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Geometry of Cell Division in *Staphylococcus aureus*

HELEN TZAGOLOFF AND RICHARD NOVICK*

Department of Plasmid Biology, The Public Health Research Institute of the City of New York, Inc., New York, New York 10016

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The process of division in *Staphylococcus aureus* was examined by phase-contrast microscopy. The organisms appeared to divide in three alternating perpendicular planes, with sister cells remaining attached to each other after division. The resulting point of attachment was usually not exactly at the point corresponding to the center of the previous septal disk. Moreover, sister cells often changed position with respect to one another while still remaining attached. These factors are apparently responsible for the irregularity of staphylococcal clumps. Studies with penicillin and the examination of thin sections in the electron microscope confirm the conclusion, based upon light microscopy, that successive divisions in *S. aureus* occur in perpendicular planes.

The growth of staphylococci is characterized by the formation of irregular clumps of varying numbers of organisms, this pattern of growth being responsible for the name of the genus. Although it is clear from microscopic observation that the clumps are formed by cells that fail to separate after division, rather than by agglutination of separate individuals, little more is known of the basis of this curious growth pattern.

This study was initiated in an attempt to define the mechanical, genetic, and other factors governing the geometry of cell division in spherical organisms, especially those that do not divide in a constant plane. Our initial hypothesis was that the point of attachment of two staphylococcal cells might define the prior division plane as a plane tangential to that attachment point (see Fig. 1). If this were indeed the case, then the irregularity of clumps could be accounted for by irregularity of the angular relationship between successive division planes, as is frequently stated in textbooks of microbiology (3, 7, 11, 21). The available data relevant to this question consist in occasional electron micrographs showing septate organisms with second septa at right angles to the first (4, 10, 12); the rarity of such data, however, makes it impossible to draw any general conclusion on division geometry from available electron micrographs. Moreover, the most that could possibly be determined by such studies, no matter how extensive, is the relationship between two, and only two, division planes.

Thus, we sought to test the hypothesis that random clusters are the consequence of random division planes, simply by following dividing organisms in the phase-contrast microscope, hoping to observe the three or more cell divisions in many different cells that we felt was necessary to permit a meaningful interpretation. The results, described here, did not support the hypothesis of random planes; instead, it appears that the organisms divide in alternating perpendicular planes, in all three dimensions, and that irregular separation of the daughter cells is what is responsible for the irregularity of the clusters. The point of attachment of two sister cells can evidently reflect any point on the prior septal disk and, moreover, the attached sister cells can change position somewhat with respect to one another while still remaining attached.

MATERIALS AND METHODS

Organisms. These studies were performed with strain 147 (pII147$^-$) of *Staphylococcus aureus*, a naturally occurring strain (20) spontaneously cured of its penicillinase plasmid.

Overnight cultures grown on GL plates (15, 16) were used to inoculate side-arm flasks containing CY broth (15). The cultures were incubated at 37°C with vigorous aeration until exponential growth phase was reached. Mass increase was followed with a Klett-Summerson colorimeter at 540 nm. Stock cultures were stored in CY broth at −75°C.

Light microscopy. Cell division was followed on agarose blocks prepared by two different methods.

(i) Two-dimensional studies. A drop of molten 1.5% agarose (Bio-Rad Laboratories, Richmond, Calif.) made up in CY broth was placed inside a well of a depression slide. After the agar had solidified, a drop of exponentially growing cells was spread over the agar surface and allowed to dry. Air pockets were created by puncturing the agar surface in several places with an inoculating loop. A cover slip

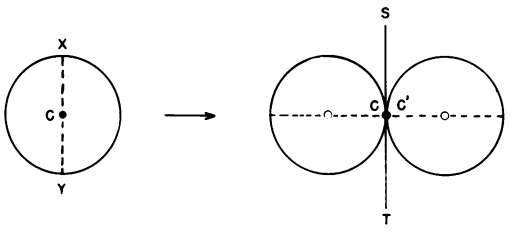

FIG. 1. *Two-dimensional representation of cell division hypothesis in S. aureus. The tangent, ST, defining the prior division plane, XY, passes through the attachment point, C/C', and is perpendicular to a line joining the centers of the two sister cells.*

