Buoyancy-driven fluid flow generated by bacterial metabolism and its proposed relationship to increased bacterial growth in space

Robert B. Brown[1], David M. Klaus and Paul Todd[2]

BioServe Space Technologies; University of Colorado; Boulder, CO 80309-0429, USA
[1] Present address: HQ USAFA Department of Astronautics, 2354 Fairchild Drive, Suite 6H223, USAF Academy CO 80840-6224
[2] Present address: SHOT, Inc., 7200 Highway 150, Greenville, IN 47124, USA

Abstract

Previous investigations have reported that bacterial growth increases in space flight; however, the underlying physical mechanisms responsible for these changes have not been fully determined. As bacteria consume nutrients, they excrete by-products whose presence can influence the onset of exponential growth and affect final cell population density. It is assumed that these metabolic processes create a reduced-density fluid zone and/or a solute gradient around each cell. On Earth, this density difference may result in local buoyancy-driven convection of the excreted by-products. The absence of convection and sedimentation in the low-gravity space flight environment, however, can be expected to alter the fluid dynamics surrounding the cells by limiting transport to diffusion only. Based on this biophysical model, it was hypothesized that acceleration affects the lag phase duration and final cell concentration of suspended bacterial cultures in a predictable, non-linear manner, due to the resultant changes incurred in the extracellular fluid composition. Eight experiments at various levels of acceleration consistently supported this hypothesis, resulting in predictable growth kinetics. In additional experiments, macroscopic plumes of fluid were observed and analyzed rising from metabolizing bacterial cultures. If similar fluid dynamics were found to occur on a microscopic level, it would help explain how acceleration affects bacterial growth kinetics.

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1. Introduction

With a few exceptions (Bouloc and D'Ari, 1991; Gasset et al., 1994), most findings indicate that space flight stimulates the growth of suspended bacterial cultures (Klaus, 2002), presumably as a result of reduced gravity. Many experiments have shown that bacteria grown in space experience a shorter lag phase (Kacena and Todd, 1997; Kacena et al., 1999b; Klaus et al., 1997; Manko et al., 1987; Mennigmann et al., 1986; Thévenet et al., 1996) and a final cell concentration significantly higher than that in comparable ground controls (Brown et al., 2002; Ciferri et al., 1986; Kacena et al., 1999a; Klaus et al., 1997; Kordium et al., 1980; Mattoni, 1968; Mennigmann and Lange, 1986). In addition, post-flight substrate analysis indicated that suspended cultures of *E. coli* utilized nutrients more efficiently while on orbit compared to ground controls (Brown et al., 2002). The underlying causal mechanisms responsible for these changes are largely unknown and have remained the objective of continued study.

It has been speculated that the absence of convection in the weightless environment of space could help explain various reports of altered cellular responses (Albrecht-Buehler, 1991; Todd, 1989, Klaus et al., 1997). Similar investigations involving protein crystal growth in space have also indirectly supported this theory. A growing crystal depletes solute from its surrounding medium, thus reducing the local medium's density relative to the bulk fluid, and, in turn, creates a small convective current that rises from the crystal in normal gravity (Brailovskaya et al., 1994; Fehribach and Rosenberger, 1989; McCay and McCay, 1994; Pusey and Naumann, 1986; Pusey et al., 1986, 1988). We propose that a similarly affected physical principle governs bacterial growth. As bacteria consume nutrients and excrete by-products, a fraction of the consumed mass is used for cellular growth and reproduction. As a result, the net mass of excreted by-products is less than the mass of consumed nutrients. Therefore, depending on the

respective diffusion rates, the medium immediately surrounding each bacterium (or some critical mass of bacterial cells) may become less dense than the bulk fluid. This reduced-density medium creates the potential for buoyancy-driven convective currents to form in the presence of gravity, causing the excreted by-products to collectively rise away from the cell while the cell concurrently sediments downward (illustrated in Fig. 1).

