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Buoyant Density Studies of Several Mecillinam-Resistant and Division Mutants of *Escherichia coli*†

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The buoyant density of wild-type *Escherichia coli* cells has previously been reported not to vary with growth rate and cell size or age. In the present report we confirm these findings, using Percoll gradients, and analyze the recently described *lov* mutant, which was selected for its resistance to mecillinam and has been suggested to be affected in the coordination between mass growth and envelope synthesis. The average buoyant density of *lov* mutant cells was significantly lower than that of wild-type cells. Similarly, the buoyant density of wild-type cells decreased in the presence of mecillinam. The density of the *lov* mutant, like that of the wild type, was invariant over a 2.8-fold range in growth rate. In this range, however, the average cell volume was also constant. Analysis of buoyant density as a function of cell volume in individual cultures revealed that smaller (newborn) *lov* mutant cells had higher density than larger (old) cells; however, the density of the small cells never approached that of the wild-type cells, whose density was independent of cell size (age). A pattern similar to that of *lov* mutant cells was observed in cells carrying the mecillinam-resistant mutations *pbpA*(Ts) and *rodA*(Ts) and the division mutation *ftsI*(Ts) at nonpermissive temperatures as well as in wild-type cells treated with mecillinam, but not in mecillinam-resistant *crp* or *cya* mutants.

The buoyant densities of several strains of *Escherichia coli* have been shown to be invariant with growth rate, cell size, and age (21–23). In contrast, the buoyant density of *Streptococcus faecium* ATCC 9790 and that of a strain of *Bacillus subtilis* have been shown to be variable with cell size (8, 10–12). In *S. faecium*, buoyant density increases as cells approach division, apparently because cytoplasmic mass increases faster than cell volume (12, 19).

Recently, a new mecillinam-resistant *E. coli* mutation, called *lov*, was isolated and characterized (4, 5). Mecillinam is a β-lactam which specifically inhibits penicillin-binding protein 2 (PBP2), which in turn is required for lateral elongation of the cell wall (28, 30). Cells bearing the *lov* mutation grew more slowly than the parent strain. However, their RNA content per unit volume, which in wild-type strains is a strong function of growth rate (24), was the same as that of *lov*$^+$ cells growing at the same rate (5). Certain *rpsL* mutations, affecting ribosomal protein S12 and conferring streptomycin resistance, were shown to restore mecillinam sensitivity to *lov* cells and result in faster growth. The allele specificity of the suppression suggested a direct interaction between the *lov* gene product and the ribosome. The intracellular concentration of ribosomes is known to increase sharply with increasing growth rate (24), and it was hypothesized that the *lov* gene product might be able to evaluate the ribosome concentration directly and transmit this information to the envelope-synthesizing machinery, including the PBPs, thus coordinating the rate of envelope growth with the mass growth rate (5).

If this hypothesis should prove to be correct, we speculate that surface and cytoplasmic growth might not be as well coordinated in *lov* mutant cells as in the parent strain, and as with *S. faecium*, this could lead to variation in buoyant density with respect to cell volume. We present here evidence that this is indeed the case.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. They are all *E. coli* K-12 derivatives with the exception of the B/r strain, kindly provided by H. Kubitschek. Bacteria were grown in either Luria-Bertani (LB) broth (Difco Laboratories) or minimal medium M63 or M9 (25). M63 and M9 were supplemented with glucose, L-alanine, or succinate at 0.4% or sodium acetate at 0.2%. Casamino acids, when used, were added at 0.4%. Amino acids (100 μg/ml), thiamine (1 μg/ml), and thymine (1 μg/ml) were added as required. The M63 medium, regardless of the carbon source, was adjusted to 250 mOsm (as determined by digimatic osmometer 3DII; Advanced Instruments Inc., Needham Heights, Mass.) by the addition of NaCl. The LB broth and M9 medium were adjusted to 200 and 220 mOsm, respectively. Mecillinam (1 μg/ml) (courtesy of J.-M. Ghuysen, University of Liège, Liège, Belgium) and cyclic AMP (cAMP, 1 mM) were added when necessary.

