Quantitative Studies of Rod–Coccus Morphogenesis in a Temperature-sensitive Rod[−] Mutant of *Bacillus subtilis*

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Cells of *Bacillus subtilis* RodB changed from rods to cocci when shifted from 20 to 42 °C in media containing no additional anions. Quantitative studies of surface growth, including cross-wall formation and pole construction, have been made from reconstructions obtained from central, longitudinal sections of cells. Measurements of surface area and volume were obtained by mathematical rotation of axial sections about their longitudinal axis. Surface markers, perhaps analogous to the wall bands of streptococci, have been used to distinguish septal from cylindrical wall. During the shape change, wall volume increased most rapidly, in relation to cell volume, at the division site. The average volume of wall distal to the septum also increased but the slopes of the lines relating distal wall volume to cell volume were the same at all stages of the shape change. The quantity of wall per distal pole gradually declined with increase of cell volume and as the cells became more coccus-like. Collectively, these features suggest that wall continues to be produced at sites of cylindrical extension but fails to become incorporated into the existing cylinder to maintain a constant diameter. Instead, wall material may be used to thicken the surface of distal poles, but the rate of addition may decline as the cells assume a coccoid morphology. The change from rods to cocci may involve a progressive dependence on septal growth for surface expansion and a modification of the time at which the cross-wall is closed. Septal closure is progressively delayed as the organisms change into cocci, so that cross-wall separation precedes septal closure, as in streptococci.

INTRODUCTION

Conditional morphological mutants of *Bacillus subtilis* (Boylan & Mendelson, 1969; Cole *et al.*, 1970; Reeve *et al.*, 1972; Rogers *et al.*, 1968, 1970, 1978; Rogers & Thurman, 1978; Shiflett *et al.*, 1977) and *B. licheniformis* (Rogers *et al.*, 1968, 1970) have been described which alter in shape from rods to cocci when placed under suitable conditions of growth. From studies of the conditions influencing the shape change in a RodB mutant of *B. subtilis*, such as temperature and the anion content of the medium, Rogers & Thurman (1978) suggested that a heat-labile membrane factor controlling cylindrical extension became modified or inactivated during the rod–coccus transformation. Morphological studies (Burdett, 1979) have suggested that the nature of the change in shape in *B. subtilis* RodB might involve a progressive dependence on septal growth sites for generating new cell surface as the organisms become coccus-shaped. During the shape transformation, in the absence of added anions, the organisms show a twofold increase in diameter, a slight reduction in average cell length and a considerable increase in cell volume (Burdett, 1979). The amount of surface area and wall volume produced by cross-wall separation and pole formation increased from about 13 % in the rods to nearly 50 % in each daughter coccus. These measurements were based on conditions of growth where cell morphology was not

grossly distorted and where naturally occurring surface markers, perhaps analogous to the wall bands of streptococci (Higgins & Shockman, 1970, 1976), enabled septal and nascent polar wall to be distinguished from wall forming the remaining portion of the cell.

In the present study, involving a substantially larger sample of cells, an analogue rotation technique (Higgins, 1976; Higgins & Shockman, 1976; Burdett & Higgins, 1978; Burdett, 1979) has been used to quantify and reconstruct the sequence of division and pole formation in relation to changes in wall volume occurring at locations distal to the plane of division. The objective is therefore to relate the morphological complexity underlying the pattern of rod-coccus morphogenesis to site-specific changes in surface growth. Changes in size and shape are most readily understood in relation to surface growth by measuring the quantity of wall actually produced. This necessitates devising a means of measuring the volume of wall at different locations on the cell surface. By means of the rotation technique, three-dimensional reconstructions of whole cells are obtained by mathematical rotation of axially sectioned cells. Assuming the cells to be circular in cross-section, surface areas, volumes and the volume occupied by the wall can be readily measured. Information obtained by this technique, concerning localized changes in size and shape, would be difficult to obtain solely by linear measurements.