In contrast to the 1 g chain of events described above, the absence of convection and sedimentation experienced in orbital space flight creates a quiescent fluid environment for non-motile bacterial cultures. The lack of sedimentation also allows the suspended cultures to remain colloidal, so consequently cells will not “fall away” from by-products excreted in their vicinity (Klaus et al., 1997). In addition, the absence of buoyancy-driven convection may allow excreted by-products to remain near the cells rather than simultaneously “rising away” (Albrecht-Buehler, 1991), as extracellular transport is limited to diffusion-only. Combined, these two phenomena are thought to reduce lag phase duration in space by allowing a higher concentration of requisite by-products (e.g. enzymes, cofactors, etc.) to accumulate in the environment immediately surrounding the cells. In turn, this local by-product accumulation may further serve to restrict nutrient uptake, helping increase the final cell concentration in a functional manner similar to that achieved in fed batch culturing (Klaus, 1997). This proposed physical model is further supported by previous reports suggesting that lag phase is reduced when cells do not have to work as hard to ‘condition’ their surrounding medium (Robinson et al., 1998 and 2001). Other studies have likewise concluded that an increased availability of dissolved carbon dioxide, a common bacterial metabolite, induces a shorter lag phase for many bacteria (Barford et al., 1982) and is beneficial for growth (Gottschalk et al., 1986).

Based upon this model, it was hypothesized that varying acceleration levels would affect the lag phase duration and final cell concentration of suspended, non-motile, bacterial cultures in a manner predictable by principles of fluid dynamics. Experiments using altered inertial acceleration were designed and carried out to test this hypothesis by characterizing the effects of acceleration on bacterial growth kinetics. The growth experiments consistently supported the predicted relationships under varying acceleration levels with lag phase duration and final cell population. . An additional set of experiments was then conducted to further investigate the possibility of bacterial metabolism creating buoyancy-driven convective flows. These experiments successfully showed macroscopic convective plumes occurring in the medium as a consequence of bacterial growth conditions. Analysis of measured plume velocities also agreed with theoretically predicted values.

2. Materials and Methods

2.1 Cells and Media

Vogel-Bonner 'Medium E' minimal growth medium (Vogel and Bonner, 1956) supplemented with 5 g l^{-1} of glucose was used to cultivate *Escherichia coli* (ATCC 4157).

2.2 Apparatus and Experimental Protocol for Growth Experiments

As in previous space flight experiments conducted in this lab, the cells were cultivated in BioServe's Fluid Processing Apparatuses (FPA), 13 mm I.D. glass barrels with rubber septa on each end and no air bubbles present (Klaus et al., 1997). An International Equipment Corporation Model HN-S centrifuge was used to provide 50, 180 and 400 g environments with estimated accuracies of ±10, ±40 and ±50 g respectively. The starting inocula were cultured in Medium E without glucose in order to promote an extended lag phase by forcing the test cultures

to reconfigure their metabolic pathways for consuming glucose. Cell concentration was measured approximately every 6 hours using a Bausch & Lomb Spectronic 20 spectrophotometer (OD_{600}) previously calibrated against cell counts in identical growth medium.

Bacterial growth experiments were conducted at 21±1°C. Growth curves (Bailey and Ollis, 1986) were determined using a least-squares fit through independent samples of cell counts (Brown, 1999; Kacena and Todd, 1997; Kacena et al., 1999b). These growth curves allowed the length of the lag phase and the final cell concentration of the experimental cultures to be determined relative to those of the matched 1 g controls. Five independent experiments were conducted at 50 g, one experiment at 180 g, and two experiments at 400 g. Each of these experiments incorporated matched 1 g controls. The n-values (defined as the total number of independent growth curves for the experimental data and their 1 g controls, respectively) were: 50 g (13, 25); 180 g (3, 8); 400 g (6, 10). Statistical significance was determined using a Student's t-test with $P < 0.05$.