Cultures were grown aerobically in Erlenmeyer flasks (medium to flask volume ratio, 1:10) with agitation (120 rpm) at 28, 37, or 42°C. Cell growth was monitored both by turbidity at 675 nm (Spectronic 20; Bausch and Lomb) and by electronic particle measurements (model ZM; Coulter Electronics, Hialeah, Fla.). For all experiments, cultures were kept under 0.2 OD (dilutions were made with warm growth medium); all cultures were monitored for at least six mass doublings, and it was verified that average cell volumes remained constant. Cultures of GC2702 were periodically sampled and tested for revertants, detected by the appear-

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TABLE 1. Strains of *E. coli* used in this study

Strain	Relevant genotype
GC2700	F^- (λ^-), prototroph
GC2702	*lov*
GC3367	*thr leu thi lacY eda*::Tn*10*
GC3442	*ftsI*(Ts) *thr pro his arg thy rpsL*
GC7344	*pbpA*(Ts) *trp*(Am) *tyr*(Am) *supD*(Ts) *ilv*
GC7345	*rodA*(Ts) *trp*(Am) *tyr*(Am) *supD*(Ts) *ilv*
GC2793	*cya*
GC2846	*crp*
B/r NC32	*fuc lac valS*(Ts) *rel*$^+$

ance of large colonies (greater than ca. 0.5 mm in diameter) on LB agar after 24 h of incubation. Any culture showing more than 1% reversion was not considered.

Fractionation of cells by gradient centrifugation. The buoyant densities of cultures were measured by layering chilled cells, concentrated by centrifugation (8,000 × *g*, 10 min, 4°C), on 35-ml linear gradients of osmotically balanced Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.), contained in tubes (25 by 89 mm; Ultra-Clear; Beckman Instruments Inc., Palo Alto, Calif.), and centrifuged for 15 min at 4°C and 22,500 × *g* in a swinging-bucket rotor (SW27; Beckman). Gradients were fractioned at 4°C into 1-ml samples, collected from the top by displacement with 65% (wt/wt) sucrose. The refractive index, number, and distribution of cell volumes were measured for each fraction. In some cases, cells were removed from gradient fractions for examination by interference light microscopy.

Percoll, used to form gradients, was made isotonic to the growth medium by the addition of NaCl. Before use, gradients were chilled (4°C) for at least 2 h. The relationship of refractive index to buoyant density was determined by previously described gravimetric procedures (8). The electronic particle counter used to measure cell distributions and volumes was calibrated by the aggregate bead method detailed by Kubitschek (21). The counterelectrolyte used was an NaCl–0.005% formaldehyde solution made isotonic with the growth medium.

Significance. The data in this paper pose the question of when the difference between the buoyant densities of two cultures is significant. For our purposes, we have accepted an empirical definition based on analysis of the buoyant densities of 12 separate cultures of GC3367 grown in M63 medium (supplemented with glucose and casamino acids) on different days. While the standard deviation of these measurements was small (±0.0013 g/ml), the maximum deviation of these cultures from the mean was ca. 0.0035 g/ml. Thus, as a working definition, we describe the buoyant densities of cultures that repeatedly differ by more than 0.0035 g/ml as being significantly different. Further improvements in the analysis of cells distributed in Percoll gradients or in the method of measuring buoyant density may well show that differences of less than 0.0035 g/ml are significant. For the present, however, we prefer this conservative approach.

Light microscopy. Cell morphology was determined via interference light microscopy with a Zeiss Photomicroscope II (Carl Zeiss, Oberkochen, Germany). Both fixed and unfixed cells were examined. Fixation was carried out by adding formaldehyde to cells to a final concentration of 0.1% for 30 min at room temperature. Cells were applied to cover slips that had previously been coated with 0.1% polylysine. The coverslips were then sealed to microscope slides by the use of vaspar. Grey-level measurements were made of photomicrographs with an image analyzer (DUMAS System; Drexel University, Philadelphia, Pa.).

Cells were also observed with a combination of fluorescence and transmitted phase contrast illumination after staining with DAPI (4′,6-diamino-2-phenyl-indole; Boehringer Mannheim, Indianapolis, Ind.). The procedure was basically that of Hiraga et al. (14, 15), with the following differences: cells were fixed in 0.1% formaldehyde (wt/vol, final concentration) for 30 min at room temperature, centrifuged at 8,000 × *g* for 5 min at room temperature, and resuspended in 10 to 100 μl of an isotonic NaCl solution; 10 μl of the suspension was applied to a coverslip treated with 0.1% polylysine. The coverslip was then inverted and placed sequentially on 200-μl aliquots of 35, 50, 70, and 95% ethanol (10 s for each treatment). Finally, the coverslips were inverted over methanol for 5 min, washed by placement on five separate drops of isotonic saline, and then inverted over a 5-μg/ml solution of DAPI for 60 min. Excess liquid was removed by touching the coverslips to a paper towel. The coverslips were then placed on a microscope slide with 5 μl of saline isotonic to the growth medium and observed.