The present study was initiated to examine more fully aspects of a model for surface growth in *B. subtilis* RodB described previously (Burdett, 1979). Several aspects of this model are confirmed by this more extensive analysis. However, a wider objective in the study of morphological mutants is the extent to which they may be used to illuminate normal processes of growth and division. In particular, Rod⁻ mutants of *B. subtilis* appear to provide a useful system for examining the relation between cylindrical wall synthesis, normally resulting in elongation of the cell, and the process of septation. For example, the type of questions that may be posed include the following: (1) if the cells fail to elongate after a shift to restrictive conditions, does cylindrical wall synthesis cease or continue in a modified form? (2) can changes in morphology, from rod to coccus, be related to the activity of localized 'growth zones'? (3) how is growth in cell diameter correlated with the pattern of septation? (4) does the sequence of septation become modified if cylindrical extension ceases? Some of these questions may be examined by morphological analysis, using the known geometrical properties of the cells, if suitable markers are available for distinguishing septal and cylindrical wall.

METHODS

Bacterial strain, media and growth conditions. *Bacillus subtilis* rodB1 leuA8 was used; details of media and growth conditions have been described previously (Rogers & Thurman, 1978; Burdett, 1979). Briefly, cells were grown at 20 °C in TRM medium (Rogers *et al.*, 1976) containing 10 mM-MgSO₄ but no supplementary anions, and then shifted to 42 °C. The temperature of incubation was chosen so as to obtain the maximum axial ratio (i.e. length/diameter) in the rods. The generation time of cells grown at 20 °C was about 7.4 h and cultures were maintained at 20 °C for 3 d (9.7 doublings in mass) prior to shift to 42 °C. Exponential growth was maintained at 42 °C by periodic dilution of the cultures with fresh prewarmed medium.

Electron microscopy. One sample of cells grown at 20 °C was taken 30 min before the shift-up to 42 °C and subsequent samples at 42 °C were taken at 15 min intervals over a period of 3 h, making a total of 12 samples. Determinations of cell numbers and absorbance were also made at these times.

Cells (5 ml in growth medium) were pipetted into formaldehyde/glutaraldehyde fixative and processed for electron microscopy by methods described previously (Burdett & Higgins, 1978; Burdett, 1979). Micrographs were taken only of sectioned cells showing a clearly tribanded wall (dark-light-dark) around all or most of the periphery and containing a partially built or completed cross-wall. Such sections were assumed to be axial sections from the middle 15 to 20 % of the cell (Higgins, 1976; Burdett & Higgins, 1978).

Quantitative analysis. Whole cells were reconstructed from axial thin sections by means of an analogue rotation technique (Higgins, 1976). As applied to *B. subtilis* (Burdett & Higgins, 1978; Burdett, 1979), this technique involves tracing profiles of axially sectioned organisms, enlarged photographically to 50000 to 100000 $\times$, on to a conductive plate interfaced to a Honeywell DDP-16 computer. Details of the plate and

Fig. 1. Diagram illustrating application of the rotation technique (see text) to sections of *B. subtilis* RodB. (a, b) Idealized central, longitudinal sections of a normal rod (a) and of a coccus-like cell (b) observed at 42 °C; the septal zone (in black) is delimited by wall bands (W). For clarity, the cross-wall in (a) is shown as only partially closed, although septal closure occurs close to the point when new polar wall is produced (see text). (c, e) Longitudinal sections at the site of septation, on one side of cell; the black areas show one half of the septal annulus (volume, S_{vol}) and nascent polar wall (volume, P_{vol}) is unshaded. The cross-hatched regions indicate the location of cylindrical wall distal to the septal zone. (d, f) Idealized figures obtained by rotation of the sections in each half cell, including surface distal to the septal zone, viewed along the plane YY': P_a is the surface area of nascent polar wall extending from the base of the cross-wall to a wall band; P_w is the surface area of wall distal to the septal zone; S_a is the area of a plane passing through the septal annulus.

the method of use have been described previously (Burdett & Higgins, 1978; Burdett, 1979; Green *et al.*, 1979). By mathematical rotation and strip integration of the image, surface areas and volumes were obtained. Measurements were taken in each half of a dividing cell, using a vertical line joining the bases of the cross-wall to bisect each cell. To aid interpretation, the location where measurements were taken (wall band, the limit of the septal annulus and the tip and base of the cross-wall) are shown diagrammatically in Fig. 1. The assumptions underlying the use of the rotation technique have been discussed elsewhere (Higgins, 1976; Burdett & Higgins, 1978). The nomenclature used generally follows that proposed by Higgins (1976).