2.3 Apparatus and Experimental Protocol for Plume Experiments

The plume measurement experiments began by inoculating 10 ml of Medium E containing glucose at 5 g l^{-1} with *E. coli* taken from cultures in exponential growth phase in the same 13 mm I.D. glass barrels used during the growth experiments, essentially a test tube with a flat bottom. The resulting inocula were established close to the maximum concentration of 5×10^8 cells ml^{-1}. These conditions were devised to allow the cells to continue metabolizing glucose, while also ensuring that they would not be glucose limited. The contents were then divided into two 5-ml samples and cultured at 21±1°C. Within a week the bacteria had reached their maximum concentration and sedimented naturally to the bottom of their containers. A second inoculation process was then repeated using the same protocol, yielding two fresh 5-ml

cultures. A day later these new cultures were near the end of their exponential phase. Both the old and new cultures were then vortexed and centrifuged at 700 g using an International Equipment Corporation Mode HN-S centrifuge. After one hour the centrifuge was quickly slowed and stopped, and the samples were removed within a minute. The medium in the tubes at that point was clear, and the bacteria were layered on the flat rubber septum bottom of the culture tubes. The tubes were then immediately placed vertically on a table at room temperature and photographed with back lighting.

3. Results and Discussion

3.1 Bacterial Growth

Based upon the proposed model discussed above, it was assumed that higher levels of acceleration experienced during centrifugation would cause the bacteria to rapidly become layered on the bottom of the culture tube, thus reducing nutrient availability to the cells (as they became concentrated on the container bottom) and also increasing buoyancy-driven convection, which would serve to more rapidly separate beneficial excreted metabolic by-products from the cells. As a result, several hypotheses were formulated relating acceleration level to various aspects of bacterial growth kinetics. First, it was hypothesized that the final cell concentration attained would decrease as acceleration increased. It was also hypothesized that acceleration would affect the lag phase in a non-monotonic manner as follows. At some point between 1 and 50 g, all of the cells were expected to quickly (< 1 hr) accumulate on the bottom of the culture tube. Under these conditions, their collective excretion of beneficial cofactors and enzymes could be expected to result in a greater local concentration surrounding the cells than in similar 1 g controls, where the cells and by-products are initially more dispersed. Concentrating

the cells on the container bottom was therefore hypothesized, on the basis of previous studies (data not shown), to result in a reduced lag phase duration for accelerations between 1 and 50 g. However, at accelerations above 50 g, where the excreted by-products were predicted to begin rising away from the cells at increasingly higher velocities due to increasing rates of buoyancy-driven convection, it was further hypothesized that lag phase duration would begin increasing again at accelerations above 50 g in a manner reflecting the corresponding increase in plume velocity.

Empirical data from this study consistently supported these hypotheses. As postulated, the cultures subjected to a constant acceleration of 50 g had an average lag phase that was 14.1 hours shorter than that of 1 g controls ($P < 0.01$). As the acceleration level increased above 50 g, the length of the lag phase began increasing as predicted (Fig. 2A). The average lag phase at 180 g was 8.0 hours shorter than in 1 g controls ($P < 0.01$), while at 400 g, the lag phase averaged only 0.8 hours shorter than the controls ($P = 0.2$). Also as hypothesized, final cell concentration was observed to be inversely related to acceleration (Fig. 2B). The average final cell concentration in samples grown at 50, 180, and 400 g was 28%, 36% and 39% less than in the 1 g controls, respectively (all $P < 0.01$).

It is proposed that the results in Fig. 2A above 50 g and in Fig. 2B can be explained by the relationship between inertial acceleration and plume velocity. As described above, it was hypothesized that the increase in the length of the lag phase above 50 g and the corresponding decrease in final cell concentration were primarily due to changes in the buoyancy force acting on excreted bacterial growth factors. Plume velocity is a function of $g^{1/2}$ (Pusey et al., 1986, 1988). Consequently, the velocity of a less dense plume of fluid rising from cells at the bottom of a culture tube increases as a function of the square root of acceleration acting on it, therefore, at

increasingly higher levels of acceleration, the resultant increase in plume velocity becomes less significant. This same trend was observed in the experimental data shown in Fig. 2A and Fig. 2B. In support of this theory, dashed lines are superimposed in Fig. 2 as a function of $g^{1/2}$, which are therefore proportional to plume velocity. These two curves illustrate trends similar to the observed changes in lag phase and final cell concentration, suggesting that the changes in these growth parameters are proportional to plume velocity. The only notable exception from the literature is for the reported change in lag phase observed at 1,000 g (Montgomery et al., 1963). However, this finding could be due to other physiological factors arising from the very high inertial forces acting on the cells or a consequence of different materials and methods used for this experiment, including an average temperature that was 9°C warmer than the experimental data presented here.