Analysis of cell volume distributions from Percoll gradient fractions. The analysis of changes in buoyant density as a function of cell size has been described previously (10, 12). Briefly, the frequency distribution of cell volumes is obtained by electronic particle counter for each gradient fraction. These distributions are normalized so that the summed frequencies of each equals 1 and then scaled to reflect the relative number of cells in the original fraction. The scaled distributions are then multiplied by the density of the corresponding gradient fraction. Finally, for every volume class, the scaled frequencies and the density weighted frequencies are summed. The summed scaled frequencies show the size distribution of the reconstructed population. The summed density weighted frequencies are divided by the scaled frequencies to calculate the average density for each volume class.

RESULTS

Studies of average buoyant density. The buoyant density of several strains of *E. coli* has been shown to be invariant with growth rate (21) and to increase with increasing osmolarity of the growth medium (2). Since our method of analysis with Percoll gradients differs from the methods that led to these conclusions (2, 22, 23), we examined the effects of growth rate and osmolarity on the buoyant density of strain B/r NC32. These measurements formed, in effect, a control for the studies reported here.

To vary the growth rate of B/r NC32, it was cultivated at 37°C in M9 salts supplemented with glucose and casamino acids, glucose alone, or L-alanine, resulting in mass doubling times of 33, 49, and 114 min, respectively. The osmolarity of each medium was adjusted (if necessary) to 220 mOsm. Our results, in agreement with those of previous reports, showed that the buoyant density of strain B/r NC32 did not differ significantly over a 3.5-fold range in growth rate. The average buoyant density was 1.1048 g/ml and varied by no more than 0.0009 g/ml; to be significantly different, in our conditions, cultures should differ by more than 0.0035 g/ml (see Materials and Methods). When cells were grown in M63 at 250 mOsm, with the same supplements as above, the buoyant density increased by about 0.0059 g/ml, to 1.1107 g/ml, again in agreement with previous reports (2).

Thus, we established that our methods lead to conclusions that parallel those of past investigations. We therefore

TABLE 2. Cell volume and average buoyant density of *lov*$^+$ and *lov* cells as a function of mass doubling time[a]

Carbon source	GC2700 (*lov*$^+$)			GC2702 (*lov*)		
	T_d (min)	Vol (fl)	Buoyant density[b] (g/ml)	T_d (min)	Volume (fl)	Buoyant density (g/ml)
Casamino acids + glucose	29	1.33	1.1052 (6)	68	0.63	1.0911 (5)
Glucose	46	1.10	1.1058 (5)	79	0.58	1.0921 (4)
Succinate	72	0.64	1.1058 (2)	100	0.62	1.0911 (1)
Acetate	157	0.54	1.1045 (2)	190	0.62	1.0923 (1)

[a] In all cases, the growth temperature was 37°C and the growth medium was M63, which was adjusted, if necessary, with sodium chloride to 250 mOsm. T_d, mass doubling time.

[b] Individual gradients with coefficients of variation of less than 0.0025 g/ml for GC2700 and 0.0035 g/ml for GC2702 were used for this analysis. The number in parentheses is the number of experiments performed for that growth condition; where $n > 1$, the reported values are averages. The maximum variation from the mean was 0.0016 g/ml, and the average variation from the mean was 0.008 g/ml.