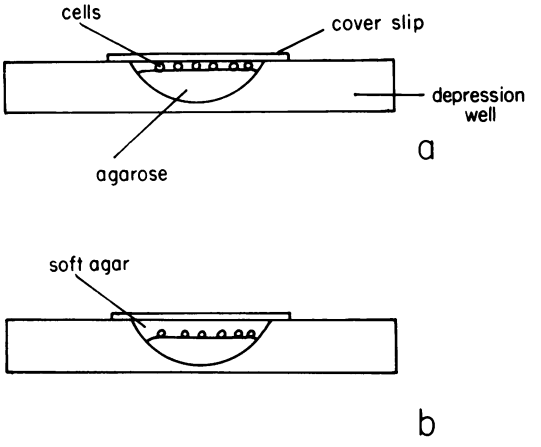

FIG. 2. *Diagrammatic representation of the growth chambers used in the study of cell division: (a) two-dimensional; (b) three-dimensional.*

(no. 0) was placed on top, and the edges were sealed with silicone grease (diagrammatically illustrated in Fig. 2a). In this method, the agar surface was slightly higher than the surface of the slide, so that on pressing the cover slip into place the cells were confined to the two-dimensional film between the cover slip and the agar. The slide was placed on a stage incubator prewarmed to 37°C, and immobile single cells were located. The division of these cells was observed with a Leitz phase-contrast microscope. Photographs were taken at various intervals with 35-mm Kodak Plus-X film. The generation time of cells growing on the agar block varied from 35 to 50 min. In aerated liquid cultures, the generation time is 35 min at 37°C.

(ii) Three-dimensional studies. The above procedure was modified to allow clumps of dividing cells to assume three-dimensional configurations. Molten agarose was placed in the depression slide so that the surface of the agar was slightly lower than that of the slide. After spreading of the cell suspension, a drop of soft (0.4%) agarose in CY broth was placed on top of the cells (Fig. 2b). Soft agar was used to immobilize the dividing bacteria. Cell division was followed during incubation at 37°C, as described above.

Electron microscopy. Cells grown in CY broth were centrifuged, and the pellets were fixed overnight with 1% OsO_4 at room temperature according to the procedure described by Kellenberger et al. (9). The specimens were then treated for 2 h with 0.5% uranyl acetate and successively dehydrated first in alcohol and then in propylene oxide. The dehydrated cells were embedded in epoxy resin as described by Luft (13). Thin sections were poststained with lead citrate and observed in a Philips 300 electron microscope.

RESULTS

Two-dimensional studies of cell division. Figure 3(a–f) shows several sequential divisions in a group of *S. aureus* cells confined to two dimensions. The field observed included, at the outset, a single cell and a pair of cells. The single cell, on the left, is large and ovoid and presumably had already formed its septum at the time the observations were begun. This morphology is observed just before division, which in this case occurred after 10 min (Fig. 3b). The second division occurred asynchronously. One daughter cell divided first (Fig. 3c), and the other divided about 20 min later (Fig. 3e). The cells then began to swell in preparation for the following division and, in doing so, apparently moved together (Fig. 3f). The pair of cells on the right (Fig. 3a) divided at right angles to their previous division septum to form a square tetrad (Fig. 3d). Upon further incubation, however, the relative positions of the cells in the tetrad changed to a rhomboid configuration (Fig. 3e, f).

We feel that in these experiments cells were truly confined to a two-dimensional plane on the basis of the following observations. (i) Tetrads always appeared flat; i.e., all cells were always in focus simultaneously. (ii) Even though cells in a tetrad underwent changes in position, there was never any overlapping of cells, which would be expected to occur, for example, in the case of tilting. (iii) Further divisions produced flat, irregular clumps, again with all cells in focus.

