Relationship between Changes in Buoyant Density and Formation of New Sites of Cell Wall Growth in Cultures of Streptococci (*Enterococcus hirae* ATCC 9790) Undergoing a Nutritional Shift-Up

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When the glutamate concentration of cultures of *Enterococcus hirae* was raised from 20 to 300 $\mu\text{g/ml}$, the mass doubling time decreased from ca. 85 to 45 min in 9 min, but balanced growth was not reestablished for 30 to 40 min. During the unbalanced period of growth, RNA and protein synthesis proceeded more rapidly than did peptidoglycan synthesis, buoyant density increased from ca. 1.1024 to 1.1075 g/ml, and the rate of formation of new cell wall growth sites transiently accelerated above the new growth rate. When studied as a function of cell size, all cultures showed buoyant density to decrease around cell separation, increase as cells increased in size, and then plateau when cells reached large volumes. Greater increases in buoyant density as a function of cell size were seen after shift-up, with the greatest increases observed at 15 to 20 min after shift-up, when the rate of formation of new sites was also maximal. In a population of cells examined by electron microscopy 15 min after shift-up, buoyant density and the frequency of cells with new sites increased as old sites approached the size of two poles. These data were consistent with a model whereby buoyant density increases in the terminal stages of the cell cycle when the surface grows slower than the cytoplasm. The greater the difference in the rates of inside to outside growth, the greater the increase in buoyant density and the more frequently new sites will be initiated.

In *Enterococcus hirae* (classified in the past as *Streptococcus faecium*), the cell wall grows in discrete annular sites. Each site of cell wall growth produces two polar caps (9). The size of these caps does not change with growth rate (4). Therefore, the amount of new surface made by a site is limited and growth rate independent. As a site grows to the size of two poles, the buoyant density of the cell increases (3, 6). In rapidly growing cells, the increase in buoyant density is larger and new sites appear well before division, whereas in slower growing cells, the increase in buoyant density is much less and sites are made just before, on, or just after division (6).

Thus, new sites are produced in parallel with an increase in buoyant density. We have suggested that new cell wall growth sites are introduced as a result of the inability of old sites to grow fast enough to accommodate continued exponential growth of the cytoplasm (6, 12). The introduction of new sites permits the surface to grow rapidly enough for buoyant density to decrease. This continues until these sites become large enough that their rates of growth become limiting and buoyant density once again begins to increase.

Here these ideas are explored by studying cultures undergoing rapid increases in growth rate upon the addition of glutamate (i.e., a nutritional shift-up). Past studies have shown that after shift-up, cells produce a burst of new sites before cultures resume balanced growth (5). This system has been restudied to test the prediction that the increase in the number of sites observed on shift-up is accompanied by a corresponding increase in buoyant density.

MATERIALS AND METHODS

Cell growth. Cultures of *E. hirae* (ATCC 9790) were grown in a chemically defined medium that initially contained glutamate (20 $\mu\text{g/ml}$) and no glutamine (13, 14). Cell growth was monitored both by turbidity measurements (675 nm) (5) and by electronic particle measurements (model ZM; Coulter Electronics, Hialeah, Fla.) (6). When cultures had undergone at least six exponential doublings in mass and the kurtosis of the distribution of cell volumes was less than 0.2, the final glutamate concentration was raised to 300 $\mu\text{g/ml}$. This nutritional shift-up was usually carried out when the cell mass reached the equivalent of 86 to 90 $\mu\text{g/ml}$ of dry mass.

The methods for the measurement of RNA, protein, and peptidoglycan synthesis by the incorporation of radioactive isotopes of uracil, leucine, and lysine, respectively, into cold acid precipitates have been described several times previously (1, 5, 7). The only modification in technique was that cells used for peptidoglycan determination were collected by vacuum filtration onto glass fiber filters (GF/C, 24 mm; Whatman International, Maidstone, England) rather than by the usual procedure of initially placing samples into 10 ml of cold trichloroacetic acid (1). After cells had been collected on filters and washed five times with 2 ml of cold trichloroacetic acid, they were treated as in the past. In our hands, the collection of cells on filters reduced the variance between duplicate samples and was used in all studies reported here.

