RADIOAUTOGRAPHIC EVIDENCE FOR EQUATORIAL WALL GROWTH IN A GRAM-POSITIVE BACTERIUM

Segregation of Choline-^{3}H–Labeled Teichoic Acid

EVE BARAK BRILES and ALEXANDER TOMASZ. From The Rockefeller University, New York 10021

INTRODUCTION

Studies of bacterial wall growth, employing fluorescent antibodies to label specific areas of the growing walls, have suggested that in Gram-positive bacteria wall growth occurs by the intercalation of newly synthesized wall materials into discrete, specific equatorial zones of the preexistent wall (2, 3, 5, 11).

In the Gram-positive bacterium, *Diplococcus pneumoniae*, choline is required for growth. It has been shown that choline becomes incorporated solely into the cell wall teichoic acid of this organism (8, 6). When deprived of choline, these cells can incorporate the amino alcohol analogue, ethanolamine, into teichoic acid in lieu of choline. Ethanolamine-grown cells exhibit several unusual properties, all of which are reversible upon restoration of choline to the culture medium (6, 9). Among these deviant properties is the inability of daughter cells to completely separate after division. Thus, continued growth with ethanolamine as the sole available amino alcohol results in the eventual formation of long coccal chains instead of diplococci. This chaining phenomenon was exploited in the experiments to be described, in which radioautographic techniques were employed to provide new evidence for zonal wall growth.

MATERIALS AND METHODS

Media

A chemically defined medium ("Cd") at pH 8 (7) was used throughout, with the following modifications:

"Choline-^{3}H medium"—methyl choline-^{3}H chloride (New England Nuclear Corp., Boston, Mass.) was substituted for choline in the medium. In a typical experiment, this was 0.007 mg/ml, sp act 0.7 mCi/mg.

"EA medium"—Ethanolamine chloride, 0.040 mg/ml, was substituted for choline.

"NAA medium"—choline was omitted (no amino alcohol).

Procedures

D. pneumoniae, strain R36A clone R6, were grown overnight at 37°C in choline-^{3}H medium. At mid-log phase, the cells were harvested by Millipore filtration, washed with 1–2 volumes of either NAA or EA medium, and resuspended in EA medium, all at 37°C. The culture was maintained at mid-log phase concentrations for the duration of the experiment by appropriate dilutions with EA medium. Samples were removed at appropriate intervals, and either (*a*) spread immediately on glass slides, using a blood-smear technique, or (*b*) fixed with ½- or 1x-volume of 9% formaldehyde-saline, and spread at some later time. Smeared preparations were air-dried at room temperature or at 60°C, and then exposed to a heat

lamp ½ m away for ½ hr, to promote adhesion to the glass. The slides were then washed extensively in distilled water, dried, and coated with Ilford L-4 gel emulsion (diluted 1:1). Exposure time was 2–9 days under a CO_2-enriched atmosphere over Drierite (W. A. Hammond Drierite Company, Xenia, Ohio) at 4°C. Development was carried out in Kodak D-19 for 2 min. Slides were viewed through a Zeiss micro-