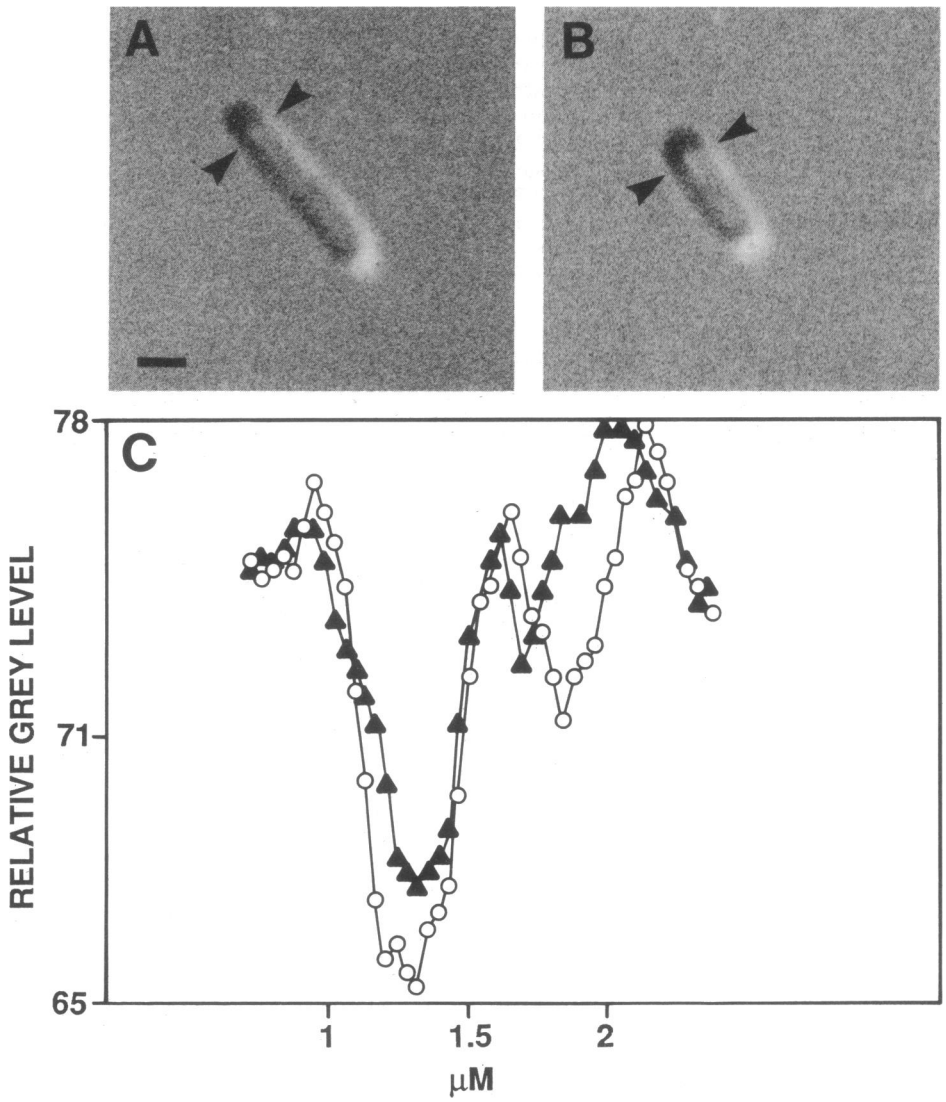


FIG. 1. Differential interference light micrographs of unfixed cells of GC2700 *lov*$^+$ (A) and GC2702 *lov* (B) and relative grey-level scans of selected portions of these micrographs (C). Before photography, cells were oriented in a similar direction with a rotating stage, which was maintained at 37°C. The grey-level scans were made by image analysis of the photographs at the locations and in the direction shown by the arrows. The cells in panels A and B were grown in M63 supplemented with glucose and glucose plus casamino acids, respectively. (A) Bar, 1 μm. In panel C, the open circles represent measurements from GC2700 cells and the solid triangles represent measurements from GC2702 cells. μM, micrometer.

TABLE 3. Buoyant density of *pbpA*(Ts), *rodA*(Ts), and *ftsI*(Ts) mutants at the nonpermissive temperature in LB broth[a]

Strain	Temp, °C (min of incubation)[b]	Vol (fl)	Buoyant density (g/ml)	Change in buoyant density[c] (g/ml)
GC7344 (*pbpA*)	28 (0)	2.26	1.0927	
	42 (60)	3.72	1.0894	0.0033
	42 (90)	5.40	1.0907	0.0020
	42 (120)	6.31	1.0901	0.0026
GC7345 (*rodA*)	28 (0)	2.26	1.0917	
	42 (60)	3.82	1.0899	0.0018
	42 (90)	5.23	1.0901	0.0016
	42 (120)	5.79	1.0899	0.0018
GC3442 (*ftsI*)	28 (0)	2.39	1.1009	
	42 (20)	3.28	1.1084	0.0075
	42 (40)	5.03	1.1014	0.0015
	42 (60)	6.42	1.0979	0.0030

[a] Since the osmolarity of LB broth was 200 mOsm (as opposed to M63, which was 250 mOsm), these buoyant densities cannot be directly compared with those reported in Table 2. LB broth was used in these experiments because it gave the clearest morphological changes when the temperature was raised to 42°C (spherical cells for GC7344 and GC7345 and filaments for GC3442).

