

# Cell Wall Growth in *Bacillus licheniformis* Followed by Immunofluorescence with Mucopeptide-Specific Antiserum

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Insertion of new wall during the growth of *Bacillus licheniformis* occurred discretely and probably at the sites of incipient cross wall formation.

The classical experiments of Cole et al. (1, 4, 6) and May (8) show the way in which the cell walls of several bacteria grow. The limitations of the immunofluorescence technique as used by these and other workers (2, 3) have been critically discussed by Cole (5). A common feature of these studies was the use of antisera directed against wall antigens other than the mucopeptide. In some cases, these antigens were known to be covalently attached to mucopeptide and presumably were synthesized in step with mucopeptide at the same sites. However, ideally cellular growth in bacteria is equated with growth of the mucopeptide matrix that provides rigidity to the cell wall and maintains cellular shape.

Recently antisera were obtained (R. C. Hughes, P. F. Thurman, and M. R. Salaman, *in press*) from rabbits immunized with pure mucopeptide preparations of *Bacillus licheniformis* NCTC 6346. The homologous reaction of the antisera with *B. licheniformis* mucopeptide was strongly inhibited by low-molecular-weight fragments of known structure prepared from this mucopeptide after dissolution with lysozyme. A full description of the preparation and properties of the mucopeptide-specific antisera will be given elsewhere. The sera, containing antibodies specific for the mucopeptide of these walls and certain related gram-positive bacilli, agglutinated at high dilution whole cells of *B. licheniformis* and *B. subtilis* strains. Presumably the mucopeptide layer present in the walls of these cells was freely available to the antibodies. Antimucopeptide serum was used to follow cell wall replication in *B. licheniformis* (*his*<sup>-</sup> *lyt*<sup>-</sup>), a poorly lytic histidine auxotroph kindly provided by C. Forsberg. All sera were heated at 56 C for 30 min to inac-

tivate complement.

*B. licheniformis* 6346 (*his*<sup>-</sup> *lyt*<sup>-</sup>) (10; Forsberg and Rogers, *in press*) was grown in minimal medium into the logarithmic phase. A sample (1.0 ml) was mixed with antimucopeptide serum (0.25 ml) and phosphate-buffered saline (PBS; 0.75 ml) and kept at 35 C for 60 min. The cells were washed with PBS, resuspended in fresh minimal medium (1.0 ml) at 35 C, and gently shaken. At this time (zero time) and at intervals thereafter, samples (0.2 ml) were removed, added to fluorescein isothiocyanate (FITC)-conjugated (9) goat antirabbit immunoglobulin (0.1 ml; Pentex Labs), and kept at 35 C for 15 min. The stained cells were washed with five 1-ml portions of PBS and examined by M. R. Young by using fluorescence microscopy under oil immersion with a Leitz Fluorite  $\times 100$  objective. The light source was a mercury vapor lamp, and dark-field illumination was used with a Chance exciter filter OX-7 and a Wratten barrier filter 2B. Photographs were made on Kodak High Speed Ektachrom X or Ilfra Pan F film. The cells were not stained by the goat serum unless they had been previously exposed to mucopeptide antiserum.

At zero time (Fig. 1a), the cells were uniformly labeled at the periphery. During outgrowth, the new wall synthesized in the absence of mucopeptide antibodies showed up as dark, nonfluorescent gaps that appeared along the periphery of the cells (Fig. 1b, c, d).

A similar pattern was obtained by the direct immunofluorescence method (5) by using FITC-conjugated (9) antimucopeptide serum. Samples (0.2 ml) of *B. licheniformis* (*his*<sup>-</sup> *lyt*<sup>-</sup>) cells were inoculated with the serum (0.1 ml) at 35 C for 60 min. At zero time, unlabeled antimucopeptide serum (0.1 ml) was added to compete with labeled antibodies for antigen sites synthesized

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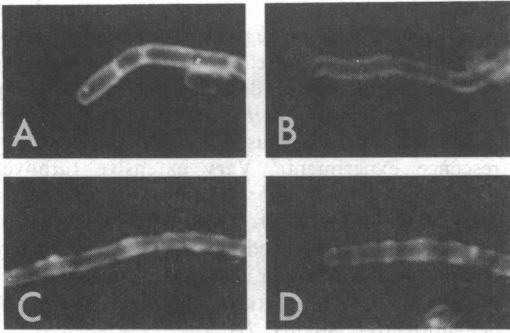


FIG. 1. *Bacillus licheniformis* 6346 (*his<sup>-</sup> lyl<sup>-</sup>*) incubated in rabbit antimucopeptide serum washed and reincubated in minimal medium. Samples were removed at (A) 0 min, (B) 60 min, (C) 120 min, and (D) 240 min after growth was restarted, and mucopeptide antibodies were visualized with goat FITC-conjugated anti-rabbit immunoglobulin.  $\times 2,700$ .

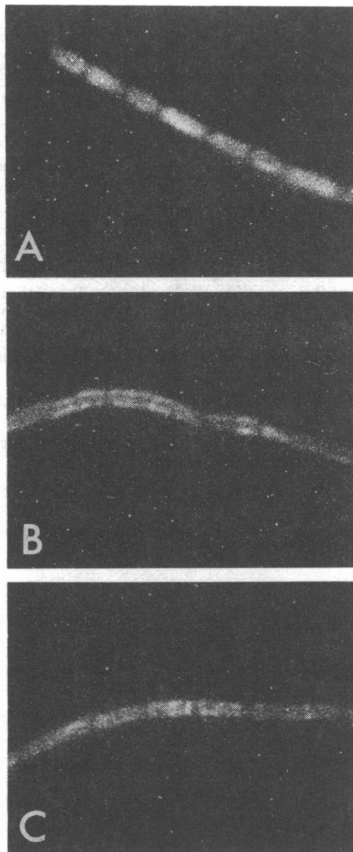


FIG. 2. *Bacillus licheniformis* 6346 (*his<sup>-</sup> lyl<sup>-</sup>*) grown in presence of FITC-conjugated antimucopeptide serum and examined at (A) 0 min, (B) 60 min, and (C) 120 min after the addition of unlabeled homologous serum.  $\times 2,200$ .

