Measurement of Live Bacteria by Nomarski Interference Microscopy and Stereologic Methods as Tested with Macroscopic Rod-Shaped Models

WILLIAM W. BALDWIN* AND PATRICK W. BANKSTON
Northwest Center for Medical Education, Indiana University School of Medicine, 3400 Broadway St., Gary, Indiana 46408

Received 15 June 1987/Accepted 18 September 1987

A new method is proposed to measure bacterial cells under growth conditions. Bacterial cells, suspended in their growth medium, were attached to a cover slip with poly-L-lysine. The cover slip was inverted and placed on a glass microscope slide. To prevent dehydration of the medium, the edges of the cover slip were sealed to the microscope slide with clear fingernail polish. The bacteria on the slide were then quickly photographed with a Leitz light microscope, using Nomarski optics. The photographic negatives were then projected at a standard distance through a lens system, and the projected images of the whole cells were outlined by hand onto graph paper. The profile images so transcribed onto the graph paper were in effect transverse sections of each of the cells. Using stereologic grid and point counting techniques, the area of the cell transverse section as well as the perimeter or circumference of the transverse section were estimated. Formulate were developed so that both the volume and surface area of the whole cell could be ascertained from these area and circumference measurements. Since the efficacy of any measurements of surface area and volume of microscopic rod-shaped bacterial cells could be questioned, macroscopic rod-shaped models were used to test the theory and formulate and to compare this method with other commonly used cell-sizing techniques. This technique could be used in any study of bacterial cell size or changes in cell size (e.g., osmotic shifts).

The sizes of growing bacteria have been measured during studies of growth rates of individual bacterial cells (2, 3, 5, 7, 8, 11, 15, 18, 22), change in size of cells exposed to osmotic shifts (1, 11, 13, 20, 23), and cell size of bacteria growing at different generation times (4, 6, 9, 12, 14, 21). These problems have been examined by light microscopy (2), electronic cell sizing (e.g., Coulter Counter) (16, 17), and electron microscopy (10, 18, 24). Each of these methods has some weaknesses in making cell size measurements.

Electron microscopy, while having the highest resolution of the techniques mentioned, has the weakness that live material cannot be observed. The cells must first be fixed, dehydrated, and also possibly embedded in a plastic, each step of which may change the size of the cells.

Electronic cell sizing has been used effectively (e.g., see references 16 and 17). However, to size bacterial cells with this technique, one must suspend the cells in an electrolyte of fixed composition. Usually, this is done by dilution into a saline or HCl solution (16, 17). This dilution procedure will produce osmotic effects on the bacterial cells and make studies of cell size in response to osmotic shifts difficult. A second problem is that the sizing orifices must be specially constructed for bacterial studies and are not commercially available. Also, since only mean cell size is measured, length and width of the individual cells cannot be determined by electronic cell sizing. If the changes in length were inverse to changes in width, the changes might compensate for each other and the difference would go unnoticed.

In light microscopy, cells can be suspended and observed in their own growth medium. The cells can also be attached to a cover slip with poly-L-lysine and shifted from one osmolality to another by submerging the cover slip in a large excess of a medium with a different osmolality. Mean cell size can be determined, as well as mean length and width. Since the limit of resolution of the light microscope is about 0.2 μm, the radius of an Escherichia coli cell (0.5 μm) approaches the resolving power of the light microscope. This could lead some to criticisms of any bacterial cell size measurements done with a light microscope.

However, we believe that accurate dimensions can be calculated if the surface area and volume of the cell are measured with the light microscope and then these figures are used to calculate the radius and length of the cell. Both the surface area and volume of the cell are much larger than the radius and length of the cell and can therefore be measured with a higher degree of accuracy when compared with the 0.2-μm resolution limit inherent in a light microscope. For example, in Fig. 1, if rod-shaped cells are assumed to be hemisphere-capped cylinders with radius r and height h (note: length = 2r + h), then the volume of the cell can be calculated by the equation V = 4/3(πr²) + πr²h. This is the volume of a sphere (two hemispheres) added to the volume of a cylinder. From this equation, the radius, which has the same dimensions for both the hemisphere and the cylinder, can be determined by taking the cube root and the square root of the measured volume. On the other hand, if radius and length (or height) are measured, then when measuring the radius, the error made in the measurement will be cubed or squared, causing it to be greatly increased, while when measuring the volume, the error made will be
reduced by the cube root or square root and accuracy will be
maximized.

Although we cannot measure surface area and volume of
cells directly, we can measure circumference and area of a
transverse section. Such a section is the image, outlined on
graph paper, of the projected light micrograph of a cell and
would appear similar to Fig. 1. An assumption is made that
the rotation of this transverse section through 360° about its
long axis will be a good approximation of the complete cell.
We also assume that these cells are hemisphere-capped
cylinders. Some of these assumptions were tested by using
rod-shaped models that were large enough to be measured
directly. In two of the three tests, the proposed method gave
better results than directly measuring the length and width
with calipers.

