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Three-Dimensional Reconstruction of Whole Cells of *Streptococcus faecalis* from Thin Sections of Cells

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A new ultrastructural technique has been developed to study the geometry of cell wall assembly in *Streptococcus faecalis*, which is believed to occur between pairs of raised bands located on the organism's surface. Three-dimensional reconstructions of these new regions of envelope growth are produced from the mathematical rotation (around a central axis) of various measurements taken from central, longitudinal thin sections of cells. These reconstructions can be used to calculate the surface area and volume of the septal and peripheral walls that were supposedly present in any given cell before sectioning. In an accompanying paper, it is shown how such surface and volume estimations, coupled with other measurements of length, thickness, and curvature, can be used to characterize a cycle of envelope growth in this organism. The validity of the assumptions used to reconstruct cells by rotation and the possible sources of error in using this technique are discussed.

Autoradiographic (3), immunofluorescence (4, 17), and ultrastructural (10, 11, 16) studies have suggested that chain-forming cocci enlarge their surfaces by the equatorial insertion of discrete zones of "new" envelope into the middle of "old" envelope. It appears that each of these envelope "growth zones" contains a cross wall or septal region from which two "new" units of peripheral wall are issued in bilateral fashion (11, 12). Each growth zone is also capable of centripetal growth, which ultimately results in the closing of the cross wall and in the final separation of the closed cross wall into two completed poles.

Characterization of this type of growth in *Streptococcus faecalis* (ATCC 9790) has been the subject of several morphological studies (8-12). This organism has proven ideal for such studies because: (i) it has naturally occurring surface markers which separate old polar wall made in past generations from the new equatorial wall being made during the current generation (10), and (ii) no detectable level of peptidoglycan turnover has been observed during any phase of growth (2). The absence of turnover is probably responsible for the observation that once a segment of the cell wall reaches a given thickness, it does not appear to be thinned during subsequent periods of growth (8, 10). This observation suggests that the thickness of the cell wall at a particular site on the cell's surface reflects the cumulative incorporation of cell wall precursors at that site, rather than being some product of the equilibrium between turnover and assembly.

Based principally on observations made from thin sections of cells fixed during exponential-phase growth (11), or during regrowth from the stationary phase (10), a model of surface growth was proposed for *S. faecalis* (11). This model suggested that cell wall precursors were used to assemble a bilayered cross wall that would subsequently separate into two layers of peripheral wall. It was further proposed that the thickness of cell wall surfaces being assembled was under the control of a separate but coupled mechanism (8, 11, 12).

In this report, a new ultrastructural method is described which has been developed to test many aspects of this model. Basically, this method consists of mathematically rotating thin sections of cells around their principal axes to form three-dimensional reconstructions of whole cells. In addition, the problems, possible errors, and assumptions on which this method is based are considered.

MATERIALS AND METHODS

Cell growth and electron microscopy. The cells used to develop the method described here were taken from cultures in midexponential-phase growth (8). Before fixation, cultures were allowed to double in mass at least seven times (T_D 31 to 33 min) in a chemically defined medium (14).

The glutaraldehyde-osmium tetroxide fixation, Epon 812 infiltration and embedding, and uranyl acetate-lead citrate post-staining have been described elsewhere (6). Silver-gray sections were examined with a Siemens Elmiskop IA electron microscope at instrumental magnifications of approximately ×30,000. The microscope was calibrated frequently with a carbon grating replica (E. F. Fullam, New York). Thin sections of cells were photographed on Kodak electron image plates and enlarged approximately ×3.3 on Kodak Kodabromide enlargement paper.

Rotational analogue method. As stated in the introduction, the rotational technique consists of rotating central, longitudinal thin sections of cells around their principal axes to form three-dimensional reconstructions of whole cells. Although any portion of the cell or its envelope may be reconstructed, to date the technique has been primarily used to study the assembly of new cell wall, which is thought to occur preferentially between raised bands of cell wall material found on the external surfaces of *S. faecalis* (Fig. 1; reference 10). The term "growth zone" is used to describe the new cell wall found between pairs of bands (Fig. 1). As shown in Fig. 1, the peripheral wall contained within a growth zone is mathematically reconstructed in two transverse halves. Since at the end of a round of synthesis each growth zone will produce two poles, each growth zone is considered to contain two nascent poles.

The technique is applied only to sections of cells where the cell wall appears tribanded around virtually the entire perimeter of the cell (Fig. 1). Such sections are assumed to be longitudinal and axial. Evidence to support these assumptions is given below.