3.1 Convective Plumes

In addition to the primary growth experiments, an interesting observation was noted during the study that led us to conduct further characterization tests. Approximately thirty seconds after the samples containing fully sedimented bacteria were removed from the centrifuge, a defined plume of streaming fluid (and containing bacterial cells) could clearly be seen rising from the bottom of the fresh cultures that were presumably still metabolizing substrate (Fig. 3, right). However, the older samples that had been inoculated one week prior to centrifugation never produced a plume, again presumably because they were no longer metabolically active (Fig. 3, left).

The biophysical model, shown in Fig. 1, suggests relevant physical mechanisms associated with these observations, which could occur routinely in certain microbiological laboratory procedures. Because bacteria in the right tube (Fig. 3) were still in the growth phase,

they were consuming glucose, thereby reducing the density of the medium in their immediate vicinity near the bottom of the container. This density gradient is suggested to have caused the lighter fluid to rise as a plume, carrying bacteria in the flow along with it. Eventually, the bacteria were distributed throughout the sample, which became uniformly turbid without physical agitation. In contrast, the sample on the left never produced a rising plume and the medium remained clear (Fig. 3). It is proposed that this occurred because these bacteria were no longer consuming substrate; therefore, no fluid density gradient was established.

The empirically measured plume velocity was in close agreement with the theoretically calculated value, which was based upon similar previous investigations of convection associated with protein crystal growth. This theoretical velocity was estimated using the governing relationship for natural (buoyant) convection (Pusey et al., 1986, 1988),

$$V = \left(gL\nu^{-1} D \Delta\rho / \rho_{\infty} \right)^{1/2}, \quad (1)$$

where g is the acceleration of gravity = 980 cm sec^{-2}; L is the diameter of the plume's source, the culture vessel = 1.4 cm, ν is the medium's kinematic viscosity = 9.9×10^{-3} cm^2 sec^{-1} (Klaus et al., 1997). The by-product diffusion coefficient, D, was estimated to be 1×10^{-5} cm^2 sec^{-1} based upon common metabolites of *E. coli* (Cussler, 1997). The density gradient divided by the density of the bulk fluid, $\Delta\rho/\rho_{\infty}$, was estimated knowing that the bulk fluid had a density of 1.011 g cm^{-3} (Klaus et al., 1997) and considering approximately 30% (Roberts et al., 1955) to 50% (Barford et al., 1982) of the glucose consumed by *E. coli* is used to supply the carbon requirements for the cells' structural components. Therefore, $\Delta\rho \approx 0.40(1.011-\rho_{H_2O}) \approx 0.0044$ g cm^{-3}. The ratio $\Delta\rho/\rho_{\infty}$ was then calculated to be 4.4×10^{-3}. This ratio was considered reasonable because the cells in Fig. 3 were determined to be layered approximately 40 bacteria thick at the bottom of the

culture tubes. It was estimated that this resulted in a solutal boundary layer of approximately 0.1 mm, with little or no glucose reaching the cells at the very bottom of the culture tube (Brown, 1999). Using these values, a theoretical plume velocity was calculated to be 0.08 cm sec^{-1}, which was within 11% of the consistently measured plume velocity of 0.09 cm sec^{-1}. This experiment was repeated five times with similar results.

4. Conclusions

The experimental findings presented support the overarching hypothesis that acceleration levels indirectly affect bacterial growth in a predictable manner. The duration of the lag phase and the final cell concentration were altered under different levels of acceleration, resulting in theoretically expected non-linear changes. In addition, mathematical analysis of the altered growth kinetics further supported the underlying physical model used to make these hypotheses. This analysis showed basic growth kinetics were correlated with inertial acceleration as a function of convective flow rate, indicating buoyancy-driven convection could be responsible for the altered growth.