[b] Time that a culture was incubated at the nonpermissive temperature.

[c] These values were calculated by subtracting the buoyant density of a culture after the given time of shift-up to the nonpermissive temperature from that measured at 28°C. The experiments shown were all performed a minimum of three times; the data represent a single typical example. However, in no case, except for *fstI* after 20 min at 42°C, did the buoyant density of any sample differ significantly from that of the cells grown at 28°C.

TABLE 4. Effects of *cya* and *crp* mutations on mass doubling time, volume, and buoyant density of cells grown in M63 with glucose and casamino acids[a]

Strain	1 mM cAMP present	T_d (min)	Vol (fl)	Buoyant density (g/ml)	Change in buoyant density (g/ml)[b]
GC2700 (*lov*$^+$)	No	30	1.41	1.1050	
	Yes	30	1.44	1.1047	0.0003
GC2793 (*cya*)	No	44	0.83	1.1035	0.0015
	Yes	37	1.09	1.1043	0.0007
GC2846 (*crp*)	No	48	0.82	1.1051	0.0001

[a] All these experiments were done at least three times; the data shown are for a single typical example. In no case did the mutant strains (with or without cAMP) differ significantly from the wild type. T_d, mass doubling time.

[b] The change in buoyant density was calculated by subtracting the buoyant density for a particular mutant growing either in the absence or presence of cAMP from that of the wild type (GC2700) grown without cAMP.

applied these methods to the *lov* mutant strain GC2702 and its wild-type K-12 parent strain GC2700. The *lov* mutant grows more slowly than its parent in all media studied (5) (Table 2). Its average buoyant density, like that of the wild-type strain, was independent of the growth rate over a 2.8-fold range, with a maximum deviation from the mean of 0.0007 g/ml (Table 2). Strikingly, however, this density was significantly lower than that of the *lov*$^+$ parent. The difference in all media was about 0.014 g/ml, well above our significance threshold of 0.0035 g/ml.

A clue to the lower density of the *lov* mutant cells was obtained by differential interference light microscopy. Micrographs of fixed and unfixed cells suggested a higher peripheral refractive index (packing density) in the wild-type cells than the *lov* mutants (Fig. 1). The apparent lower central density seen in both cell types reflects a proportional enrichment of DAPI-staining material (data not shown). This is also consistent with the lower density of the *lov* mutant strain being in part due to an alteration in the configuration of cytoplasmic materials or in their state of hydration.

The *lov* mutant was originally isolated by its resistance to the β-lactam mecillinam (4). To determine whether the lower buoyant density of *lov* mutant cells was a general characteristic of mecillinam resistance, we examined several other mecillinam-resistant mutants. We first studied a *pbpA*(Ts) strain, mutated in the structural gene for PBP2, the target of mecillinam, and a *rodA*(Ts) strain, with a mutated RodA protein, which is thought to act together with PBP2 (16, 29). These two proteins are required for lateral elongation of the cell wall and maintenance of rod shape (29). The mutants grow as bacilli at 30°C and as cocci at 42°C (9). Neither mutant showed a significant change in buoyant density after the shift to the nonpermissive temperature, even at times when the morphological change was complete (Table 3). However, the average buoyant density observed was consistently less than that of a wild-type strain (GC2700) grown in LB broth (see Table 5) by 0.0076 to 0.0120 g/ml, slightly less than the decrease observed in the *lov* mutant.

PBP3 is required for septation but not for lateral elongation of the cell wall (9, 30), so in this sense it is complementary to PBP2. Inactivation of PBP3, whether by mutation in the *ftsI* structural gene or by specific β-lactams, results in filamentous growth. However, inactivation of PBP2 by mecillinam can also lead to a cell division block (18, 26), and it has been suggested that this may reflect a regulatory circuit in which the *lov* protein connects PBP2 and PBP3 activities (5, 9a). We therefore examined the buoyant density of an *ftsI*(Ts) mutant. The *ftsI* mutant did not exhibit a significant change in density when shifted to 42°C, with the possible exception of a slight transient increase after about 20 min (Table 3). It was notable, however, that the mecillinam-sensitive *ftsI* mutant was about 0.0080 g/ml denser than the *pbpA* and *rodA* strains (Table 3) and not significantly different from GC2700 grown in the same medium (see Table 5).