Estimation of the surface area contained by the peripheral and septal walls of nascent poles. The first step in the reconstruction of the peripheral wall involves the fitting of trapezoids to the outside perimeter of the peripheral wall between pairs of wall bands (Fig. 1B, C). These trapezoids are then mathematically converted into their three-dimensional analogues, namely, the frustra of right cones (Fig. 1D). Therefore, in this example, the surface areas of these cones are taken as an estimate of the external surface area of the peripheral wall (Pa) found in the two nascent poles before sectioning. In most cases, the Pa values calculated for each nascent pole of a growth zone are plotted and analyzed separately. As presented in the accompanying paper (13), this is done so that the assembly of a single pole can be studied from the beginning to the end of a cycle of growth and so that, for model building purposes, the amounts of peripheral wall found on either side of the septum can be compared. Further details and equations used for these transformations are given in the legend of Fig. 1.

The peripheral wall segments seen in Fig. 1B (i.e., P_1, P_2, P_3, and P_4) are approximately linear in section and show no detectable curvature. This is approximately the situation during very early phases of growth zone development (see reference 13). However, as the length of the peripheral seg-

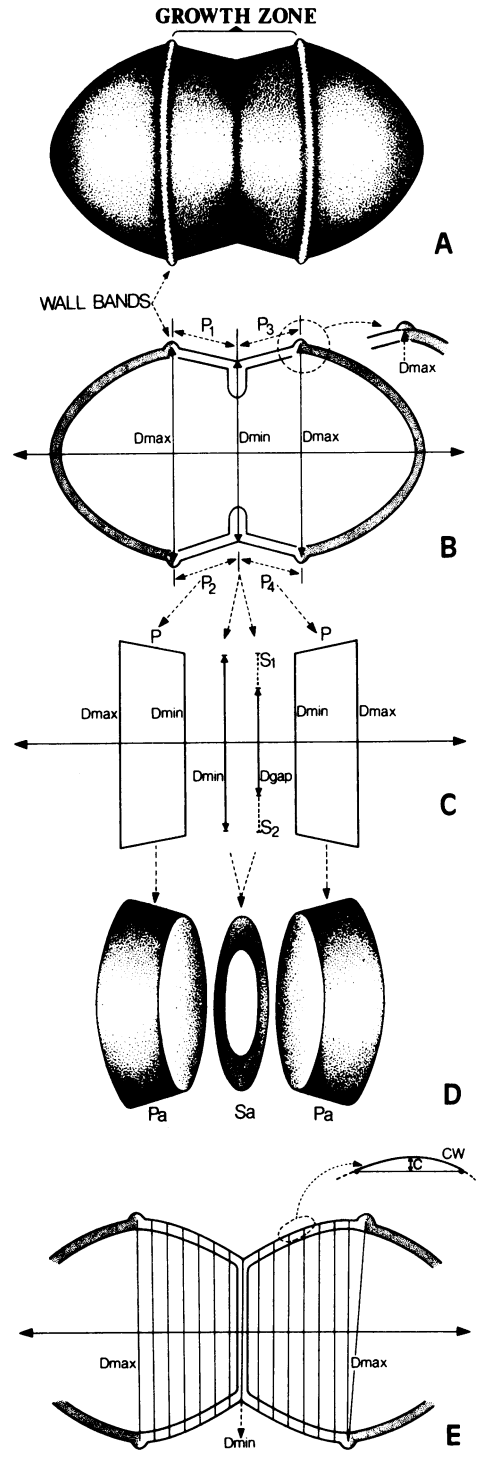

FIG. 1.

ments increases, curvature is introduced (Fig. 1E). As this occurs, the short sides of a single trapezoid (i.e., P in Fig. 1C) can only approximate the curved perimeter of the peripheral wall over very short stretches. This problem is overcome by increasing the number of trapezoids fitted to a growth zone (Fig. 1E). The actual number is determined by noting how far the short side of each trapezoid can be extended and still offer a reasonable fit to the outer perimeter of the wall. The reasonableness of this fit can be quantitated by measuring the maximum distance between the short side of the trapezoid and the outer perimeter of the cell wall (Fig. 1E). In practice, the number of trapezoids used is sufficient to keep this distance undetectable by eye (in enlargements of cell sections where the total magnifications are about ×100,000).

In thin sections of nascent poles, it is not uncommon to find one segment of the peripheral wall slightly longer or shorter than the other. This has been shown in exaggerated fashion in the nascent pole on the right side of the cross wall seen in Fig. 1E. The legend of Fig. 1E describes how the surface area and volume of asymmetric segments are estimated.

The surface area of the cross wall is calculated by following the same principles as applied to the reconstruction of the peripheral wall. Measurements are used to determine the length of two lines (i.e., the diameter of the septal region [Dmin] and the diameter of the "gap" [Dgap] between the leading edges of a nonseptated cross wall [Fig. 1C]), which are mathematically converted into two discs by rotation. The surface area of the septal wall (Sa) is then taken to equal the area remaining after the area of septal gap has been subtracted from the total area of the septal region (Fig. 1D).