Additional experiments, which showed centrifuged bacterial cultures creating buoyancy-driven convective flows on a macroscopic level, also supported the assumed physical model. In repeated experiments, a clearly visible rising plume was consistently observed immediately following centrifugation of bacterial cultures that were assumed to be actively consuming glucose. The measured velocity of the observed plumes agreed in general with theoretically derived values. Although, we were not able to directly observe plumes rising from a single cell, this does indicate that bacterial metabolism can create a distinct convective fluid flow in the medium.

The results of the growth experiments and the plume experiments both lend support to a new physical model associated with bacterial growth. This model assumes that bacterial metabolism decreases the solute concentration in the medium surrounding each cell, or some critical mass of cells. This less dense fluid then creates convective currents that help may separate excreted by-products from their cells. Because some of these by-products have been shown to be beneficial for initiation and continuation of growth, buoyancy-driven convection was hypothesized to affect the length of the lag phase as well as the final cell concentration of suspended cultures. Furthermore, based on this physical model, the combined absence of buoyancy-driven convection and sedimentation in non-motile bacterial cultures grown onboard an orbiting spacecraft may prove to be the primary underlying cause of the shorter lag phase and higher final cell concentrations that have been reported in the literature.

The identification of buoyancy-driven convection associated with bacterial growth may also help explain other observed effects of space flight on microorganisms, such as reports of reduced antibiotic effectiveness on orbit (Tixador et al., 1985, 1994) or enhanced antibiotic production in space (Klaus, 1998; Lam et al., 1998, Lam et al., 2002). This insight might allow improvements to be made in the design of suspended-cell bioreactors, which to our knowledge have not taken into consideration the detailed convective phenomena characterized in this work.

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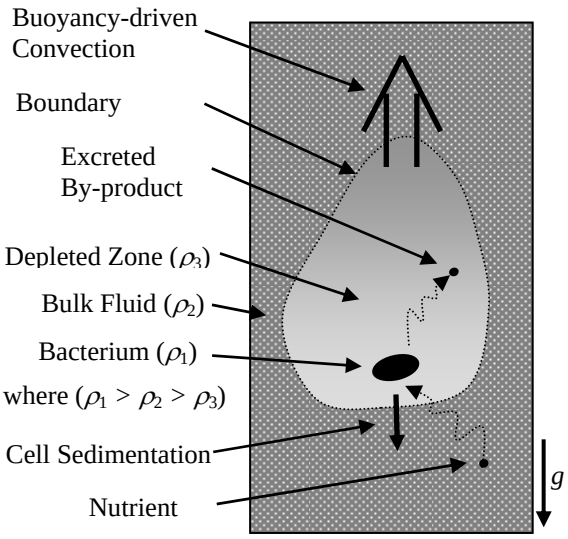
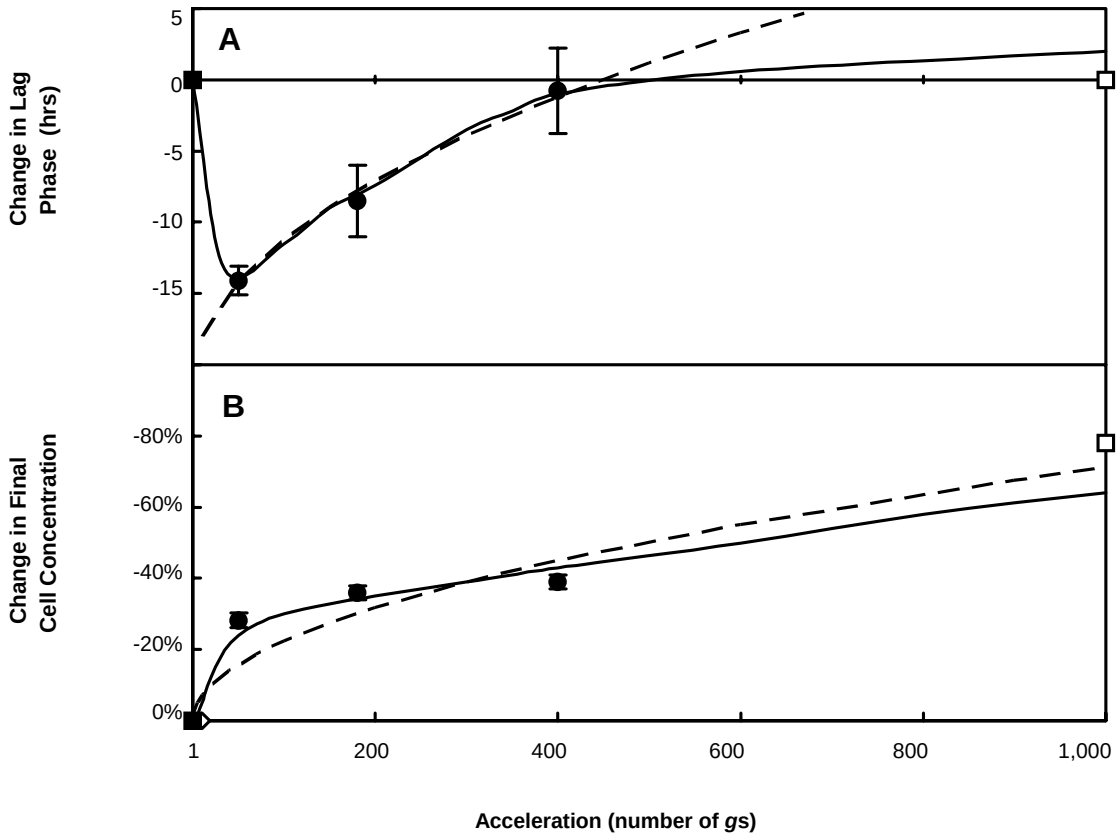
Fig. 1. Graphical representation of the proposed model. As a bacterium consumes nutrients and excretes by-products, the medium immediately surrounding the cell becomes less dense than the bulk fluid (ρ_2). On Earth, this density gradient causes a buoyant plume to rise from each bacterium as the cell simultaneously sediments. In one minute, an *E. coli* bacterium (ρ_1) sediments a distance of 3.6 μm (Klaus et al., 1997) and the rising plume (ρ_3) moves approximately 40 μm (Brown, 1999). This combined gravity-dependent motion separates by-products from their cells, which affects the length of the lag phase and the final cell concentration.

