Can genetically modified *Escherichia coli* with neutral buoyancy induced by gas vesicles be used as an alternative method to clinorotation for microgravity studies?

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Space flight has been shown to affect various bacterial growth parameters. It is proposed that weightlessness allows the cells to remain evenly distributed, consequently altering the chemical makeup of their surrounding fluid, and hence indirectly affecting their physiological behaviour. In support of this argument, ground-based studies using clinostats to partially simulate the quiescent environment attained in microgravity have generally been successful in producing bacterial growth characteristics that mimic responses reported under actual space conditions. A novel approach for evaluating the effects of reduced cell sedimentation is presented here through use of *Escherichia coli* cultures genetically modified to be neutrally buoyant. Since clinorotation would not (or would only minimally) affect cell distribution of this already near-colloidal cell system, it was hypothesized that the effects on final population density would be eliminated relative to a static control. Gas-vesicle-producing *E. coli* cultures were grown under clinostat and static conditions and the culture densities at 60 h were compared. As a control, *E. coli* that do not produce gas vesicles, but were otherwise identical to the experimental strain, were also grown under clinostat and static conditions. As hypothesized, no significant difference was observed in cell populations at 60 h between the clinorotated and static gas-vesicle-producing *E. coli* cultures, while the cells that did not produce gas vesicles showed a mean increase in population density of 10\(^{-5}\) % (\(P = 0.001\)). These results further suggest that the lack of cumulative cell sedimentation is the dominant effect of space flight on non-stirred, *in vitro* *E. coli* cultures.

INTRODUCTION

The effects of space flight on bacterial cell cultures have been studied since the 1960s, with many differences in metabolic activity having been reported (Klaus, 2002). Most findings have demonstrated higher final cell populations in space compared to ground controls (Mattoni, 1968; Mennigmann & Lange, 1986; Klaus et al., 1994, 1997; Mennigmann & Heise, 1994; Thévenet et al., 1996; Kacena et al., 1999a; Brown et al., 2002). In addition, lag phase has been shown to be shortened during space flight experiments (Mennigmann & Lange, 1986; Thévenet et al., 1996; Kacena & Todd, 1997; Klaus et al., 1997; Kacena et al., 1999b), conjugation efficiency to be increased (Ciferri et al., 1986), antibiotic effectiveness reduced (Tixador et al., 1985; Moatti et al., 1986), and secondary metabolite production increased (Lam et al., 1998, 2002).

It is postulated that the primary aspect of space flight affecting microbial cell suspension growth characteristics is that of weightlessness, rather than radiation (Klaus, 2002). Furthermore, it has been hypothesized that space flight indirectly causes the observed differences in bacterial cell suspension cultures as a consequence of altering the distribution of cells in their fluid environment. This altered distribution is thought to affect the extracellular environment, hence triggering a physiological response (Mattoni, 1968; Thévenet et al., 1996; Kacena et al., 1997, 1999a; Klaus et al., 1997).

Several factors caused by the weightless environment of space flight are likely to alter the fluid environment appreciably. For example, the lack of sedimentation of cells in suspension may contribute to the accessibility of nutrients simply due to better cell distribution (Klaus et al., 1997). In addition, a decrease in the density of the medium immediately surrounding a population of actively metabolizing bacterial cells has been observed to create buoyant plumes in a 1 g environment (Brown, 1999). Albrecht-Buehler (1991) pointed out that the lack of microconvective currents around a growing bacterial cell in microgravity might mean that the cell would resemble a growing protein
crystal in microgravity, McPherson (1993) suggested that the lack of mixing in weightlessness creates a quasistable ‘depletion zone’ around the nucleus of a growing crystal. The result is that crystal surfaces interface with a micro-environment of lower protein concentration than the bulk solution, which is ideal for the formation of crystals of higher order and larger size. Klaus et al. (1997) postulated that a similar phenomenon may occur around growing bacterial cells in a weightless environment with respect to nutrients and by-products, and that this depletion zone may be partially responsible for the observed differences in space flight experiments. The net effect is that an individual cell may become separated from its immediate surroundings at a higher rate when sedimentation is present (1 g) than in conditions of weightlessness.

Interestingly, higher final biomass yields are commonly achieved in bioreactor engineering by controlled addition of a reduced carbon source, such as glucose, in what is known as ‘fed-batch’ culturing (Robbins & Taylor, 1989; Yee & Blanch, 1992). This technique prevents excessive accumulation of byproducts such as acetate and allows for a more prolonged growth phase (Frude et al., 1994). If, as previously discussed, a ‘depletion zone’ is formed around growing bacterial cells in microgravity, the effect of reducing mass transport might essentially be the same as that of controlled addition of glucose to the cell, thus similarly contributing to higher final cell density (Klaus, 1998, 2002). Todd & Klaus (1996) explored this notion with theoretical calculations and predicted higher final cell densities if the glucose level at the cell boundary is reduced from the bulk solution concentration.