A total of 429 cell profiles were analysed, of which 70 consisted of micrographs of rods grown at 20 °C and the remaining 359 were of cells incubated at 42 °C.

Analysis of data. Graphical relationships were established by means of least squares regression analysis, commencing with a first degree (linear) equation then adding polynomials of successively higher degree (Armitage, 1977). Curve-fitting procedures and statistical analyses (Armitage, 1977) were performed on Honeywell DDP-16 or Hewlett-Packard 3000 computers. Results are shown as means $\pm$ one standard error of the mean.

Fig. 2. Relationship between the area (S_a) of septal annulus and the surface area (P_a) of nascent pole in cells of sample 1 (a), sample 7 (b) and sample 12 (c) obtained by rotation (see text). In this and subsequent figures, sample 1 consisted of rods grown at 20 °C, and samples 7 and 12 were taken after 75 min and 150 min at 42 °C. In this figure (and Figs. 3, 5 and 6) the diagram indicates the location where measurements were taken. The means $\pm$ one standard error are shown for increments of P_a corresponding to 0.025 μm^2 (sample 1) or 0.1 μm^2 (samples 7 and 12).

RESULTS AND DISCUSSION

Morphologically, the shape change is initiated by formation of a centrally located bulge some 40 to 60 min after the shift to 42 °C (Burdett, 1979). In the following sections a comparison has been made of linear, area and volume measurements in the rods and in cells transitional in morphology between rods and cocci. For convenience the measurements are shown for the rods grown at 20 °C (sample 1) and for organisms kept at 42 °C for 75 min (sample 7) and 150 min (sample 12). Prolonged incubation at 42 °C led to the production of organisms with aberrant cross-walls and distorted shapes, as described previously (Rogers *et al.*, 1970; Burdett, 1979). It was not therefore possible to compare cells of different morphology (rod and coccus) which had been grown in steady-state conditions.

Pole formation

Area measurements. The relationship between centripetal synthesis of the cross-wall, septal closure and the production of new polar surface is shown by a plot of S_a (the area of a plane passing through the cross-wall, Fig. 1) against P_a , the surface area of the nascent pole. Surface area, rather than pole volume, was used as a measure of pole size because, particularly in the coccal cells, there was much variation in pole volume with increasing curvature (data not shown).

For sample 1, a curvilinear relationship was obtained by regression analysis (Fig. 2a). This graph would also be expected to reflect the pattern relating centripetal synthesis of the cross-wall to pole production in the rods during the cell cycle because the cell poles have been ordered with respect to increasing P_a and decreasing S_a . That is, in a steady-state

Fig. 3. Linear measurements from sample 1 (*a*), sample 7 (*b*) and sample 12 (*c*). During pole formation, $D_{\min}$ (the distance between the bases of the cross-wall) decreases continuously with increase of P_a ; septal closure (obtained from the sum of septal lengths, i.e. $S_1 + S_2 = 2S$) is shown by the intersection of $2S$ with $D_{\min}$. Septal closure occurs at $P_a < 0.05 \mu\text{m}^2$ in sample 1 (*a*), at $P_a = 0.35 \mu\text{m}^2$ in sample 7 (*b*) and at $P_a = 1.0 \mu\text{m}^2$ in sample 12 (*c*).

population, S_a would decrease as the cross-wall is gradually severed and the nascent poles increase in surface area.

