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Growth and division of *Escherichia coli* under microgravity conditions

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SUMMARY

The growth rate in glucose minimal medium and time of entry into the stationary phase in pepton cultures were determined during the STS 42 mission of the space shuttle Discovery. Cells were cultured in plastic bags and growth was stopped at six different time points by lowering the temperature to 5°C, and at a single time point, by formaldehyde fixation. Based on cell number determination, the doubling time calculated for the flight samples of glucose cells was shorter (46 min) than for the ground samples (59 min). However, a larger cell size expected for more rapidly growing cells was not observed by volume measurements with the electronic particle counter, nor by electron microscopic measurement of cell dimensions. Only for cells fixed in flight was a larger cell length and percentage of constricted cells found. An optical density increase in the peptone cultures showed an earlier entry into the stationary phase in flight samples, but this could not be confirmed by viability counts. The single sample with cells fixed in flight showed properties indicative of growth stimulation. However, taking all observations together, we conclude that microgravity has no effect on the growth rate of exponentially growing *Escherichia coli* cells.

Key-words: *Escherichia coli*, Microgravity, Cell division, Cell growth; Space shuttle, Exponential growth rate.

INTRODUCTION

Observations in previous space experiments with microorganisms supported the assumption that microgravity or cosmic radiation could stimulate bacterial growth (Gmünder and Cogo-

li, 1988). Bacteria like *Bacillus subtilis*, for instance, were reported to attain a higher growth rate and biomass yield under microgravity conditions (Mennigman and Lange, 1986), whereas *Escherichia coli* was found to acquire increased resistance to antibiotics (Lapchine *et al.*, 1986),

which was ascribed to an overall growth stimulation. The mechanism for a gravity effect on bacteria is difficult to envisage, but observations like the gravity sensitivity of a mammalian signal transduction pathway (de Groot et al., 1991) point to an interference of microgravity even at the macromolecular level.

Recently, the possible influence of microgravity on bacterial growth was studied in a simple experiment by Bouloc and D'Ari (1991) carried out in the Soviet satellite Biocosmos 2044. In that experiment, the final E. coli biomass concentration reached after attainment of stationary growth was determined in various minimal medium cultures containing limiting amounts of glucose and glycerol. That study showed no significant difference in cell mass and cell number between flight and ground control cultures. Because the average cell volumes after growth arrest also remained the same, the authors deduced that exponential growth rate had not been affected.

The performance of growth experiments in a space program is greatly influenced by time schedules and hardware requirements. For instance, cells have to be stored at low temperatures for several days (e.g. Bouloc and D'Ari, 1991), and the possibilities for taking samples during the experiment are limited, as they usually require human interaction. In view of these restrictions, we determined the optimal storage condition for E. coli cells at low temperature. We observed that the use of glucose-starved cells and of a relA$^+$ strain contributed to rapid recovery of exponential growth at 37°C (Van Bakel et al., 1991). We had the opportunity of applying these conditions in a space shuttle mission in January 1992, in which samples could be taken during the growth experiment. Growth was stopped in the samples by lowering the temperature to 5°C, but in one sample, cells could immediately be fixed for microscopic observation. This experimental set-up, for the first time, enabled the determination of both exponential growth rate and the size and shape of bacteria grown under space conditions. In a concomitant experiment, the antibiotic sensitivity as previously studied (Lapchine et al., 1986) was again tested (Tixador et al., 1993).

MATERIALS AND METHODS

Strains

For growth in glucose minimal medium, an isogenic relA1$^+$ derivative of strain E. coli K12 MC4100 was used, which was described previously (Van Bakel et al., 1991). For growth in peptone medium the same K12 strain (ATCC 25922) was used as in a previous space experiment (Lapchine et al., 1986).

Media

The minimal medium used was M9 and contained 1 g NH$_4$Cl/l, 7.5 g NaH$_2$PO$_4$.2H$_2$O/l, 3 g KH$_2$PO$_4$/l, 0.5 g NaCl/l, 2 mg aneurine/l, 0.5 mM MgSO$_4$ and 0.4 % (w:v) glucose as carbon source. The osmolality was adjusted to 300 mOsM with 1 M NaCl and the pH to 7.4 with 1 N NaOH. M9 medium without glucose, used for storage of the cells, was likewise brought up to 300 mOsM by adding 1 M NaCl to a final concentration of 26 mM. Peptone medium (Difco) consisted of 1 % peptone and 0.5 % NaCl, pH 7.2.

