Cell Wall Replication in Streptococcus pyogenes

Immunofluorescent methods applied during growth show that new wall is formed equatorially.

Roger M. Cole and Jerome J. Hahn

Abstract. Group A streptococci (*Streptococcus pyogenes*) of several different serological types were grown in fluorescein-labeled homologous type or group-specific globulins, thereby labeling the antigen-containing cell walls. Specific precipitation or inhibition of the labeled antibody, followed by continued incubation and examination at intervals by ultraviolet, phase, and dark-field microscopy, showed that new cell wall was then nonfluorescent. These nonfluorescent portions were differentiated by a reverse technique of culture in unlabeled globulin, followed by antibody precipitation, further growth, and fluorescent-antibody staining. This technique of differential labeling of cell wall has permitted following, for the first time in a living system, the fate of cell wall formed at different times. The results suggest that cell wall synthesis in actively growing cultures usually occurs simultaneously at at least two sites per coccus, each site representing stages in successive divisions, and that cell wall growth in *Streptococcus pyogenes* is not by diffuse intercalation with old wall, but is initiated at and extends both peripherally and centripetally from the coccal equator.

There is ". . . no precise knowledge of the location and manner of synthesis of the bacterial cell wall" (*1*). Information concerning cell wall replication during bacterial division has been obtained principally by indirect and retrospective methods, with preparations examined, after fixation, by tannic-acid-crystal violet staining (*2*) or by electron microscopy with (*3*) or without (*4*) thin sectioning. These methods have established that, in general, an equatorial thickening appears on the dividing cell and grows centripetally to form an annular cross-wall. The mode of peripheral cell wall elongation prior to cross-wall initiation, however, is not subject to verification by such methods, although the use, in addition, of dark-field and phase microscopy has led some investigators (*5*) to claim that cell wall growth occurs diffusely by intercalation. The points of new wall and cross-wall origin, relative to "old" wall—and the directions of new wall growth—are also subjects about which little is known.

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Labeling Cell Walls

If bacterial cell wall could be differentially labeled during growth to distinguish portions of different relative ages, answers to some of these questions might be forthcoming.

We have so labeled the cell walls of Group A streptococci by growth in fluorescein-labeled globulin from homologous antisera. Gram-positive cocci, and *Streptococcus pyogenes* in particular, grow freely in homologous antisera in the absence of leukocytes (*6*). Indistinguishable, apparently normal, growth occurred when mouse-virulent, M^+ strains of types 1, 18, and 19 were incubated at 37°C in their respective homologous immune rabbit globulins labeled with fluorescein isothiocyanate (*7*). Similar results were obtained whether globulins from either group-specific or type-specific antisera, prepared by Lancefield's methods (*8*), were used.

Rubber-stoppered tubes, 9 × 100 mm, containing 0.20 ml of labeled globulin, were inoculated with 0.05 ml of a 10^{-2} dilution of a 24-hour streptococcal culture. At the end of 90 minutes of stationary incubation (zero time), a culture was removed from the water bath, iced or heat-killed, and the streptococci were washed three times at 5°C by centrifugation and resuspension in cold phosphate-buffered saline (*p*H 7.4) containing 10-percent normal rabbit serum. Smears of these organisms, mounted in buffered glycerol (*p*H 8.6) under No. 1 cover slips after drying on glass slides, were examined under oil immersion with a Leitz Ortholux microscope. The light source was an Osram HBO 200 mercury vapor lamp, and dark-field illumination was employed with a Schott exciter filter BG 12 and barrier filter OG 1. Photographs were made on Kodak Panatomic X film, exposed for 1 to 2 minutes and developed 2½ to 3 minutes at 68°F in Acufine (*9*).

As a result of binding of fluorescent antibody by streptococcal cell wall antigens—either type-specific M protein or group-specific C polysaccharide—there was complete labeling of the cell walls as seen in optical section (Fig. 1 and Fig. 2*a*). Growth in fluorescein-labeled normal or heterologous rabbit globulins did not result in fluorescent cell walls.