Over 100 cells were followed through two divisions by using this method. Cells were never observed to divide parallel to the previous division, but generally appeared to divide at right angles. Inasmuch as the third division occurs in the third dimension (see below), in these two-dimensional experiments no attempt was made to follow more than two cell divisions. The significant results here were: (i) although the cells appeared to divide at right angles, the attachment point was usually not at the point corre-

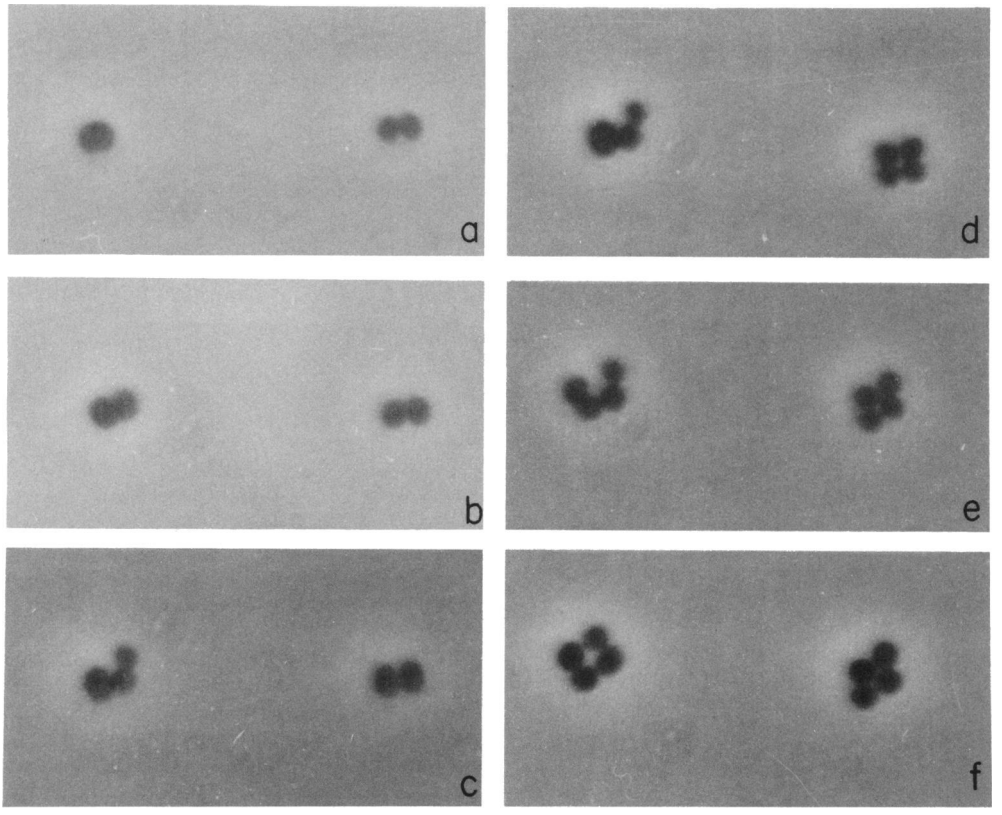

FIG. 3. *Division of S. aureus apparently confined to two dimensions, as observed in phase contrast. (a) 0 time; (b) 10 min; (c) 20 min; (d) 30 min; (e) 40 min; (f) 50 min. ×3,400.*

sponding to the center of the previous septal disk, and so one could not infer from that point the location of the previous septal plane; and (ii) although sister cells remained attached, they were often observed to rotate with respect to one another, and so the attachment point did not appear to be fixed.

Three-dimensional studies of cell division. Observations of dividing clumps that were allowed to assume three-dimensional configurations were more difficult to interpret but were necessary if more than two successive divisions were to be followed.

Figure 4(a–h) illustrates the division of a single cell of *S. aureus*. A diagrammatic representation of the formation of the clump is included in the upper right-hand corner in the photographs. At 0 time (Fig. 4a), the cell had a readily visible transverse septum. After 15 min, the cell divided (Fig. 4b), and 25 min later the two daughter cells moved slightly apart (Fig. 4c). The second division occurred asynchronously (Fig. 4d, e). After a total of 85 min, three of the four cells divided again (Fig. 4f, g, h) in a new plane. One of the daughter cells in each case is slightly out of focus and therefore lies either below or above the second division plane. Evidently the third division occurred in a plane that lies parallel to the surface of the agar, and so one of the daughter cells ended up below the other.