Estimation of the volumes occupied by the pe-

FIG. 1. *Reconstruction of a growth zone of S. faecalis observed in a longitudinally axial thin section of a cell. (A) A diagrammatic representation of a cell of S. faecalis. A "growth zone" is made up of the envelope found between a pair of raised bands of cell wall material known as "wall bands," and is considered to be the "new" wall surface assembled exclusively during the current round of cell wall growth (10). (B) A diagrammatic representation of a longitudinally axial thin section of the cell shown in (A). The three-dimensional reconstruction of the peripheral wall of the growth zone seen in this diagrammatic thin section is produced as follows. To the peripheral wall seen in the growth zone and flanking the cross wall, two trapezoids are fitted. The sides of the trapezoids are defined as follows: Dmin, the shortest outside diameter of the cell; Dmax, the largest outside diameter of the cell; and P_1, P_2, P_3, and P_4, the shortest lines that will connect Dmin and Dmax. Dmax is determined by placing one point in each of two opposing wall band markers. Each point is defined by the intersection of two lines, namely one that passes through the middle of the opposing wall bands and a second that estimates the continuance of the outer perimeter of the cell wall if the raised wall band markers were removed. (C) This figure shows the dimensions of the two trapezoids and two lines (Dmin and Dgap) taken from (B) that are mathematically rotated to estimate the surface areas of the cross and peripheral walls of the growth zone that supposedly were present before sectioning. In addition, S_1 and S_2, which are the lengths of the sectioned cross wall segments measured from tip to base, are also shown. The measurements used to calculate peripheral wall surface area can also be used to estimate the volume of the growth zone. The exposed surface area of the peripheral wall (Pa) and volume (VOL, not shown but equaling that volume occupied by the frustra of the two right cones shown here) found in each half of the growth zone (i.e., in each nascent pole) are calculated as follows:*

$$Pa = \frac{\pi}{2}(Dmin + Dmax)\,P \qquad (1)$$

$$Vol = \frac{\pi}{12}\sqrt{P^2 - \frac{(Dmax - Dmin)^2}{4}} \times \cdot(Dmax^2 + Dmin\,Dmax + Dmin^2) \qquad (2)$$

where P would be an average of the lengths of the shortest sides of the trapezoids fitted to either side of the growth zone (i.e., an average of P_1 and P_2, and P_3 and P_4, respectively), and where the length of Dmax would depend on which trapezoid is being rotated. The surface area of the septal wall (Sa) is determined by subtracting the area of the central "gap" (if present) from the total septal area as follows:

$$Sa = \left(\frac{Dmin^2 - Dgap^2}{4}\right)\pi \qquad (3)$$

where Dgap is the distance between the leading edges of the cross wall (see [C]). (D) Diagrammatic representation of the three-dimensional figures produced by the application of equations 1, 2, and 3 to the linear measurements shown in (C). (E) Diagrammatic representation of the application of the rotational-analogue technique to growth zones that have curved perimeters. In this case, a series of trapezoids are used to approximate the curvature of the perimeter. This is done by fitting a series of P lengths by eye to the curved peripheral wall. The maximum length of each P segment is determined by judging how far each segment can be extended without deviating from a reasonable fit of the curved surface (see insert). The amount of deviation from a perfect fit can be quantitated by measuring the distance C, which is the maximum distance, from the shortest side of the trapezoid fitted to this segment of the wall to the outer perimeter of the cell wall. In sections of cells magnified to about × 100,000, C is kept below levels detectable by eye. The Pa and VOL of each nascent pole are thus determined by summing the individual surface areas and volumes contained by the rotation of each trapezoid by equations 1 and 2, respectively. Note that in the right side of this growth zone the length of the last segment is asymmetric. In such cases, the surface and volume of the last segment are estimated by substituting into equations 1 and 2 a Dmax value taken as shown and a P value that is an average of the asymmetric P lengths.

ripheral and septal walls of nascent poles. The volumes occupied by the septal and peripheral walls of nascent poles can also be calculated by rotation (see Fig. 2 for details and equations). The volume of the peripheral wall (Pvol) is obtained by rotating trapezoids, fitted to longitudinal thin sections of cells, to estimate (i) the total volume of a nascent pole (VOL), (ii) its cytoplasmic volume (CYTvol), and (iii) its total septal volume (SEPvol). Pvol can then be calculated by subtracting from the total volume of the nascent pole (VOL) those volumes not directly occupied by the peripheral wall (i.e., CYTvol and SEPvol). Just as in the case of determining the Pa, the number of trapezoids required to calculate Pvol increases with increasing peripheral wall curvature. The volume occupied by the septal wall (Svol) of a nascent pole is also estimated by using trapezoids. However, in this case they are used to determine one-half of the septal volume based on the average thickness of the cross wall and to define the volume of one-half of the central gap (if present). The volume of the septal wall (Svol) thus results from subtracting the gap volume (GAPvol) from the total septal volume (SEPvol) of a nascent pole.