At this time (zero time) in replicate tubes remaining in the water bath, free fluorescent antibody was either (i) precipitated, or (ii) inhibited, in the following fashions: (i) Acid extracts (*10*) of homologous streptococci, or purified homologous M protein (*11*), were added. (ii) Homologous unlabeled globulin, prepared from the same antiserum as the fluorescein-tagged globulin, was added. This competed successfully, through a mechanism ascribed to "avidity" [as first noted by Goldman (*12*) in fluorescent antibody systems], with the labeled globulin for the antigen sites on the cell wall.

Either of these methods, which give the same visual result to be described, is referred to herein as the *direct* method. Following either procedure, preparations made and washed as described above at intervals of 15 minutes additional incubation were examined. New portions of the cell wall, because of their formation in the absence of labeled antibody, were nonfluorescent. The fluorescein label, however, remained as a marker on the older portions of the wall, so that the labeled halves of each original coccus or diplococcus became separated by nonfluorescent or "blank" gaps. Seen in optical section, the terminal halves of the end cells of each chain then appeared as fluorescent "C's," whereas the original halves located back-to-back within the chains appeared as a series of fluorescent "X's."

After fluorescent antibody precipitation or inhibition at zero time, the

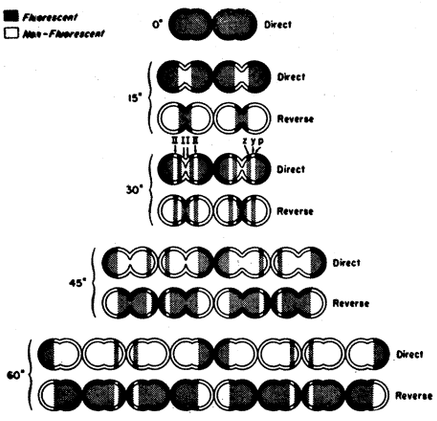

Fig. 1. Diagrammatic representation of stages shown in Fig. 2.

nonfluorescent gaps appeared very rapidly and enlarged as incubation proceeded (Fig. 2, *b–e*). They were small up to about 15 minutes (Fig. 2*b*); between 15 and 30 minutes after zero time, they enlarged and a new, nonfluorescent segment (*y* in Fig. 1) appeared at a point "p" which was usually within the originally labeled portion of cell wall (three-dimensionally, *p* is actually an infinite series of points on an equator). The new nonfluorescent segment enlarged, appearing to grow away from the oldest cell wall of the originally labeled half. As it did, it pushed away a fluorescent segment (*z* in Fig. 1), the size of which varied with the stage of division of the cell at which fluorescent antibody was precipitated or inhibited. This segment then appeared as a fluorescent band, densest at each end, because it was actually a narrow ring of cell wall viewed in optical section. It became a part of the cell wall of the new hemisphere, which in turn represents only half of the cell wall of the daughter (or, preferably, sister) cell being formed.

During this study, the same streptococcal chains were examined by ultraviolet, and phase and dark-field microscopy. To verify further and interpret the state of the cocci seen, a *reverse* technique was devised. The cocci were incubated first in unlabeled homologous globulin, after which the free unlabeled antibody was precipitated by method (i). They were then removed at intervals of 15 minutes additional incubation, and washed and smeared as described. The smears were then stained with the same globulin, fluorescein-labeled, in the conventional manner. Portions then fluorescent represent the unstained gaps seen in the direct technique, whereas the now blank areas are covered by unlabeled antibody and represent the fluorescent portions seen in the direct technique. Results are shown in photographs, taken at corresponding time intervals, Fig. 2, *f–i*, and in diagrammatic interpretation in Fig. 1.

