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Independence of Buoyant Cell Density and Growth Rate in *Escherichia coli*

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The relationship between growth rate and buoyant density was determined for cells from exponential-phase cultures of *Escherichia coli* B/r NC32 by equilibrium centrifugation in Percoll gradients at growth rates ranging from 0.15 to 2.3 doublings per h. The mean buoyant density did not change significantly with growth rate in any of three sets of experiments in which different gradient conditions were used. In addition, when cultures were allowed to enter the stationary phase of growth, mean cell volumes and buoyant densities usually remained unchanged for extended periods. These and earlier results support the existence of a highly regulated, discrete state of buoyant density during steady-state growth of *E. coli* and other cells that divide by equatorial fission.

Early studies of buoyant cell density in exponential-phase cultures of *Escherichia coli* indicated that the density variation of individual cells is small (5, 9). Accurate measurements became possible with the subsequent introduction of the use of colloidal silica particles in equilibrium gradient centrifugation. Percoll (a suspension of colloidal silica particles coated with polyvinyl pyrrolidone; Pharmacia Fine Chemicals, Piscataway, N.J.) has very little effect on the osmolarity of gradient solutions. With Percoll gradients, Woldringh et al. (11) found that the density variation among cells in an exponentially growing culture was well within 1% of the mean buoyant density, and Martínez-Salas et al. (8) observed similarly narrow bands in gradients of another silica colloid, Ludox, mixed with polyvinyl pyrrolidone. Our own recent experiments also showed that the density variation in exponentially growing cultures was remarkably small, with a coefficient of variation of about 0.15% (7). Even this small value may be an overestimate because all experimental errors in equilibrium density banding act to broaden cell distributions.

Woldringh et al. determined the mean buoyant densities of *E. coli* cells as a function of culture growth rate and observed an increase of ca. 1% over the growth rate range of 0.5 to 3.0 divisions per h (11). From similar experiments, Martínez-Salas et al. (8) concluded that mean buoyant density was almost constant over the same range of growth rates for three different *E. coli* strains. However, our analysis of their data showed that the exponential-phase cultures of their three strains had significantly different average buoyant densities and therefore no common mean, as was assumed in their conclusions. Furthermore, in their experiments, density increased significantly with growth rate in one strain, B/rA, rather than remaining constant. With the other two strains, the data presented were insufficient to establish that either slope was significantly different from zero or from the value for B/rA. Therefore, their experimental results do not support their conclusion of density constancy for the three strains over a range of growth rates.

In this paper, we present experimental evidence for the constancy of mean buoyant cell density at different growth rates in cultures of an *E. coli* strain and show that it is unlikely that any such change with growth rate can be more than 0.001 g ml^{-1} h^{-1}. Our results suggest that in these *E. coli* cells, growth is characterized by discrete cellular states of buoyant density.

MATERIALS AND METHODS

Culture conditions. *E. coli* B/r NC32 [*fuc lac valS*(Ts) *rel*$^+$] (obtained from J. Jagger, University of Texas at Dallas) was cultured as described earlier (7) at 37°C in minimal M9 salts supplemented with glycerol (1%), glucose (0.4%), or alanine (0.1%) to obtain growth rates less than 1.4 doublings per h or in Luria broth without added glucose, with glucose (0.4%), or with glucose plus Casamino Acids (Sigma Chemical Co., St. Louis, Mo.) added to 0.1% to obtain more rapid growth rates. All media also contained 0.01% Hoagland's trace element salt solution (2) without selenium. For rapidly growing cultures, cells from a colony on Luria broth agar were inoculated into 30 ml of medium in sidearm flasks and grown overnight at 37°C with vigorous shaking. For media providing slowly growing cultures, inocula were taken from a culture that was grown in the same medium earlier during the week. Growth was measured as the increase in turbidity with a Klett-Summerson colorimeter provided with filter no. 66. In a few experiments, even slower growth rates were obtained in glucose-limited chemostat cultures.