Method by which measurements are taken and calculations are performed. The rotational analogue technique was initially developed and applied to thin sections of cells by using measurements, taken with calipers, from photographic enlargements of the thin sections. Recently, a Numonic digitizer (Numonic Corp., North Wales, Pa.) interfaced with an ASR 33 teletype has been used to increase the speed by which measurements are made. The probe of this instrument is placed on the desired locations on a photographic enlargement of a thin section of a cell (total magnification of about ×100,000), and upon command an appropriate series of x,y coordinates are punched into a paper tape. The data on this tape are subsequently fed into a PDP8/m computer (Digital Equipment Corp., Maynard, Mass.), where the desired calculations are performed.

RESULTS AND DISCUSSION

Validity of the assumptions on which the rotational-analogue technique is based. To reconstruct a cell by rotation, several assumptions must be made. Among the most important are: (i) that the primary glutaraldehyde fixation rapidly "freezes" all active physiological and biosynthetic processes of growing cells; (ii) that the fixation, embedding, and thin-sectioning processes do not appreciably alter the size or shape of cells that are associated with the living state; (iii) that a longitudinal section of a cell in which the entire cell wall appears tribanded is considered central as well as longitudinal; and (iv) that cells of *S. faecalis* show longitudinal radial symmetry. In the text which follows, the validity of these assumptions is discussed in light of present evidence.

(i) The primary glutaraldehyde fixation rapidly "freezes" all active physiological and biosynthetic processes of growing cells. Previous studies have shown that the addition of glutaraldehyde (2.5% final concentration) to exponential-phase cultures of *S. faecalis* (T_D 31 to 33 min) rapidly inhibits lysis of both cells and isolated cell wall fractions (9), deoxyribonucleic acid, ribonucleic acid, and protein synthesis, and the incorporation of glycerol into membrane components (7). In each case, the effects of glutaraldehyde appear to be virtually instantaneous, and it seems that both biosynthetic and degenerative processes could be active only for very short periods of time after the addition of fixative.

(ii) The fixation, embedding, and sectioning processes do not appreciably alter the shape or size of cells that are associated with the living state. The collective processes to which cells are subjected before and after sectioning are quite drastic. It is conceivable that changes in the geometry of cells could occur during these manipulations. For example, in *Escherichia coli* the average diameter of cells measured in thin sections is about 35% less than comparable measurements taken from freeze fractures of unfixed cells (1). This suggests that at some point during the preparation of cells for thin-sectioning, significant reductions in size occur. However, when similar measurements were made from thin sections and unfixed freeze fractures of cells of *S. faecalis*, no large differences in the average diameters of cells in either preparation could be detected (Table 1). Apparently, the larger amount of highly crosslinked peptidoglycan in the cell walls of *S. faecalis* (5) protects these organisms from gross reductions in diameter.

From the diameter data shown in Table 1, it is not to be inferred that all structures in cells of *S. faecalis* remain unaltered during dehydration and embedding. Recent evidence gathered in this laboratory (H. C. Tsien, personal communication) suggests that while the diameters of cells are not appreciably influenced by dehydration and embedding, cell walls of *S. faecalis* observed in thin sections are about 33% thinner than the cell walls seen in freeze fractures of unfixed cells. Apparently, during the dehydration process required for thin-sectioning, the wall contracts into a somewhat more compact state. The question raised by this study is whether the cell wall volumes derived by the rotational-analogue technique (which in part are based on thickness measurements taken from sections of cell walls [Fig. 2]) are related to the actual amounts of wall material present before dehydration. Fortunately, in *S. faecalis* it has been observed that when protein synthesis of exponential-phase cells was inhibited

TABLE 1. *Average diameters of cells of S. faecalis as observed in thin sections and freeze fractures*

Type of prepn	Fixation	Avg diam (μm)[a]	Standard deviation
Thin sections[b]	Glutaraldehyde/osmium tetroxide	1.02	0.05
Freeze fractures[c]	None	0.98	0.06
	Glutaraldehyde	0.99	0.05

[a] Calculated from the measurement of Dmax (see Fig. 1B) in 50 longitudinal thin sections of cells showing completely tribanded wall profiles and from the same number of longitudinal freeze fractures of cells that were judged to be from the center of cells (7).

[b] Fixation of cells in 2.5% (final concentration) glutaraldehyde and then in 1.0% (final concentration) osmium tetroxide, followed by the infiltration and embedding of cells in Epon 812, is described in reference 6.

[c] Freeze fracture techniques and fixation of cells in 2.5% (final concentration) glutaraldehyde for 60 min before freezing has also been previously described (7). The glutaraldehyde fixation was carried out at 37°C. The measurements shown for the unfixed cells were from cultures that were chilled to ice bath temperature before cells were concentrated by centrifugation and quenched (7).

with several antibiotics, parallel increases were noted in (i) the average thickness of cell wall seen in thin sections and (ii) the amount of peptidoglycan found on a per-cell basis (8). Thus, this study suggested that increases in the peptidoglycan content produced proportional increases in the thickness of cell walls.