In *S. aureus* the separation of the two daughter cells occurs with a rather abrupt popping motion, as has been described by Previc (17). When this occurs within a clump of cells, there is often displacement of adjacent cells. Because of such displacements we have been unable to follow the formation of the clump through the fourth division.

Effect of penicillin on cell division. In *E. coli* it has been shown that at low concentrations, penicillin acts preferentially at sites of the cell wall where new septa are formed, causing the formation of characteristic bulges (6, 19). Similar treatments were tried with *S. aureus* in an attempt to locate the sites of septum initiation.

Growth chambers were prepared as for two-

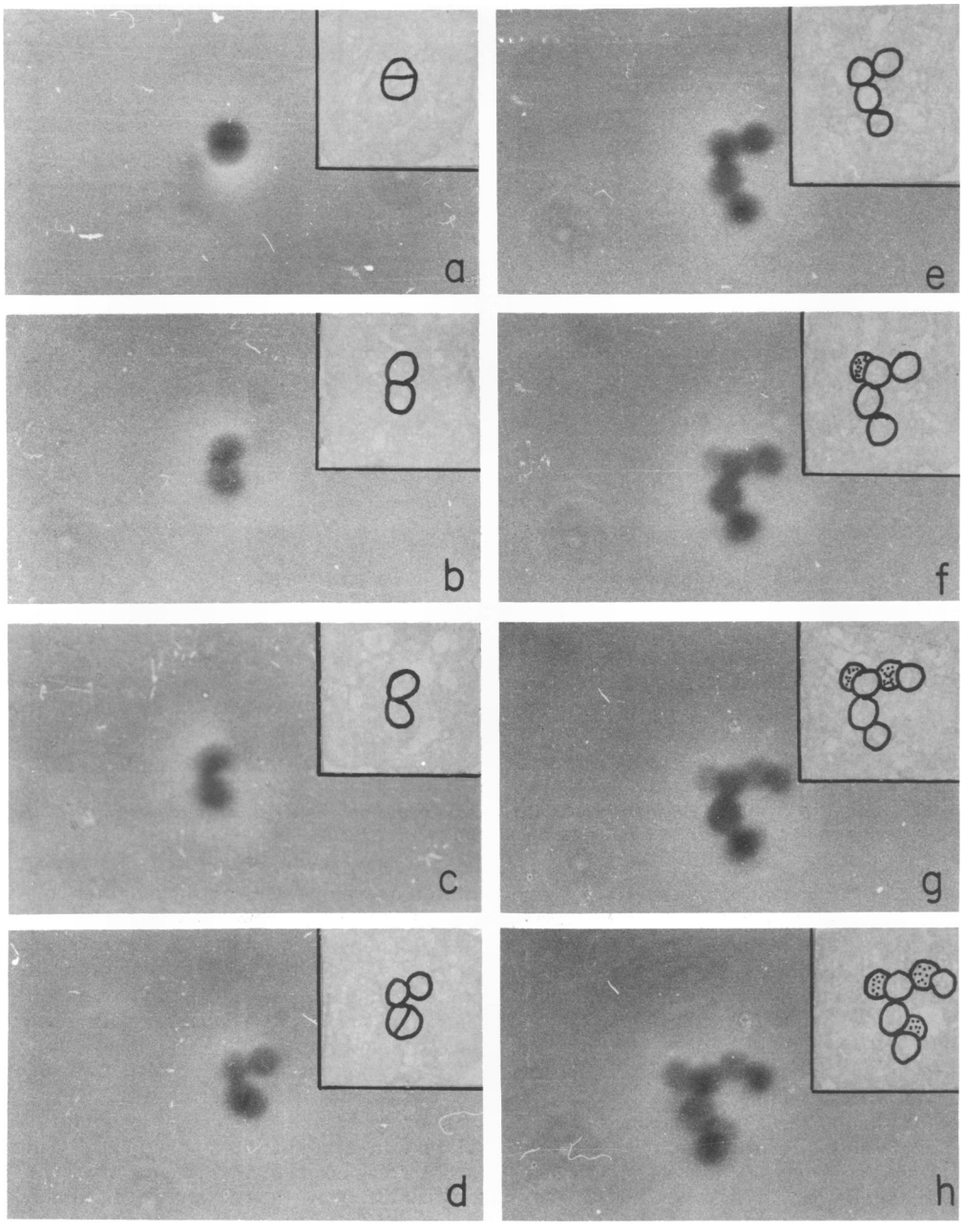

FIG. 4. *Division of S. aureus in three dimensions, as observed in phase contrast. The formation of the clump is shown diagrammatically in the insert next to the photomicrographs. (a) 0 time; (b) 15 min; (c) 25 min; (d) 50 min; (e) 55 min; (f) 85 min; (g) 95 min; (h) 100 min.* ×3,400.

dimensional studies, except that the agarose block was supplemented with 12% sucrose, 0.2% magnesium sulfate (19), and penicillin G (gift of Charles Pfizer & Co.).

Several different concentrations of penicillin G were tried until one was found that allowed the cells to continue to increase in mass. In Fig. 5 is shown the effect of penicillin at 0.5 ng/ml on the division and morphology of *S. aureus* after 3 h of incubation at 37°C. The cells are seen to be large and, in many, cross-septa at right angles are visible.

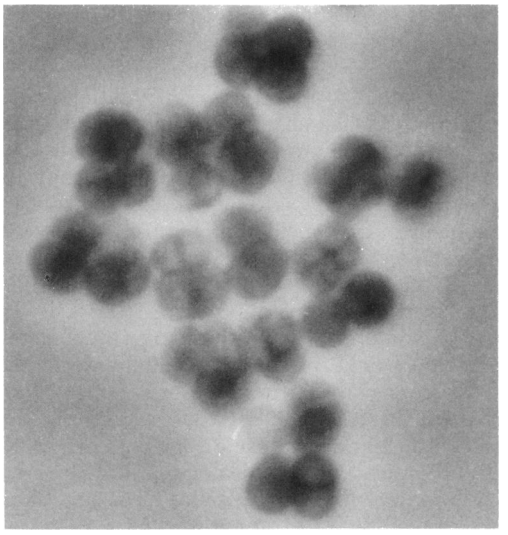

FIG. 5. *Effect of penicillin on the division of S. aureus. Phase-contrast photomicrograph shows cells after 3 h of incubation with 0.5 ng of penicillin G per ml. ×3,400.*