The buoyant density of cultures was measured by placing chilled cells, concentrated by centrifugation ($8,000 \times g$, 10 min, 4°C), on 35-ml linear gradients of Percoll (1.085 to 1.142 g/ml; Pharmacia Fine Chemicals, Piscataway, N.J.) contained in 25- by 89-mm tubes (Ultra-Clear; Beckman Instruments, Inc., Palo Alto, Calif.) and centrifuging the cells for 10 min at 4°C and $22,500 \times g$ in a swinging-bucket rotor

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FIG. 1. Turbidity, cell number, new cell wall growth sites and dividing cells per milliliter, and buoyant density during a nutritional shift-up. At zero minutes, the glutamate concentration of cultures was raised from 20 to 300 $\mu\text{g/ml}$. Cell growth was monitored by turbidity measurements, and samples were taken at the times indicated for cell number (A), electron microscopy measurements of new cell wall growth sites and dividing cells per milliliter (B), and buoyant density determination (C). Small or newly initiated cell wall growth sites (SS; insert to Fig. 1B) were classified as cells with sites greater than zero but smaller than $0.046 \mu\text{m}^3$ and those with central site with furrows (F) less than $0.36 \mu\text{m}$, respectively. The number of Percoll gradients analyzed to make panel B were, starting at zero time, 18, 12, 18, 10, 12, and 4, respectively. Symbols: Δ , turbidity; $\blacktriangle$, cell number; $\blacksquare$, small sites per ml; $\square$, dividing cells per ml; $\bullet$, buoyant density plus and minus 1 standard deviation.

(SW27; Beckman). Fractions of 1 ml each were collected from the top of each tube by displacement with 65% (wt/wt) sucrose (3, 6). The refractive index, number, and distribution of cell volumes was measured for each fraction. In some cases, cells were removed from gradient fractions for examination by electron microscopy as described below.

Electron microscopy. Preparation of carbon-platinum replicas of cells has been described (4, 5). Measurements were made directly from negatives of electron micrographs of cells with a Dumas image analysis system (Drexel University, Philadelphia, Pa.) (6). For this study, we defined a small or newly made site of cell wall growth (SS; insert to Fig. 1B) as having between wall bands a volume greater than zero but less than $0.046 \mu\text{m}^3$ and cells in the division phase as having a central furrow (F; Fig. 1B) less than $0.36 \mu\text{m}$. For each sample studied, at least 200 cells were measured.

Analysis of cell volume distributions from Percoll gradient fractions. By rearranging the data obtained from the study of Percoll gradient fractions, we have devised a method of graphically showing changes in buoyant density as a function of cell size. The procedure is shown in a semidiagrammatic form in Fig. 2. Figure 2A is a modified frequency distribution of a population of cells fractionated on a Percoll gradient. For purposes of clarity, the number of fractions shown has been reduced from the 11 usually analyzed to 5. The frequency distribution of cell volumes obtained by electronic particle counter for each gradient fraction is given in Fig. 2B. Note that the distributions in Fig. 2A and 2B have been normalized so that their summed frequencies each equals 1. To scale the distributions in Fig. 2B so that they reflect the relative number of cells that were in the original population, each distribution is multiplied by the frequency in Fig. 2A (y axis) that describes the portion of population that was in the fraction that produced a given size distribution. These scaled distributions are shown in Fig. 2C and are used to produce a set of weighted density distributions (not shown). The weighted distributions are made by multiplying the scaled distributions in Fig. 2C by the density of the gradient fractions (taken from the x axis of Fig. 2A) that are used to produce each scaled volume distribution. The last part of the process requires that for every volume class shown in Fig. 2C, two values are summed. These are a sum of the scaled frequencies (Fig. 2C) and a sum of the weighted density frequencies. The summed scaled frequencies show the size distribution of the reconstructed population (solid line in Fig. 2D); by dividing the summed weighted frequencies by the scaled frequencies, the average density for each size class can be calculated (filled circles in Fig. 2D).

