Unit Cell Hypothesis for *Streptococcus faecalis*

EUGENE M. EDELSTEIN, MARTIN S. ROSENZWEIG, LOLITA DANEO-MOORE, AND MICHAEL L. HIGGINS

Department of Microbiology and Immunology, and Department of Biometrics, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

The mass doubling times of exponential-phase cultures of *Streptococcus faecalis* were varied from 30 to 110 min by omitting glutamine from a defined growth medium and providing different concentrations of glutamate (ranging from 300 to 14 μg/ml). After Formalin fixation, cells were dried by the critical point method, and carbon-platinum replicas were prepared. The surface area and volume of cell poles seen in these replicas were estimated by a computer-assisted, three-dimensional reconstruction technique. It was found that the amount of surface area and volume of poles seen in these replicas were independent of the growth rate of culture from which the samples were taken. These observations were consistent with the unit cell model hypothesis of Donachie and Begg, in which a small number of surface sites would produce a constant amount of new cell surface regardless of the mass doubling time of the culture. However, measurements of the thickness of the cell wall taken from thin sections of the same cells showed that the cell wall increased in thickness as a function of the increase in cellular peptidoglycan content which occurs when the growth rate of this organism is slowed down by a decrease in glutamate concentration. Thus, it would seem that although the size of polar shells made by *S. faecalis* is invariant with growth rate, the amount of wall precursors used to construct these shells is not.

In 1970, Donachie and Begg proposed a model in which, at the slowest possible exponential-phase growth rate, a cell of *Escherichia coli* would be enlarged by a single growth site producing a “unit cell” of new membrane (4). As the growth rate of a culture increased, the number of growth sites per cell would also increase, but the amount of envelope produced per site would remain constant (i.e., one unit cell). Thus, the model predicted that: (i) the amount of envelope produced per site would be independent of the growth rate, and (ii) the smallest average size of a newborn cell, grown under the slowest possible conditions, would be equal to a unit cell. Unfortunately, the testing of this and other models in rod-shaped bacteria such as *E. coli* has had to rely on indirect methods to assess the number, location, and output envelope growth sites (15).

In these types of studies, chain-forming cocci such as *S. faecalis* offer the distinct advantage that their envelope growth sites are localized between pairs of raised bands of wall material which can easily be seen in electron micrographs (see Fig. 1A) (8-10). Such sites are initiated by a nascent cross wall being assembled on the interior surface of the wall beneath a pre-existing band. Two new bands are formed as this nascent cross wall begins to split to form two new layers of peripheral wall. The further enlargement of these two peripheral wall surfaces (as a result of the continued constructive cleavage of the cross wall) causes the bands to be pushed from the center of a site. The peripheral wall produced from the complete symmetric severance of the cross wall results in the formation of two polar caps. Thus, unlike rod-shaped bacteria, each envelope growth site in a coccus produces only two new poles after a round of growth.

Here we have tested the unit cell model of Donachie and Begg (4) in *S. faecalis* by proposing that a unit cell in this organism is equivalent to the size of two poles (i.e., the amount of envelope surface produced by a single growth site) and by asking whether the size of a pole is constant in cultures showing a wide range of mass doubling times.

**MATERIALS AND METHODS**

Cell growth. Cells of *S. faecalis* ATCC 9790 were allowed to go through at least six mass doublings in a chemically defined medium (14) (reaching the equivalent of 100 μg of dry mass per ml) before being used for study. Mass doubling times (T_d) between 30 and 110 min were obtained by removing glutamine and providing a range of concentrations of glutamate between 300 and 14 μg (16).

Electron microscopy. Formaldehyde prepared from the hydrolysis of paraformaldehyde was added
Fig. 1. Examples of carbon-platinum replicas of exponential-phase cells of S. faecalis used in this study to characterize the size and shape of poles from cells that had come from cultures with $T_D$ ranging from 30 to 110 min. The presence of wall bands (b) in these cells allowed their polar surfaces (i.e., the surfaces external to the bands) to be differentiated from the envelope growth sites of these cells (i.e., surfaces internal to the bands). The series of pictures shown (A to C) is arranged to show the proposed development of a single envelope growth site which will eventually produce two poles. The replicas shown were cast from cells which were doubling in mass every 60 min before fixation. Bar in C applies to all micrographs and equals 0.1 µm.