These results and similar observations reported by Lorian (12) suggest that this concentration of penicillin affects preferentially the synthesis of the peripheral wall, and as a consequence the septa become more frequent as well as more visible in the phase microscope. The cells shown in Fig. 5 had undergone one to two divisions, but the daughter cells in most cases did not go through the final separation stage. One of the reasons for the poor separation of these cells could be the loss of cofactors necessary for the activity of autolysins that have been implicated in cell separation (8) through the much more permeable penicillin-damaged cell membrane (18). Another possibility is that the septa that are synthesized are abnormal and this in some manner reduces the efficiency of autolysin activity. Septa synthesized in *S. aureus* in the presence of penicillin have been reported to be of abnormal morphology (12, 14).

Electron microscopy. We examined sections of *S. aureus* in the electron microscope with the hope of finding cells that would have a fully completed transverse septum and also incipient septa initiated for the following division. Out of the thousands of cells that were examined, only a few such cells were found (Fig. 6a, b). This phenomenon appears to be a very rare event in *S. aureus*.

In all cases where incipient cross-septa were observed, these were at right angles to completed septa, thus confirming the conclusion reached earlier on the basis of light microscopic observations that successive divisions occur at right angles. Similar patterns can be seen in electron micrographs of sections of *S. aureus* published by Klainer and Geis (10) and by Lorian (12).