For cells transitional between rods and cocci, the relationship between S_a and P_a was used to follow the change in the pattern of pole production. The maximum P_a increased from about $0.35 \mu\text{m}^2$ in sample 1 to $0.7 \mu\text{m}^2$ in sample 7, whereas in sample 12 the increase was approximately 4-fold, to $1.5 \mu\text{m}^2$ (Fig. 2*b, c*). The average S_a prior to cross-wall separation (i.e. $P_a = 0$) for samples 1, 7 and 12 was, respectively, 0.185 , 0.32 and $0.545 \mu\text{m}^2$. In samples 7 and 12, S_a showed a steep increase following the initial separation of the cross-wall, i.e. when P_a increased from 0 to $0.2 \mu\text{m}^2$ and to $0.4 \mu\text{m}^2$, respectively.

Linear measurements. These were obtained to provide additional information on the relationship between septal closure, separation of the cross-wall and increase in pole surface area. In each sample (Fig. 3), the curve for $D_{\min}$ (the diameter between the bases of the cross-wall) suggested that a decreasing amount of P_a was produced for every reduction in $D_{\min}$. The formation of a nascent pole by separation of the cross-wall can be regarded as occurring by the continuous removal of wall material from the outer circumference of the septum. As the cross-wall is cleaved the material so removed from around the constricting division site is converted into two hemispherical poles. On purely geometric grounds, this process involves an expansion in area. Calculations of the estimated amount of expansion (see Burdett & Higgins, 1978) showed that the P_a produced by separation was greater than that accounted for solely by removal of wall from the circumference of the septum. The

Fig. 4. Electron micrographs from central, longitudinal sections of cells from sample 1 (*a*), sample 7 (*b*) and sample 12 (*c*), showing wall bands (W) and cross-wall (CW). In sample 12 (*c*) a gradient of wall thickness extends from the base of the cross-wall to the wall band; the gradient is less conspicuous in (*a*) and (*b*). Note the thickening at the central cross-wall, in (*c*), which narrows towards separating layers of polar wall (arrows). Bar markers represent 0.1 μm .

Fig. 5. (a) Linear regression of wall volume on cell volume in sectioned cells (see text). The volume of wall at sites distal to the septal zone (i.e. total wall volume – septal wall volume, the unshaded area in the diagram at top left) shown in relation to the cell volume for sample 1 (■), sample 7 (△) and sample 12 (○). These lines have the same slope although the cells change from rods to cocci. (b) Diagrams illustrating how this relationship could be influenced by wall thickness and hence wall volume: in (1), the wall is of thickness 'a' in the rod; on a shift-up to 42 °C, the wall thickness increases (2) to 'a + b' to maintain the constancy of the relation between pole volume and wall volume; in (3) the wall remains at the same thickness as in the rod although the cell has increased in volume. The dashed line in (a) shows the expected slope for cells of sample 12 if wall thickness had remained as in sample 1. Plots of the volume of wall within the septal zone (black area in the diagram at top left) on cell volume for sample 1 (□), sample 7 (▲) and sample 12 (●) have different slopes (see text); the mean values lie at intervals of 0.1 μm^3 and S.E. bars have been omitted for clarity.

amount of expansion at $P_a < 0.1 \mu\text{m}^2$ varied from 2.5-fold in the rods to 4.4-fold in the cocci, but was gradually reduced to values slightly greater than 1.0 as cross-wall separation proceeded (data not shown). If no expansion mechanism was involved, the relationship between $D_{\min}$ and P_a would be linear with a slope of -1.0 ; no evidence of a linear relationship was obtained. One possibility to account for the postulated expansion is that wall material is added to the separating layers of polar wall (Higgins & Shockman, 1976; Burdett & Higgins, 1978). In the cocci, but to a lesser degree in transitional cells and rods, a conspicuous gradient of wall thickness was observed (Fig. 4), increasing in width towards the wall band, as in streptococci (Higgins & Shockman, 1970, 1976). Measurements showed that, with increase of P_a , wall thickness increased at sites adjacent to the wall bands but remained relatively constant at the base of the separating layers (data not shown). At this location, the width of the cross-wall was almost exactly twice the thickness of the separating layers, despite a considerable increase in thickness at the centre of the cross-wall (Fig. 4). These observations suggested that the thickness of the separating layers of nascent polar surfaces was closely regulated.