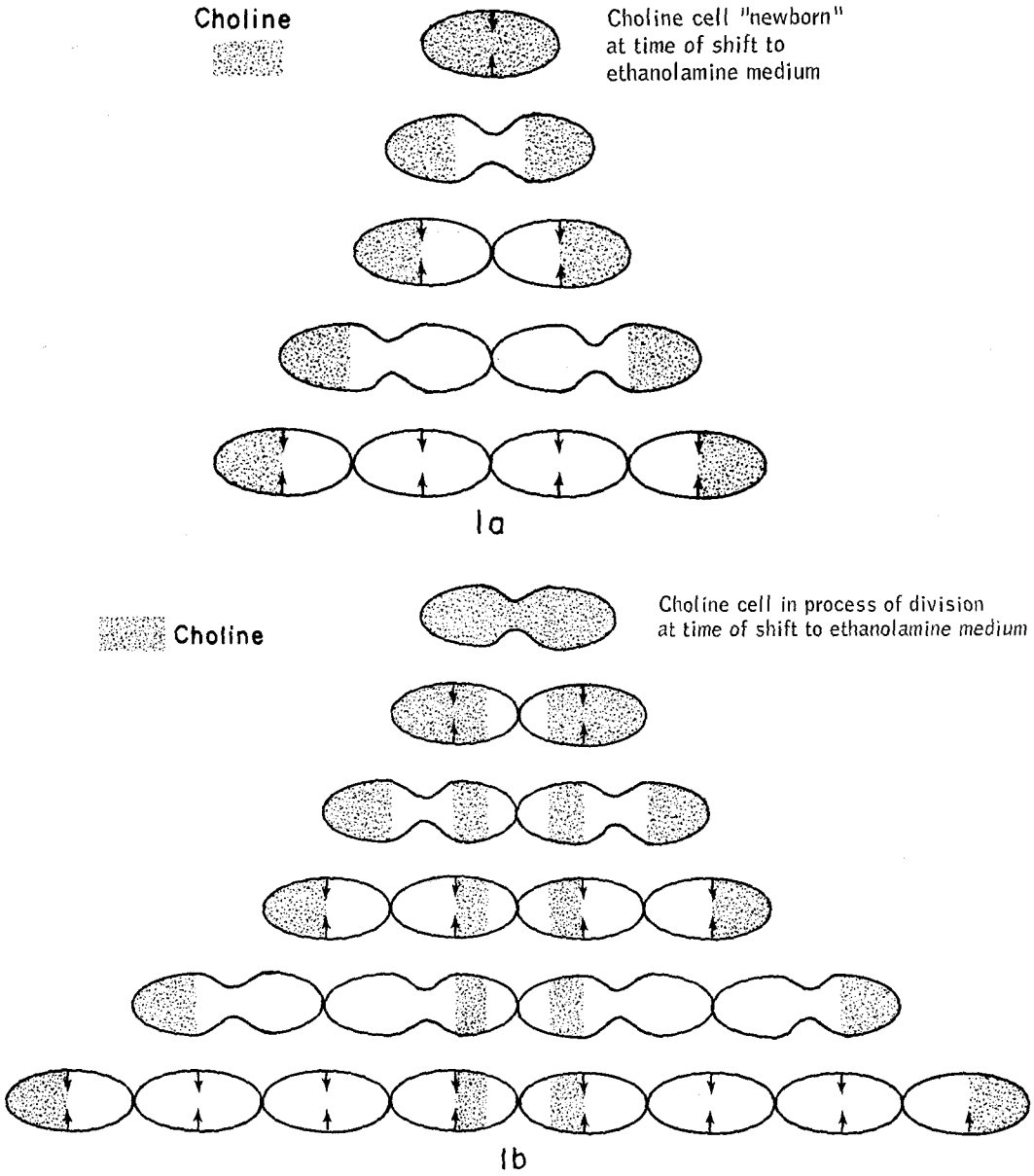

FIGURE 1 Diagram of wall segregation after shift from choline to ethanolamine (EA) media, assuming zonal growth. Choline-containing wall is shown in speckled regions, EA-grown wall in white regions. Arrows indicate equatorial growing zones. Successive stages in growth and division of a single cell are shown from top to bottom. Nonseparation of cells begins immediately upon shift to EA medium. Fig. 1 *a*, Cell is "newborn" at time of shift. Choline-containing wall segregates to distal regions of the ethanolamine-grown chain of cells. Fig. 1 *b*, Cell is in process of division at time of shift. Choline-containing wall segregates to distal and central regions of the chain.

scope fitted with a Planachromat 100/1.25 phase-contrast oil immersion objective, and photographed with Kodak Panatomic -X film.