DISCUSSION

The experiments described here support the conclusion that *S. aureus* cells divide in successive perpendicular planes. Thus, this organism seems to be closely related to the anaerobic sarcinae (1, 2) with respect to cell division geometry. The formation of irregular clumps is apparently due to irregularities that occur during the separation of sister cells and so may be a less fundamental property of the species. Although these results show that the geometry of the clump cannot be used by itself to infer anything about the placement of septal planes during the divisions that gave rise to the clump, they have revealed several features of cell division in *S. aureus* that seem to permit at least a phenomenological description of the process.

Unlike streptococci, which constrict at the equator prior to division (8), the staphylococci remain spherical or nearly so until the precise moment of division. Such spherical or slightly elliptical cells with complete transverse septa have been seen repeatedly in the electron mi-

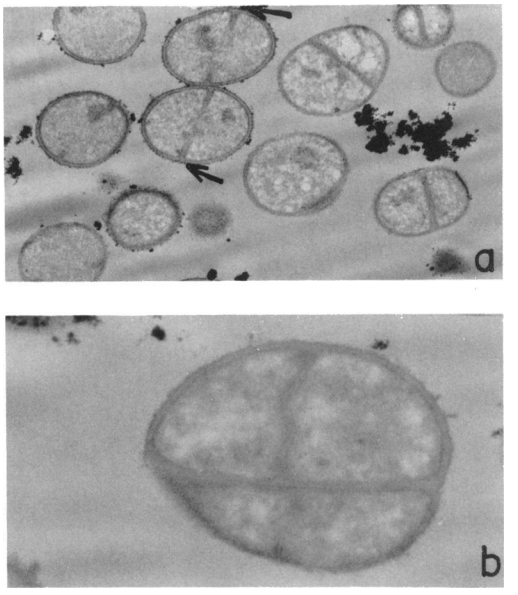

FIG. 6. *Electron micrographs of sections of S. aureus cells. Arrows point to incipient septa being formed at right angles to the previously completed transverse septum. (a) ×12,900; (b) ×33,500.*

croscope and are exemplified in Fig. 6a. As the division process is completed, presumably by autolysins acting along the septum and especially at the periphery of the septal disk, a moment is reached when the remaining septal bonds can no longer withstand the force of surface tension. At this point, the sister cells separate with a snap as they rapidly assume their spherical shape. This evidently occurs without rupturing all of the remaining bonds, and so the sister cells remain attached to one another. However, there seems to be considerable variability in the location of the attachment point with respect to the prior septal disk. The attachment point is most commonly at or near the center, since most of the tetrads that one sees are square or nearly square. With diminishing probability the attachment may lie away from the center, ultimately producing tetrads that are more or less linear (see Fig. 7). These, however, are quite rare. One could attempt to generate a probability distribution for this situation; however, there is an additional complication that would largely negate the validity of such a distribution. This is that the sister cells can often be observed to rotate with respect to one another so that the attachment point appears not to be rigidly fixed. This curious effect remains unexplained, as the attachment is quite resistant to chemical or enzymatic disruption (unpublished observations of the authors).

Figure 7 is a diagrammatic representation of how clumps of various configurations may be generated. For the sake of simplicity only two dimensions are considered. Figure 7a shows a cell with a complete septum, XY, and points A and B representing two of the many possible attachment points of the future sister cells. In Fig. 7b and d are shown the two pairs of sister cells that would result if attachment were centric, i.e., at point A (Fig. 7b), or eccentric, i.e., at point B (Fig. 7d). In these diagrams the former peripheral wall (e.g., XPY) is drawn as a double line, and the former septal wall (e.g., XAY) is drawn as a single line. The junctions are marked by X, X', Y, or Y'.

The relationship between septa in sister cells may be understood as follows. If attachment is centric as in Fig. 7b, then sister cells will have coplanar septa (e.g., P P'). If attachment is eccentric as in Fig. 7d, then formation of a septum perpendicular to the previous one

