future, long-term space exploration by humans will require closed-loop life support systems containing plants and micro-organisms (Dixon *et al*. 2005). Presently, there is a lack of knowledge on the physiological responses of diverse micro-organisms in the absence of gravity (Klaus *et al*. 1997; Kacena *et al*. 1999) under randomized microgravity (Gassett *et al*. 1994; Nickerson *et al*. 2000; England *et al*. 2003; Lynch *et al*. 2004) and under low pressure or hypobaric conditions. Genigeorgis (1985) reviewed the literature in 1985 from a food safety perspective and briefly commented on hypobaric conditions. Information on membrane fluidity was not part of the review. One study on hypobaric conditions with plants researched gene expression in *Arabidopsis* (Paul *et al*. 2004). For example, exposure of this plant to 10 kPa resulted in the expression of more than 200 genes compared to ambient growth conditions of 101 kPa.

Because microbes will be present on space missions we researched some physiological responses for two different species under two hypobaric values (67 and 33 kPa). *Escherichia coli* and *Bacillus subtilis* were chosen as model

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bacterial species as they are two of the most studied microorganisms. They are, however, different in that *E. coli* is Gram-negative and *B. subtilis* is Gram-positive and lacks an outer membrane, and can form spores.

The physiological responses selected were growth, as it is the sum of all physiological activities, and cytoplasmic membrane polarization was used as an estimate of membrane fluidity (Barbaro *et al.* 1999; Trevors 2003). The bacterial cytoplasmic membrane remains in a fluid state under normal growth conditions, but may be more fluid under conditions of high temperature or alternatively become more rigid or less fluid under conditions of low temperature (Denich *et al.* 2003a). Membrane polarization is an excellent parameter to measure under hypobaric conditions using a fluorescent lipid probe such as 1,6-diphenyl-1,3,5-hexatriene (DPH), which readily inserts into the cytoplasmic membrane of live bacterial cells and provides virtual real-time data on the fluidity of the membrane (Trevors 2003). The relationship between probe polarization and cytoplasmic membrane fluidity is an inverse one. As the bacterial cytoplasmic membrane fluidity decreases, the polarization value increases, and *vice versa*. Rotation of the fluorescent probe in the cytoplasmic membrane is dependent on the structure of the cytoplasmic membrane. In a gel or rigid-state membrane, the probe does not freely rotate, due to increased interactions between acyl chains. In the liquid-crystalline membrane, the acyl chain interactions are reduced, allowing more motion of the chains, and therefore more rotation of the probe. When polarized light excites the inserted probe in a fluid cytoplasmic membrane, the light is emitted in all directions as the probe rotates.

Total cellular fatty acids were measured using the MIDI method (Microbial ID, Inc., Newark, DE, USA) as reported in our previous research (Denich *et al.* 2005). This analysis provides information on the bacterial cellular physiology as well as total cellular fatty acids by Microbial ID, Inc. Since these are the first experiments conducted on these bacterial species under hypobaric conditions, we selected measurements that could be made immediately after the cultures were removed from the hypobaric chambers, to prevent any changes upon return to ambient kPa conditions. Pagan and Mackey (2000) reported on membrane damage and cell death in *E. coli* under high pressure (up to 600 MPa) and concluded that membrane damage is an important physiological event in the death of the cells under high pressure. To the best of our knowledge, the experiments in the present study are the first of this type to study these bacteria under hypobaric conditions.

Materials and methods

Bacterial species

Escherichia coli JM109 and *Bacillus subtilis* (our laboratory culture collection) were grown overnight (about 14 h) at 25 °C in the dark in 75 mL of Luria Bertani (LB) broth (Difco, USA) in an Erlenmeyer flask with shaking at 200 rpm. 3 mL of these exponential cultures were used to inoculate 75 mL of sterile LB medium in 250 mL Erlenmeyer flasks plugged with a sterile foam stopper. The O.D.600 nm of the cell suspension was about 0.12–0.15. All triplicate flasks were placed in a plastic container and immediately placed into one of the three hypobaric chambers at two different low pressures and one ambient pressure for 18 or 19 d, depending on the experiment.