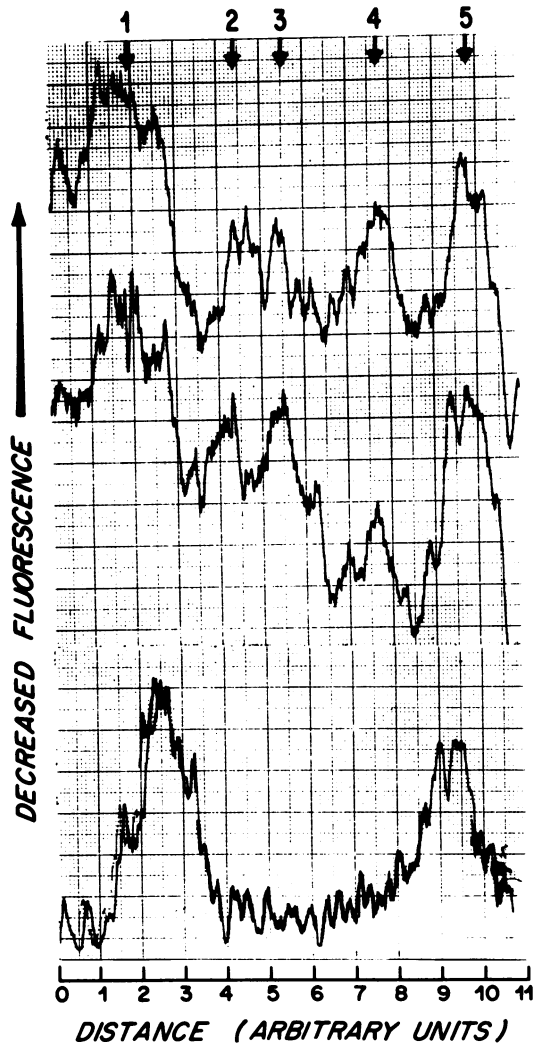


FIG. 3. Densitometer trace taken of selected cells from Fig. 2. From bottom to top: a cell at zero time (the traces obtained from the two extreme longitudinal edges of the cell are superimposed); a cell after 120 min (the traces obtained from the two extreme longitudinal edges are shown separately). The arrows (top) indicate the nonfluorescent bands referred to in the text.

during subsequent growth at 35 C. At intervals, washed cells were examined as before. The fluorescent staining obtained by the direct method (Fig. 2) was poorer than that shown for the indirect method (Fig. 1). In the latter method, the mucopeptide-specific antibody molecules bound to the surface of the cells were localized with FITC-conjugated goat antirabbit immunoglobulin. Each mucopeptide antibody molecule attached to the cell wall was capable of binding several FITC-conjugated antirabbit immunoglobulin molecules. Therefore, an amplification effect

was produced that was not possible with the FITC-conjugated antimucopeptide serum alone, as in the direct method. In particular, in the direct method the cross walls fluoresced very poorly (Fig. 2a). Presumably the cross walls contained fewer antigenic sites compared to the peripheral wall or these sites were not fully available to the antimucopeptide molecules. Again, the uniform fluorescence of the peripheral cell surface found at zero time (Fig. 2a) was replaced by a banding pattern of fluorescent (i.e., old) wall and nonfluorescent (i.e., new) wall. The relative positions of the new nonfluorescent bands were measured on plates of  $\times 1,800$  magnification by densitometry performed along the straightest stretches of the chains of unseparated cells. A Joyce-Loebl microdensitometer was used, and runs were made along both of the extreme edges of selected stretches. The fully formed cross-septa of cells at zero time were easily identified in traces as zones of poor fluorescence (Fig. 3, bottom). After 120 min (Fig. 3, top and middle), a new nonfluorescent band (band 3) appeared approximately halfway along the periphery of the cell at the presumed site of the incipient cross wall. Two other new nonfluorescent zones (bands 2 and 4) appeared at approximately the positions at which new cross wall would be formed if synthesis at these sites were initiated before completion of the original cross wall and complete compartmentalization of the daughter cells had occurred.

In summary, immunofluorescence techniques strongly suggest that in *B. licheniformis* 6346 (*his<sup>-</sup> lyt<sup>-</sup>*) mucopeptide synthesis occurs normally at very few sites during growth. Very similar results were obtained by Cole (5) in other gram-positive bacteria. Results obtained by Chung and co-workers (2) for *B. cereus* and *B.*

*megaterium* were also consistent (5) with localized wall formation in the regions of developing cross-septa. It should be noted that very little turnover of mucopeptide was detected in these poorly lytic cells of *B. licheniformis* (*his<sup>-</sup> lyt<sup>-</sup>*) grown under the conditions used for the immunofluorescence experiments. Very extensive turnover was reported in *B. subtilis* strains under certain growth conditions (7). Obviously turnover of mucopeptide, if proceeding, would have greatly complicated the proper interpretation of the immunofluorescence pictures obtained with *B. licheniformis* 6346 (*his<sup>-</sup> lyt<sup>-</sup>*).

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