Hardware

All bacterial cultures were grown in plastic bags (polyethylene) packed in containers as previously described (Tixador et al., 1981). For the minimal medium cultures, 700 μl of glucose-starved cells were placed in the bags at a concentration of 5.7×10^5 cells/ml together with a glass ampoule containing 33 μl of 10 % glucose to start growth at 37°C. For the peptone medium cultures, cell samples containing an overnight culture were sealed in glass ampoules which were placed in the plastic bags together with the peptone medium. After breakage of the ampoules, the starting concentration was 4.5×10^4 cells/ml. Filling of ampoules, sealing of bags and packing the containers used for incubation were performed as previously described (Tixador et al., 1983). The bags were sealed without any air bubbles remaining. After growth, no aggregation or settling of cells on the walls of the bags was observed. In total, 8 ESA type I containers were used, 6 of which were placed in a static rack and 2 on a 1 g centrifuge in the Biorack facility. This centrifuge represented a 1.4 g force on the ground. For every culture and time-point, four individual plastic bags were used. An identical setup was used for a synchronous experiment on the ground at Kennedy Space Center (KSC).

Growth measurements

At the start of the experiments, the glass ampoules containing either glucose or bacterial suspension were

broken by an astronaut, and the containers were transferred to the incubators at 37°C on either the static rack or the centrifuge. They were subsequently replaced at 5°C to stop further growth (see below). After landing and about 30 h after the growth experiment, all bags of both flight and ground experiment were recovered from the containers, coded and handed over to an observer for "blind" measurement with respect to the nature (flight or ground) of the samples. The plastic bags containing the minimal medium cultures were cut open and the cells were fixed by adding 500 μl suspension to 125 μl 1.2 % formaldehyde in an Eppendorf tube. The number and volume of these cells were determined with an electronic particle counter (equipped with a 30 μm orifice), which actually measures the relative resistance of cells. The peptone-grown cells were analysed by measuring their optical density at 530 nm with a microsample (200 μl) "Secomam" spectrophotometer (type S500P) and by the determination of viable counts with a "Spiral" inoculator (Interscience, France).

Chronology of the experiment

As schematized in figure 1, cells were prepared and packed at KSC two days before launch of the
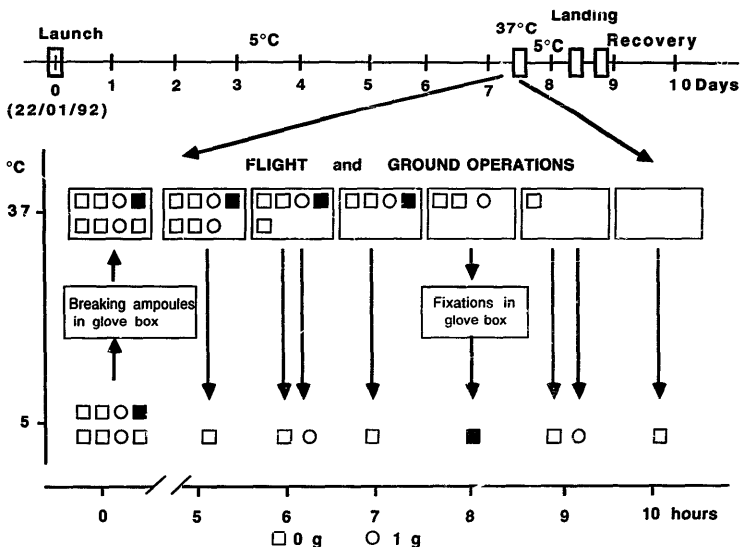
Fig. 1. Chronology and flow chart of the different operations carried out by astronauts during the experiment which was performed on day 7 after launch at Kennedy Space Center.