This same approach can be used for measurements from electron micrographs except that in this case the volume of the central growth site (CS; insert to Fig. 1B) is used to size cells (6). For this analysis, 200 cells were measured from the Percoll gradient fraction containing the greatest number of cells and the two fractions on either side of this peak fraction.

RESULTS AND DISCUSSION

Comparison of buoyant density with new site production and macromolecule synthesis. When the glutamate concentration was raised from 20 to 300 $\mu\text{g/ml}$ in cultures of *E. hirae* growing in the absence of glutamine, the mass doubling time decreased after about 9 min from 85 to 45 min (Fig. 1 and Table 1). Below we describe the effect of shift-up on (i) buoyant density, (ii) the number of new sites and number of cells in the division phase, and (iii) macromolecular synthesis and cell division.

Buoyant density. On the addition of glutamate, buoyant density increased from ca. 1.1024 to 1.1075 g/ml (Fig. 1C). The increase began virtually immediately and was approximately linear for about 20 min. Thereafter, the rate of increase slowed until it reached a higher constant value by 30 to 40 min.

New sites and cells in the division phase. As we have shown before (5), the addition of excess glutamate results in a very large temporary increase in the rate of formation of new sites of cell wall growth. Our current measurements indicate that this increase is apparently without lag and is initially much more rapid than any other measured element of growth (Fig. 1B). After 15 to 20 min it slowed, and by 30 to 40 min the rate of formation of new cell wall growth sites had reached the new value appropriate for the new, higher rate of mass

FIG. 2. Graphic representation of steps used in calculating the average buoyant density of a culture of cells. (A) Hypothetical distribution of cells fractionated from a Percoll gradient. Each fraction has a different symbol, which is used below to refer to cell volume distribution obtained from analyzing a given fraction. (B) Volume distributions obtained from studying the five Percoll gradient fractions in panel A with a Coulter counter. (C) Result of multiplication of each frequency distribution in panel B by the frequency given in panel A which applies to a given distribution of cell volumes. The result in panel C is a series of scaled frequencies

TABLE 1. Time that elements of macromolecular synthesis continue to be synthesized at the rate observed before glutamate addition^a

Determination	Time ^b (min)	SD	No. of observations
Mass	9.1	4.5	43
Cell no.	12.6	6.8	34
Incorporation of radioactive:			
Uracil	1.6		2
Leucine	2.0	0.6	7
Lysine into peptidoglycan	12.1 ^c	2.2	8

^a At zero time, the glutamate concentration of cultures was raised from 20 to 300 $\mu\text{g/ml}$. This resulted in a decrease in the mass doubling time from 84.5 ± 16.4 to 39.1 ± 4.8 min ($n = 43$).

^b All times were based on regression analysis.

^c After glutamate addition, peptidoglycan was synthesized at the pre-shift-up rate for ca. 12 min and then slowly accelerated to the new shift-up rate for mass over a 20- to 30-min period.

increase. After an initial lag of about 10 min, the number of cells in the division phase showed acceleration-deceleration kinetics (Fig. 1B) similar to that observed earlier for new site production (Fig. 1B).

Macromolecular synthesis and cell division. For purposes of comparison, Table 1 gives the periods after excess glutamate was added before RNA, protein, and peptidoglycan synthesis and cell division reached the new, more rapid rate of balanced growth.