Gradients and centrifugation. Gradients were composed of Percoll (Pharmacia Fine Chemicals; density 1.130 g ml^{-1}) and saline (0.15 M NaCl) or Percoll and growth medium, at starting concentrations of 95% Percoll. Linear 6-ml gradients were prepared in 15-ml Corex glass centrifuge tubes (Corning Glass Works, Corning, N.Y.) between selected density levels by diluting starting concentrations with growth medium or saline. Samples (1 ml) of cells in the exponential-growth phase were layered upon the gradients and centri-

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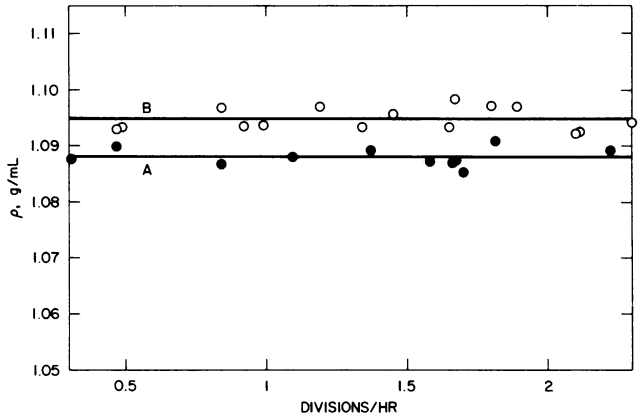

FIG. 1. Mean buoyant densities (ρ) of cells of *E. coli* B/r NC32 as a function of growth rate as determined in gradients of Percoll and growth medium. Symbols: (A) ●, centrifuged at 19 to 21°C; (B) ○, centrifuged at 1 to 4°C. The horizontal lines indicate the respective mean values of 1.0881 and 1.0949 g ml^{-1}.

fuged in an angle-head rotor (SS-34) of a Sorvall RC2-B centrifuge for 10 min at 14,000 rpm (23,000 × *g*) at either 1 to 4°C or 19 to 21°C. All band positions were measured in millimeters from the bottom of each tube.

To determine buoyant cell densities, 100-μl samples were extracted from the top, middle, and bottom of each band with a blunt-ended hypodermic needle attached to a calibrated syringe (The Hamilton Co., Reno, Nev.). Because of the sharpness of the visual bands, the precision of this method was equivalent to that obtained through the more tedious method of fractionating the gradient and determining cell numbers (7, 8, 11). The refractive index of each sample was measured with an Abbe refractometer. Buoyant densities were determined from linear calibration curves of density versus refractive index for each of the different Percoll-medium gradients used, as established by pycnometry.

Cell volumes. Mean cell volumes were determined from cell size distributions measured with a Coulter counter-multichannel analyzer system employing an aperture 17 μm in diameter and about 50 μm deep.

RESULTS

Figure 1 shows mean buoyant densities (ρ) of cells in exponentially growing cultures as determined in Percoll-medium gradients centrifuged at 19 to 21°C (line A, filled circles) or at 1 to 4°C (line B, open circles). The slopes of the linear regressions fitted to these data were very small and numerically less than their standard errors (see Table 1). Assuming constant buoyant density, mean values of the density were calculated for each temperature (Table 1) and are shown as the straight lines in Fig. 1. The results of a similar experiment using Percoll-saline gradients at 1 to 4°C are shown in Fig. 2. Although the results in this experiment were more variable than those shown in Fig. 1, the slope of the linear regression for these data was again indistinguishable from zero (Table 1), so these results also are consistent with a hypothesis of constant buoyant density at all growth rates.

Comparison of buoyant densities for cells centrifuged at the two different temperatures in Percoll-growth medium gradients (Fig. 1) shows that mean buoyant density was greater at the lower temperature, in agreement with earlier observations (11). This increased density presumably reflects the decreased osmotic pressure expected at lower temperatures, but the difference is too large to be explained by temperature shift alone. Because the density of water goes through a maximum at 4°C, the observed increase in buoyant density might also reflect a change in the molecular interactions between water and cell components. The difference in mean cell densities in the two different gradients at 1 to 4°C (Table 1) probably reflects changes in osmotic pressure that occurred when cells were centrifuged out of their original growth medium and into saline. The increased variability observed with Percoll-saline gradients also seems to be due, at least in part, to changes in cell volume that occurred when cells were removed from growth media of various osmolarities to saline.

The apparent independence of buoyant density and growth rate (Fig. 1 and 2), coupled with our earlier observations of the similar independence of cell-cycle phase and the narrow range of buoyant densities in exponentially growing cultures of this strain (7), suggested that growth might be characterized by one or more discrete states of buoyant density. To examine that possibility, we also measured buoyant cell densities in cultures that were allowed to enter the stationary growth phase. Figure 3 shows typical results obtained for an M9-glucose culture. As indicated by the initial increase in turbidity, this culture was in the exponential-growth phase at the beginning of the experiment. The growth rate began to decrease after about 1 h, and was markedly reduced by 4 h. Nevertheless, there were no significant changes either in mean cell volume or in buoyant density during the course of this experiment. In similar experiments with rapidly growing cultures in Luria broth medium, with doubling times of ca. 30 min, mean buoyant densities remained constant for about 2.5 h after the onset of stationary-growth phase and then slowly decreased. These experiments show, therefore, that mean buoyant densities remained unchanged in stationary phase cultures of NC32 for periods corresponding to several generations of growth in the exponential phase.

