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Passage of a Membrane Protein Through the Walls of Toluene-Treated *Bacillus megaterium* Cells

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Based on autoradiographic and microscopic evidence, it seems likely that a membrane protein essential for peptidoglycan synthesis can be extracted from unlysed toluene-treated *Bacillus megaterium* cells. Furthermore, this protein can be added back to the membrane through the wall to reconstitute peptidoglycan synthesis. Autoradiograms also show that peptidoglycan is synthesized from externally added nucleotide precursors over the entire length of the toluene-treated bacteria. The amounts of peptidoglycan made is too small to be visible by thin section electron microscopy.

Peptidoglycan of the bacterial cell wall could be made by toluene-treated *Bacillus megaterium* cells from the externally added precursors uridine 5′ diphosphate (UDP)-GlcNAc and UDP-MurNAc-L-ala-D-glu-*meso*-diaminopimelyl-D-ala-D-ala (UDP-MurNAc-pentapeptide) (6). Furthermore, at least one protein essential for peptidoglycan synthesis could be removed from the membranes of toluene-treated cells by extraction with 2 M LiCl (7). This protein has no affinity for cell walls but does bind to membranes (7). This membrane-associated protein, which we propose to call factor PG-I, has now been purified 230-fold (A. Taku, personal communication).

After LiCl extraction, peptidoglycan synthesis by toluene-treated cells was reduced 2- to 10-fold (7). Then the extracted factor PG-I could be added back to the toluene-treated cells to reconstitute the original rate of peptidoglycan synthesis (7). In these experiments, the toluene-treated, the LiCl-extracted, and the reconstituted cells all looked approximately the same in the light microscope (7). In all cases, the majority of the cells looked unchanged while a small minority, usually not more than 5%, looked lysed. A possible explanation was that only the lysed cells participated in the wall synthesis from the externally added precursors. On the other hand, the majority of the cells might have synthesized wall from the added precursors. This latter result would suggest that factor PG-I could be extracted from the cell membrane through a relatively intact cell wall. Similarly, factor PG-I could have passed through undisrupted cell wall during the reconstitution with the membrane. Thus, the possibility of a membrane protein traversing intact cell wall could be supported by finding that a majority of the toluene-treated, LiCl-extracted, and the reconstituted cells could all synthesize peptidoglycan from externally added precursors. Consequently, the synthesis of peptidoglycan by individual cells is explored in the present communication. Also, ultrastructural examinations are discussed.

MATERIALS AND METHODS

Preparation of toluene-treated, LiCl-extracted, and factor PG-I reconstituted cells. *B. megaterium* 899 was grown at 30 C in antibiotic medium no. 3 (Difco). The cells were harvested and shaken with 1% toluene at room temperature for 30 min to prepare toluene-treated cells. These cells were further incubated in 2 M LiCl for 10 min at 30 C to produce the LiCl-extracted cells. To obtain reconstituted cells, factor PG-I partially purified (80-fold) from an extract of LiCl-treated cells (kindly provided by A. Taku), was mixed with the LiCl-extracted cells. After 5 min in an ice bath, the resulting reconstituted cells were collected by centrifugation. The details of all these techniques have all been described previously (6, 7).

Incubation for peptidoglycan synthesis from externally added precursors. Samples of toluene-treated, LiCl-extracted, and reconstituted cells were all incubated for 1 h at 30 C for peptidoglycan synthesis using externally added UDP-MurNAc-pentapeptide (0.8 mM final concentration) and UDP-[^{3}H]GlcNAc (0.7 Ci/mmol, 0.3 mM final concentration, New England Nuclear Corp.). As a control, an identical incubation was performed using toluene-treated cells with the UDP-[^{3}H]GlcNAc replaced by water. The incubation conditions have already been described (5).

Analysis of samples. At the end of the incubation, fractions of all samples were precipitated with 5% trichloroacetic acid and counted in a scintillation

counter for incorporation of [^{3}H]GlcNAc into peptidoglycan. Simultaneously, a fraction of each sample was not acid precipitated but was fixed and examined autoradiographically using Ilford L4 Nuclear Emulsion according to the procedures of Caro and van Tubergen (2). Before and after the incubation for peptidoglycan synthesis, each sample was also fixed, embedded, and sectioned for electron microscopy according to the method of E. Hagen (Ph.D. dissertation in preparation).