Therefore, it seems that wall volumes derived by the rotation-of-thickness measurements (Fig. 2) can be used with caution to describe the relative amounts of wall material present in a nascent pole.

(iii) A longitudinal section of a cell in which the entire cell wall appears tribanded is considered central as well as longitudinal. The validity of assuming longitudinal sections of cells, which show tribanded cell walls around their entire perimeters, to be axial was tested by studying cells that had been serially sectioned. On the average, about 11 to 14 longitudinal silver-gray serial sections were required to pass completely through a single cell of *S. faecalis*, and of these usually one, and rarely two, sections showed a complete tribanded profile. These tribanded sections were invariably found in slices that passed through the most central portion of the cells. It was also observed that the maximum amount of the cell's perimeter appearing tribanded in any serial section was determined by how closely the section was aligned with the longitudinal axis of the cell. Deviations from this axis caused portions of the cell wall seen in the central slice to appear fuzzy or diffuse.

To demonstrate the effect that the plane of sectioning has on the results obtained by rotation, growth zones seen in serial sections of single cells were reconstructed with the rotational technique. An example of this study is shown in Fig. 3, where the surface areas and volumes obtained by rotating the growth zone observed in the mid-eight serial sections of a single cell are given. In this series, only one of the sections showed a completely tribanded cell wall, and, as would be predicted, this growth zone generated the largest exposed surface area and cell volume (data circled in Fig. 3).

In summary, these observations are consistent with the assumption that longitudinal sections of cells with tribanded walls are approximately axial.

(iv) Cells of *S. faecalis* show longitudinal radial symmetry. If cells of *S. faecalis* show radial symmetry, one would expect the four segments of peripheral wall seen in longitudinal sections of growth zones to be approximately equivalent in length (i.e., the lengths of P_1, P_2, P_3, and P_4 in Fig. 1B should be equivalent). Variation in these lengths would, therefore, be one index of the asymmetry found in a particular growth zone. Such asymmetry within a population of exponential-phase growth zones can be studied by plotting the average lengths of the peripheral wall found in the "top" portion of a sectioned growth zone (i.e., an average of P_1 and P_3 in Fig. 4A) against the average lengths of the peripheral wall found in the "bottom" portions of the same growth zone (i.e., the average of P_2 and P_4 in Fig. 4A). The theoretical line in Fig. 4A indicates the expected relationship between peripheral wall lengths if growth zones are perfectly symmetrical; thus, the distance of each point from the theoretical line is an index of top to bottom asymmetry. In Fig. 4B the treatment is repeated by plotting the average length of the peripheral wall in the "left" side of each growth zone (i.e., the average of P_1 and P_2 in Fig. 4B) against that found in the "right" side (i.e., the average of P_3 and P_4 in Fig. 4B). A comparison of the scatter of points around the theoretical lines in Fig. 4A and 4B suggests that most growth zones enlarge their peripheral walls in a fairly symmetrical fashion. However, there appears to be more deviation in longitudinal sections of growth zones between the top and bottom measurements than those seen between the left and right sides. The greater equivalence between the left and right was further suggested when the standard error of estimate for the left to right comparison was calculated to be 11.66, whereas the top to bottom error of estimate was 12.45.