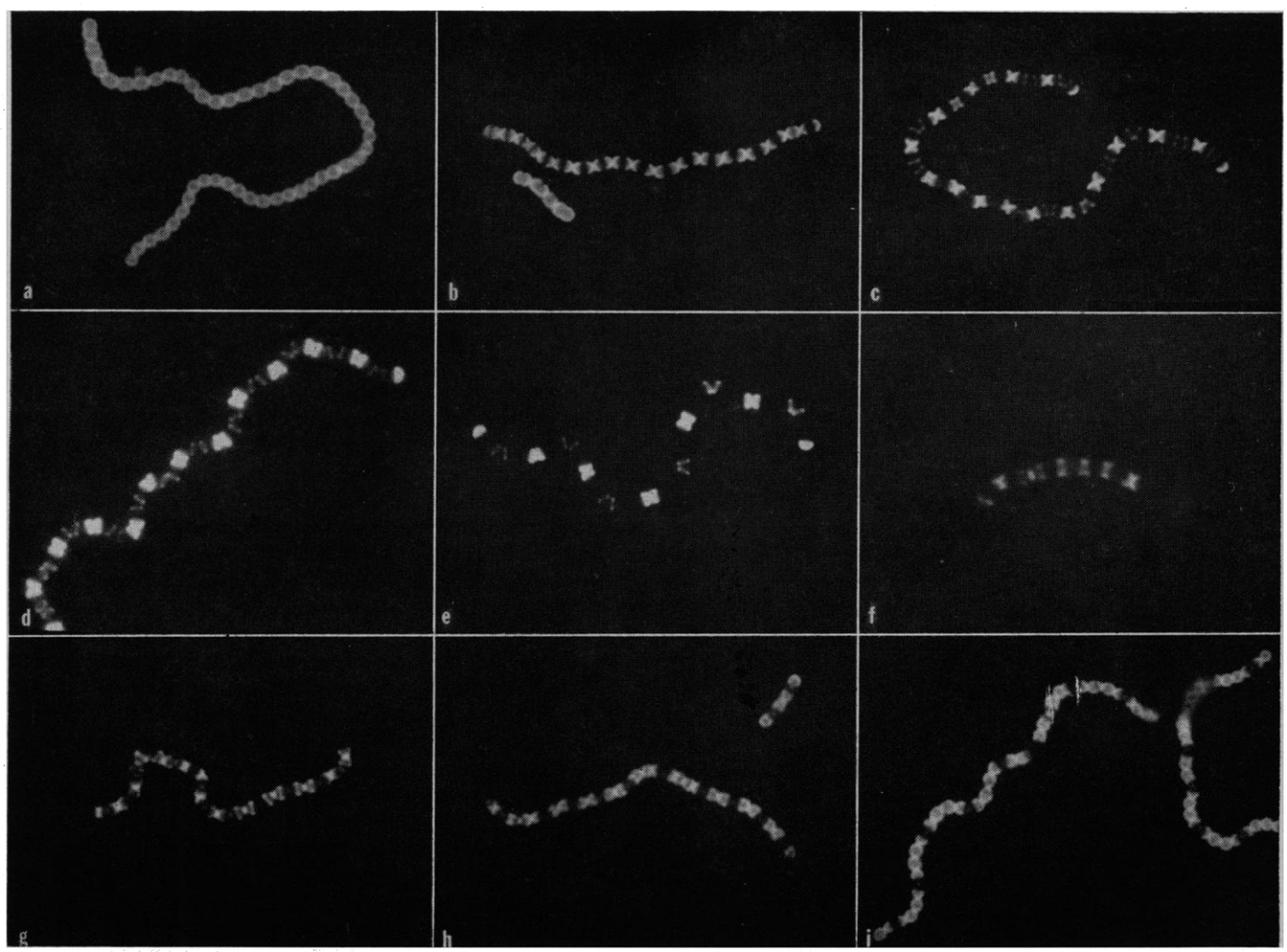

Fig. 2. Ultraviolet photomicrograph. (Magnification approximately 2100 ×.) *a–e*: Group A streptococcus, Type 19, grown in homologous fluorescein-labeled globulin and examined at intervals after addition of unlabeled homologous globulin (Direct Method). *a*, 0 minutes; *b*, 15 minutes; *c*, 30 minutes; *d*, 45 minutes; *e*, 60 minutes. *f–i*: Group A streptococcus, Type 19, grown in unlabeled homologous globulin, removed at intervals after precipitation of antibody, and stained with homologous fluorescein-labeled globulin (reverse method). *f*, 15 minutes; *g*, 30 minutes; *h*, 45 minutes; *i*, 60 minutes.