Fig 2. Effect of acceleration on the length of the lag phase (A) and the final cell concentration (B). As predicted, accelerations of 50 g significantly reduced the length of the lag phase duration relative to that of 1 g controls. It is assumed that this was due to the collective contribution of cells and by-products that were accumulated at the bottom of the culture tube. At higher levels of acceleration, the length of the lag phase began to increase and final cell concentration decreased. Based upon the proposed mode, it is proposed that these changes were due primarily to the effect of acceleration on plume velocity, which altered the concentration of metabolic by-products surrounding the cells. The new experimental data from the eight experiments presented in this paper (●) are shown with one standard deviation error bars; ◇ shows previous results at 10 g (Lapchine et al., 1990); □ indicates previous results at 1,000 g (Montgomery et al., 1963); ■ shows the 1 g baseline. The solid lines indicate suggested trends based upon existing data. The superimposed dashed lines are a function of $g^{1/2}$, and are therefore proportional to plume velocity.

Fig. 3. Plume rising from growing bacteria. The photograph, taken ~30 seconds after the samples were removed from 700 g centrifugation, shows a plume of bacterial cells rising 2.8 cm, with an average velocity of 0.09 cm sec^{-1}. The plume continued to be visible in the right tube for about 10 minutes. After this time the sample was too cloudy to continue observing a defined plume. In contrast, the tube on the left, which contained older, presumably non-metabolizing cells at roughly the same concentration, never produced a plume.


Buoyancy-driven
Convection
Boundary
Excreted
By-product
Depleted Zone (ρ_3)
Bulk Fluid (ρ_2)
Bacterium (ρ_1)
where ($\rho_1 > \rho_2 > \rho_3$)
Cell Sedimentation
Nutrient
g


A
B
Change in Lag Phase (hrs)
Change in Final Cell Concentration
5
0
-5
-10
-15
-80%
-60%
-40%
-20%
0%
1
200
400
600
800
1,000
Acceleration (number of gs)