PBP2 and RodA are intact and functional in *lov* mutant cells, which are rod-shaped in the absence of mecillinam and spherical in its presence (5). A similar phenotype has been observed in *cya* and *crp* mutants (7), defective in adenyl cyclase and the cAMP receptor protein, respectively. Both of these mutants are resistant to mecillinam (1, 17). The cAMP-catabolite gene activator protein (CAP) complex is known to activate or repress the transcription of a large number of operons in *E. coli* (3, 27, 31), one or more of which may be involved in the cell's normal sensitivity to mecillinam. The buoyant density of the *cya* and *crp* mutants was not significantly different from that of the wild-type strain, and the presence of 1 mM cAMP in the medium had no effect on density in the *cya* mutant or parental strain (Table 4).

The above results show that the lower buoyant density of the *lov* mutant is not obligatorily associated with mecillinam resistance or with the morphological changes (rods to spheres) caused by inactivation of PBP2 or the RodA protein. However, the analysis is not entirely clear, since the *pbpA*(Ts) and *rodA*(Ts) mutants used are able to grow at 42°C, whereas complete inactivation of PBP2 or RodA is lethal in LB broth (25a, 26). This lethality can be suppressed by *lov* and *cya* mutations, and it is possible that the *pbpA*(Ts) and *rodA*(Ts) strains used harbor additional mutations of this type.

TABLE 5. Effects of mecillinam treatment on buoyant density of *lov*$^+$ and *lov* cells

Strain	Medium[a]	Treatment time (min)	Vol (fl)	Buoyant density (g/ml)	Change in buoyant density (g/ml)	% Viable cells[b]
GC2700 (*lov*$^+$)	LB broth	0	2.65	1.1003		100
		30	3.10	1.0923	0.0081	99
		60	4.33	1.0860	0.0143	45
		70	4.89	1.0834	0.0170	44
	M63 + glucose + casamino acids	0	1.36	1.1046		100
		40	1.71	1.0965	0.0080	88
		60	2.20	1.0955	0.0090	57
GC2702 (*lov*)	M63 + glucose + casamino acids	0	0.61	1.0893		100
		70	1.02	1.0875	0.0019	100

[a] The effects of mecillinam (1 μg/ml) over time could not be observed with the *lov* mutant in LB broth because in this medium the cells were too low in buoyant density to penetrate the gradients used in this study.

[b] Viability was established from plate counts on LB agar. These experiments were carried out at least three times; data are shown for a single typical example. In every experiment the buoyant density of the *lov*$^+$ strain decreased significantly after 30 min of treatment, whereas that of the *lov* mutant strain did not show significant decreases even after 70 min of treatment.

To evaluate the effect of PBP2 inactivation on buoyant density in well-defined genetic backgrounds, we examined the effect of mecillinam on density. For wild-type cells grown in LB broth or M63 glucose, exposure to mecillinam (1 μg/ml) produced a significant decrease in buoyant density, which approached the buoyant density of untreated *lov* mutant cells (Table 5). This density decrease was accompanied by a loss of viability. With the *lov* mutant, in contrast, no significant density change was observed, at least over the 70-min period of observation, and viability was not affected by mecillinam (Table 5).

Studies of changes in buoyant density as a function of cell volume. While measurements of average buoyant densities are instructive, they cannot reveal possible variations in buoyant density with cell size during the cell cycle. Therefore, for each of the cultures studied above, the buoyant density was measured as a function of cell volume (see Materials and Methods).

Using this approach, we confirmed that buoyant density is essentially invariant with increasing cell volume for two wild-type strains (B/r NC32 and GC2700 [*lov*$^+$]; Fig. 2A and B, respectively). In contradistinction, a significant decrease in buoyant density was observed with increasing cell volume in the GC2702 (*lov*) cultures (Fig. 2C). Usually, the largest decreases were observed in *lov* cells that were below the modal volume. The magnitude of this decrease was greatest in rapidly growing cells but was observable in cultures at all mass doubling times studied.