Such results are in good agreement with our

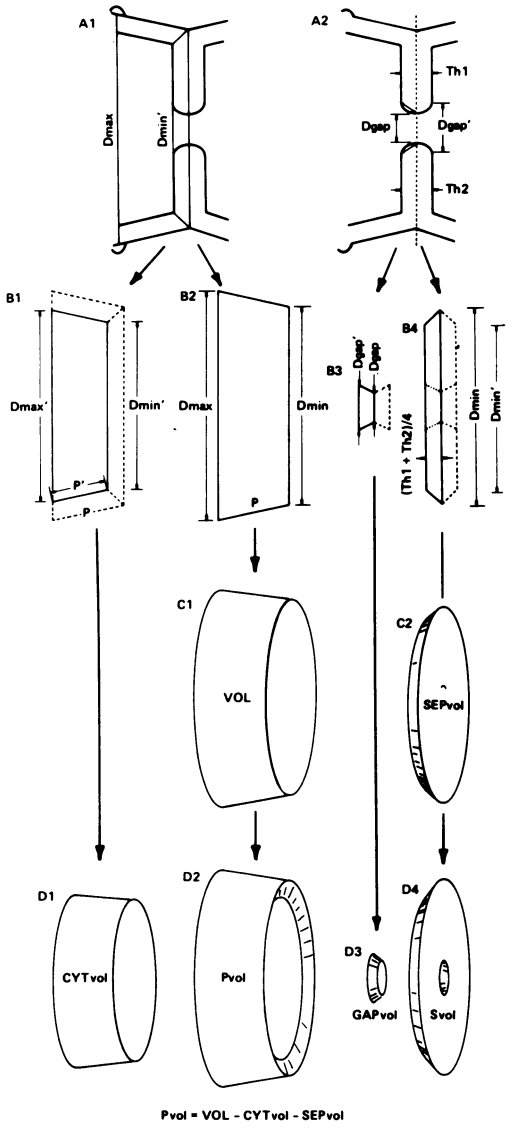

FIG. 2. *Estimation of the volumes occupied by the peripheral wall (Pvol) and septal wall (Svol) by rotation. (A1) and (A2) are identical diagrammatic representations of a nascent pole seen in a central, longitudinal thin section (used here to illustrate the measurements needed to calculate Pvol [D2] and Svol [D4]). Pvol is determined by first fitting trapezoids to: (i) the outside perimeter of the peripheral wall (B2); (ii) the inside perimeter of the peripheral wall (B1); and (iii) the septal region of a nascent pole (B4). Trapezoids (B1) and (B2) are then transformed into three-dimensional figures by the basic equation 2, described in the legend of Fig. 1, to give the total volume (VOL, [C1]) and the cytoplasmic volume (CYTvol, [D1]), respectively, of a nascent pole. The height of the trapezoid fitted to the septal region (B4) is based on the average thickness of the septal region of the nascent pole (Th, which equals [Th 1 + Th 2]/*

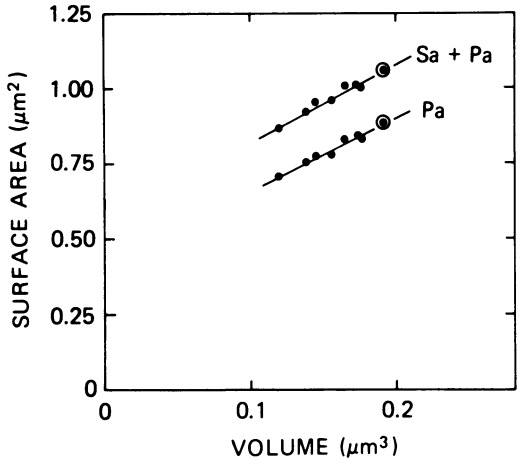

FIG. 3. *Total (Sa plus Pa) and exposed (Pa) surface and volume characteristics of the growth zone found in the mid-eight serial sections taken from a single cell. In this plot, each pair of points shown is an average of the surface areas and volumes obtained from the rotation of measurements taken from the two nascent poles observed in each serial section.*

previously proposed model for the surface growth of *S. faecalis* (11), which predicted that peripheral wall would be made by a bilayered cross wall separating into two equal layers of peripheral wall. Thus, the right and left sides of a growth zone should be mirror images. The greater top to bottom deviations could be explained by proposing that the amount of cross

4, [A2]) and is transformed into a three-dimensional figure by the following equation to give septal volume (SEPvol, [C2]):

$$SEPvol = \pi \frac{Th}{12} (Dmin^2 + Dmin\ Dmin' + Dmin'^2) \quad (4)$$

In nascent poles with closed cross walls, SEPvol (C2) is also equivalent to the septal wall volume (Svol) of a nascent pole. However, in nascent poles where cross walls contain central perforations (A1 and A2), the volume of the gap region (GAPvol, [D3]) between the leading edges of the cross wall must first be subtracted from SEPvol (C2) before Svol (D4) can be determined. In this case, GAPvol is calculated by rotating, by the following equation, a trapezoid fitted to this region whose height is also determined by the average septal thickness of the nascent pole (Th).

$$GAPvol = \pi \frac{Th}{12} (Dgap^2 + DgapDgap' + Dgap'^2) \quad (5)$$

The dashed lines in (B3) and (B4) represent the additional trapezoids that would be required to reconstruct the total septal wall volume of a growth zone.

