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Effects of Space Flight, Clinorotation, and Centrifugation on the Substrate Utilization Efficiency of *E. coli*

Cultures of Escherichia coli grown in space reached a 25% higher average final cell population than those in comparably matched ground controls ($p < 0.05$). However, both groups consumed the same quantity of glucose, which suggests that space flight not only stimulated bacterial growth as has been previously reported, but also resulted in a 25% more efficient utilization of the available nutrients. Supporting experiments performed in "simulated weightlessness" under clinorotation produced similar trends of increased growth and efficiency, but to a lesser extent in absolute values. These experiments resulted in increases of 12% and 9% in average final cell population ($p < 0.05$), while the efficiency of substrate utilization improved by 6% and 9% relative to static controls ($p = 0.12$ and $p < 0.05$, respectively). In contrast, hypergravity, produced by centrifugation, predictably resulted in the opposite effect - a decrease of 33% to 40% in final cell numbers with corresponding 29% to 40% lower net growth efficiencies ($p < 0.01$). Collectively, these findings support the hypothesis that the increased bacterial growth observed in weightlessness is a result of reduced extracellular mass transport that occurs in the absence of sedimentation and buoyancy-driven convection, which consequently also improves substrate utilization efficiency in suspended cultures.

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1 Introduction

With a few exceptions [1, 2], most findings to date indicate that microgravity stimulates the growth of bacterial cultures and their production of byproducts. Numerous experiments have shown that cultures grown in space experience a shorter lag phase [3-6] and attain a final cell population that is significantly higher than in comparable ground controls [5-10]. Similar growth kinetics have also been reported for *E. coli* cultivated on a clinostat, which simulates "functional weightlessness" on Earth by minimizing the effects of sedimentation and convection through rotation [11]. In addition to these findings of improved growth, recent space-flight experiments have also shown that microbial and fungal cultures produce significantly more antibiotics when compared with similar ground controls [12, 13]. The underlying mechanisms responsible for these changes remain largely unknown, however, and are the objective of continued study.

In particular, it has not yet been determined whether these observed increases in cell growth and secondary metabolite production are accompanied by proportionally higher nutrient consumption, or if space flight allows a more efficient use of an equal amount of consumed nutrients. Previous studies investigating nutrient consumption by cells on orbit produced inconclusive findings [1, 14, 15]. One experiment that examined bacterial metabolism found no difference in nutrient consumption by *E. coli* on orbit [1]. However, contrary to most other investigations, no difference was reported in bacterial growth in space either. Therefore, it could not be feasibly determined if increased growth on orbit was accompanied by a corresponding increase in nutrient consumption. It has also been reported that microgravity does not affect the metabolism of yeast [14]. In contrast to these two findings indicating that the metabolism of single cells was not affected by microgravity, another previous study reported that 37% less glucose was consumed by human embryonic lung cell cultures maintained on orbit when compared with similar ground controls, with no other differences

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noted [15]. This suggested that the lung cells might have metabolized glucose more efficiently in space.

It is proposed here that improved bacterial growth in space results from the combined absence of cell sedimentation and buoyancy-driven convection of the nutrient-depleted, reduced-density fluid volume immediately surrounding the cell. These phenomena are hypothesized to slow nutrient transport from the bulk fluid into cells and to simultaneously permit greater concentrations of excreted byproducts to remain near the bacterial cells in weightlessness. Increased final cell mass yields produced in fed-batch culture systems on Earth have been shown to result from the slow addition of nutrients [16, 17]. In these experiments, the gradual addition of nutrients was found to minimize pH increases and prolong the growth phase, resulting in higher final cell populations than were achieved in comparable batch-grown controls. It has been hypothesized that the enhanced growth observed in space may indirectly result in part from a similarly slowed nutrient uptake rate as a consequence of reduced fluid mixing [13]. In addition, since the presence of certain excreted byproducts is also known to be beneficial to bacterial growth, their increased accumulation around the cell in a weightless environment may facilitate more efficient utilization of available nutrients. This hypothesis is corroborated by previous findings showing that an increased level of dissolved carbon dioxide, a common bacterial metabolite, was beneficial to bacterial growth [18] and induced a shorter lag phase preceding the exponential phase of growth for many types of bacteria [19]. It is postulated that a similar effect occurs on orbit as follows. Because there is no density-driven sedimentation in a weightless environment, cells do not "fall" away from their excreted byproducts. Likewise, due to the absence of convection, excreted byproducts that surround each cell in a less dense nutrient-depleted zone do not "rise" away from cells. This quiescence is proposed to reduce the transport rate of nutrient molecules to the cell, and also allow beneficial excreted byproducts to remain near the cell, which is believed to shorten the lag phase and stimulate growth, resulting in a higher final cell population than the ground controls.