The point of cross-wall closure was measured by the intersection of the line connecting the sum of septal lengths (i.e. $S_1 + S_2 = 2S$, Fig. 3) with $D_{\min}$. Closure occurred at $P_a < 0.05 \mu\text{m}^2$ in sample 1, at $P_a = 0.35 \mu\text{m}^2$ in sample 7 and at $P_a = 1.0 \mu\text{m}^2$ in sample 12. Although centripetal synthesis of the cross-wall appeared to accelerate initially, as shown

Fig. 6. The ratio of distal pole wall volume/distal pole volume as a function of distal pole volume in sample 1 (■), sample 7 (□) and sample 12 (●). The slope of the line for the rods (sample 1) is not different from zero, but in the cells transitional between rods and cocci the slope is negative. The overall trend, pooling the data from all samples (---) and fitted by a second degree curve, shows a decline in the volume of wall/distal pole after the inflection point, $1.3 \mu\text{m}^3$ of distal pole volume (see text).

by a rise in S_a (Fig. 2), actual septal closure did not occur until comparatively late in pole construction in samples 7 and 12.

The diameter between the wall bands (D_{max} , Fig. 3) remained relatively constant during pole construction. Although the diameter increased at the centre of the cells shifted to 42°C , the bulk of the increase occurred prior to formation of the cross-wall. Thus, in dividing cells intermediate in morphology between rods and cocci, D_{max} remained constant whilst increases occurred in the diameter at the centre of each daughter coccus.

Changes in wall volume at the septum and at sites distal to the division site during the shape transformation

Although it has been shown above that the proportion of wall assembled at the division site increased during the shape transformation from rod to coccus, it may be asked whether wall synthesis was modified at sites distal to the plane of division on the shift to 42°C . Several possibilities may be envisaged: (i) wall synthesis along the cylindrical portion of the rods ceases abruptly or is gradually reduced on the shift-up; (ii) cylindrical wall synthesis continues unmodified but is not used to extend the cell in length, only to thicken existing surfaces. Regression analysis showed that, for the septate organisms, wall volume was linearly related to cell volume (correlation coefficient, $r > 0.9$), both for the rods and for cells at all stages of the shape change. This basic observation was used to examine changes in wall volume (i) within the cross-wall and nascent poles and (ii) at sites distal to the septum, in relation to cell volume. By this means it was possible to analyse whether increase of cell volume was accompanied by an increase of wall volume at different surface locations.

Linear regression of the volume of wall within the septum (i.e. $S_{\text{vol}} + P_{\text{vol}}$, Fig. 1) on cell volume showed that the slopes, varying from 0.063 to 0.111, were significantly non-parallel ($F = 5.8$, $P < 0.001$). For ease of comparison, the lines are shown for samples 1, 7 and 12 (Fig. 5). The graph indicated that, in relation to cell volume, the rate of increase in wall volume occurred most rapidly at the division site, an observation which is supported by the measurements described above and by morphological studies (Burdett, 1979).

In contrast, linear regression of the volume of wall distal to the site of septation (i.e. total wall volume – septal wall volume) on cell volume in all samples showed that the slopes, varying from 0.179 to 0.260, were parallel and not significantly different ($F = 0.9$, $P > 0.20$). The best estimate of the common slope was 0.221. Therefore, the rate of increase in wall volume relative to cell volume at sites distal to the cross-wall was the same at all

stages of the shape change (Fig. 5). One interpretation would be that wall continued to be added to the distal poles at the same rate even though the organisms, on a shift to 42 °C, gradually ceased to extend in length and changed from rods to cocci. The dashed line in Fig. 5 shows the expected slope in the cocci if wall synthesis at the distal poles had ceased abruptly so that the wall remained at the same thickness as in the rods but the volume of the distal poles had increased.