One substantial obstacle in determining how space flight affects bacterial cell cultures is the relatively small number of samples obtained over a large array of experiments. Flight opportunities to conduct experiments are not only infrequent and expensive, they are also subject to a number of unique hardware and operational constraints, and opportunities to exactly repeat an experiment are rare. In addition, results are sometimes contradictory. For example, no significant difference between final cell population density of flight and ground samples was reported by Boulouc & D’Ari (1991), Gasset et al. (1994), Thévenet et al. (1996) or Kacena & Todd (1997). In some cases this lack of a difference was attributed to a specific parameter, such as cell motility (Thévenet et al., 1996) or growth on agar substrate (Kacena & Todd, 1997). These latter two experiments actually corroborate the concept that a quiescent fluid environment and lack of cell sedimentation affect cell growth in microgravity. Specific test conditions, growth medium and assay methods may account for other contradicting results (Klaus et al., 1997). In addition to studies conducted in space, various ground-based methods have been designed to mimic certain aspects of microgravity. Two devices, the clinostat and the rotating wall vessel bioreactor, are used extensively for experiments with bacteria and other cell cultures (Klaus, 2001).

A clinostat is a device that partially simulates the microgravity conditions of space flight for suspension cell cultures. It does so by rotating a cylinder completely filled with a liquid medium (i.e. no air bubbles) and cells at a constant velocity. After initial start-up of the clinostat, the rotational velocity of the cylinder wall is transferred radially inward as a function of the viscosity of the medium. This continues until solid body rotation of the fluid medium occurs and virtually no relative fluid motion exists (Klaus et al., 1998). The cells in this situation are still under the direct influence of gravity; however, the gravity vector is continually being reoriented as the cells and fluid rotate together. If the clinostat is operated at an appropriate rotation rate, the cells will neither sediment nor be appreciably centrifuged to the container wall. Therefore, the lack of net sedimentation of the cells and the reduction of relative fluid motion represent functional features of space flight experiments that are mimicked with the clinostat in 1 g. Bacteria grown under clinorotation typically exhibit trends similar to comparable space flight data (Mattoni, 1968; Mennigmann & Lange, 1986; Klaus et al., 1998; Kacena et al., 1999a; Brown et al., 2002). Although bacteria do not sediment to the container bottom during proper clinorotation, they still move, thus making it difficult to quantify exactly how much fluid mixing occurs as a result.

Rotating wall vessel (RWV) bioreactors are also used to maintain low-shear suspensions of cell cultures in a similar fashion to clinorotation. A review of several RWV applications has recently been published (Nickerson et al., 2003). For bacterial RWV experiments, findings pertaining to final cell number or dry cell weight, when reported, show varying results (Fang et al., 1997a, b, 2000; Huitema et al., 2002; Wilson et al., 2002; England et al., 2003; Baker & Leff, 2004). This unpredictability further emphasizes the assertion that specific experimental parameters must be considered before general conclusions can be drawn.

A novel method is proposed here for evaluating the effect of reduced cell sedimentation in a suspension culture (without imparting resultant motion from rotation) using Escherichia coli cultures that have been genetically modified to be neutrally buoyant. These bacteria produce gas vesicles, which are intracellular, protein-coated, hollow organelles that are normally found in cyanobacteria and halophilic archaea (Li & Cannon, 1998). The vesicles are permeable to ambient gases by diffusion and provide the carrier cell with buoyancy, allowing the cells to move upwards in liquid. Cultures of genetically modified E. coli that produce gas vesicles sediment at a considerably reduced velocity, thus approximating neutral buoyancy. The modified cells were investigated as an alternative experimental model for further characterizing how the lack of cell sedimentation and reduction of related fluid motion, common to space flight and clinorotation, affect bacterial growth. This experimental model does not, however, reduce convective fluid mixing, which is also an important feature of microgravity (Albrecht-Buehler, 1991). The study described here directly
compared the growth of matched buoyant or non-buoyant E. coli strains in static controls versus clinorotated suspension cultures of each strain.

**HYPOTHESIS**

The hypothesis consists of two related elements:

1. Non-buoyant E. coli grown under clinorotation will achieve a higher population density at or near the onset of stationary phase as compared to matched (non-buoyant) static controls.