RESULTS

Distribution of cells synthesizing peptidoglycan. In order to study the removal of factor PG-I and reconstitution of wall synthesis by its readdition, *B. megaterium* cells were first harvested from the exponential growth phase and treated with toluene. Then, a portion of the toluene-treated cells was extracted with 2 M LiCl and a fraction of the LiCl-extracted cells was reconstituted using a partially purified preparation of factor PG-I. UDP-[^{3}H]GlcNAc of very high specific activity was added to the toluene-treated, the LiCl-extracted, and the reconstituted cells. After a 60-min incubation period at 30 C, the cells were fixed and prepared for autoradiography. Simultaneously, the cells were precipitated with trichloroacetic acid and counted for acid-insoluble radioactivity in peptidoglycan. A fraction of each sample was also counted microscopically in a counting chamber to determine the total number of cells in the reaction mixtures.

These data (Table 1) showed that LiCl extraction decreased peptidoglycan synthesis to 10 to 20% of the level in the toluene-treated cells. The residual synthesis could be increased four- to eight-fold by reconstitution with factor PG-I. There was general agreement for the extent of extraction and reconstitution regardless of whether the computations were based on information from autoradiography or acid precipitation. This agreement indicated that the fixation procedure used for the autoradiography probably gave as good an indication of peptidoglycan synthesis as acid precipitation. In two additional experiments, no significant amounts of radioactive peptidoglycan were observed using either autoradiography or acid precipitation if UDP-[^{3}H]GlcNAc was added but UDP-MurNAc-pentapeptide was omitted.

The distributions of cells synthesizing peptidoglycan from externally added precursors are presented in Fig. 1 and 2. These distributions showed that essentially all toluene-treated cells could perform the synthesis. After the LiCl extraction, all cells seemed less capable of synthesizing peptidoglycan. There did not seem to be a small class of cells continuing to synthesize peptidoglycan at the original rate. Then, after reconstitution, most cells seemed to have recovered their ability to make peptidoglycan. The data in Fig. 2 were obtained from autoradiograms of the LiCl-extracted cells of Fig. 1 developed after a longer exposure for radioactive decays. Figure 2 was included to show from a significant number of grains per cell that most of the LiCl-extracted cells were still capable of synthesizing peptidoglycan at the same low level.

If all cells behaved identically through all the treatments, the observed distributions should have followed the Poisson distributions (also plotted in Fig. 1 and 2). However, the observed distributions seemed more disperse suggesting some heterogeneity among the various cells. Part, but probably not all, of the heterogeneity could be explained by differences in cell size. The newly born cells would have been about half as large as cells about to divide. For the distributions in Fig. 1 and 2, any one cell was counted as a cell regardless of size. The distribution for the toluene-treated cells seems to follow the Poisson distribution more closely than the distributions for the LiCl-extracted and the reconstituted cells. Thus, the toluene-treated cells were probably more homogeneous than the extracted or the reconstituted cells. This fact was not surprising because of the extra

TABLE 1. *Synthesis of peptidoglycan by toluene-treated, LiCl-extracted, and reconstituted B. megaterium cells*[a]

Cell in incubation	Acid-precipitable radioactivity		Autoradiography	
	nmol/10^8 bacteria	% of toluene-treated cells	Avg grains/cell	% of toluene-treated cells
Toluene treated	2.8	100	16.5	100
LiCl extracted	0.2	8	3.4	21
Reconstituted	1.9	68	12.3	75

[a] Bacteria treated and incubated were analyzed for peptidoglycan synthesized from externally added UDP-MurNAc-pentapeptide and UDP-[^{3}H]GlcNAc. In one set of measurements, trichloroacetic acid-precipitable radioactivity was divided by the number of cells counted by using the light microscope. In another set of measurements, cells in the samples were examined by autoradiography after 7 days of exposure of the photographic emulsion. An average number of grains per cell was computed from individual measurements on approximately 200 cells. The distribution grains for the cells measured are presented in Fig. 1.