The growth experiment was started by breaking the glass ampoules and placing the type I containers (squares and circles) into the static rack (squares) or on the 1-*g* centrifuge (circles) at 37°C. After 5 h, the containers were sequentially placed back at 5°C to stop further growth, representing six consecutive time samples, t5 to t10. Container t8 (filled square) was packed with double-sized plastic bags containing a second glass ampoule for immediate fixation of the cells (see filled symbols in subsequent figures). An identical experiment was performed with a 1-h delay on the ground.

space shuttle Discovery. Verification of steady state growth and preparation of the *E. coli* cells by glucose starvation for low-temperature storage was performed as described by Van Bakel *et al.* (1991). The type 1 containers with the plastic bags were kept at 5°C until the experiment was started eight days after launch by breaking the glass ampoules and placing the containers at 37°C. After about 5 h of growth recovery at 37°C, the first time sample (t5) was taken by replacing one container at 5°C in order to stop further growth. This was repeated for 5 subsequent samples (t6-t10) at intervals of about 1 h (square symbols in fig. 1). At the start of the experiment, two ESA type 1 containers were placed on a 1 *g* centrifuge in flight, to serve as a gravity control. (On the ground, the 1 *g* centrifugal force adds up together with gravitation to 1.4 *g*, but no effects of hypergravity on the growth of *E. coli* cells has been detected; unpublished observations). Growth in the centrifuged samples was stopped at six (t6) and nine (t9) hours (round symbols in fig. 1) after start. For one time point (t8) the container was packed with two (instead of four) double-sized plastic bags (1,400 μl) in which an additional ampoule with 6 % formaldehyde was placed (filled symbol in fig. 1) and subsequent figures). By breaking this second ampoule, the cells in this sample (t8) were fixed with a final concentration of 0.24 % formaldehyde under flight conditions. All other samples were fixed with the same concentration of formaldehyde after landing on day 9.

Preparation of cells for fluorescence and electron microscopy

Cell suspensions of 225 μl fixed with 0.24 % formaldehyde were additionally fixed by adding 25 μl of 1 % OsO₄ and prepared by the agar filtration method (Woldringh *et al.*, 1977) for electron microscopic determination of cell dimensions. Measurements were carried out as previously described (Van Bakel *et al.*, 1991; Trueba and Woldringh, 1980). Fluorescence microscopy of glucose-grown cells fixed with formaldehyde and OsO₄, was performed as published elsewhere (Mulder and Woldringh, 1989). Peptone-grown cells were fixed with glutaraldehyde for embedding and thin sectioning.

Statistical tests

For determination of the increase in cell number, regression analysis was performed as described by Sokal and Rohlf (1969). For comparison of the two regression coefficients, the F test was applied. For the determination of differences in cell size measured with the electronic particle counter, in cell length and in percentage of constricted cells, a three and a two

level nested anova with unequal sample sizes were used according to Sokal and Rohlf (1969). To determine possible differences between growth curves of peptone-grown cells, a non-parametric rank sum test (Van Elteren, 1960) was applied.

RESULTS

Exponential growth in minimal medium

Experimental conditions during the present space mission necessitated prolonged storage of cells at low temperature and permitted only a relatively short period for growth measurement. Preparatory experiments had shown (Van Bakel *et al.*, 1991) that glucose-starved *relA*⁺ cells could reach their final steady state size after low-temperature storage within 3 h after growth recovery. In addition, simulation tests indicated that the growth rate reached in the plastic bags (calculated from cell number) was the same as that reached in flask cultures (calculated from cell numbers and OD measurements). The doubling time in 12 control experiments varied from 53 to 62 min, showing an average value of 57 min (SD = 3 min). In these simulation experiments, the coefficient of variation in cell number counts of the 4 individual plastic bags belonging to one time sample had an average value of 15 %, ranging between 5 and 36 %. By contrast, for both flight and ground cultures, the present space shuttle experiment (fig. 2) showed a much larger variation, with an average of 47 % and ranging between 19 and 88 %. This may indicate how difficult it is to exactly simulate such a complex experiment, especially with respect to medium composition and temperature fluctuations. In spite of this considerable scatter, restriction lines through the two sets of points show, in an F test a significant difference ($F_s = 45.53$; $F_{(0.05)} = 5.32$) in cell number increase, with doubling times of 46 min for the flight and 59 min for the ground cultures (fig. 2A). Omitting the values of the differently treated t8 sample did not change this result.