MATERIALS AND METHODS

Strains and culture conditions. E. coli MC4100 lysA phiB
was grown in 1% tryptone (Difco Laboratories, Detroit,
Mich.)-0.5% yeast extract (Difco)-1% glucose. NaCl was
added to the culture medium to give the appropriate experi-
mental osmolarity. Only exponentially growing cells were
used.

Rod-shaped models. Our requirements for rod-shaped
models were as follows: large enough to be measured with
calipers; some variation in size within each group to be like
normal cells; at least three different size groups to test the
flexibility of the method; suitable for measurement of water
displacement volume (e.g., not readily water soluble or
readily absorbing water); and finally, easily available. We
chose breakfast sausages (rod-shaped model one), hot dogs
(rod-shaped model two), and Polish sausages (rod-shaped
model three) as the three different size groups.

Measurement of models. Models were photographed with a
Polaroid MP4 Land camera copy stand. A metric ruler was
also photographed at the same focal length. These photo-
graphic negatives were then projected at a standard distance,
and the images of the models (the so-called transverse
sections) were outlined on graph paper. The projected image
of the ruler, at the same standard distance, was used to
determine the appropriate conversion factor. The area and
circumference of each outlined image were estimated by
counting the total number of squares inside the image (area)
as well as the number of horizontal and vertical lines on the
graph paper that were intersected by the image outline
(circumference) (25) (Fig. 1). The estimates of circumference
could also easily be measured with a planimeter; however, a

planimeter was not used in these studies. Various stereologic
methods of measuring surface areas and volumes have been
reviewed by Williams (25).

Models were also measured with metric calipers. The
length and width of each model were measured, and mean
values were determined. The models were also submerged in
water in a graduated cylinder to determine the water dis-
placement volume. The volume of water in the graduated
cylinder after the models were removed was the same as
before submersion (e.g., no water was absorbed).

Measurement of bacterial cells. Bacterial cells were pho-
tographed onto Kodak Tri-X film with a Leitz microscope
equipped with Nomarski optics, using a 100× oil immersion
objective, and the photographic negatives were projected
and measured in the same stereologic manner as described
above. Control experiments were conducted to measure the
osmolarity at which plasmolysis occurred in these cells
under our experimental conditions. No plasmolysis could
be observed at the osmotic concentrations used in these ex-
periments. Latex spheres of 0.9-μm average diameter (Poly-
sciences, Inc., Werrington, Pa.) were also studied as a control
with this microscopic system to determine whether the
osmolarity of the suspending medium changed the apparent
size of the spheres. When they were photographed and
measured according to the methods above, no size change
was noted. Nomarski imaging utilizes two prisms of the
Wollestone type that results in a pseudo three-dimensional
image of biological objects suspended in medium. It has the
advantage over phase-contrast microscopy of not introduc-
ing halos around the object (19). The duplicated image has
shadows and highlights in a constant direction relative to the
image. Since the bacteria are randomly oriented and only
those held in the single plane of focus are used, the optical
technique should not introduce any measurement errors.

Statistical analysis. The data were analyzed by one-way
analysis of variance (one-way ANOVA) with all values being
compared with the water displacement value for each rod-
shaped model. The calculations were done by a personal
computer.

RESULTS

Figure 2 shows photographs of the rod-shaped models as
well as E. coli cells grown in 150 mosM Luria broth. Figure
2A shows rod-shaped model one (smallest), panel B shows
rod-shaped model two (medium sized), panel C shows rod-
shaped model three (largest), and panel D shows E. coli.

Table 1 shows the results of volume measurements of the
rod-shaped models determined by three independent meth-
ods. The three methods used were measurement of the
length and width with metric calipers; measurement by
water displacement; and measurement of area and circum-
ference of the transverse section (called stereology in the
Table). The formulae and equations used in this study as well
as a computer program to solve the equations are outlined in
the appendix to this paper. Using calipers to measure the
volume of the models gave results that in two of the three
cases were significantly different from the water displace-
ment volume at the 0.01 level by one-way ANOVA. These
differences were seen with rod-shaped models two and
three. When stereologic methods were used to measure the
volume of the models, results in one of the three cases were
significantly different from the water displacement volume at
the 0.05 level by one-way ANOVA. This difference was seen
with rod-shaped model one.
BACTERIAL SIZE MEASUREMENTS

When volume was determined by the caliper method, two of the values were significantly different from the values determined by the water displacement method. These differences might represent an error in the assumption that the model cells are hemisphere-capped cylinders. Since the assumption that the model cells are hemisphere-capped cylinders was also used in the stereologic method, this argument seems unlikely. Another possible explanation for the data is that measuring length and width is not a good method to determine volume even in these large rod-shaped models.

When volume was determined stereologically, one of the values was significantly different from the values determined by the water displacement method. This difference might be explained by a close examination of rod-shaped model one (Fig. 2A) which shows that the models are not hemispheres on the ends. If this is the correct explanation for the significant difference seen by ANOVA, an interesting possibility is created that could explain why rod-shaped model one was the only model whose volume as measured by calipers was not significantly different from the water displacement volume. That possibility is that the error in the assumption of hemispherical ends offsets the errors in measurement to produce a nearly correct value for volume.