In the past, complex kinetics (as in references 10 and 11) were reported for the increase in cell numbers for *E. hirae* after shift-up (5). About 40 min was required for cultures to reach the new rate. By adding glutamate to cultures that have slightly higher initial turbidities (i.e., equivalent to 90 rather than 77 $\mu\text{g/ml}$ of dry weight), we no longer see the complex kinetics observed previously, and the post-shift-up lag, or rate maintenance period, is about 12 min or less. It is possible that these results are due to the increase in autolytic capacity that occurs in *E. hirae* as culture turbidity increases (L. Daneo-Moore, personal communication).

We previously reported that on shift-up, peptidoglycan synthesis continued at the old, pre-shift-up rate for about 20 min (5). On restudy, we now find that the most reproducible pattern is a period of synthesis at the pre-shift-up rate of about 12 min, with the rate of synthesis slowly accelerating to the new growth rate in about 20 to 30 min.

Upon shift-up, RNA and protein synthesis and the frequency of new wall growth sites begin to increase before peptidoglycan synthesis and before the number of cells in the division phase increases.

Interpretation. These data suggest that on shift-up there is a close linear increase in buoyant density until balanced growth is reestablished.

If RNA and protein synthesis are correlated with cytoplasmic mass growth, and if peptidoglycan synthesis is corre-

lated with cell size, then the data suggest that the buoyant density of cells more accurately reflect the distribution of cell sizes of each fraction in the total population. The sum of the areas under all five curves is 1. (D) Final result of this procedure, showing the summation of the scaled frequencies in panel C (—) and the average buoyant density of cells in each size class (●). The average buoyant densities were obtained by multiplying each distribution in panel C by the buoyant density of the Percoll gradient fraction used to make the distribution (taken from panel A), summing these products for each distribution for each volume class, and dividing this sum by the sum of the scaled frequencies for each size class.

lated with surface growth, then our incorporation data are consistent with the idea that after shift-up, cytoplasmic mass increases faster than surface area, leading to an increase in buoyant density, which is correlated with a burst of initiation of new cell wall growth sites.

Obviously caution must be applied in the interpretation of radioactive precursor data during unbalanced growth. For example, one does not know the rates at which peptidoglycan precursors are being incorporated into various portions of the surface (i.e., into old sites relative to new sites, or into surface growth relative to cell wall thickening) during this period.

The initial overshoot kinetics for the rate of formation of new sites (Fig. 1B) supports the idea that the sites that existed before glutamate addition could not grow fast enough to house the cytoplasm made after shift-up. The decline in rate of formation of new sites after 15 min probably is due to the new sites growing faster than those made before glutamate addition.

Buoyant density as a function of cell size. Figure 3 shows the frequency distributions and average buoyant densities of cells before and 10, 20, and 40 min after shift-up as a function of cell volume. The four plots resulted from the analysis of volume distributions from electronic counter measurements of cultures separated on Percoll gradients. The method of analysis used to create Fig. 3 is given in Materials and Methods and shown in semigraphic form in Fig. 2. In each panel in Fig. 3, the common pattern is a slight initial decrease in buoyant density, followed by a close to linear increase and then a final plateau. On shift-up, the extent and slope of the increase per unit volume increase. These increases in extent and slope reach maximum values at 15 to 20 min and decrease thereafter but are higher than before shift-up.

Buoyant density as a function of cell size and the initiation of new sites of cell wall synthesis. Electron microscope measurements of carbon-platinum replicas (Fig. 4) agreed qualitatively with electronic measurements (Fig. 3) of cells from Percoll gradients. In both cases, density decreased and then increased as cell size increased. In the case of the electron microscope measurements, we found changes in density as a function of the volume of the central growth site of cells (CS; insert to Fig. 1B). For this purpose, we chose a population of cells 15 min after shift-up, at which time new sites are initiated at the maximum rate (Fig. 1A). In Fig. 4, cells with central sites that are less than about $0.15 \mu\text{m}^3$ are assumed to be in newborn cells, whereas sites that are longer than about $0.30 \mu\text{m}^3$ are assumed to be in the process of division (6). This plot indicates that small cells have high buoyant densities which fall as the cells increase in size. Finally, as the site approaches the size of two finished poles, density begins to increase. Comparison of Fig. 4A with Fig. 4B shows a very good correlation between the terminal increases in buoyant density and the appearance of new sites.