2. Induced buoyant E. coli grown under clinorotation will not experience an increased cell population density at or near the onset of stationary phase relative to matched (buoyant) static controls.

**METHODS**

Bacterial strains and plasmids. The E. coli parental strain DH5α (Clontech) with either plasmid pNL29 (which contains a 6040 bp cluster of 11 putative gas vesicle genes from Bacillus megaterium) or the non-recombinant, cloning vector pBluescriptII SK (Stratagene) were used for all experiments. The E. coli strain containing pNL29 was demonstrated to express gas vesicles in all cells of a culture when induced with isopropyl β-D-thiogalactopyranoside (IPTG), while the E. coli strain with only the cloning vector produced no gas vesicles (Li & Cannon, 1998). These E. coli strains were graciously provided by Maura Cannon from the University of Massachusetts in Amherst.

Experiment design. The induced E. coli DH5α(pNL29) was used as the experimental culture; for simplicity it will be referred to as the 'experimental' strain. Growth of E. coli DH5α(pNL29) without induction by IPTG was also used as a control, but the DH5α host strain of E. coli does not have tight regulation of the plasmid genes; therefore, the non-induced E. coli DH5α(pNL29) still produces some gas vesicles, resulting in partial buoyancy. This strain will be referred to as 'control I'. Finally, the E. coli with the pBluescriptII SK cloning vector, which produced no gas vesicles, will be referred to as 'control II'. It was expected that the relative difference in culture density at 60 h between static and clinorotated cultures for the control I strain would fall between the experimental strain (smallest difference) and the control II strain (largest difference).

Media and growth conditions. Cultures were inoculated from 15% (v/v) glycerol freezer (−80 °C) stock in 5 ml liquid Luria–Bertani Miller (LBM) broth (Sigma L-3152) with 100 µg ampicillin ml−1 (AMP100) (Fischer BP1760). The 5 ml cultures were grown for 12 h (to early exponential phase) in a 15 ml conical tube tilted at a 30° angle, incubated at 37°C, and aerated by rotation at 225 r.p.m. (2 cm diameter). A volume of 1 ml of each culture was then transferred to 50 ml of a defined minimal growth medium (medium E; Vogel & Bonner, 1956) supplemented with 5 g glucose l−1 (autoclaved separately) and AMP100. Each culture was grown in a 250 ml Erlenmeyer flask, aerated by rotation at 225 r.p.m. (2 cm diameter), and incubated at 37°C for 12 h. OD600 readings were taken with a Genesys 10 Series Spectrophotometer (Spectronic Unicam) and 10 mm path-length cuvettes. Cells were counted on a Hauser Scientific Improved Neubauer Haemocytometer with a minimum of 200 cells counted in 16 squares. An appropriate volume of culture was diluted to 1×107 cells ml−1 in fresh 1× medium E plus 5 g glucose l−1 and AMP100. The E. coli experimental culture was induced to activate gas vesicle production by adding an appropriate volume of 1000× (1 M) stock of IPTG (Sigma I-5502) to obtain a final concentration of 1 mM IPTG. Experimental cultures were pipetted in 4 ml volumes into one end of a fluid processing apparatus (FPA) and 4 ml of either control culture (non-induced control I strain or control II strain) was added to the other end, such that each FPA contained an experimental and a control culture. The FPA is a glass barrel that has an inner diameter of 1.35 cm and is 11.5 cm long. It is a specially designed test tube for use with the clinostat described below and for use on space flights (Klaus et al., 1997). Rubber septa were used to seal each fluid chamber of the FPA such that no air space existed; however, the cultures were not rendered anoxic prior to filling of the FPA. Initial dissolved oxygen levels in the freshly filled FPAs were calculated to be 0–23 mM. It was anticipated, based on previous unpublished data, that the dissolved oxygen in the FPAs would be depleted in approximately 24 h. Fourteen FPAs were loaded in this fashion for each experimental run. Seven were placed on the clinostat and seven were placed in a test tube rack that was laid horizontally next to the clinostat at room temperature. The temperature was recorded every 15 min and the overall mean was 20.6±0.85 °C. Due to the limited capacity of FPAs on the clinostat, comparison of culture growth was performed at only one time point in order to readily obtain a sufficient number of samples. The appropriate time for population comparison was determined from the preliminary growth curve data (with duplicate samples only) illustrated in Fig. 1. It can be seen in Fig. 1(a) that the control II cultures reached stationary phase on the clinostat at ~60 h; however, the static cultures appeared to enter stationary phase sooner (~48 h). As such, 60 h was chosen as the time point for sampling the control II cultures. The experimental cultures showed nearly identical growth curves for both clinostat and static cultures, with stationary phase not completely reached until 72 h (Fig. 1b). At 60 h, however, there appeared to be the greatest difference between the clinostat and static cultures. Therefore, by choosing the sample time point of 60 h, we were investigating a 'worst-case scenario', since we expected no difference in cell population density. Also, this allowed for all cultures to be harvested at the same time point.