Conclusion

Our observations indicate that, in *Streptococcus pyogenes*, new cell wall is not diffusely intercalated with the old, but its formation is instead initiated equatorially along a circumference which is the site of the next cross-wall formation. These findings, made by labeling of living cells, confirm the conclusions expressed by Bisset (*13*) concerning the site of the main growth of the cell wall in septate bacteria. The new cell wall growth, in actively dividing cultures, is well under way, and the cross-wall may have progressed centripetally halfway, before there is complete separation by cell wall of the two previously formed cocci; as a result, the predominant forms in a chain appear as diplococci. The method at present does not indicate the presence or absence of cytoplasmic membrane septa, and does not therefore confirm or deny the activity of any part of such a membrane in secreting cell wall. It is obvious, however, that there are at least two sites of simultaneous activity—membranous or other—within the bounds of any one coccus as defined by cell wall furrowing: at one (*I*, Fig. 1) cross-wall to complete the previous division is still being formed, and at the other (*II*, Fig. 1) peripheral cell wall and cross-wall for the current division are being initiated.

Our findings and interpretations apply only to *S. pyogenes*. The methods described, however, should be widely applicable to any microorganism, with antigenic cell wall components, which can be grown in the presence of homologous antibody (*14*).

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Index for Measurement of Synchronization of Cell Populations

Abstract. An index for the estimation of synchronization in a microbial population is proposed and discussed. The index is equal to the fraction of cells that divide during a measured interval in excess of the fraction that would divide during random growth in the same interval.

In studying the effects of various treatments on the synchronization of cell populations, it is desirable to have a quantitative measure for comparing the amounts of synchrony obtained. Such an index should have the following characteristics. It should reach an upper limit if all the cells simultaneously carry out the particular reaction used as the criterion for synchrony. It should be proportional to the fraction of the cells undergoing the reaction and inversely proportional to the length of time in which the synchronized fraction of the culture carries out the reaction. It should permit positive identification of logarithmic growth even if the treatments have caused a lag or an acceleration of growth rate.

A number of indexes have appeared (*1*), but none seems to meet these requirements satisfactorily. We wish to propose an index that seems to be a reasonable compromise among these criteria. We have used cell division for the measurement of synchrony, but certain other parameters of growth could be used.

If the number of cells in a culture increases from N_0 to N in an interval t less than one generation time g, then

$$N/N_0 - 1$$

is the fraction of cells in the culture which divide during t, and

$$2^{t/g} - 1$$

is the fraction of cells in the culture which would have divided during t if the culture had been growing logarithmically. The quantity

$$F = (N/N_0 - 1) - (2^{t/g} - 1)$$
$$= N/N_0 - 2^{t/g}$$

measures the fraction of the population which divides during t in excess of that expected to divide during logarithmic growth in the same interval.

F has a maximum value of $+1$ if the entire population divides during an infinitely small time interval. It has positive values less than 1 if doubling takes a finite time (which, of course, it must), or if less than the entire population divides during the measured interval. After a synchronized burst of divisions, the population must increase at a rate less than that of normal logarithmic growth, and F falls to negative values. Thus the criterion for synchronized cell division is a positive value followed by a negative value. (The index should never fall below -1.) A logarithmically growing culture has an index of zero over any interval. An index of 0.8 (followed by a negative index in the next interval measured) would indicate excellent synchrony (*2*).

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Hydrostatic Pressure Has a Selective Effect on the Copepod Tigriopus

Abstract. High hydrostatic pressures have been found to be an agent in producing a shift in the sex ratio in populations of the marine copepod *Tigriopus.*

The study of the effect of hydrostatic pressure on the harpacticoid copepod *Tigriopus* was initiated in an attempt to obtain morphological mutations in this organism. These experiments revealed a definite shift in sex ratio of the pressure-treated organisms and are therefore being reported here.

The first experiments consisted of collecting adults from their natural habitat in the splash pools of the supralittoral tidal zone and subjecting them to various levels of hydrostatic pressure to determine a survival curve. At pressures from 1 to 500 atm and short exposure intervals 100-percent survival was observed. In view of the relative