When taken in toto, our results indicate that the stereologic method, which does not require physical contact with the cells, may provide an accurate measurement of cell parameters. This technique has been applied to the measurement of cell size changes in response to osmotic shifts in E. coli (1a). The stereologic method is capable of detecting small shifts in cell size in a large population of cells; furthermore, circumference and area values can be compared directly without any assumptions being made about the final cell shape.

APPENDIX

We assumed the cell is a cylinder with hemispherical caps. To be able to solve for the radius (r) and height (h) (h = length - 2r) of a cell from the transverse section, the area, A, of the transverse section image is:

\[ A = \pi r^2 + 2\pi r \]  

and circumference, C, is:

\[ C = 2\pi r + 2h \]  

Solving equation 2 for h yields:

\[ h = C - 2\pi r/2 \]  

Substituting equation 3 in equation 1, we get:

\[ A = \pi r^2 + [2((C - 2\pi r)/2)r \]  

which can be rearranged to:

\[ \pi r^2 - Cr + A = 0 \]  

which is a quadratic equation and can be reduced to:

\[ r = (C \pm \sqrt{C^2 - 4\pi A})/2\pi \]  

and solved for r since A and C are known.

The height (h) is found by substituting the values of r in either equation 1 or 2. Volume, V, and surface area, SA, can be found by using r and h from above in the equations: 

\[ V = 4/3(\pi r^3) + 2\pi rh \]  

SA = 4\pi r^2 + 2\pi rh

Using the above equations, the following program was written to solve the equations. The program is written in Applesoft BASIC.
1 VT = 0; ST = 0; DA = 0; DB = 0
3 PRINT CHR$(4);"PB#1"
5 PRINT "Would you like a hard copy? If so type a y for yes and an n
or no."; INPUT AA
7 IF AA = "Y" THEN PRINT CHR$(4);"PB#1"
10 INPUT "How many cells were sized?"; N
11 N = N + 1
20 DIM C(N),A(N),RA(N),RB(N),HA(N),HB(N),SA(N),V(N),D(N),DS(N)
21 N = N - 1
30 FOR I = 1 TO N STEP 1
31 PRINT "Circumference of cell "I"?"
32 INPUT C(I)
33 PRINT "Area of cell "I"?"
34 INPUT A(I): NEXT
40 PI = 3.1415927
50 FOR I = 1 TO N STEP 1
51 RA(I) = (C(I) + SQR(C(I)^2 - 4 * PI * A(I))) / (2 * PI)
52 RB(I) = (C(I) - SQR(C(I)^2 - 4 * PI * A(I))) / (2 * PI)
53 HA(I) = (A(I) - PI * RA(I)^2) / (2 * RA(I))
54 HB(I) = (A(I) - PI * RB(I)^2) / (2 * RB(I))
55 PRINT "Circumference of "I": PRINT "Area of "I": PRINT "Height of "I": PRINT "Radius of "I""
100 FOR I = 1 TO N STEP 1
110 IF RA(I) < 0 GOTO 250
115 IF HA(I) < 0 GOTO 290
120 IF HB(I) < 0 GOTO 290
125 IF RB(I) < 0 GOTO 290
130 VT = 0; ST = 0;
140 FOR I = 1 TO N STEP 1
150 V(I) = (4 / 3 * PI * (RB(I)^3 - RA(I)^3)) + (PI * (RB(I)^2 - RA(I)^2) * HB(I))
160 PRINT "The volume of cell "I" is "V(I)
170 ST = ST + SA(I); GOTO 170
180 V(I) = (4 / 3 * PI * (RA(I)^3 - RB(I)^3)) + (PI * (RA(I)^2 - RB(I)^2) * HA(I))
190 PRINT "The volume of cell "I" is "V(I)
200 VT = VT + V(I)
210 SA(I) = (4 * PI * (RA(I)^2 - RB(I)^2)) + (2 * PI * RA(I) * HB(I))
220 PRINT "The surface area of cell "I" is "SA(I)
230 ST = ST + SA(I); GOTO 170
240 D(I) = (V(I) - VA) / 2
250 DA = DA + D(I); NEXT I
260 SD = SQR(DA / (N - 1))
270 SM = SD / SQR(N)
280 PRINT "The standard error of the mean volume = + or -" SM
290 FOR I = 1 TO N STEP 1
300 DS(I) = (GA(I) - SA(I)) / 2
310 DB = DB + DS(I); NEXT I
320 DS = SQR(DB / (N - 1))
330 MS = DS / SQR(N)
340 PRINT "The standard error of the mean surface area = + or -" MS
350 GOTO 240

ACKNOWLEDGMENT

This work was supported in part by funds from the Lake County (Indiana) Medical Center Development Agency (LCCMDA).

LITERATURE CITED