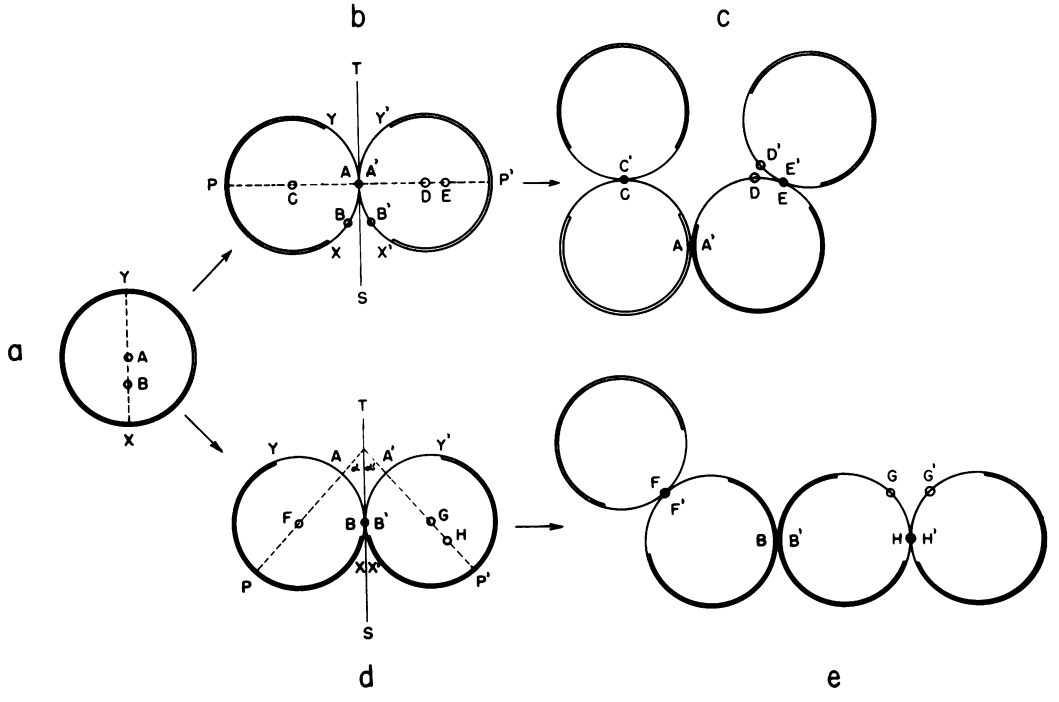

FIG. 7. *Two-dimensional representation of the generation of staphylococcal clumps. Circles represent cells, dashed lines represent septa, double lines represent prior peripheral wall, and single lines represent prior septal wall. The distinction between peripheral wall and septal wall is made for illustrative purposes only and is not intended to imply that it has any biological significance. Possible past or future attachment points are represented by open circles; actual ones are represented by solid circles.*

means that the septal plane must pass through the center of the cell (i.e., points F and G in Fig. 7d) and a point representing the centers of the prior septal disk (i.e., points A and A′ in Fig. 7d). These septal planes must then make equal angles α and α', with a tangent, ST, drawn through the actual attachment point, B/B′.

Depending solely upon the relationship of successive attachment points to the center of the prior septal disk, virtually any tetrad configuration can be generated, from linear to square. Two examples are shown in Fig. 7c and e, where the left-hand pair of cells have centric attachments at C/C′ and at F/F′, respectively, and the right-hand pair have eccentric attachments at E/E′ and H/H′, respectively. Note that in Fig. 7e the eccentricity is in the same direction as that in the first division, B/B′. Additional irregularity can be generated by rotation of the attached sister cells.