DISCUSSION

Our results show that the mean buoyant densities of cells in exponential phase cultures of *E. coli* NC32 are remarkably constant at all growth rates. We found no evidence for dependence of buoyant density upon growth rate in each of three sets of experiments in which different gradients or different temperatures were used (Table 1). In each case, there was no significant departure from density constancy, with standard errors of each slope of about 0.001 g ml^{-1} h^{-1} (Table 1). It is, therefore, unlikely that the mean buoyant density of these cells varies by more than this value over the entire range of growth rates. This degree of constancy of buoyant density was unexpected because the physical and chemical characteristics of *E. coli* cells are known to change

TABLE 1. Mean buoyant density of *E. coli* B/r NC32 as determined under different gradient and temperature conditions

Gradient	Temp (°C)	n[a]	Slope[b] (± SE) (g ml^{-1} h^{-1})	ρ[c] (± SE) (g ml^{-1})
Percoll-medium[d]	19–21	11	2.24 (± 9.07) × 10^{-4}	1.0881 (± 0.0005)
Percoll-medium	1–4	15	2.89 (± 9.88) × 10^{-4}	1.0949 (± 0.0005)
Percoll-saline	1–4	30	1.77 (± 1.15) × 10^{-3}	1.0919 (± 0.0008)

[a] Number of determinations.
[b] Slope of linear regression of ρ versus divisions per hour.
[c] Mean buoyant density, assuming ρ is independent of growth rate.
[d] Medium in which cells were cultured.

with growth rate. For example, cell volumes are known to increase by a factor of three or more over the range of growth rates used in these experiments (6), and, correspondingly, the cellular content of stable RNA, a dense macromolecule, increases by a factor of eight or more (3). On the other hand, two other relatively dense macromolecules, peptidoglycan and phospholipid, do not increase as rapidly as cell volume and would therefore tend to compensate for the increase in RNA, but this compensation is insufficient to explain density constancy.

Our results for buoyant density constancy differ from those of Woldringh et al. (11), who observed an increase of ca. 1% in mean buoyant density as growth rates were increased from 0.5 to 3.0 doublings per h. That increase, however, occurred almost entirely in a single step at about 1 doubling per h. Thus, when their values at low growth rates are excluded, the results of Woldringh et al. also appear to be constant. Recently, we found that buoyant cell density increases with the osmolarity of the growth medium (W. W. Baldwin and H. E. Kubitschek, manuscript in preparation), and this factor might account for the stepwise results of Woldringh et al. (11). In addition, the increase in buoyant density that they observed in a shift-up experiment would be expected on the basis of an increase in osmolarity, due to their additions of glucose and Casamino Acids to the medium.

Buoyant density constancy also was claimed in an earlier report by Martínez-Salas et al. (8), who concluded that buoyant-cell densities were almost constant over a wide range of growth rates for three strains of *E. coli*, B/rA, B/rF, and OV-2. Unfortunately, analysis of their experimental results (see Table 1, reference 8) does not support that conclusion. In strain B/rA they measured buoyant densities both in exponentially growing and age-selected cultures and averaged those values. However, the mean value (± standard error) for the age-selected cultures [1.1103 (±0.0013) g ml^{-1}] was significantly greater than that of the remaining B/rA cultures [1.1028 (±0.0009) g ml^{-1}] as determined by the *t* test for the means for populations composed of different numbers of observations. In addition, the mean buoyant density for strain OV-2 [1.1093 (±0.0026) g ml^{-1}] was significantly different from those for exponentially growing cultures of strain B/rA (see above) and for strain B/rF [1.1015 (±0.0010) g ml^{-1}]. Thus, their results do not support their conclusion that a common mean buoyant density exists for these strains, thus invalidating their quoted mean value.