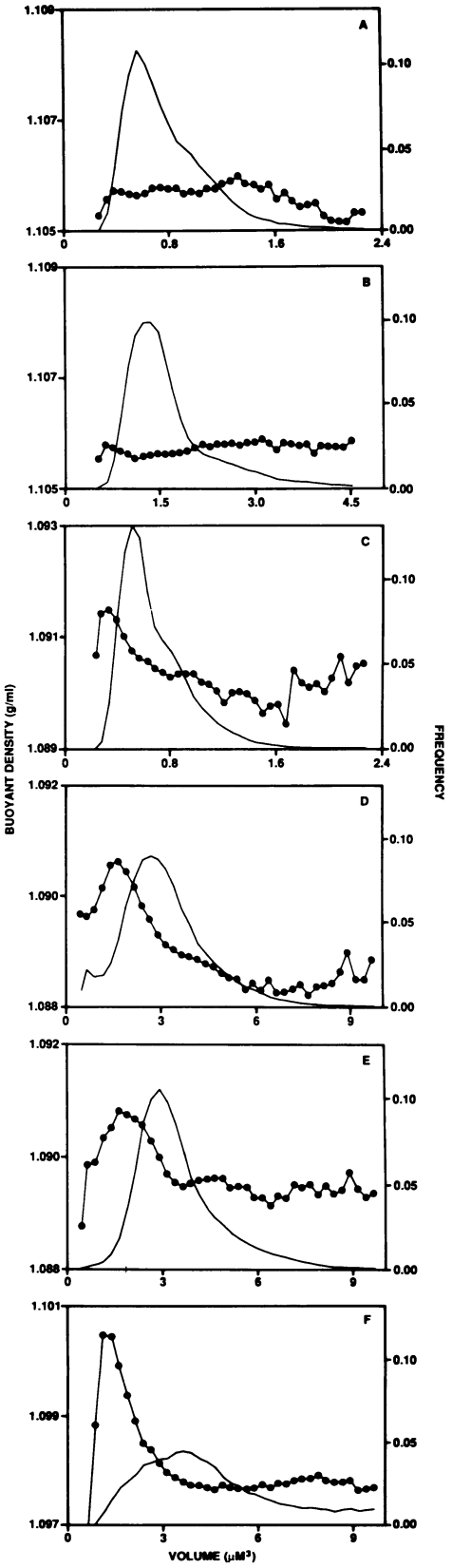
As stated earlier, it has been proposed that the *lov* gene product might be a mediating factor in coordinating surface and cytoplasmic growth and might in some way interact in a regulatory fashion with "morphogene" products, such as PBP2, RodA, and PBP3 (5, 9a). If this model or some variation is valid, one prediction might be that mutations in genes such as *lov*, *pbpA*, *rodA*, or *ftsI* might disrupt the normal coordination between surface and cytoplasmic growth. This might in turn disturb the usual constancy seen in *E. coli* between buoyant density and cell size. The data in Fig. 2D, E, and F are consistent with this prediction. In each case, temperature-sensitive mutants carrying *pbpA*(Ts), *rodA*(Ts), and *ftsI*(Ts), when elevated to the nonpermissive temperature (but not at the permissive temperature), showed a significant increase in the average buoyant density of small cells (similar in pattern to that seen in *lov* cultures). This pattern was also observed when mecillinam was added to wild-type GC2700 (*lov*$^+$) cells. However, it was not seen in the mecillinam-resistant *cya* and *crp* mutants. We must qualify this last statement in that in about one out of three experiments a slight increase in buoyant density was seen in the smallest *cya* cells. However, this increase was not consistent, and the increase was in range of the largest increases observed in wild-type cells. Thus, at this time, we suggest that the possible variation in the buoyant density of the *cya* mutant is an open question which the sensitivity of our detection methods does not allow us to answer.

We conclude that buoyant density decreases with increasing volume in *lov* cells, in wild-type cells treated with mecillinam, and in *pbpA*(Ts), *rodA*(Ts), and *ftsI*(Ts) mutants elevated to the nonpermissive temperature, but probably not in *cya* and *crp* mutant cells. The comparative constancy of the buoyant density of the *cya* and *crp* mutants implies that buoyant density variability is not an obligatory phenotypic characteristic of mecillinam resistance.