to 100 ml of cell cultures until a final concentration of 8.3% was reached. After the addition of formaldehyde, a membrane filter with a pore diameter of 0.45 µm (Millipore Corp., Bedford, Mass.) was used to separate the cells from the growth medium. When most of the culture fluid had passed through the filter, the vacuum to the apparatus was reduced, and a balanced salt solution (BBS; 0.09 M KCl, 0.01 M magnesium acetate, 0.05 M phosphate buffer, pH 6.2) containing 8.3% formaldehyde was allowed to “percolate” over the cells for 20 min. After this period of percolation, cells were resuspended for 1 h in BBS containing 8.3% formaldehyde, centrifuged (8,000 × g; 2 min), washed four times in distilled water, and suspended in 1.0 ml of 0.05 M magnesium acetate. Carbon-platinum replicas of these cells were made by a modification of the
EVIDENCE FOR CELL UNIT HYPOTHESIS

Data Celloscope (Particle Data Co., Chicago, Ill.) An equation relating cell count per microgram (dry weight) to growth rate (Lancy et al., submitted for publication) was used to estimate peptidoglycan and rhamnose polysaccharide per cell at various growth rates.

RESULTS

Cultures with \( T_D \) ranging from 30 to 110 min were established; cells were taken from each of these cultures, and carbon-platinum replicas of their cell surfaces were made. The surface area and volume of the poles observed in electron micrographs of each population of cell replicas were estimated by using a rotational technique described in Materials and Methods. The cells were allowed to go through at least six doublings in mass before study to ensure that virtually all of the poles analyzed within a given population were produced in envelope growth sites of cells that had been growing at the stated \( T_D \). Figure 3 shows the frequency distributions of the volume measurements obtained from analyzing the poles found in each of the 11 cultures studied. In addition, for comparative purposes, the figure also shows a distribution of measurements from a group of unfixed cells (Fig. 3A). The symmetry of the distributions of polar volume measurements about the means shown in Fig. 3 was examined under the hypothesis that under symmetry one half of the measurements would be expected to be greater than (and one half to be less than) the mean. This hypothesis was tested by applying a large sample test of the binomial proportion (p) to each set of measurements. The results of this test are shown in Table 1 and indicate that at a 95% confidence level the hypothesis of symmetry can only be rejected in the case of the cells that had a \( T_D \) of 83 min before fixation. This finding caused some concern, since the 83-min distribution was based on roughly four to five times more observations than any

---

Vol. 143, 1980
other sample analyzed (n; Table 1). This concern was strengthened when closer examination of the data in Table 1 indicated that in 10 of the 11 samples the proportion of the observations greater than the mean was slightly less than 0.5. The probability of this number of samples showing p values less than 0.5 in truly symmetric populations was calculated to be 0.006. Thus, we conclude that (i) the distribution of measurements shown in Fig. 3 is similar, but most are skewed slightly toward the smaller volume measurements, and (ii) that the large sample test used in Table 1 is too insensitive to detect this level of skewness with the number of observations available.

Measurements have shown that the poles of S. faecalis continue to enlarge on the average of about 13% in volume and 10% in surface area after they are initially formed by cross-wall closure (11). Thus, the predominance of poles with smaller volumes can be explained on the basis that in any population in exponential growth there should be twice as many newly formed poles with smaller volumes as there are larger poles produced one or more generations in the past.

To determine whether the mean pole volumes shown in Fig. 3 were dependent upon growth rate, each of these volumes was plotted in Fig. 4 as a function of its culture's exponential growth rate constant (\( \alpha = \ln 2/T_D \text{[h]} \)). Similarly, to determine whether some of the other shape-related measurements made from these poles were growth rate dependent, the average polar height, the equatorial radius, and surface area of each population (Fig. 2) were plotted in the same manner (Fig. 4). The lines shown in Fig. 4 are derived from a regression analysis of the mean values in each panel. Examination of the residuals obtained from this analysis indicated that a straight line provided an adequate description of each measurement (Table 2). A test was then applied in each case to determine whether the slopes of the fitted lines differed from zero. The results of these tests are also shown in Table 1 and suggest that only the slope of the line passing through the average measurements of pole height differed significantly (at the 95% confidence level) from zero. This indicates that poles elongate slightly (i.e., increase in pole height) as