This more rapid exponential growth rate in flight cultures should be reflected in an increased mean size of the exponentially growing cells and
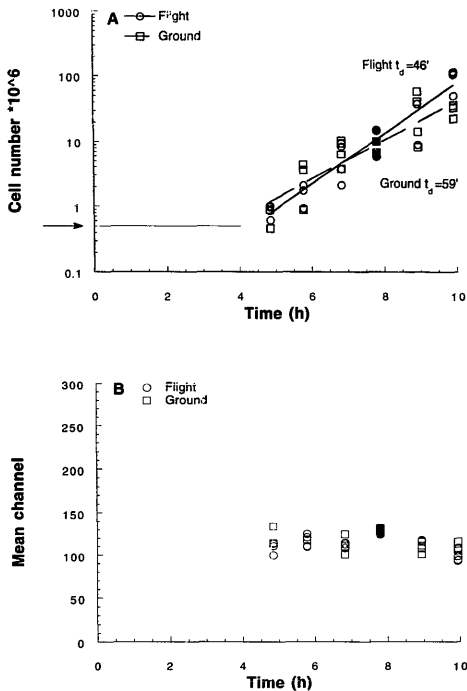
Fig. 2. Cell number (A) and relative cell size (B) as determined with the electronic particle counter during growth at 37°C in glucose minimal medium for the six time samples indicated in figure 1.

Each time sample includes the measurements of 4 individual plastic bags, except for t8, which contained two double-sized bags. In addition, the following samples showing no increase in cell size and number, were omitted: 2 at t5 (ground samples); 1 at t6 (ground sample) and 2 at t9 (flight samples). The straight lines in A represent regression lines (regression coefficients were 0.390 and 0.304 for flight and ground measurements, respectively). The arrow in A indicates the cell concentration of glucose-starved cells in the bags before breaking the glucose ampoule. t_d, doubling time. Because no significant difference between the flight and ground values was obtained in B, lines have been omitted.

Table I. Cell dimensions and percentage of constricted cells as determined by electron microscopic observation of individual plastic-bag cultures [*] grown in flight (F) or on the ground (G).

Sample	Culture condition F or G	Number of cells measured	Mean length μm (SD)	Mean volume [+] μm³ (SD)	Percentage constricted cells (SD)
t6	F	1,159	1.97(0.09)	1.53(0.16)	17(7)
	G	981	1.91(0.02)	1.45(0.10)	16(2)
t7	F	1,419	1.79(0.11)	0.67(0.15)	26(4)
	G	1,573	1.82(0.08)	0.67(0.09)	23(5)
t8	F	1,374	1.88(0.19)	0.95(0.18)	42(6)
	G	778	1.68(0.04)	0.80(0.06)	32(2)
t9	F	1,063	1.81(0.30)	1.08(0.54)	29(5)
	G	2,305	1.76(0.15)	0.97(0.25)	24(4)
	FC	1,115	1.75(0.28)	1.08(0.51)	22(5)
	GC	1,439	1.55(0.13)	0.70(0.17)	25(5)
t10	F	1,257	1.91(0.08)	1.19(0.14)	21(3)
	G	1,193	1.90(0.02)	1.35(0.10)	21(4)

The t5 samples contained too few cells for representative measurements. The t6 samples from cultures placed on the centrifuge were lost during preparation.

[*] The plastic bags were coded in such a way that the observers would only know the time of the sample, but not whether it concerned a flight or ground culture. The results of only one observer are presented. Samples t8 and t9 were also measured by a second observer who, apart from a systematic deviation, obtained the same results.

[+] Calculated from average values for length and diameter, assuming the cell to be right cylinder with hemispherical polar caps. The large variation is caused by diameter variations, probably due to the fact that cells had to be prefixed with formaldehyde before OsO_4-fixation and agar filtration.