The hypothesis was evaluated using three approaches- one experimental set was cultured on orbit with corresponding, matched ground controls; and two separate sets of experiments were performed in the lab; one using a clinostat to simulate the quiescent fluid environment of space and another using a centrifuge to create a 50*g* environment. Each of the lab experiments included their own matched groups of static 1*g* controls. It was hypothesized that cultures grown in microgravity (and simulated microgravity) would not only achieve a higher final cell population relative to their controls, as has been reported in previous experiments, but would also utilize available nutrients more efficiently, resulting in a higher ratio of new cells per mass of glucose consumed. In contrast, at 50*g* it was hypothesized that the higher rates of convection and sedimentation would more quickly separate "falling" cells from their lighter "rising" byproducts accumulated in the less dense surrounding fluid. This was speculated to impair nutrient uptake by reducing overall access to the glucose available in the bulk solution as the cells sedimented on the bottom and by inhibiting accumulation of beneficial byproducts around the cells due to rapid separation under the higher level of acceleration. These effects, in turn, would reduce the final cell population, along with reducing the ratio of new cells per mass of nutrient consumed, relative to 1*g* controls.

2 Materials and Methods

2. 1 Cells and Media

All experiments used a minimal growth medium [20] supplemented with glucose to cultivate *Escherichia coli* (low-motility original Escherich strain ATCC 4157). An inoculum concentration of 10^6 cells/ml was obtained from a saturated culture grown without glucose to induce a lag phase upon inoculation.

2. 2 Supporting Hardware

As in earlier space experiments [6], *E. coli* were cultivated anaerobically in sterile glass barrels called FPAs (Fluids Processing Apparatus, Fig. 1). Movable rubber septa were used to initially confine and subsequently mix the fluids on orbit. For the flight experiments, FPAs were contained in Lexan™ sheaths, which provided a second level of containment. A third level of containment was provided by an automated Group Activation Pack (auto-GAP), which was used to autonomously inoculate the cultures by translating the septa in the FPAs 2.5 hours after launch. The auto-GAP was placed in a Commercial Generic Bioprocessing Apparatus-Isothermal Containment Module (CGBA-ICM). The CGBA-ICM was installed into a shuttle

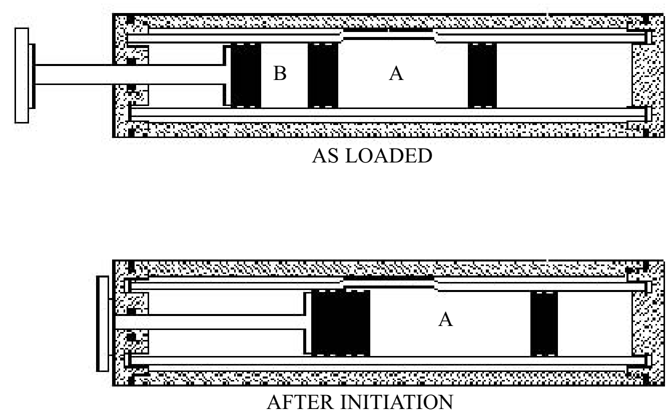

Fig. 1. Each Fluids Processing Apparatus (FPA) included an inner glass barrel with a fluid bypass (shown on the top of each FPA). Fluids were contained with moveable septa, and the FPA was housed in a Lexan™ outer sheath. Prior to launch chamber A contained 3.5 ml of sterile growth medium, and chamber B held 0.5 ml of inoculum. Inoculation occurred 2.5 hours after launch when the contents of chamber B were injected into chamber A via the bypass in the glass barrel.

middeck-locker and provided the samples with temperature control at approximately 24°C. The temperature set point was dictated by other experiments contained in the CGBA-ICM, and was found to provide adequate conditions for bacterial growth.

A clinostat was used to simulate "functional weightlessness". The clinostat rotated horizontal, fluid-filled FPAs at 8 rpm along their axis, which minimized the net effects of cell sedimentation and buoyancy-driven convection of depletion zones in the medium. As a result of the constant rotation, sedimenting bacterial cells and rising convection currents experience small circular motions, which are considered negligible when compared with concurrent cell and byproduct displacement due to Brownian motion. Therefore, clinorotation is considered an approximation of the near-motionless state of microgravity [11].