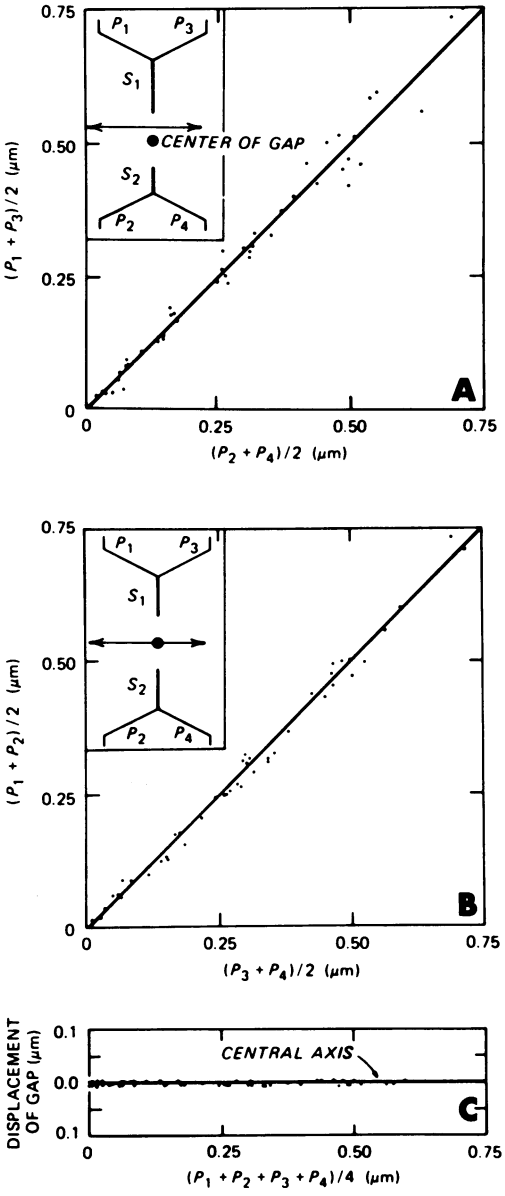

FIG. 4. *Asymmetries observed in central, longitudinal thin sections of growth zones as measured by comparing the lengths of various wall segments. (A and B) If growth zones of S. faecalis show perfect radial symmetry, the four lengths of the peripheral wall segments observed in sections should be equal (i.e., the lengths of P_1, P_2, P_3, and P_4 in the inserts to [A] and [B] should be equivalent). To describe the deviation from symmetry, the average lengths of the peripheral wall segments observed in the top halves ($[P_1 + P_3]/2$, [A]) of 53 exponential-phase growth zones (due to merged points and points that lie on the theoretical line, only 45 measurements are visible) as seen in thin sections were plotted against the average lengths in the bottom halves ($[P_2 + P_4]/2$, [A]). In addition, the average left sides ($[P_1 + P_2]/2$, [B]) were plotted against the right sides ($[P_3 + P_4]/2$, [B]). The solid lines indicate the expected values if growth zones show perfect symmetry with regard to peripheral wall lengths. Thus, the distance of an individual point from the theoretical lines in (A) and (B) is an index of asymmetry within a growth zone. (C) Asymmetry in the location of the hole in the cross walls of 50 growth zones (see inserts to [A] and [B]) was determined by measuring the distance separating the center of each cross wall (i.e., the center of a disc described by the rotation of Dmin; Fig. 1C) from the center of the cross wall gap (i.e., the center of a disc produced by the rotation of Dgap; Fig. 1C). The points in (C) show the position of the center of the gap in relation to the center of the cross wall (solid line).*

wall separation around the outer circumference of the cross wall is subject to localized variations. Therefore, the cross wall may be separating slightly faster at one location along the outer circumference of the cross wall than at another location found on the other side of the cell.

When viewed from an end-on position, the septal region of *S. faecalis* resembles either a disc or a circular perforated disc (i.e., a flat washer [Fig. 1D]). In terms of defining the symmetry of the growth zone, it is also important to know, in terms of model building, whether the central hole found in open cross walls is placed exactly in the center of the septum. This question has also been approached graphically. In Fig. 4C the points show the displacement of the center of the hole of 50 open cross walls (i.e., the center of the disc created by the rotation of Dgap in Fig. 1C) from the center of the septal region of these growth zones (i.e., the center of the disc created by the rotation of Dmin; see Fig. 1C). In Fig. 4C the points, therefore, show the distance in micrometers of the center of the open gap of these cross walls from the center of the septal region (shown by the solid line). These data indicate that the amount of asymmetry in the placement of the central hole in open cross walls is very small.

Thus, most growth zones conform fairly well with expected predictions of structures showing longitudinal radial symmetry. Since the rotational technique can be used to compensate for small asymmetries such as those observed in Fig. 4 (see legend of Fig. 1E), it seems that asymmetry does not contribute a significant source of error to the results obtained by the rotational technique.

Sources of error. To a degree, the accuracy and precision of the rotation technique is influenced by the number of trapezoids that are fitted to the peripheral walls of growth zones.

However, a point of diminishing return is approached where further increases in the number of trapezoids fitted to a growth zone will no longer result in a significant increase in the precision or accuracy of the estimations obtained. The error in estimating the surface area and volume of curved structures by the rotation technique was studied by fitting trapezoids to a circle that had a diameter similar to that of the cells analyzed in this paper. The number of trapezoids fitted to the circle was determined by the same criteria applied to curved peripheral wall segments seen in growth zones (see legends to Fig. 1E for details). The surface area and volume of the sphere calculated by the rotation of these trapezoids were then compared with the surface and volume values obtained by substituting the known radius of the circle into standard equations. This analysis indicated that the rotational analogue method produced average errors for the estimation of surface area and volume of less than 2 and 4%, respectively.

A far larger problem than determining the number of measurements used for a reconstruction of growth zones is finding the suitable numbers of thin sections of cells that have completely tribanded cell walls. As shown in Fig. 3, small deviations in the plane of sectioning from the longitudinal axis can produce quite significant errors.