Hypobaric chambers

The hypobaric chambers are located at the Controlled Environment Systems Research Facility (CESRF) at the University of Guelph, Guelph, ON, Canada. Each chamber has an internal volume of 4.5 m^3 and dimensions of 1.0 m in length, 1.8 m in width and 2.5 m in height. The three treatment conditions were 1, 2/3 and 1/3 ambient atmospheric pressures that are equivalent to 101, 67 and 33 kPa total pressure, respectively. 67 and 33 kPa hypobaric pressure values were selected because they coincided with the values currently being tested for the growth of edible plants under similar conditions. Each chamber was supplied with lighting from high intensity discharge (HID) lamps on a 16 h light/8 h dark cycle at 300 $\mu mol\ m^{-2}\ s^{-1}$. The air temperature was maintained at 25 °C and the CO_2 level was maintained at 1200 $\mu mol\ mol^{-1}$ (0.12 kPa). The vapour pressure deficit (VPD) was maintained at a constant 0.9 kPa (65% relative humidity). The experiment was conducted for 19 days under the above conditions in triplicate in each hypobaric chamber. The chambers were then slowly repressurized to 1 kPa over a period of about 1 h. All samples were immediately removed and placed on ice and processed immediately to avoid any physiological changes. The volume of LB growth medium in each flask was measured to estimate any loss of liquid under the incubation conditions over the 19 d. The amount lost over 18 days ranged from about 3 to 5 mL. A schematic of the hypobaric chambers was published in Dixon *et al.* (2005). Note that the elevated CO_2 level simulated a closed system in which plant growth was occurring under an elevated CO_2 concentration. This parallel experiment was also being conducted in the chambers.

Bacterial colony forming units

Colony forming units (CFU/ml) were estimated using the drop plate method (5 μl of sample per drop on the Petri plate) of Cassidy *et al.* (2000) with the serial dilution made in sterile 0.75% (w/v) saline. All triplicate plates were incubated at 37 °C in the dark overnight and CFU were counted before they became overgrown.

Cytoplasmic membrane polarization

The fluorescent membrane probe, DPH (Molecular Probes, Eugene OR, USA) was dissolved in tetrahydrofuran to a final concentration of 12 mM. 10 mL of the culture were removed from each flask and centrifuged at 3500 *g* for 10 min at 25 °C. The supernatant fluid was discarded and the bacterial pellet washed with 1 mL of sterile, 15 mM Tris-HCl buffer (pH 7.0). The sample was centrifuged again, and resuspended in a sterile Tris-HCl buffer to an O.D. at 600 nm of 0.2. From this suspension, 3 mL were incubated with 1 μl. of 12 mM DPH for 10 min in the dark at room temperature. Kim *et al.*

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(2001) verified that 10 min was sufficient time for the optimal incorporation of the probe into the cytoplasmic membrane.

Fluorescence polarization was measured using a spectrofluorometer (Photon Technology International Inc. London, ON, Canada) equipped with a temperature-controlled cuvette holder and stirrer set at 140 rpm (Kim *et al*. 2001; Denich *et al*. 2003a, b, 2005; England *et al*. 2003; Trevors 2003; Vincent *et al*. 2004). The sample temperature was maintained at 25 °C in the fluorometer cuvette holder. Excitation and emission wavelengths were set at 358 and 428 nm, respectively. Polarizers were set in either a vertical or a horizontal position. The slit widths for excitation and emission beams were 12 and 10 nm, respectively. The voltages for the horizontal and vertical polarized light amplifiers were 1005 and 1040 V, respectively. Data was recorded using FeliX software (version 1.4, Photon Technology International, London, ON, Canada). The degree of polarization (P) was calculated from the emission fluorescence intensities measured parallel (I_1) and perpendicular (I_2) to the plane of excitation light according to the following equation:

$$P = I_1 - GI_2/I_2 - GI_1.$$

The correlation factor G was calculated as the ratio of parallel and perpendicular emitted light when the excitation light was horizontal, as described by Kim *et al*. (2001) and Vincent *et al*. (2004). All the analyses were repeated in triplicate.

Total bacterial cellular fatty acid analysis

Total cellular fatty acids were extracted according to the protocol recommended by Microbial ID (Sasser 1990). Fatty acids were saponified, methyl esterified and the fatty acid methyl esters analysed by GC-FID using the MIDI microbial identification system (Kim *et al*. 2002; Denich *et al*. 2003b).

Statistical analysis and experimental replication

An ANOVA followed by a SNK multiple range test was performed using InStat software (GraphPad Software Inc.).