Hypergravity experiments were conducted using FPAs placed in an International Equipment Corporation Model HN-S centrifuge operating at 500 RPM to achieve an average acceleration of $50 \pm 10g$.

Cell population count was determined using a Bausch & Lomb Spectronic 20 spectrophotometer (OD_{600}) calibrated against microscopy cell counts measured in identical growth medium. Glucose concentrations were determined by chromatography using a Hewlett-Packard model 1090 HPLC with an evaporative light scattering detector (gain = 8) calibrated with a glucose standard. A YMC-Pack, Polyamine II, S 5-µm-particle column (250×4.6 mm) was used with a solvent system of water and acetonitrile (1:3 by volume) and a flow rate of 1 ml/min. The volume of each injection was 0.02 ml.

2. 3 Experimental Protocols

Space Flight: The flight experiment consisted of four samples that flew on the STS-95 shuttle mission and four matched ground control samples. In an attempt to minimize variables between flight and ground, all eight samples underwent the same handling, shipping and loading procedures. The inoculum was kept initially isolated from the growth medium by movable septa in the FPA. Chamber A contained 3.5 ml of sterile growth medium with 7.8 *g*/l of glucose. Chamber B held the inoculum of 8×10^6 cells/ml in growth medium without glucose (Fig. 1). After the samples were loaded, all eight FPAs were shipped together at 4°C from Boulder, Colorado to the Kennedy Space Center. Forty hours before launch, the flight samples were loaded on the shuttle in the CGBA-ICM, and the ground samples were placed in an incubator, with both groups held at 24°C. Approximately 2.5 hours after launch, the flight and ground-control experiments were simultaneously initiated when a plunger pushed the septa forward, allowing the inoculum to mix with the growth medium via the FPA bypass. After dilution, chamber A in the four ground and four flight samples contained growth medium with 6.8 *g*/l of glucose and a starting concentration of ~10^6 cells/ml, which continued to grow through landing nine days later.

Five hours after the shuttle landed in Florida, the flight and ground-control samples were placed together at 4°C and flown overnight to Boulder, Colorado. The samples were shaken vigorously to distribute the cells, and concentrations were measured by optical density (OD_{600}). The bacteria were then removed using a 0.22 µm filter and the supernatant samples were frozen and shipped overnight to Bristol-Myers Squibb Pharmaceutical Research Institute in Wallingford, Connecticut for blind glucose analysis. The concentration of glucose in each sample was subtracted from an average concentration of glucose in three sterile samples from the same batch of medium saved before launch. This difference gave the net mass of glucose consumed by each bacterial culture. Statistical significance between experimental and control data was determined using the Student's one-tailed *t*-test ($p < 0.05$).

Clinostat and Centrifuge: The clinostat and centrifuge experiments were conducted independently from the flight experiments with only minor differences in protocol. A separate glucose solution was prepared for these experiments at 5.9 *g*/l, which was 0.9 *g*/l less than that used on orbit. Control and experimental cultures for all clinostat and centrifuge tests had identical glucose concentrations of 5.9 *g*/l and these experiments were not directly compared with the flight samples. In an effort to ensure all matched sets of samples were identical, the cultures were first inoculated in a single vial with 10^6 cells/ml and then divided into varying numbers of experiment and control FPAs to match the flight hardware and protocol as closely as possible. After 50 hours of elapsed growth, samples were periodically collected from the centrifuge, clinostat, and 1*g* controls for interim analysis. Since the sampled FPAs had to be shaken in order to be measured, they were removed from the experiment at the time of collection.

3 Results

As summarized in Table 1, the four samples flown on STS-95 produced an average of 25% more cells than the four ground controls ($p < 0.05$). After nine days of growth, the bacteria had consumed all of the glucose in each of the eight (four flight and four ground) samples. Therefore, the increase in bacterial growth was not a direct consequence of more nutrients having been consumed, rather apparently a result of more efficient utilization of the nutrients available. Because the ground and space samples ultimately consumed the same quantity of glucose, the growth efficiency, measured by new cells/mg nutrient consumed, also averaged 25% higher in space than in the ground controls ($p < 0.05$). The ground controls were maintained close to the desired temperature of 24ºC, averaging 23.3ºC during the nine-day experiment. Due to a software problem experienced with the payload on orbit, the flight samples went through periodic temperature fluctuations with an average of 26.6ºC between inoculation and landing. However, multiple post-flight sensitivity experiments performed using the flight and ground temperature profiles as well as other profiles with average temperatures between 20ºC and 30ºC indicated that these differences did not affect the final cell population or final glucose con-