For electron microscopy, formaldehyde-fixed samples were used (see above). Copper grids, 200-mesh, precoated with Formvar and carbon, were floated briefly on drops of the samples, partially drained, and allowed to dry. They were then coated with Ilford L-4 gel emulsion, according to the method of Caro and van Tubergen (1). Exposure time was 29 days under a CO_2-enriched atmosphere over Drierite at 4°C. Development was carried out for 5 min in Kodak Microdol X (Eastman Kodak Co., Rochester, N. Y.). Specimens were viewed at 75 kv in a Type HU-11C-1 Hitachi electron microscope.

As controls, similar procedures were carried out for light microscopy, as follows:

(*a*) Choline-^{3}H medium prelabeling, followed by growth in C_d medium containing nonradioactive choline.

(*b*) EA-^{3}H medium prelabeling (ethan-1-ol-2-amine-2T, Amersham-Searle Corp., Arlington Heights, Illinois), followed by growth in unlabeled EA medium.

RESULTS

Expected

If one assumes that the zonal growth model of cell-wall synthesis is correct, the pattern of wall segregation to be expected after a shift from choline to EA media is indicated in Fig. 1.

Fig. 1 *a* shows a single cell, "newly-born" at the time of the shift, with *one* growing zone (equatorial). After two division cycles in EA medium, the choline-containing wall parts are seen to be conserved in two discrete half-cell units which have become segregated to the distal ends of the chains. In Fig. 1 *b*, again only one growing zone is indicated, but this time the cell was in the process of division at the moment of shift to EA medium. Here, the choline-containing wall parts are seen to segregate into three regions: the two distal ends, and the center of the chain. It is not difficult to envision the pattern that would result if, instead of just one zone of growth, there existed both a *primary* growth region at the equator of the cell, and secondary or *premature* growth zones at sites

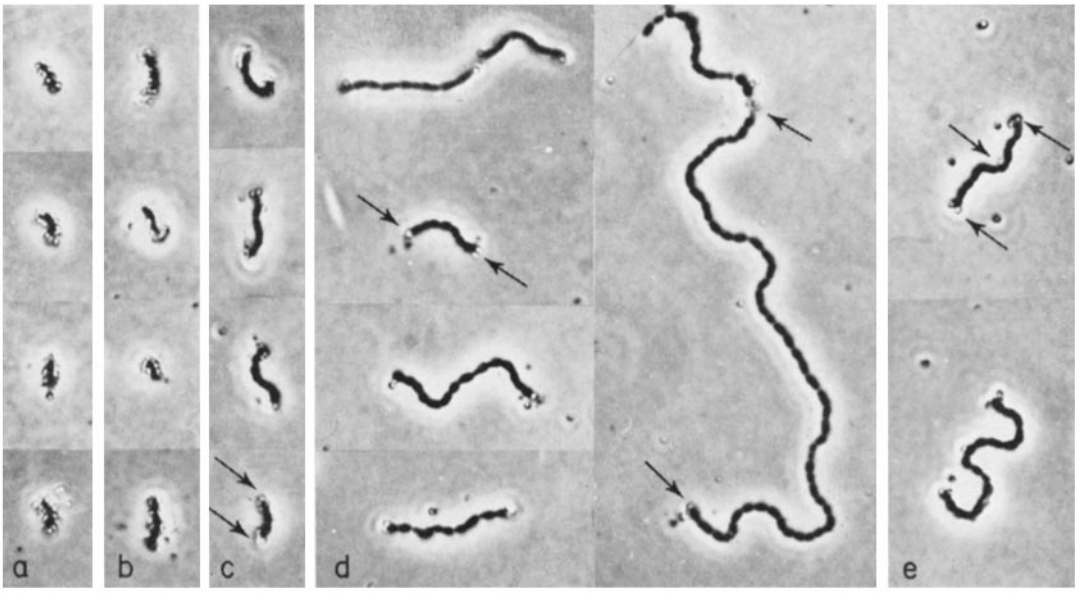

FIGURE 2 *D. pneumoniae* cells prelabeled with choline-^{3}H, then shifted to EA medium. Light-microscope radioautography; phase-contrast optics. Arrows indicate silver grains. × 1,600. Fig. 2 *a*, 0–1 generation's growth after shift. Fig. 2 *b*, 1–2 generations' growth after shift. Fig. 2 *c*, 2–3 generations' growth after shift. Fig. 2 *d*, Several generations after the shift. Compare localization of grains to pattern of segregation of choline-containing wall shown in Fig. 1 *a*. Fig. 2 *e*, Several generations after the shift. Compare localization of grains at ends and middles to segregation pattern in Fig. 1 *b*.