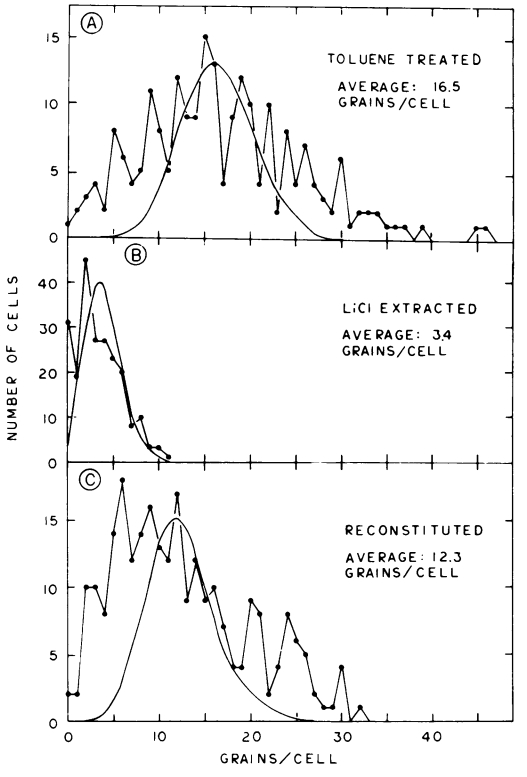

FIG. 1. *Distribution of grains over various B. megaterium cells in developed autoradiograms. The preparation and incubation for peptidoglycan synthesis and the counting of the silver grains after autoradiography are described in the legend of Table 1. All autoradiograms were developed after 7 days of exposure. In a sample where no radioactivity was included in the incubation of toluene-treated cells, only three grains were seen over each of three cells in a total of 250 cells examined. The curved line gives the Poisson distribution corresponding to the average number of grains determined by microscope observation.*

steps required for making the extracted and the reconstituted cells.

Sites of peptidoglycan synthesis. Autoradiography experiments also provided information about the sites of peptidoglycan synthesis. Light microscope observations were made on the cells incubated in the experiments of Fig. 1 and 2 to determine if peptidoglycan synthesis was localized to particular regions of the cell such as the septa or the hemispherical caps. The results (Fig. 3) showed that peptidoglycan was formed over the entire length of the toluene-treated, the LiCl-extracted, and the factor PG-I reconstituted cells.

Ultrastructural studies. As discussed in the introductory passage in this paper, it seemed possible that factor PG-I could traverse the walls of relatively intact cells. Support for this contention came from the data in Fig. 1 and 2 in which it was found that factor PG-I could be extracted from and added back to the membranes of the majority of the cells. Most of these cells were not lysed as seen in the light microscope so their walls were probably not greatly degraded. Additional information about the intactness of the walls could be obtained from ultrastructural studies. Therefore, toluene-treated, LiCl-extracted, and reconstituted cells were compared to untreated cells (Fig. 4). So far as the wall was concerned, no important changes could be observed in all the four samples suggesting that the walls did stay relatively unaltered during the various treatments. The most striking differences between the samples was the greater dispersion of nuclear material in the untreated cells.

Another question which could be addressed ultrastructurally was whether the peptidoglycan made from externally added precursors could be visualized. Thus, the cells used in the experiments of Fig. 1 to 3 were also examined in thin sections after the 60-min incubation. No newly made wall material was discernible in electron micrographs of any of the samples (Fig.

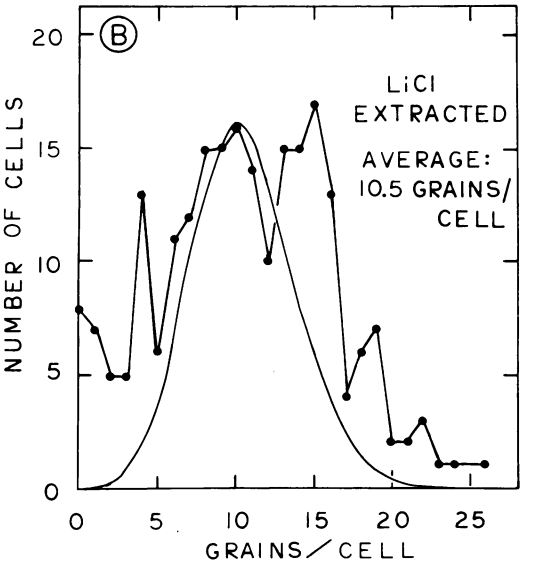

FIG. 2. *Distribution of grains over various B. megaterium cells in developed autoradiograms. The autoradiograms for the LiCl-extracted bacteria described in Fig. 1 were incubated for 23 days instead of 7 days before developing. The curved line gives the Poisson distribution corresponding to the average number of grains determined by microscope observation.*