![Fig. 3. Distributions of the volumes of poles determined from the analysis of populations of cells from cultures with \( T_D \) that ranged from 30 to 110 min. (A) represents the distribution of measurements from a culture \( (T_D = 30 \text{ min}) \) which was not fixed before it was replicated, whereas the remainder of the distributions (B to M) were based on the analysis of the poles of cells that had been fixed in an identical manner before replication. Dashed line indicates the average volume of each population. In the latter case, the \( T_D \) of the respective cultures were: B = 30 min, C = 33 min, D = 37 min, E = 43 min, F = 47 min, G = 55 min, H = 60 min, I = 70 min, J = 83 min, K = 105 min, M = 110 min.](image)

<table>
<thead>
<tr>
<th>Culture ( T_D ) (min)</th>
<th>( n^a )</th>
<th>( p^b )</th>
<th>( Z^c )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>240</td>
<td>0.467</td>
<td>-0.937</td>
<td>NS(^d)</td>
</tr>
<tr>
<td>33</td>
<td>194</td>
<td>0.485</td>
<td>-0.321</td>
<td>NS</td>
</tr>
<tr>
<td>37</td>
<td>258</td>
<td>0.465</td>
<td>-1.065</td>
<td>NS</td>
</tr>
<tr>
<td>43</td>
<td>180</td>
<td>0.449</td>
<td>-1.300</td>
<td>NS</td>
</tr>
<tr>
<td>47</td>
<td>206</td>
<td>0.495</td>
<td>-0.074</td>
<td>NS</td>
</tr>
<tr>
<td>55</td>
<td>182</td>
<td>0.512</td>
<td>0.250</td>
<td>NS</td>
</tr>
<tr>
<td>60</td>
<td>228</td>
<td>0.447</td>
<td>-1.543</td>
<td>NS</td>
</tr>
<tr>
<td>70</td>
<td>210</td>
<td>0.499</td>
<td>0.040</td>
<td>NS</td>
</tr>
<tr>
<td>83</td>
<td>1,016</td>
<td>0.453</td>
<td>-2.980</td>
<td>0.0014</td>
</tr>
<tr>
<td>105</td>
<td>192</td>
<td>0.469</td>
<td>-0.788</td>
<td>NS</td>
</tr>
<tr>
<td>110</td>
<td>180</td>
<td>0.482</td>
<td>-0.409</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^a\) \( n \), Number of poles in the population.  
\(^b\) \( p \), Observed proportion of observations greater than the mean.  
\(^c\) \( Z = (p - 0.5) - 1/2n) / \sqrt{p (1 - p)/n} \).  
\(^d\) NS, Not significant.
the doubling time of the culture decreases from 110 to 30 min; however, no significant growth rate-dependent changes in the volume, surface area, or equatorial radius could be detected.

Recent investigations have shown that the amount of peptidoglycan found per cell in *S. faecalis* increases when the growth rate of the culture is reduced by the same glutamate supplementation method as that used in the present study (P. Lancy, Ph.D. thesis, Temple University, Philadelphia, 1976). Results in Fig. 5 show that the thickness of the cell wall observed in the poles of thin sections of cells increased as the growth rate of the culture was decreased. To compare the average thickness of the cell walls of these cells with the cellular peptidoglycan and rhamnose content, equal numbers of measurements were included from the envelope growth sites and poles of each group of cells. This inclusion of growth site measurements was necessary to place both the thickness and chemical determinations on a per cell basis. This comparison (Fig. 6) indicates that the increase in cell wall thickness can be correlated with an increase in the amount of peptidoglycan per cell (rather than to any change in the amount of rhamnose accessory wall polymer).

**DISCUSSION**

Our measurements suggest that the size of the poles of *S. faecalis* are invariant in cultures with mass doubling times ranging from 30 to 110 min. This finding is consistent with the unit cell model of Donachie and Begg (4), which proposed that a single site of envelope growth would produce a fixed amount of membrane independently of growth rate. In *S. faecalis*, this fixed amount of membrane would be assumed to be equivalent to the surface area of two poles produced by one of its envelope growth sites. The model of Donachie and Begg also proposed that this unit cell would be equivalent to the size of the smallest average cell at birth in the slowest growing culture. In *S. faecalis*, a unit cell would consist of two poles attached at the equator by a single band, and would be about 0.309 μm³ in size (i.e., the average volume of two poles at any growth rate). Such cells can in fact be observed in slowly

**FIG. 4.** The mean height (H), equatorial radius (R), surface area (Pa), volume (Vol), and surface area-to-volume ratio obtained from the analysis populations of poles seen in replicates of cells taken from cultures with T₀, ranging from 30 to 110 min plotted as a function each culture's exponential growth rate constant α, where α = ln2/T₀ and the units of T₀ are in hours.