The complete data sets including replicate values for experiment 1 are presented in the tables. The means and standard deviations for the 18 day repeat experiment 2 are presented in the text at the appropriate locations to allow comparisons to the experiment 1 data. A third 18 day experiment was conducted with only *B. subtilis* to determine whether or not a resolution to the differences in membrane polarization was possible and to increase the amount of data.

Results and discussion

Table 1 contains the log CFU/ml values at the termination of the first 19 day experiment. With decreasing hypobaric pressure *E. coli* exhibited a slight increase in CFU (log 10.15 at 67 kPa and log 9.34 at 33 kPa) compared to the 101 kPa ambient control (log 9.34) (Table 1). However, the trend was not linear as the CFU had slightly higher values at 67 kPa than at 33 kPa. *B. subtilis* exhibited about the same final mean CFU values (log 7.6–7.8) under both ambient and hypobaric conditions (Table 1). *B. subtilis* may be the more robust bacterial species because of the thicker peptidoglycan structure in the cell wall and also its ability to form spores under less than optimal environmental conditions. This experiment was repeated to ascertain whether a distinct trend in the data would emerge or not. In a repeated experiment for 18 days under the same conditions as in the first experiment, *E. coli* reached mean log CFU/ml values of 9.3, 9.3 and 9.0 after 18 days under ambient 67 and 33 kPa conditions, respectively. *B. subtilis* reached mean log CFU/ml 9.0, 9.2 and 8.6 after 18 days under ambient 67 and 33 kPa conditions, respectively. The hypobaric conditions did not affect the final mean CFU counts in a manner where a distinct trend emerged. Both species were capable of growth and survival for 19 days under 67 and 33 kPa hypobaric conditions. Growth rates were not estimated as the chambers were not repeatedly opened and closed to remove samples. Moreover, both shorter and longer duration experiments will need to be completed in the future. The 19 day experiment was used as it coincided with a hydroponic plant growth experiment in the same chamber.

Table 1. *log (base 10) colony forming units (CFU/ml) of bacteria after being statically grown in LB broth for 19 days at 25 °C under ambient and hypobaric conditions*

	log CFU/ml under different pressures (kPa)		
	101	67	33
E. coli			
Replicate			
1	9.05	10.43	9.52
2	8.26	9.68	9.34
3	8.18	10.42	9.16
Mean ± S.D.	8.50 (0.48) a	10.15 (0.47) b	9.34 (0.18) c
B. subtilis			
Replicate			
1	7.56	7.50	7.86
2	7.75	7.68	7.31
3	8.06	7.94	7.56
Mean ± S.D.	7.78 (O.256) a	7.71 (0.220) a	7.59 (0.241) a

Means followed by the same letter within each experiment are not significantly different at the 95% level using ANOVA followed by an SNK test.

Both bacterial species were also grown in 1/10 strength LB broth for 18 days (in experiment 2) under the same hypobaric conditions to determine the effect of a lower nutrient growth condition. *E. coli* grew and survived with mean CFU values of about log 10 in the ambient and hypobaric treatments. *B. subtilis* reached mean log CFU values of about log 8 in the ambient and both hypobaric treatments. This is in agreement with the results obtained using full-strength LB broth where *E. coli* also exhibited the higher mean CFU counts. It is not known if a hypobaric value of less than 33 kPa and/or

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Table 2. *Cytoplasmic membrane polarization (P is unitless 0–1) values for bacteria cultures statically grown in LB broth after 19 day incubation at 25 °C under ambient and hypobaric conditions. The lower the P value the more fluid is the bacterial cytoplasmic membrane*

	Cytoplasmic membrane polarization (*P*) under different pressures (kPa)		
	101	67	33
E. coli			
Replicate			
1	0.283	0.301	0.266
2	0.274	0.288	0.274
3	0.285	0.274	0.290
Mean ± S.D.	0.281 (0.050) a	0.287 (0.0130) a	0.277 (0.013) a
B. subtilis			
Replicate			
1	0.234	0.259	0.332
2	0.243	0.271	0.320
3	0.256	0.263	0.331
Mean ± S.D.	0.248 (0.011) a	0.265 (0.006) b	0.327 (0.006) c

Means followed by the same letter within each experiment are not significantly different at the 95% level using ANOVA followed by an SNK test.

over a longer time period will produce a detectable effect on bacterial growth estimated by the CFU counts. These experiments need to be conducted in the future if the chamber can be held at a much lower hypobaric value for longer time periods.