Interpretations. Figure 4 indicates that the smallest cells have densities as high as those of the largest cells, whereas Fig. 3 gives the impression that the smallest cells are considerably less dense than the largest. This difference is easily explained, for with the electron microscope we can determine whether a cell has divided but has not separated. Also, the preparative methods for electron microscopy often separate cells that are in the terminal phases of division. Therefore, in these cases we count such cells by electron microscopy as two, whereas with the electronic particle counter these cells would be scored as one. Thus, to interpret Fig. 3 and 4, one must remember that most of the smallest cells seen in Fig. 4 are in the higher-volume classes

FIG. 3. Average distribution and buoyant density of cells as a function of cell volume. (See Fig. 2 and text for the method used for these graphs.) Panels A, B, C, and D give a typical set of results for a culture at zero time and 10, 20, and 40 min after shift-up, respectively. In each case, the solid line shows the distribution of cells and the circles represent the average density.

FIG. 4. Analysis of the change in buoyant density and the appearance of new or small sites in electron micrographs of carbon-platinum replicas of cells 15 min after shift-up. (A) Average change in buoyant density (+95% confidence limits); (B) distribution of measurements (—) and appearance of new sites of cell wall growth as a function of central growth site volume (see CS in insert to Fig. 1). Symbols: ●, buoyant density; ○, new sites; —, frequency of measurements. Figure was calculated by using 200 measurements of cells from each of five Percoll gradient fractions (the fraction containing the most cells plus two others on either side of this one), representing 96% of the population.

of Fig. 3. The advantage of using electron microscopy is that we obtain a view of what is happening in the separation phase of the cell cycle as well as information regarding new site production.

Conclusions and Speculation. In summary, upon nutritional shift-up, the buoyant density of *E. hirae* increases linearly for about 20 to 30 min and then plateaus. Comparing this increase in buoyant density with macromolecular synthesis, the simplest interpretation is that buoyant density increases because the inside of cells grows faster than their surfaces. Also, this differential growth model offers a working explanation of the great increase in rate of formation of new sites upon shift-up. Analysis of volume distributions of cells separated on Percoll gradients indicates that buoyant density increases almost linearly with volume as cells go from birth to division (Fig. 3) and also suggests that an increasing amount of work may be required to enlarge the surface as the cell proceeds to division. This is not to suggest that the growth of mass in *E. hirae* is linear. To the contrary, data has been presented at the 95% confidence level (8) that the mass of *E. hirae* grows exponentially. However, our measurements do suggest that there is differential packing of the mass that is made per unit volume during the cell cycle.

Figure 4 clearly indicates that new sites are made in concert with increases in buoyant density on a per-cell basis. Furthermore, these new sites are made over the same cell

size range as observed in electron micrographs of balanced cultures (6). Therefore, it seems that the frequency of initiation of new sites is related to the magnitude of the increase in buoyant density as old sites approach the size of two poles.

Does the rate of growth of old sites speed up on shift-up? Two lines of arguments suggest it does not. First, if pre-shift-up sites could greatly increase their rate of growth, there would be little need for the immediate increase in new sites. Second, on shift-up there is no increase in rate of formation of cells in the division phase for over 10 min. Thus, we suggest the following model of cell growth in *E. hirae*. Within 15 min after shift-up, levels of RNA and protein synthesis increase, leading to an increase in buoyant density. This sudden increase in buoyant density is correlated with a burst of initiation of new peripheral cell wall growth sites. Soon thereafter, peptidoglycan synthesis increases, leading to an increase in the rate of surface growth. The number of predivisional cells increases, and soon thereafter the rate of increase of cell numbers follows suit.

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