Regression analysis was also used to examine whether the volume of wall per distal pole decreased during the shape change. For this, linear regression was performed of the distal pole wall volume/distal pole volume on distal pole volume (Fig. 6). For the rods, the slope of this line (0.0029) was not significantly different from zero at the 10 % level of the *t*-test. That is, a constant ratio of wall volume/distal pole volume was maintained at all values of distal pole volume because the organisms were extending only in length without change of diameter or wall thickness. Comparison of the slopes of all samples showed no evidence for them being non-parallel ($F = 0.52$, $P > 0.20$). However, all samples incubated at 42 °C for more than 1 h, with the exception of sample 9 (105 min), had negative slopes varying from -0.036 to -0.0893 . Sample 9 had a slope of 0.023, not significantly different from zero at the 10 % level of the *t*-test. When the data were pooled, the relationship was best represented, not by a linear function, but by a second degree curve (Fig. 6). The point of inflection occurred where the distal pole volume was $1.3 \mu\text{m}^3$ (Fig. 6). Therefore, when the organisms became fully coccal in morphology, the amount of wall added to the distal poles eventually decreased.

Pattern of surface growth in rods and cocci

The results described above may be summarized as follows: (i) after the shift-up to 42 °C, wall synthesis continues at sites distal to the cross-wall in a manner which matches the increase in distal pole volume; (ii) only when the organisms are fully coccal in morphology does the quantity of wall added to the distal poles begin to decline; (iii) the point of cross-wall closure is progressively delayed as the quantity of wall assembled at the septum increases, such that septal closure occurs prior to separation in the rods, but separation precedes septal closure in the cocci.

Previous morphological studies (Burdett, 1979) suggested that the shape change in *B. subtilis* RodB could be interpreted in terms of modification in the activity of a central wall growth site. In rods, this site would be concerned with elongation of the organism prior to cross-wall formation. After the temperature-shift, the site would continue to remain active but wall would fail, for reasons as yet unknown, to become incorporated into the existing surface so as to maintain a cylinder of constant diameter. As the organisms fail to extend in length but grow only in diameter, the wall produced from this site would be added to the poles distal to the division site. The present results support this hypothesis and would explain the constancy of the proportion of wall at the distal poles as a function of cell volume (Fig. 5). The eventual decay in the activity of the growth site would presumably be reflected in the decrease in the quantity of wall at each distal pole (Fig. 6).

The results also indicate very clearly that although an increasing proportion of the surface is produced by separation of the cross-wall during the shape change, the division site is not the exclusive site of incorporation of newly synthesized wall. For example, the gradient of wall thickness at the nascent poles (Fig. 4) may persist after cell separation and confer a definite polarity to the cell, one distal pole being much thinner and more rounded than the other (Burdett, 1979). Gradual modification of shape to a more elongated structure, effectively equalizing the size of each distal pole, may occur by localized wall thickening.

The delay in cross-wall closure which occurs during the morphological shape change can be used to relate the pattern of surface growth of the rods to the cocci. In rods, it is assumed that cylindrical growth consists only of extension in length. Therefore, both the diameter D_{max}^* and the area (S_a^*) of an annulus produced by passing a plane through the cylinder

the transition in shape from rods to cocci, the amount of P_a^* would decrease as length extension ceased and more new surface would be generated from septal sites (increase of P_a).

In the cocci (Fig. 7), much of the cycle could conceivably depend on growth from septal sites and new cell surface would arise from separation and expansion of the cross-wall. The initial separation of the cross-wall at its base, prior to closure, might represent an adaptation to the modification of the cylindrical wall growth mechanism. This initial period of pole construction could conceivably be used to effect nuclear segregation in the absence of the elongation mechanism. In this respect the model resembles the cycle of pole formation in *S. faecalis*, when surface growth also arises from septal sites (Higgins & Shockman, 1970, 1976). However, in *S. faecalis* a plateau level of S_a is maintained for two-thirds of the cycle until cross-wall closure. In fact, about one-third as much ($1.4 \mu\text{m}^2$) surface area is generated in *S. faecalis* before septal closure than in coccal forms of *B. subtilis* RodB (about $1.0 \mu\text{m}^2$, Fig. 3; the two organisms are of similar diameter).

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