The fluorescent DPH probe is used for obtaining information on the physiological status of bacteria under a variety of growth or environmental conditions (Trevors 2003). Also, the cytoplasmic membrane is one cellular structure that provides knowledge on the physiology of bacterial cells (Trevors 1983). *E. coli* exhibited similar mean cytoplasmic membrane polarization values (*P*) of about 0.28 under the 101 kPa ambient control and two hypobaric conditions (Table 2). There was no statistical significance (95% level) between the mean control and hypobaric treatments (Table 2). However, the mean *B. subtilis* membrane polarization values were all significantly different (95% level) from each other (Table 2) in experiment 1. At hypobaric pressures of 67 and 33 kPa the cytoplasmic membrane polarization in *B. subtilis* became less fluid or more rigid, with mean *P* values of 0.265 and 0.327, respectively.

The membrane polarization experiment was repeated again in the hypobaric chambers (randomized for their pressure values) and the following data was obtained. *E. coli* exhibited mean *P* values of 0.28 ± 0.01, 0.29 ± 0.01 and 0.29 ± 0.02 ($n = 3, \pm$ S.D.) at ambient 66 and 33 kPa, respectively, after 18 days at 25 °C. *B. subtilis* exhibited mean *P* values of 0.22 ± 0.003, 0.21 ± 0.04 and 0.24 ± 0.02 ($n = 3 \pm$ S.D.) at ambient 66 and 33 kPa, respectively. This experiment revealed that the *E. coli* membrane fluidity was not altered and was in agreement with the findings of the first experiment. The *B. subtilis* cytoplasmic membrane fluidity was not altered under the 33 kPa hypobaric conditions when compared to mean values observed under ambient and 67 kPa conditions. The mean values were not significantly different (at 95% level) as observed in experiment 1.

A third experiment was conducted with *B. subtilis* for 18 d. The membrane polarization values at ambient, 67 and 33 kPa conditions were 0.34 ± 0.012, 0.30 ± 0.013 and 0.34 ± 0.018 ($n = 3$), respectively. An obvious trend in membrane polarization due to decreased hypobaric pressure was not observed. It is possible that the same species responds differently over the 18–19 day incubation period. For example, a portion of the inoculum may have responded differently in each of the experiments and the cells that grew and divided exhibited different *P* values. Possibly, the hypobaric conditions selected for cells that were able to grow even with a less fluid membrane as in experiment 1, while the other cells became non-viable and died. The reason the experiments yielded different results with respect to membrane fluidity in *B. subtilis* is still unknown, whereas *E. coli* gave consistent results in both experiments 1 and 2. One possibility is different gene expression levels by the same genes or different genes in the experiments by cells that are selected as best able to grow and divide under hypobaric conditions, from the total inoculum added to each flask.

The lower the hypobaric pressure, the cytoplasmic membrane became less fluid or more rigid only in *B. subtilis* cells in experiment 1. Rigid cytoplasmic membranes may allow cytoplasmic contents to leak to the outside of the cells. For example, the predominant intracellular ion K^+ can pass through the membrane to the external medium or environment. The polarization experiments confirmed the significant effect of hypobaric pressure on cytoplasmic membrane fluidity; the ability to function as viscous two-dimensional fluids within their physiological temperature range. The fluid membrane state is present when the membrane phospholipids are free to flex and rotate and the phospholipids molecules can undergo lateral motion. A fluid cytoplasmic membrane is also necessary for membrane curvature as bacterial cells have many different sizes and morphologies and when undergoing cell division must maintain a sealed, continuous membrane with the correct curvature for the shape of the cell. Both lipids and protein accomplish the structural changes needed to deform cellular membranes for curvature (see the review by Holthius 2004). In both bacterial species and in both experiments the membrane *P* values were not associated with inhibited growth and vice versa. To the best of our knowledge, the range of cytoplasmic membrane *P* values that each bacterial species can have and still grow and divide is not known.