**TABLE 2. Test of slopes of regression lines**

<table>
<thead>
<tr>
<th>Class</th>
<th>Equation of regression line</th>
<th>r²</th>
<th>t</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pole ht</td>
<td>y = 0.035x + 0.465</td>
<td>0.53</td>
<td>3.17</td>
<td>0.01</td>
</tr>
<tr>
<td>Pole Rx</td>
<td>y = -0.015x + 0.417</td>
<td>0.30</td>
<td>-1.99</td>
<td>NS</td>
</tr>
<tr>
<td>Pole Pa</td>
<td>y = -0.016x + 1.120</td>
<td>0.04</td>
<td>0.58</td>
<td>NS</td>
</tr>
<tr>
<td>Pole vol</td>
<td>y = -0.004 + 0.154</td>
<td>0.06</td>
<td>0.75</td>
<td>NS</td>
</tr>
<tr>
<td>Pole Pa/vol</td>
<td>y = 0.106x + 7.240</td>
<td>0.08</td>
<td>0.92</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Pa*, Surface area; Rx, equatorial radius; ht, height; vol, volume.

r², Coefficient of determination; measures linear dependence of y values on x values.

t, Experimentally determined t value.

NS, Not significant. n = 11.

**FIG. 5.** Average thickness of the cell wall of the poles of *S. faecalis* measured from axial thin sections of cells from cultures with a range of mass doubling times between 30 and 110 min.
growing cultures where rounds of envelope synthesis do not overlap (Fig. 7).

Although pole size appeared to be independent of growth rate, the poles appeared to become slightly less elongated and their cell walls thicker at slow growth rates. The increase in cell wall thickness was correlated with an increase in the peptidoglycan content of cells (Fig. 6). It has been shown that if the envelope growth sites in a culture of exponential-phase cells of *S. faecalis* are inactivated (such as by the addition of an inhibitor of protein synthesis), surface growth is inhibited and cell wall precursors accumulate in cells in the form of cell walls of greater than normal thickness (7). In slowly growing exponential-phase cultures where the mass doubling time is greater than \( C + D \) minutes (i.e., greater than about 80 min), studies of synchronous cultures have shown that the rate of synthesis of cell wall precursors declines, but still continues in the early phases of a cell cycle (12). However, it has been proposed that envelope growth sites would be introduced and would function in such cells only in the latter phases of the cell cycle (15). This suggests that precursors made in the early part of the cycle of slowly growing cells could be involved primarily in thickening, whereas precursors produced in the latter phases of the cycle (after envelope growth sites have been introduced) would be used primarily in enlarging the cell surface. As cultures increase, their growth rate so that they are dividing faster than \( C + D \) minutes, the number of envelope growth sites per cell should increase in a manner similar to DNA replication initiations, and surface expansion would occupy an increasing fraction of the cell division cycle. The decrease in cell wall thickness observed in this study as the growth rate increases can be explained by proposing that an increasing number of envelope growth sites appearing in the rapidly growing cells would outcompete the thickening process for available cell wall precursors.

In summary, it seems that the sizes of poles in *S. faecalis* are apparently independent of growth rate (\( T_D \) between 30 and 120 min); however, the geometry and the thickness of these structures are affected by changes in growth rate. The constancy of pole size in *S. faecalis* is remarkable enough to hypothesize that it might be a regulatory element in determining the size of cells at division. In this model, each surface growth site would produce a specific amount of surface area equivalent to that found on two cell poles.

**ACKNOWLEDGMENTS**

We thank M. Case for the discussions and many of the ideas that led to this approach; S. Wagner and U. Sleytr for developing the carbon-platinum replica technique; A. Koch for his criticisms and suggestions, especially concerning the conclusions of this study; G. D. Shockman for his ideas, comments, encouragement, and help in the preparation of this manuscript; M. P. O'Connor for her help in electron microscopy; and J. Miller for her digitization of some of the electron micrographs.

This work was supported by a research grant, GB 31920, from the National Science Foundation, and by Public Health
EVIDENCE FOR CELL UNIT HYPOTHESIS

Service grant AI 10971 from the National Institute for Allergy and Infectious Diseases.

LITERATURE CITED