Table 1. Summary of Experimental Data. The table shows the percent change in number of new cells, nutrients consumed, and ratio of new cells/mg of nutrient consumed for all five experiments. Data was taken from the final sample time during the stationary phase of growth. The n-values indicate the number of independent samples collected from the experiment and 1g controls, respectively. The new cells/mg nutrient values are mean ± SD. Results indicate cultures grown in space or on a clinostat produce more new cells, and do so more efficiently than in 1g controls. In contrast, samples grown in hypergravity produced fewer cells, and required more nutrients per new cell than in control samples.

Experiment	*n*-values	%Δ New Cells	%Δ Nutrient Consumed	New Cells [10^7]/mg Nutrient			
				Experiment	Control	%Δ	*p*
Space Flight	4-4	25[1]	0	14.1±0.81	11.3±1.40	25[1]	<0.05
Clinorotation	6-3	12[1]	6	24.3±0.18	22.8±0.15	6	0.12
	2-3	9[1]	0	23.4±1.10	21.5±7.20	9[1]	<0.05
Hypergravity	4-3	-33[1]	-6	16.3±0.05	22.8±0.15	-29[1]	<0.01
	3-3	-40[1]	0	12.9±0.40	21.5±7.20	-40[1]	<0.01

1 Indicates statistically significant compared to control ($p < 0.05$).

Table 2. Clinostat Experimental Data. Comparison of average new cells, nutrient consumption, and growth efficiency of clinostat and control samples for every sampling time during both clinostat experiments. The n-values indicate the number of independent samples collected from the clinostat and 1g controls, respectively. Results suggest clinorotation improves cell growth, resulting in more new cells produced and more cells per nutrient consumed than in 1g controls.

	Hours of Growth	*n*-values	%Δ New Cells	%Δ Nutrient consumed	New Cells/mg Nutrient	
					%Δ	*p*
1	58	3-2	1	7	-8	0.36
	71	6-3	12[1]	6	6	0.12
2	50	2-2	6	-4	10	0.25
	62	2-2	11	8	13	0.40
	84	2-2	17	7	10	0.22
	108	2-3	20[1]	0	19[1]	<0.05
	171	2-3	9[1]	0	9[1]	<0.05

1 Indicates statistically significant compared to control ($p < 0.05$).

centration [7].

The two similarly performed clinostat experiments produced results comparable to the flight data, albeit with less significant differences relative to their respective controls (Table 1). The first experiment concluded after 71 hours of growth, when the remaining samples were collected from the clinostat and static controls. In this experiment samples cultivated on the clinostat had 12% more new cells on average than the control samples ($p < 0.05$), yet had only consumed 6% more glucose ($p = 0.16$). This resulted in a 6% increase in bacterial growth efficiency as measured by new cells/mg of nutrient consumed ($p = 0.12$). In

Table 3. Centrifuge Experimental Data. Comparison of average new cells, nutrient consumption, and growth efficiency of centrifuge and control samples for every sampling time during both centrifuge experiments. The n-values indicate the number of independent samples collected from the centrifuge and 1g controls, respectively. Results suggest hypergravity suppresses cell growth, resulting in fewer new cells produced, and fewer cells per nutrient consumed than in 1g controls.

	Hours of Growth	*n*-values	%Δ New Cells	%Δ Nutrients consumed	New Cells/mg Nutrient	
					%Δ	*p*
1	58	2-3	-35[1]	-17	-22	0.12
	71	4-3	-33[1]	-6	-29[1]	<0.01
2	50	2-2	-27[1]	22	-39	0.07
	62	2-2	-41[1]	-2	-40[1]	<0.01
	84	2-2	-42[1]	1	-42[1]	<0.05
	108	3-3	-41[1]	0	-41[1]	<0.01
	171	3-3	-40[1]	0	-40[1]	<0.01

1 Indicates statistically significant compared to control ($p < 0.05$).

the second clinostat experiment, the final samples were collected after 171 hours of growth. The bacteria in the two clinostat and three control samples had consumed all of the available glucose, yet the cultures subjected to clinorotation averaged 9% more cells than the controls, resulting in a 9% higher ratio of new cells per mass of consumed nutrient ($p < 0.05$). Additional samples were periodically collected throughout both of the clinostat experiments. As shown in Table 2, at all seven sampling times during the two experiments, the cultures grown on the clinostat had higher cell counts than the controls. In addition, for six of these seven sampling times the ratio of new cells per mg of consumed glucose was also higher in the clinostat samples.