The total saturated to unsaturated fatty acids ratios for *E. coli* decreased as the hypobaric pressure decreased, suggesting that the lower the hypobaric pressure the lower the saturated to unsaturated ratio of total cellular fatty acids. The ambient control (mean ratio = 0.95) had a ratio significantly different (95% level) from the two hypobaric treatments (0.87 for 67 kPa and 0.82 for 33 kPa pressures). However, the two hypobaric treatments were not significantly different from

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Table 3. *Percentages of total cellular fatty acids in bacterial cultures statically grown in LB broth after 19 day incubation at 25 °C under ambient and hypobaric conditions*

	Percentage ($n=3\pm$S.D.) total cellular fatty acids under different pressures (kPa)		
	101	67	33
E. coli			
12:0	3.8 (0.07) a	5.8 (0.81) b	6.37 (0.34) b
13:0	0.31 (0.02) a	0.45 (0.07) b	0.41 (0.03) b
14:0	5.6 (0.11) a	6.7 (0.59) a	7.0 (0.23) a
15:0	2.6 (0.12) a	2.2 (0.16) b	1.8 (0.20) c
16:0	41.3 (0.12) a	33.8 (2.8) b	32.3 (1.43) b
16:1	0.15 (0.01) a	0.11 (0.09) a	0.14 (0.01) a
17:0	6.22 (0.17) a	5.9 (0.78) b	5.7 (0.07) b
17:1	0.15 (0.02) a	0.12 (0.11) a	0.11 (0.09) a
18:0	0.47 (0.04) a	0.43 (0.02) a	0.42 (0.02) a
18:1	20.7 (0.78) a	20.7 (0.23) a	21.6 (0.51) a
19:0	1.3 (0.28) a	1.01 (0.30) a	0.92 (0.14) a
Total saturated	61.6 (0.92) a	55.8 (1.9) b	55.7 (0.87) b
Total unsaturated	66.9 (1.9) a	64.2 (1.5) a	67.0 (1.8) a
Saturated to unsaturated	0.95 (0.04) a	0.87 (0.02) b	0.82 (0.03) b
B. subtilis			
14:0	0.35 (0.32) a	0.66 (0.10) a	0 (0) a
15:0	53.9 (1.29)	55.2 (2.6)	61.2 (1.2)
16:0	6.2 (0.51) a	6.1 (1.0) a	5.1 (0.76) a
16:1	1.1 (0.21) a	0.54 (0.58) a	0.17 (0.29) a
17:0	33.5 (0.83) a	32.4 (1.3) a	28.4 (0.82) b
17:1	1.7 (0.87) a	1.4 (0.40) a	2.0 (0.46) a
18:0	0.58 (0.20) a	0.45 (0.05) a	0.16 (0.28) a
19:0	0 (0) a	0.29 (0.29) a	0 (0) a
19:1	0 (0) a	0.27 (0.46) a	0 (0) a
Total saturated	94.6 (3.1) a	95.1 (5.3) a	94.9 (3.0) a
Total unsaturated	2.7 (0.30) a	2.3 (1.4) a	2.2 (0.76) a
Saturated to unsaturated	34.7 (10.5) a	42.2 (3.7) a	43.4 (4.1) a

each other (Table 3). This data suggested an effect of low pressure on total cellular fatty acids in *E. coli* over the 19 day period. The changes in the fatty acids may have been in non-membrane fatty acids or fatty acids that did not alter membrane fluidity. This experiment was repeated and the mean saturated to unsaturated ratios for ambient 67 and 33 kPa pressures were, 0.95 ± 0.36, 0.87 ± 0.02 and 0.79 ± 0.27 ($n=3\pm$S.D.) respectively, similar to the results from the first experiment. Significant differences (95% level) were observed between the ambient and 67 kPa treatment, and the ambient control and the 33 kPa treatment, but not between the 33 and 67 kPa treatments. The differences in saturated to unsaturated fatty acid ratios were not accompanied by differences in membrane polarization changes. This was also observed in experiment 1.

In the ambient control and two hypobaric treatments the total mean saturated fatty acids content ranged from 94.6 to 95.1 for *B. subtilis*. The range for the unsaturated fatty acids was 2.2–2.7 for *B. subtilis*. The saturated to unsaturated ratios of fatty acids increased to 43.4 in the 33 kPa hypobaric treatment for *B. subtilis*. The control *B. subtilis* culture (101 kPa) had a saturated to unsaturated ratio of 34.7, but the standard deviation was also high (10.5) (Table 3). The ratio of total saturated to unsaturated cellular fatty acids did not significantly change under hypobaric conditions compared to the ambient control (Table 3). When the experiment was repeated for 18 d, *B. subtilis* exhibited mean saturated to unsaturated total fatty acids ratios of 43.1 ± 10.5, 43.5 ± 5.9 and 27.8 ± 8.7, respectively at ambient 67 and 33 kPa pressure values. Values are means$\pm$S.D. for $n=3$. These values were not significantly different (at 95% level) when tested using ANOVA as observed in experiment 1.

