METHOD FOR STANDARDIZING STAPHYLOCOCCAL SUSPENSIONS: RELATIONSHIP OF OPTICAL DENSITY TO VIABLE CELL COUNT

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Quantitative assays of antimicrobial activity require well-dispersed bacterial suspensions. Staphylococcal suspensions are especially difficult to prepare, and most reported methods are inadequate. The method reported here produces finely dispersed staphylococcal suspensions that give reproducible viable counts (pour plates) correlating well with spectrophotometric readings.

Screw-cap tubes (16 × 125 mm) containing 5 ml of Trypticase Soy Broth (BBL) and 0.5% polyoxyethylene sorbitan monooleate (Tween 80) were inoculated with one loopful of a 24-hr staphylococcal culture from a Trypticase Soy Agar (BBL) slant. The inoculated broths were then incubated at 36 C for 5 hr on a Roller Tube Culture Apparatus (Aloe Scientific Co., catalogue no. 59818) modified to rotate at 20 rev/min. After the 5-hr incubation period, the cultures were centrifuged at $1,000 \times g$ for 10 min in an International centrifuge, size 2, model R. The supernatant broth was poured off, and 10 ml of normal saline with 0.5% Tween 80 were added to the bacterial pack. This was mixed and poured into a heavy-walled 100-ml centrifuge tube, closed with a no. 6 rubber stopper, and shaken

TABLE 1. *Optical density values versus bacterial colony counts*

Expt no.	Optical density*				
	0.195	0.268	0.392	0.497	0.592
1	8.0	9.5	15.8	—	30.0
2	8.2	9.6	15.5	20.0	29.4
3	8.0	8.8	—	18.8	30.0
4	7.7	8.7	16.4	18.9	—
5	8.0	9.0	16.2	18.5	30.2

* Results expressed as colony count $\times 10^7$.
Correlation coefficient = 0.965.

15 min in a Red Devil Paint Conditioner (Red Devil Tools, Irvington, N.J., catalogue no. 30). The tube was kept stoppered for 20 min to permit the aerosol to settle; then the staphylococcal suspension was poured into a Coleman tube, and the optical density read at 650 m μ by use of a Coleman Universal spectrophotometer. The staphylococcal suspensions were tenfold serially diluted, and pour plates were made to determine viable-cell counts.

Typical results achieved by the application of this technique may be seen in Table 1. These data gave a correlation coefficient of 0.965.

SPHEROIDAL AND POLYHEDRAL FORMS OF *CLOSTRIDIUM BUTYRICUM* INDUCED BY PENICILLIN

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As clostridia are generally resistant to lysozyme, neither protoplasts nor spheroplasts have been produced by treatment with the enzyme in a hypertonic medium. However, Diens (Proc. Soc. Exptl. Biol. Med. **75**:412, 1950) obtained an L-type culture from clostridia on solid media with the help of penicillin. The present communication reports formation of spheroidal and

irregular polyhedral bodies from *Clostridium butyricum* in a liquid medium containing penicillin.

Cells of *C. butyricum* strain 3315, supplied by the Institute for Fermentation, Japan, were grown in a hypertonic thioglycolate medium (1% polypeptone, 0.7% Na₂HPO₄·2H₂O, 0.5% glucose, 0.5% yeast extract, 0.15% sodium thio-

FIG. 1. Spheroidal form obtained after 30 min of incubation. All figures are electron micrographs of *Clostridium butyricum* 3315 grown in a hypertonic thioglycolate medium containing penicillin (100 units/ml). The cells were fixed with formaldehyde and shadowed with palladium.

FIG. 2 and 3. Developing polyhedral bodies obtained after 15 min of incubation. In Fig. 2, the cytoplasm is protruding through the rupturing cell wall. In Fig. 3, the cytoplasm is transformed to a spearheadlike form at the end of the cell.

FIG. 4. Small polyhedral form obtained after 30 min of incubation.