In contrast to the space and clinostat experiments, the average cell population and growth efficiency for the hypergravity samples at 50*g* were consistently lower than those of their corresponding 1*g* controls. As summarized in Table 1, the final samples from the first centrifuge experiment had a 33% lower final cell population than in the controls, yet consumed only 6% less glucose. This resulted in 29% reduced growth efficiency ($p < 0.01$). In the second centrifuge experiment, no glucose remained in any of the samples, and the final cell population and growth efficiency of the 50*g* cultures averaged 40% lower than in the controls ($p < 0.01$). Similar results were found during five other sampling times during both centrifuge experiments (Table 3). In all of the sampling times, the 50*g* cultures consistently had a lower average cell population and reduced growth efficiency than the control samples, with 12 of these 14 differences being statistically significant ($p < 0.05$).

4 Discussion

The results from all three experiments (space flight, clinostat

and centrifuge) further support the proposed hypothesis by establishing a systematic relationship between bacterial growth and corresponding inertial environment. The physical mechanisms suggested to be responsible for the observed increases in bacterial growth in a weightless environment - a combination of decreased nutrient transport rate from the bulk fluid into the cell and greater concentrations of excreted byproducts remaining near the cell - are consistent with those hypothesized to similarly govern the outcome under conditions of clinorotation and hypergravity. Each experimental approach produced statistically significant differences in the average final cell population, as predicted, for bacterial cultures subjected to various conditions of inertial acceleration when compared with their respective 1*g* undisturbed controls. Furthermore, the data also indicated differences in the ratio of new cells/mg of consumed nutrient, which suggests that inertial force may also affect the efficiency of substrate utilization.

The flight experiment corroborated previous findings that space flight stimulates the growth of *E. coli*, resulting in a higher final cell population than in ground controls. More importantly, this experiment was the first investigation to establish that the observed increase in population growth was not accompanied by a correspondingly proportional increase in nutrient consumption, but was instead suggestive of more efficient bacterial metabolism.

The second set of experiments found that *E. coli* grown on a clinostat resulted in similar growth kinetics to those of cultures grown in orbit, as has been previously reported [11]. Again, in addition to a higher final cell population being attained, an increased ratio of new cells/mass of consumed nutrient was also observed compared to the control samples. However, the relative differences were not as great in the clinostat samples as they were for the space flight cultures, as could be expected, since the simulated weightless environment is not as quiescent as conditions experienced on orbit. Small accelerations due mechanical vibration and/or varying rotation speeds prevent a perfect simulation of actual weightlessness. These limitations offer plausible rationale for the less significant increase in final cell population and growth efficiency for the clinostat experiments when compared with the results obtained on orbit. It is also possible that some variations occurred due to unintended differences in the experimental protocol between the clinostat and space flight experiments; and it remains feasible that cellular stress, caused by the gravitational force that is continually reoriented during clinorotation, might have affected growth for the clinostat samples. However, given their small size, and uniform internal composition, it is unlikely that these different inertial environments can act directly on cellular components inside the bacterial cell [21].

The final set of hypergravity experiments conducted on a centrifuge further supported the proposed hypothesis in showing a predicted reversal of trends. In these studies, samples cultivated at 50*g* had significantly lower average final cell populations as well as lower efficiency of glucose utilization than in 1*g* controls. This was expected because the higher rates of cell sedimentation and increased convection of depletion zones would serve to more quickly separate cells from their excreted byproducts, some of which are beneficial to growth, and to reduce overall access to nutrients remaining in suspension.

While not yet conclusively proven, collectively, these findings and their causal interpretations logically support the hypothesis that the absence of sedimentation and convection experienced on orbit, and reduced under clinorotation, allow excreted byproducts to remain near the bacterial cells, which is hypothesized to enhance metabolism as indicated by higher final cell populations and improved substrate utilization efficiency when compared to 1*g* controls. In contrast, hypergravity, which increases the rate of cell sedimentation and buoyancy-driven convection in the medium, accelerates the separation of cells from their excreted byproducts, thereby reducing the efficiency of bacterial metabolism. These experiments further affirm the probable underlying physical mechanisms by which microgravity stimulates bacterial growth, and the results may also eventually help to explain other observed effects of space flight on microorganisms, such as reports of enhanced antibiotic production [12, 13] or reduced antibiotic effectiveness [22, 23]. Continued investigation into this topic is warranted.

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