SD = standard deviation of the measurements from individual cells or from 4 plastic-bag cultures; for exceptions, see legend to fig. 2.
FC = flight centrifuge; GC = ground centrifuge.

in populations showing an increased percentage of constricted cells (Nanninga and Woldringh, 1985). However, estimation of mean cell size with the electronic particle counter, as shown in figure 2B, shows no significant difference between flight and ground cells. In table I, the results of electron microscopic analysis are summarized for flight (F) and ground (G) cultures. Because the diameter measurements fluctuated strongly, the values for mean volumes also showed large variations. Therefore, in figure 3A, only the mean length values were compared. Figure 3B shows the values obtained for the percentage of constricted cells for the same populations. Because cells in the samples taken after eight hours (t8) were immediately fixed, these samples (filled symbols in fig. 3) give the most accurate representation of the actual flight condition in which the cells were grown. In the case of the t8 sample, the size of flight cells as determined by electron microscopy appeared to be larger (table I, fig. 3A). The flight cells also showed a significantly higher percentage of constricted cells than the ground cells (fig. 3B). In the other samples, the percentages were much lower because low-temperature storage in the presence of glucose after the 37°C-growth period allowed residual divisions to take place, as well as some additional increases in size (or length) as previously observed (Van Bakel et al., 1991). In spite of these changes, however, we can still make a comparison between flight and ground samples. Using all data collected in table I, a statistical test (Sokal and Rohlf, 1989; box 10.4) showed no significant difference in cell size or percentage of constricted cells between flight and ground cultures.

Figure 3 and table I also include the flight (FC) and ground (GC) cultures placed on the centrifuge. The values obtained for these gravity control cultures do not point to any compensating effect by gravity.
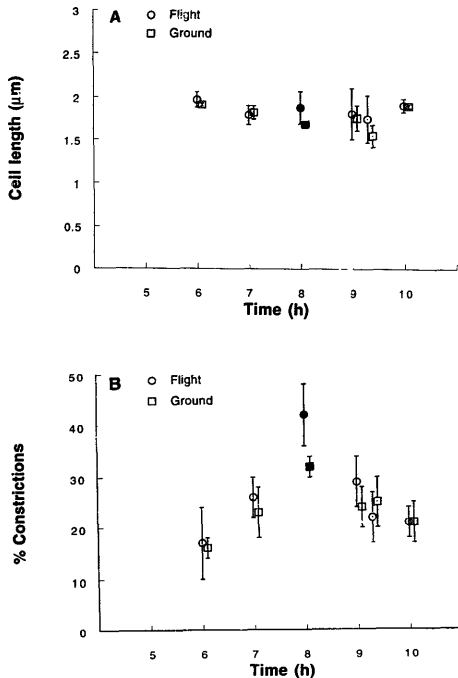
Fig. 3. Average cell length calculated from the pooled length distributions (A) and percentage of constricted cells calculated for the populations in the four bags (B) as obtained after electron microscopic measurement of cell dimensions.

See also table I. Vertical lines indicate the SD found for the individual cells (A) or the plastic bags (B). The dark symbols indicate the t8 samples directly fixed with formaldehyde. Open symbols with point at t9 were displaced for clarity and represent cultures placed on 1 g centrifuge.

Entering stationary phase in peptone medium

Due to the more rapid growth rate or a shorter lag period in peptone medium (doubling time 25 min), the cells in these cultures were just entering the stationary phase of growth during the experimental period. The absorbance measurements of the samples in figure 4A suggested that the flight cultures entered the stationary phase earlier than the ground cultures. Application of a non-parametric rank sum test (Van Elteren, 1960) shows that the curve of the ground

samples ran, in a significant way, below that of the flight samples (p < 2×10^{-3}). However, for viable cell counts (fig. 4B) results were not significant (p = 6.1×10^{-2}). After 9 h, flight and ground curves joined (fig. 4A and B), indicating that the growth yield reached in the stationary phase was the same.

Fluorescence and electron microscopy

Fluorescence and electron microscope investigations carried out on cells of the t8 samples did not show any differences in nucleoid segregation or cell shape, nor in the ultrastructure, between flight and ground cells.
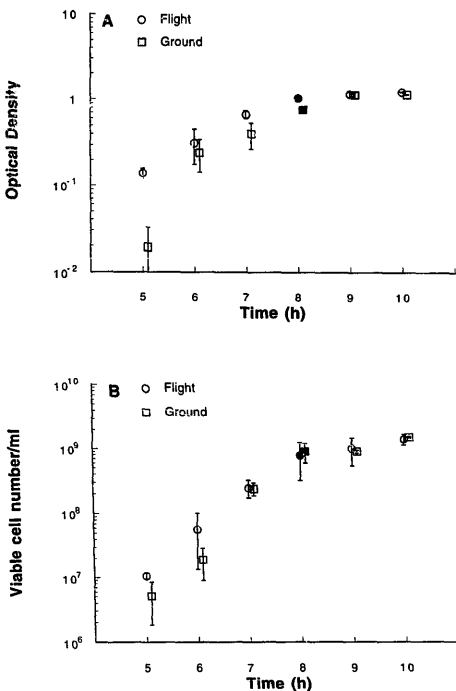

Fig. 4. Increase in optical density (A) and viable cell count (B) during growth at 37°C in pepton medium.