However, there were significant changes in the *B. subtilis* membrane polarization values under hypobaric conditions in experiment 1, but not in experiment 2. There were no significant changes in membrane polarization in both experiments 1 and 2 with *E. coli* under any ambient and hypobaric conditions. The membrane polarization values increased in *B. subtilis* indicating a more rigid or less fluid membrane. However, the saturated to unsaturated fatty acids were similar in the control and both hypobaric treatments. Again, other molecules such as proteins may have been altered in the membrane that decreased the fluidity. It is important to note for example, that some changes did occur in the percentages of 15:0 and 17:0 fatty acids in *B. subtilis* under hypobaric conditions (Table 3).

The 15:0 values increased under hypobaric conditions while the 17:0 values decreased. In experiment 1, the percentages of 15:0 fatty acids at ambient 67 and 33 kPa were 53.9, 55.2 and 61.2, respectively. In experiment 2, the percentage values were 55.9, 55.4 and 62.9 at ambient 67 and 33 kPa. These values are very similar in both experiments. The 17:0 fatty acids percentages were 33.5, 32.4 and 28.4 at ambient 67 and 33 kPa in experiment 1. In experiment 2, the percentage values were 30.5, 32.1 and 25.3 at ambient 67 and 33 kPa, respectively. Again these values are very similar between experiments.

The 15:0 and 17:0 values both decreased in *E. coli* cells grown under hypobaric conditions. In experiment 1, the 15:0 fatty acids values were 2.6, 2.2 and 1.8 at ambient 67 and 33 kPa, respectively. The same general trend was observed in the second experiment with *E. coli* where the 15:0 fatty acids at ambient 67 and 33 kPa were 3.0, 2.6 and 2.6, respectively. The 17:0 fatty acids percentages were 6.2, 5.9 and 5.7 at ambient 67 and 33 kPa, respectively, in *E. coli* in experiment 1. In experiment 2, the 17:0 fatty acids percentages at ambient 67 and 33 kPa were 6.6, 5.8 and 5.8, respectively. The response of different bacterial species to hypobaric conditions may be complex and will require research on numerous diverse species.

There were overall changes in the membrane fluidity of *B. subtilis* under hypobaric conditions in experiment 1. Fluorescent polarization provided an overall estimate of the membrane fluidity, while the total cellular fatty acids analysis provides data on all fatty acids in the cells, not just those in the membrane. For example, Gram-positive bacteria do not contain appreciable amounts of hydroxy fatty acids while Gram-negative bacteria do contain

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varying combinations of straight-chain, unsaturated, hydroxy and cyclo fatty acids as well as iso and/or anteiso fatty acids (MIDI Inc.). Gram-positive bacteria contain combinations of straight-chain, unsaturated, iso and anteiso fatty acids only.

Bacteria that have desaturase enzymes have a synthetase that produces only saturated fatty acids. The only way for these bacteria to produce unsaturated fatty acids, most commonly palmitoleic (16:1*n*-7) and oleic (18:1*n*-9) is by the desaturase enzyme, which makes a double bond in the saturated acyl chains by removing two hydrogen atoms and transferring them to oxygen (Russell 1984). Desaturation is a post-synthesis modification processes occurring after lipid biosynthesis (Russell 1984). Since existing lipids are the substrate, changes in the saturation to unsaturation ratio can occur rapidly by this mechanism. Once the membrane fluidity has been adjusted by desaturation, the enzyme is down-regulated. Membrane fluidity alterations can also inactivate the enzyme through conformational changes to its proteins (Russell 1984). Within seconds after perturbation (e.g., decrease in temperature) the rate of fatty acid biosynthesis is altered. Although fatty acid biosynthesis changes quickly, time is required for the new fatty acids to be incorporated into the cytoplasmic membrane if the bacterium is not growing (Russell 1984).

If micro-organisms are to be used for applications in space such as waste bioreactors, or if they are challenged with antibiotics (human medicine) or antimicrobial compounds in space, it will be necessary to know whether or not their membranes remain normal from structural and functional perspectives and if the micro-organisms can adapt to the hypobaric and low gravity conditions. For example, gene expression microarray analysis and other parameters such as mutation rates, antibiotic resistances/sensitivities, gene transfer frequencies, starvation survival responses in dilute media, changes in cell sizes, shapes, biofilm growth, the use of reporter genes and specific biochemical reactions can also be measured in future experiments.