halfway between the equator and distal ends of a cell. Such a pattern of growth would give rise to additional bands of choline-containing wall halfway between the distal ends and centers of the chains. The size of these choline-containing bands, however, would be small compared to the size of the choline-containing regions at the distal ends, and the number of silver grains resulting from exposure to such a low concentration of tritium would not be expected to be appreciably above background under the radioautographic conditions employed. Premature growth zones have been observed in *D. pneumoniae*, although they do not appear to be very common. Premature growth zones have also been observed in other Gram-positive cells (see reference 2, page 330, for an example in *Streptococcus pyogenes*).

Observed

Fig. 2 shows the results of experiments performed with light-microscope radioautographic techniques, and Fig. 3 shows the results of electron-microscope radioautography. Silver grains occur over regions containing the tritium-labeled choline. In Fig. 2, they appear as bright granules, and in Fig. 3 as electron-opaque threads. In each case, as well as in the controls, the results are perfectly compatible with the model illustrated in Fig. 1.

DISCUSSION

Segregation of wall materials in a pattern suggesting a zonal mode of growth was first demonstrated independently by Cole and Hahn (3) and by May (5), and later for *D. pneumoniae* by Wagner (11). These studies employed immunofluorescence techniques. However, these same techniques, when applied to certain other bacteria (e.g., *Salmonella*) suggested a uniform incorporation of new wall materials over the entire wall surface (2). Van Tubergen and Setlow, by following the distribution of DAP-^{3}H in *Escherichia coli* walls by radioautography (10), also concluded that old wall ma-