DISCUSSION

We show here that the buoyant density of *E. coli* cells with and without the *lov* mutation is growth rate invariant; however, the density of *lov* cells is consistently about 0.014 g/ml less than that of the parent at all growth rates studied. At this time we have no explanation for the lower buoyant densities of the *lov* cells. Since *lov* cells have approximately the same amount of RNA and protein per cell volume as do the parent cells when grown with the same mass doubling time (5), the lower densities of the *lov* cells might be due to some component(s) of these cells being more hydrated or to *lov* cells differing in pool content or cellular composition. Observation by differential interference contrast microscopy does suggest that the lower densities of the *lov* cells may result from some of their components being arranged in a different manner and/or in a different hydration state.

Also, besides having lower buoyant densities, *lov* cells differed from parent cells by showing decreases in buoyant density with increasing cell volume (i.e., increasing cell age). These decreases were greater in *lov* cells grown in rich than in poor medium. Since past studies as well as those reported here have concluded that the buoyant density of wild-type strains of *E. coli* is invariant with cell size (21), the observation that *lov* cells demonstrate a decrease in buoyant density with increasing cell volume is significant. Such



fluctuations were also seen at nonpermissive temperatures in *ftsI*(Ts), *pbpA*(Ts), and *rodA*(Ts) mutants as well as in *lov*$^+$ cells treated with mecillinam (but not reproducibly in *cya* or *crp* cells). As discussed, it has been postulated that the *lov* gene product, by some unknown mechanism, may coordinate cytoplasmic growth and envelope enlargement, perhaps by interating in some manner with the *pbpA-rodA* and/or *ftsI* gene products to affect cell cycle-related changes in the rates of cylindrical and septal wall growth (5, 9a). This would be consistent with the idea that coordination of rod and septal growth is needed for the buoyant density of *E. coli* to remain constant normally (6).

Our data are consistent with these ideas, since they suggest that whenever one part of the proposed shape-regulatory matrix experiences a lesion or is blocked by an inhibitor (e.g., by a mutation in *pbpA*, *rodA*, or *ftsI* or by the addition of mecillinam), surface and cytoplasmic growth become uncoupled and buoyant density is no longer invariant with cell volume. We do not imply that this list of components is complete. We also cannot say whether the *lov* gene product is the major regulatory element or merely an element involved in coordinating surface and cytoplasmic growth.

The final question we discuss is why the buoyant density of supposedly deregulated mutants is highest in cells with the lowest volumes (newborn cells).

In *S. faecium* ATCC 9790 (also known as *Streptococcus faecalis* and *Enterococcus hirae*), buoyant density is not constant in wild-type cells but increases with increasing cell size (8, 10, 12). It has been proposed that this pattern results from the organism's being able to increase its size only by discrete septal sites undergoing constrictive division (13). In this case, as the division furrow becomes progressively narrower, less and less new volume is made per unit of new cell surface (13, 19). Thus, buoyant density may increase because the cytoplasmic mass increases faster than the new cell volume. This increase in density has been suggested to be directly or indirectly related to the production of new septal sites (8, 10, 12).

Applying this model to *E. coli*, the suggestion would be that *lov* mutants and mecillinam-treated cells and mutants carrying *pbpA*, *rodA*, *ftsI*, etc., have problems in producing the proper number of new envelope growth sites at the proper time or that new sites might not produce envelope material fast enough, perhaps because these cells do not have an accurate estimate of how fast their cytoplasmic components are being made. The result might be that early in the cycle, the cell surface grows more slowly than the cytoplasm, resulting in small cells with higher buoyant densities.

FIG. 2. Relationship between average buoyant density and cell volume in wild-type and mutant cultures. In each panel, the solid line shows the distribution of cells, and the solid circles represent the average density. The method used to construct Fig. 2 is described briefly in Materials and Methods and in more detail in reference 12. Note that the volume scales of the figures are not all the same. Panels show typical results from exponential-phase cultures of B/r NC32, GC2700 (*lov*$^+$), and GC2702 (*lov*) grown at 37°C (A, B, and C, respectively) and temperature-sensitive mutants GC7344 (*pbpA*), GC7345 (*rodA*), and GC3442 (*ftsI*) after 60 min of growth at 42°C (D, E, and F, respectively). Cultures used for these studies were grown in either M9 growth medium (A, B, and C) or LB broth (D, E, and F). μM, micrometer.

This model is not proposed as an exclusive solution but rather as a stimulus for further experimentation.

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