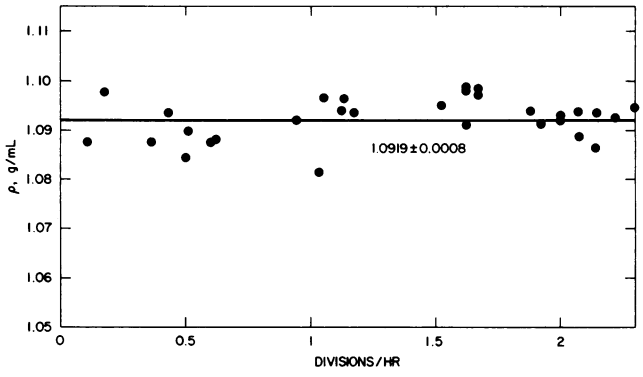

FIG. 2. Mean buoyant densities of cells of *E. coli* B/r NC32 as a function of growth rate, as determined in gradients of Percoll and 0.15 M NaCl at 1 to 4°C.

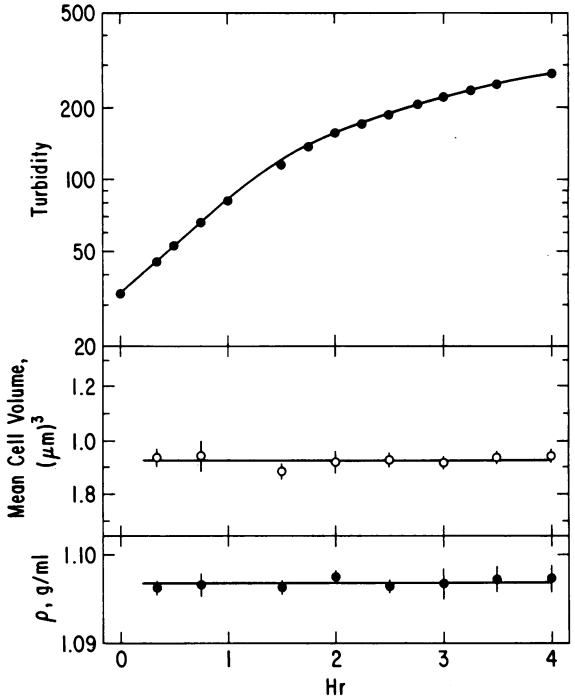

FIG. 3. Mean cell volumes and buoyant densities of cells of *E. coli* B/r NC32 in late-exponential and early-stationary phase in M9-glucose medium. Densities were measured in Percoll-saline gradients.

In addition, their values of mean buoyant densities for exponentially growing cultures of strain B/rA increased with growth rate, rather than remaining constant; the rate of increase [2.82 (±1.37) × 10^{-3} g ml^{-1} h^{-1}] was significantly greater than zero ($P < 0.01$). For the other two strains, neither slope differed significantly from zero nor from the value for the exponentially growing cultures of strain B/rA. Thus, their results support a growth-rate-dependent increase in density for nonselected cultures of one strain, B/rA, and provide no clear evidence for the dependence of density upon growth rate for the other two strains.

Earlier, we observed that the range of buoyant cell densities in exponentially growing cultures of *E. coli* NC32 was remarkably small and, in addition, that mean buoyant-cell densities appeared to be independent of age during the cell cycle (7). These results along with the independence of buoyant density and growth rate reported here suggest that in *E. coli* B/r NC32 there is a single, highly regulated state of buoyant density during the exponential-growth phase. Our measurements of buoyant density in stationary phase cultures support this possibility. Usually, there was no observable change in the mean buoyant density and mean cell volume in stationary-phase cultures for a period of several generation times after the first observable decrease in culture growth rate (Fig. 3), that is, until growth rates decreased three- or fourfold. These results show that control of buoyant density can be very stable, because a constant density was maintained well beyond the exponential-growth phase.

In contrast, buoyant density is known to vary during the cell cycle of the budding yeast *Saccharomyces cerevisiae* (4, 10; Baldwin and Kubitschek, unpublished data). This cyclic variation, however, is associated with a very different mode of cell division than that for *E. coli* in which daughter cells are formed by equatorial binary fission.

Discrete density states with narrow distributions of cell density and constant mean density during the cell cycle also have been observed for exponential-phase cultures of other kinds of cells that divide by equatorial fission, namely, Chinese hamster ovary cells (1) and cells from three murine lines (M. R. Loken and H. E. Kubitschek, J. Cell. Physiol., in press). In addition, preliminary observations in this laboratory indicate that the buoyant densities of cells of the fission yeast *Schizosaccharomyces pombe* are narrowly distributed and that mean buoyant densities are independent of growth rate. The sharp, discrete states of buoyant density maintained by each of these very different kinds of cells reveal the operation of highly accurate regulatory systems and, furthermore, suggest that very different kinds of cells may use the same or similar mechanisms for control of buoyant density.

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