FIG. 5 and 6. Irregular polyhedral forms obtained after 3 hr of incubation.

FIG. 7. Ghost of the polyhedral body obtained after 3 hr of incubation.

glycolate, 0.075% L-cystine, 15% sucrose, and 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), superlayered with liquid paraffin, at 37 C for about 3 to 5 hr. Crystalline penicillin G potassium (in final concentrations of 100 to 1,000 units/ml) was added to the cultures during the exponential growth phase. From 15 min to 5 hr, the normal rod-shaped cells were quantitatively converted to spheroidal (Fig. 1) and curious polyhedral bodies (Fig. 4 to 6), as shown in electron micrographs. These bodies could be distinguished from each other by careful observations on wet preparations under a light microscope, and they gradually increased in size with age. These forms possessed flagella (Fig. 1 and 5), but seemed to be devoid of cell walls, since they burst immediately after being resuspended in water. Figures 2 and 3 illustrate the intermediate stages in the formation of polyhedral bodies from rod-shaped cells. Figure 7 shows a ghost of the polyhedral form that occasionally occurred during preparation for electron microscopy. This ghost appeared to be the cytoplasmic membrane. The spheroidal and poly-

hedral bodies could not be recovered in the original rod-shaped form after being transferred into a hypertonic thioglycolate medium without the antibiotic.

Penicillin-induced spheroplasts were first produced from *Escherichia coli* by Lederberg (Proc. Natl. Acad. Sci. U.S. **42**:574, 1956). Since Park and Strominger (Science **125**:99, 1957) demonstrated that penicillin inhibits cell-wall synthesis in *Staphylococcus aureus*, recent workers have confirmed their findings in other bacteria (Rogers and Mandelstam, Biochem. J. **84**:299, 1962; Roberts and Johnson, Biochim. Biophys. Acta **59**:458, 1962). In *C. butyricum*, the mechanism of formation of the spheroidal forms seems to be similar to that in *E. coli* (Lederberg and St. Clair, J. Bacteriol. **75**:143, 1958), but it is not known why many cells treated with penicillin take the polyhedral shape in the hypertonic medium. This phenomenon in *C. butyricum* does not appear to be specific for penicillin, since glycine induces similar bodies.

HIGH MUTATION FREQUENCY OF BACTERIA DEVELOPING RESISTANCE TO BACTERIOPHAGE

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The mutation frequency of bacteria for developing phage resistance has been determined by various authors. Most of the work so far reported on the mutation frequency of bacteria shows a very low mutation frequency, as low as 10^{-8} per cell per generation (Demerec and Fano, Genetics **30**:119, 1945). In the present report, an unusually high mutation frequency for phage resistance in *Escherichia coli* var. *communior* G has been presented.

An atypical strain of *E. coli* var. *communior* G and its phage were employed in the present study. The morphology of the bacteriophage has been described previously (Guha, Nucleus **2**:119, 1959). The bacteriophage is very specific to this particular strain of *E. coli* var. *communior*, and does not produce any lysis even with other strains of bacteria of the same group. The bacteria were grown in M & E liquid medium (Morton and Engley, J. Bacteriol. **49**:245, 1945), and

the phage suspension used was also obtained in M & E medium. Bacteria from a single colony were allowed to grow for 2.5 hr in an incubator. Bacteriophage (1 ml), in the proportion of 1 bacterium to 70 phage particles, was added to 1 ml of bacterial suspension of a desired dilution. The mixture was kept for 12 min in an incubator at 37 C, which is the time for maximal phage adsorption. The same procedure was followed for the control samples, except bacteriophage was not added. Samples of 0.1 ml each from the phage plus bacteria mixture, as well as from the control, were plated for assay in M & E agar plates, and incubated overnight at 37 C. The ratio of the number of bacterial colonies obtained in the agar plates of phage plus bacteria mixture, henceforth called experimental plates, to that obtained in the control plates gives the frequency of mutation per cell per generation of the bacteria.

A total of 27 experiments were done, and the