Finally, these studies may constitute yet another example of the fact that in bacteria, the biological life cycle of the organism transcends the event of cell division (5). In the case studied, the organisms appear to divide in the three perpendicular planes in a regular sequence. For example, in the group of cells followed in Fig. 4, at the four-cell stage all of the observable cells divide in the same plane. Thus, the plane of every division may be determined geometrically at least two divisions earlier—so that the individual cell may "remember" instructions for division received from its great-grandparent and may in turn pass such instructions on at least to its great-grandchild. If, indeed, division occurs in no more than three alternating planes, then continuity of instruction for three successive divisions is sufficient to ensure regularity. Alternatively, it is possible that the plane of one cell division determines only that of the next succeeding one; however, to account for coplanar division among sister cells, one must postulate either that there is some form of intercellular communication or else that the cell has some internal indicator of its geometrical orientation such that, for example, division in the XY plane is always followed by division in the YZ plane, and the latter is always followed by division in the XZ plane. Since our data do not go beyond three divisions, we cannot extend this scheme further. The genetic nature of this type of control, in which some event associated with cell division determines the plane of a septum for a division that will occur from one to three generations later, is a matter of great interest, and we have begun an approach to it through an exploration of cell division mutants. One relevant finding so far is of a conditional mutant in which the geometry of septum placement is totally disrupted at the restrictive temperature but is relatively normal at the permissive temperature (unpublished observations of the authors). A provisional conclusion from the mere fact of occurrence of this mutant is that the geometry of septum placement is indeed genetically controlled in a direct manner. Further studies of this and other mutants may help to define precisely this interesting genetic control system.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Canale-Parola, E.** 1970. Biology of the sugar-fermenting sarcinae. Bacteriol. Rev. 34:82-97.
2. **Chapman, G. B.** 1960. Electron microscopy of cellular division in *Sarcina lutea*. J. Bacteriol. 79:132-136.
3. **Clifton, C. E.** 1958. Introduction to the bacteria, 2nd ed., p. 19. McGraw-Hill Book Co., Inc., New York.
4. **Cole, R. M., A. N. Chatterjee, R. W. Gilpin, and F. E. Young.** 1974. Ultrastructure of teichoic acid-deficient and other mutants of staphylococci. Ann. N.Y. Acad. Sci. 236:22-53.
5. **Cooper, S., and C. E. Helmstetter.** 1968. Chromosome replication and the division cycle of *Escherichia coli* B/r. J. Mol. Biol. 31:519-540.
6. **Donachie, W. D., and K. J. Begg.** 1970. Growth of the bacterial cell. Nature (London) 227:1220-1224.
7. **Frobisher, M.** 1968. Fundamentals of microbiology, 8th ed., p. 412. W. B. Saunders Co., Philadelphia.
8. **Higgins, M. L., and G. D. Shockman.** 1971. Procaryotic cell division with respect to wall and membranes. Crit. Rev. Microbiol. 1:29-72.
9. **Kellenberger, E., A. Ryter, and J. Séchaud.** 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4:671-687.
10. **Klainer, A. S., and I. Geis.** 1973. Agents of bacterial disease, p. 29. Harper and Row, Hagerstown, Md.
11. **Lamanna, C., M. F. Mallette, and L. Zimmerman.** 1973. Basic bacteriology, 4th ed., p. 72. The Williams & Wilkins Co., Baltimore.
12. **Lorian, V.** 1975. Some effects of subinhibitory concentrations of penicillin on the structure and division of staphylococci. Antimicrob. Agents Chemother. 7:864-870.
13. **Luft, J. H.** 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409-414.
14. **Murray, R. G. E., W. H. Francombe, and B. H. Mayall.** 1959. The effect of penicillin on the structure of staphylococcal cell walls. Can. J. Microbiol. 5:641-648.
15. **Novick, R. P.** 1963. Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. J. Gen. Microbiol. 33:121-136.
16. **Novick, R. P., and R. Brodsky.** 1972. Studies on plasmid replication. I. Plasmid incompatibility and establishment in *Staphylococcus aureus*. J. Mol. Biol. 68:285-302.
17. **Previc, E. P.** 1970. Biochemical determination of bacterial morphology and the geometry of cell division. J. Theor. Biol. 27:471-497.

18. **Rogers, H. J.** 1967. Killing of staphylococci by penicillin. Nature (London) 213:31-33.
19. **Schwarz, A., A. Asmus, and M. Frank.** 1969. Autolytic enzymes and cell division of *Escherichia coli*. J. Mol. Biol. 41:419-429.
20. **Segalove, M.** 1947. The effect of penicillin on growth and toxin production by enterotoxic staphylococci. J. Infect. Dis. 81:228-243.
21. **Wilson, G. S., and A. A. Miles.** 1946. Topley and Wilson's principles of bacteriology and immunity, vol. 1, 3rd ed., p. 36. The Williams & Wilkins Co., Baltimore.