Vertical bars indicate SD found for the measurements of the populations in the 4 plastic bags.

DISCUSSION

The present space shuttle experiment enabled, for the first time, chemical fixation of *E. coli* cells growing exponentially under microgravity conditions. Cells in this single time sample (t8; filled symbols) showed some properties (*i.e.* increased average dimensions and higher percentage of constricted cells; see table I and fig. 3) indicative of growth stimulation under flight conditions. Growth stimulation was also suggested by the electronic counting of cell number in the individual plastic bags, presented in figure 2A. If this decrease in doubling time of the minimal medium cultures from 59 to 46 min did indeed occur, the average size of the cells in the various samples should have increased by about 20 %, assuming the applicability of the Cooper-Helmstetter model for bacterial growth (Helmstetter *et al.*, 1968). According to this model, more rapidly growing cells are larger because they initiate DNA replication at a constant mass (M_i), but they grow faster during the subsequent constant period, (C + D) minutes, between initiation of chromosome replication and cell division, reaching a larger size at division.

As demonstrated in table I, the average volumes for the respective samples showed an increase of less than 10 % for the flight cultures (although 19 % in the case of t8!). However, an increase in average size for the flight cultures of the t8 and other samples was not confirmed by measurement of cell volume with the electronic particle counter (fig. 2B). In addition, the samples placed on a centrifuge to serve as gravity controls did not show the decrease in average size expected if microgravity had stimulated the growth rate and caused a cell size increase. It should, however, be noted that these controls were not placed on the centrifuge during the long storage period (9 days) preceding the 37°C growth period and could therefore also have been influenced by possible microgravity effects.

In this study, some individual observations (*e.g.* on the t8 sample) suggested that bacterial growth may have been stimulated by space conditions. However, taking all observations together, we conclude that *E. coli* cells show no deviation in exponential growth rate due to

microgravity conditions. Nor did we observe an effect on growth yield, in accordance with the observations of Bouloc and D'Ari (1991).

Croissance et division de *Escherichia coli* en microgravité

Le taux de croissance en milieu glucosé minimal et le moment d'entrée en phase stationnaire de *Escherichia coli* cultivé en milieu peptoné, ont été déterminés lors de la mission STS42 de la navette spatiale Discovery. Les cellules ont été cultivées dans des sachets en plastique et la croissance a été stoppée à six temps différents par abaissement de la température à 5°C, à l'exception d'une seule fois où la fixation a été réalisée par le formaldéhyde. L'augmentation cellulaire, le temps de doublement calculé pour les échantillons «vol» des cellules en milieu glucosé, a été plus court (46 min) que celui des échantillons «sol» (59 min). Cependant, l'augmentation de la taille de la cellule qui accompagne une croissance cellulaire plus rapide, n'a pas été observée lors des mesures volumétriques effectuées avec le compteur électronique de particules, ni des mesures des dimensions cellulaires faites en microscopie électronique. Seules les cultures en vol présentent un accroissement de la longueur cellulaire et un pourcentage de cellules en constriction plus élevé. L'augmentation de la valeur de la densité optique pour les cultures en milieu peptoné a montré l'existence d'une entrée plus précoce en phase stationnaire pour les échantillons «vol», mais cette observation n'a pu être confirmée par la numération des cellules viables. L'unique échantillon contenant les cellules fixées en vol a présenté des propriétés qui traduisent la stimulation de la croissance cellulaire. Toutefois, le regroupement de l'ensemble des observations nous amène à conclure que la microgravité n'a pas d'effet sur le taux de croissance de *E. coli* en phase exponentielle.

Mots-clés: Microgravité, Croissance cellulaire, *Escherichia coli*, Division cellulaire; Phase exponentielle, Navette spatiale.

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