Finally, one source of error that is unrelated to either the precision of the rotation technique or the selection of thin sections of cells is the precision with which cells grow even under carefully controlled conditions. As shown in an accompanying paper (13), the cells of *S. faecalis* are much more variable in the surface-to-volume ratios they show as they enlarge and enter the last third of a cycle of growth.

It appears that the assumptions upon which the rotational-analogue technique are based appear to be valid within the present limits of our measurements and the reservations expressed. Clearly, future use of the method (i.e., investigating cultures where the average cell volume and macromolecular composition have been altered by unbalanced synthesis) will allow further evaluation of the accuracy and precision of the technique by determining changes in the amount of cell wall material in these populations. However, studies to date (see reference 13) suggest that the method is potentially capable of yielding site-specific information concerning the geometry and assembly of bacterial envelopes that cannot be approached at this time by available biochemical or biophysical techniques.

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LITERATURE CITED

1. **Bayer, M. E., and C. C. Remsen.** 1970. Structure of *Escherichia coli* after freeze-etching. J. Bacteriol. 101:304-313.
2. **Boothby, D., L. Daneo-Moore, M. L. Higgins, J. Cozette, and G. D. Shockman.** 1973. Turnover of bacterial cell wall peptidoglycans. J. Biol. Chem. 248:2161-2169.
3. **Briles, E. B., and A. Tomasz.** 1970. Radioautographic evidence for equatorial wall growth in a Gram-positive bacterium. Segregation of choline-^{3}H-labeled teichoic acid. J. Cell Biol. 47:786-790.
4. **Cole, R. M., and J. J. Hahn.** 1962. Cell wall replication in *Streptococcus pyogenes*. Science 135:722-724.
5. **Ghuysen, J.-M., E. Bricas, E. Lehy-Bouille, M. Lache, and G. D. Shockman.** 1967. The peptide N$^{\alpha}$-(L-alanyl-D isoglutaminyl)-N$^{\epsilon}$-(D-isoasparaginyl)-L-lysyl-D-alanine and the disaccharide N-acetyl-glucosaminyl-β-1, 4-N-acetylmuramic acid in cell wall peptidoglycan of *Streptococcus faecalis* strain ATCC 9790. Biochemistry 6:2607-2619.
6. **Higgins, M. L.** 1973. A fixation and embedding procedure for thin-sectioning bacteria, p. 686-689. *In* A. I. Laskin and H. Lechevalier (ed.), CRC handbook of microbiology, vol. 1. CRC Press, Cleveland.
7. **Higgins, M. L., and L. Daneo-Moore.** 1974. Factors influencing the frequency of mesosomes observed in fixed and unfixed cells of *Streptococcus faecalis* (ATCC 9790). J. Cell Biol. 61:288-300.
8. **Higgins, M. L., L. Daneo-Moore, D. Boothby, and G. D. Shockman.** 1974. Effect of inhibition of DNA and protein synthesis on the direction of cell wall growth in *Streptococcus faecalis* (ATCC 9790). J. Bacteriol. 118:681-692.
9. **Higgins, M. L., H. M. Pooley, and G. D. Shockman.** 1970. Site of initiation of cellular autolysis in *Streptococcus faecalis* as seen by electron microscopy. J. Bacteriol. 103:504-512.
10. **Higgins, M. L., H. M. Pooley, and G. D. Shockman.** 1971. Reinitiation of cell wall growth after threonine starvation of *Streptococcus faecalis*. J. Bacteriol. 105:1175-1183.
11. **Higgins, M. L., and G. D. Shockman.** 1970. Model for cell wall growth of *Streptococcus faecalis*. J. Bacteriol. 101:643-648.
12. **Higgins, M. L., and G. D. Shockman.** 1971. Procaryotic cell division with respect to wall and membranes. CRC Crit. Rev. Microbiol. 1:29-72.
13. **Higgins, M. L., and G. D. Shockman.** 1976. Study of a cycle of cell wall assembly in *Streptococcus faecalis* by three-dimensional reconstructions of thin sections of cells. J. Bacteriol. 127:1346-1358.
14. **Shockman, G. D.** 1962. Amino acids, p. 567-673. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press Inc., New York.

15. **Shockman, G. D., L. Daneo-Moore, and M. L. Higgins.** 1974. Problems of cell wall and membrane growth, enlargement, and division. Ann. N.Y. Acad. Sci. 235:161–197.
16. **Swanson, J., K. C. Hsu, and E. C. Gotschlich.** 1969. Electron microscopic studies on streptococci. I. M antigen. J. Exp. Med. 130:1063–1091.
17. **Wagner, M.** 1964. Studien mit fluoreszierenden Antikörpen an wachsenden Bakterien. I. Die Neubildung der Zellwand bei *Diplococcus pneumoniae*. Zentralbl. Bakteriol. Parasitenkd. Infekttionskr. Hyg. 195:87–93.