Understanding the effects of hypobaric conditions (and also microgravity) on micro-organisms is necessary if less massive structures will be used in space missions where microbial bioreactors, regenerative life support systems such as water and waste treatment are used and where humans and edible plants may become diseased with micro-organisms that are accidentally or purposely taken into space. Secondly, it is not known how diverse microbial species will respond to hypobaric pressures and less than 1 *g* environments at the same time as being under hypobaric pressure. The effects of low pressure and less than 1 *g* environments in combination have not been adequately researched. Additional future research on these unique environmental conditions can also be researched using gene expression analysis (Saleh-Lakha *et al.* 2005). For example, the complete genomes of *E. coli*, *P. aeruginosa* and *B. subtilis* have been arrayed and would be very useful in future studies of gene expression under hypobaric conditions as well as any other conditions.

To the best of our knowledge, this is the first research reported on these bacterial species under hypobaric conditions. Both *E. coli* and *B. subtilis* grew and survived under the hypobaric conditions in both dilute and full strength LB broth. This verifies that the necessary genes for cell growth and division were able to function and that the cytoplasmic membrane remained fluid enough for growth. Much more research will need to be completed to determine the responses of numerous diverse bacterial species to hypobaric conditions. Gene expression array analysis will also provide useful knowledge. This information is valuable for microbial waste reactors that may be operated under hypobaric conditions in space missions or for other future space experiments. It will be necessary to study more species of micro-organisms as there is currently a paucity of information on how other microbial species will respond to hypobaric and/or microgravity conditions. We also suggest that fluorescent polarization is a good biophysical approach to studying overall bacterial membrane fluidity under hypobaric and other environmental conditions. The method is rapid, non-destructive, is used with viable cells and the measurements can be made as soon as the cultures are removed from the hypobaric chambers. There was no effect of hypobaric conditions on membrane polarization in *E. coli* under the conditions we tested in all experiments. This was not the situation with *B. subtilis* where a decrease in membrane fluidity was observed in one experiment but not in repeated experiments, for which we have no explanation at this time.

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References

Barbaro, S.E., Trevors, J.T. & Inniss, W.E. (1999). Fatty acid composition, membrane changes and surface tension in a psychrotrophic *Acinetobacter* sp. HH1-1 during cold shock. *World J. Microbiol. Biotechnol.* **15**, 683–692.

Cassidy, M.B., Leung, K.T., Lee, H. & Trevors, J.T. (2000). A comparison of culturable enumeration methods for *Pseudomonas fluorescens* marked with green fluorescent protein. *J. Microbiol. Meth.* **40(2)**, 135–145.

Denich, T.J., Lee, H. & Trevors, J.T. (2003a). Effect of selected environmental and physico chemical factors on bacterial cytoplasmic membranes. *J. Microbiol. Meth.* **52**, 149–182.

Denich, T.J., Beaudette, L.A., Cassidy, M.B., Lee, H. & Trevors, J.T. (2003b). Membrane fluidity of the pentachlorophenol-mineralizing *Sphingomonas* sp. UG30. *J. Fluor.* **13**, 385–391.

Denich, T.J., Beaudette, L.A., Lee, H. & Trevors, J.T. (2005). Fluorescent methods to study DNA, RNA, proteins and cytoplasmic membrane polarization in the pentachlorophenol mineralizing bacterium *Sphingomonas* sp. UG30 during nutrient starvation in water. *J. Fluor.* **15**, 143–151.

Dixon, M., Wehkamp, C.A. & Stasiak, M. (2005). Physiological responses of lettuce (*Lactica sativa*) to reduced atmospheric pressure. 051CES-294,

https://doi.org/10.1017/S1473550405002727 Published online by Cambridge University Press

SAE International, in press and additional information can be obtained at www.ces.uoguelph.ca.

England, L.S., Gorzelak, M. & Trevors, J.T. (2003). Growth and membrane polarization in *Pseudomonas aeruginosa* UG2 grown in randomized microgravity (RMG) in a high aspect ratio vessel (HARV) *Biochim. Biophys. Acta Gen.* **624(1–3)**, 76–80.

Gasset, G., Tixador, R., Eche, B., Lapchine, L., Moatti, N., Toorop, P. & Woldringh, C. (1994). Growth and division of *Escherichia coli* under microgravity conditions. *Res. Microbiol.* **145**, 111–120.