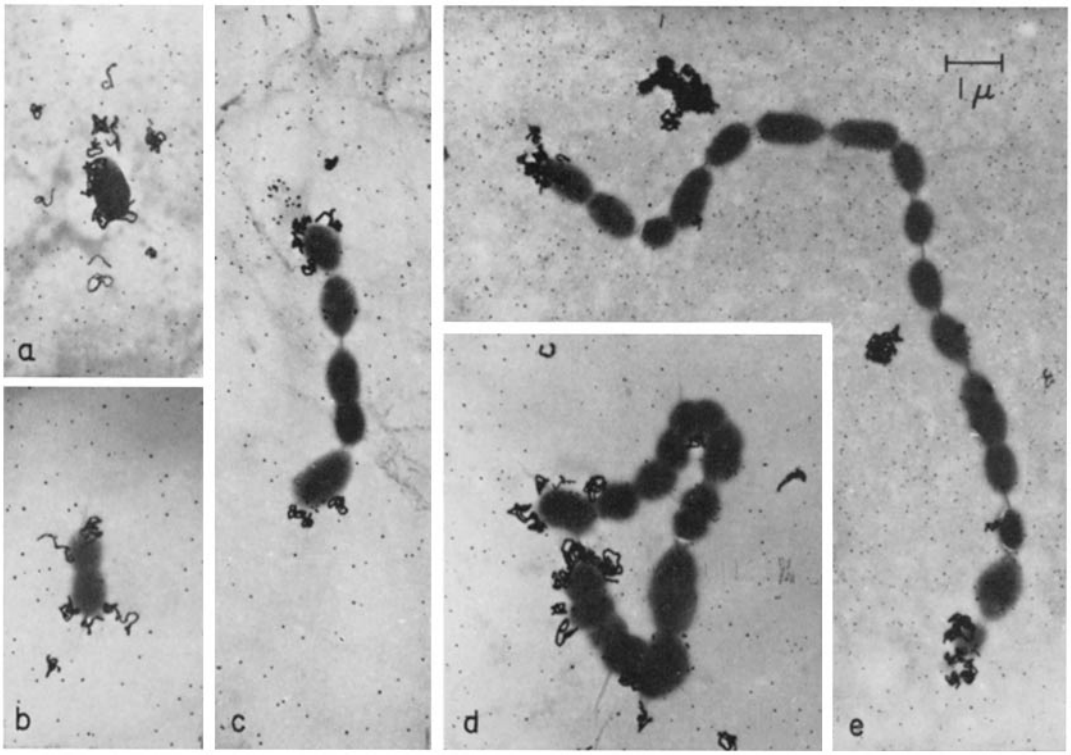

FIGURE 3 *D. pneumoniae* cells prelabeled with choline-^{3}H, then shifted to EA medium. Whole-mount electron microscope radioautographs. Unstained. × 8,000. Fig. 3 *a*, Immediately after shift. Fig. 3 *b*, Less than one generation (1 hr) after shift. Fig. 3 *c*, *d*, and *e*, Four to five generations (7 hr) after shift. Note localization of silver grains at distal ends of the chains.

terial must segregate uniformly and randomly among progeny cells.

The use of antibodies to label the wall in such studies is not entirely satisfying. Cole (2) points out the need to assume that the antigenic site to which the antibody is binding is actually segregating along with the entire wall. One must also assume that the binding of the antibody does not directly affect the normal growth of the wall.

The use of tritiated wall precursors as tracers eliminates the need for both of these assumptions. Here, a chemical constituent of the wall material provides the label, in the absence of extraneous bound substances.

Since choline is a structural component of the pneumococcal teichoic acid (6), our data demonstrate for the first time the zonal growth and conservative segregation pattern of a bacterial teichoic acid. Furthermore, there is good evidence that, in *D. pneumoniae*, the choline-containing teichoic acid is *covalently* linked with the peptidoglycan of the wall (6), and thus the segregation of choline coincides with the distribution of the peptidoglycan itself. In this respect, our experiments are directly comparable to those in which DAP-^{3}H was used to label walls in *E. coli* (see above) (10), and to those in which antibody to the C-polysaccharide (4) was used to label walls in *S. pyogenes* (2).

The results of the experiments presented in this report provide clear evidence that, in *D. pneumoniae*, cell wall growth occurs by the addition of new wall materials at distinct equatorial zones, thus confirming the result obtained with immunofluorescence methods. The differences noted in both radioautographic and immunofluorescence studies between Gram-positive and Gram-negative cells might thus reflect a genuine difference in the manner in which these cells increase their surface area during growth and division.

The authors are indebted to Dr. George E. Palade and members of his laboratory for their help and use of their facilities.

This investigation was supported in part by a grant from the National Institute of Health, United States Public Health Service.

A preliminary report of portions of this work was presented at the April–May 1970 meetings of the American Society for Microbiology.

Received for publication 14 May 1970, and in revised form 9 June 1970.

REFERENCES

1. Caro, L. G., and R. P. Van Tubergen. 1962. *J. Cell Biol.* **15**:173.
2. Cole, R. M. 1965. *Bacteriol. Rev.* **29**:326.
3. Cole, R. M., and J. J. Hahn. 1962. *Science (Washington).* **135**:722.
4. Liu, T. Y., and E. C. Gotschlich. 1967. *J. Biol. Chem.* **242**:471.
5. May, J. W. 1962. *Exp. Cell Res.* **27**:170.
6. Mosser, J. L., and A. Tomasz. 1970. *J. Biol. Chem.* **245**:287.
7. Tomasz, A. 1964. *Bacteriol. Proc.* **64**:29.
8. Tomasz, A. 1967. *Science (Washington).* **157**:694.
9. Tomasz, A. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **50**:86.
10. Van Tubergen, R. P., and R. B. Setlow. 1961. *Biophys. J.* **1**:589.
11. Wagner, M. 1964–1965. *Zentralbl. Bakteriol. Parasitenk.* **195**:87.