Cell sedimentation rate. Cell sedimentation rate was estimated by resuspending cells in 4 ml 1× medium E in screw-cap vials and measuring (with callipers) the distance between the top of the fluid...
and the interface between turbid and clear medium over time (see Fig. 2). The distance measurements were performed in duplicate with a precision of approximately 1 mm. Based on the measured cell sedimentation rates, and accounting for the cylindrical geometry of the FPA, the approximate percentage of cells to reach the container bottom after 60 h was calculated. These calculations are included as supplementary data with the online version of this paper (at http://mic.sgmjournals.org).

Clinostat. The clinostat used (shown in Fig. 3) has the capacity to concurrently rotate seven FPAs. The rotation rate (~8 r.p.m.) was determined by using the relationships published by Klaus et al. (1998) to calculate upper and lower limits, then selecting a value near the middle of this range. Important parameters in these equations are the density of the particle and the medium, the viscosity of the medium, the effective Stokes radius of a bacterial cell, and the radius of the container (FPA).

Statistical analysis. The \( P \)-values between the matched clinorotated and static cultures were calculated using the two-tailed \( t \)-test with unknown, unequal variances. It should be noted that all comparisons were made within, not across, the three strains, which were inoculated from a common starting stock to ensure initial consistency between growth on the clinostat and the static controls.

RESULTS

As predicted by hypothesis 1, the control II strain (no gas vesicles) showed statistically significant differences on the clinostat compared to matched, static cultures, with a mean increase in cell population density at 60 h of 10·5\% \( (P=0·001) \). The experimental (neutrally buoyant) strain, which produced the most gas vesicles, showed a mean increase of 0·3\% \( (P=0·39) \) on the clinostat at 60 h, thus indicating that there was no significant difference in cell population density compared to the static controls and confirming hypothesis 2. Also of interest, the control I strain (some cells with gas vesicles) showed a mean population increase at 60 h of 5·6\% \( (P=0·01) \) on the clinostat, falling in between the two primary hypothesized results. The findings were consistently repeated in three independent trials for each strain (consisting of seven or eight matched pairs each). The mean differences are illustrated graphically in Fig. 4.

The sedimentation distances at 60 h for the three cell types resuspended in static screw-cap vials were measured to be approximately 0·6 cm for the experimental cultures, 1·0 cm for the control I cultures, and 1·8 cm for the control II cultures. Since the diameter of the FPA is only 1·35 cm, the control II cultures were estimated to sediment to the bottom of a static FPA in ~45 h. The corresponding percentages of cells calculated to be sedimented to the container bottom at 60 h are overlaid on Fig. 4, indicating an apparent correlation between percentage sedimentation and the relative population difference achieved between clinostat and static culture densities.

Certain factors impaired the ability to directly compare growth between the different \( E. \) \( coli \) strains. For example, one complication is the possibility of the production of gas
The findings presented here indicate that preventing cell significant, support the two stated hypotheses. While the clinostat typically produces the same trend of increased growth as reported for comparable space flight experiments, the magnitude of the difference is usually less pronounced. This may be a function of the complete lack of buoyancy-induced convective flow that occurs in the extracellular fluid under true microgravity conditions, thus reducing metabolic byproduct transport in addition to causing a lack of cell sedimentation (Brown, 1999).

The mean 5.6% difference of clinorotated and static control I E. coli cultures fell between the 10.5% difference of the control II strain and the insignificant difference of the experimental strain. This was also expected since the control I strain showed a relatively high percentage of cells with gas vesicles, resulting in some buoyancy, presumably due to leaky operon control in the E. coli DH5α host cells as discussed.

The higher final cell population density achieved in the clinorotated samples compared to static cultures of the E. coli control II strain (no gas vesicles) compares favourably with differences observed in the literature for a variety of different bacteria (Mattoni, 1968; Mennigmann & Heise, 1994; Klaus et al., 1998; Kacena et al., 1999a; Brown et al., 2002).

Gas-vesicle-producing E. coli that achieve neutral buoyancy offer a novel ground-based method for isolating the role of reduced cell sedimentation on bacterial suspension cultures. Plans for future work in this area include developing an optical monitoring system to allow continuous recording of clinostat and static culture population densities through all phases of growth.

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