Genigeorgis, C.A., (1985). Microbial and safety implications of the use of modified atmospheres to extend the storage life of fresh meat and fish. *Int. J. Food Microbiol.* **1**, 237–251.

Holthius, J.C.M. (2004). Regulating membrane curvature. In *Topics in Current Genetics*, ed. Keranen, S. & Jantti, J. ch. 10, pp. 39–64. Springer, Berlin.

Kacena, M.A., Merrel, C.A., Manfredi, B., Smith, E.E., Klaus, D.M. & Todd, P. (1999). Bacterial growth in space flight: logistic growth curve parameters for *Escherichia coli* and *Bacillus subtlis*. *Appl. Environ. Microbiol.* **51**, 229–234.

Kim, I.S., Beaudette, L.A., Cassidy, M.B., Lee, H. & Trevors, J.T. (2001). Effect of 2, 2′,5,5′-tetrachlorobiphenyl and biphenyl on membrane fluidity in *Ralstonia eutrophus* H850. *FEMS Microbiol. Lett.* **200(1)**, 17–24.

Kim, I.S., Lee, H. & Trevors, J.T. (2002). Alterations in the fatty acid composition and fluidity of cell membranes affect the accumulation of PCB congener 2,2′,5,5′-tetrachlorobiphenyl by *Ralstonia eutropha* H850. *J. Chem. Technol. Biotechnol.* **77**, 793–799.

Klaus, D., Simske, S., Tood, P. & Stodieck, L. (1997). Investigation of space flight effects on *Escherichia coli* and a proposed model of underlying physical mechanisms. *Microbiol.* **143**, 449–455.

Lynch, S.V., Brodie, E.L. & Matin, A. (2004). Role and regulation of σ^s in general resistance conferred by low-shear simulated microgravity in *Escherichia coli*. *J. Bacteriol.* **186**, 8207–8212.

Nickerson, C.A., Ott, C.M., Mister, S.J., Morrow, B.J., Burns-Keliher, L. & Pierson, D. (2000). Microgravity as a novel environmental signal affecting *Salmonella enterica* Serovar Typhimurium virulence. *Infect. Immun.* **68**, 3147–3152.

Pagan, R. & Mackey, B. (2000). Relationship between membrane damage and cell death in pressure-treated *Escherichia coli* cells: differences between exponential- and stationary-phase cells and variation among strains. *Appl. Environ. Microbiol.* **66**, 2829–2834.

Paul, A.-L., Schuerger, A.C., Popp, M.P., Richards, J.T., Manak, M.S. & Ferl, R.J. (2004). Hypobaric biology: arabidopsis gene expression at low atmospheric pressure. *Pl. Physiol.* **134**, 215–223.

Peterson, B.V., Hummerick, M., Roberts, M.S., Krumins, V., Kish, A.L., Garland, J.L., Maxwell, S. & Mills, A. (2004). Characterization of microbial and chemical composition of shuttle waste with permanent gas and volatile organic compound analyses. *Adv. Sp. Res.* **34**, 1470–1476.

Russell, N.J., (1984). Mechanisms of thermal adaptation in bacteria: blueprints for survival. *Trends Biochem. Sci.* **9**, 108–112.

Saleh-Lakha, S., Miller, M., Campbell, R., Schneider, K., Elahimanesh, P., Hart, M.M. & Trevors, J.T. (2005). Microbial gene expression in soil: methods, applications and limitations. *J. Microbiol Meth.* **63(1)**, 1–19.

Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. *MIDI Technical Note 101*. MIDI Inc., Newark, DE, USA.

Trevors, J.T. (1983). Effect of pentachlorophenol on the membrane fluidity of *Pseudomonas fluorescens*. *FEMS Microbiol. Lett.* **16**, 331–334.

Trevors, J.T. (2003). Fluorescent probes for bacterial cytoplasmic membrane research. *J. Biochem. Biohys. Meth.* **1693**, 1–18.

Vincent, M., England, L.S. & Trevors, J.T. (2004). Cytoplasmic membrane polarization in selected Gram-negative and Gram-positive bacteria grown in the absence and presence of tetracycline. *Biochim. Biophys. Acta Gen.* **1672–3**, 131–134.

https://doi.org/10.1017/S1473550405002727 Published online by Cambridge University Press