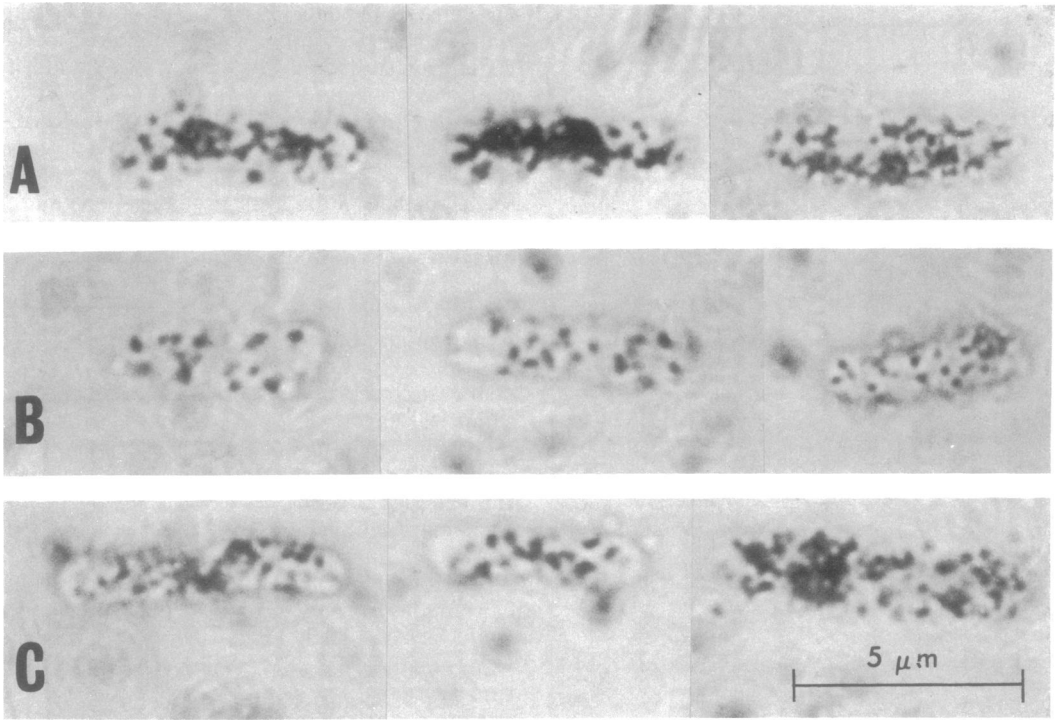

FIG. 3. *Light micrographs of grains over individual B. megaterium cells in developed autoradiograms. The autoradiograms for the toluene-treated (A) and reconstituted cells (C) of Fig. 1, and the autoradiogram for the LiCl-extracted cells (B) of Fig. 2 were photographed.*

5). However, significant lysis and disintegration of the cells was observed in all cases. From other ultrastructural experiments where the incubation was prolonged to 1.5 and 4 h, it was clear that the deterioration of cell structures continued as incubation times were extended.

The inability to see peptidoglycan made from external precursors was not surprising in light of the following computation. The results presented in Fig. 1 and 2 suggested that essentially all cells synthesized the same approximate amounts of peptidoglycan from externally added precursors. Of all the cells tested, the toluene-treated cells were the most efficient at synthesizing peptidoglycan with 10^8 cells incorporating about 3 nmol of glucosamine during the incubation. For comparison, Bricas et al. (1) found 1 mg of isolated *B. megaterium* walls to contain 725 nmol of glucosamine. From this information, it could be calculated that 10^8 cells have approximately 300 nmol of glucosamine in their walls. Thus, the peptidoglycan made by the toluene-treated cells constituted only 1% of the amount already present in previously formed walls. Furthermore, the data in Fig. 3 suggested that all the newly synthesized peptidoglycan was uniformly distributed over the entire surface of the cells. This small proportion of new peptidoglycan could easily remain undetected in the electron microscope.

DISCUSSION

Most of the toluene-treated, LiCl-extracted, and factor PG-I reconstituted *B. megaterium* cells behaved reasonably homogeneously with respect to peptidoglycan synthesis from externally added precursors (Fig. 1-3). Thus, factor PG-I could pass through the walls of the cells studied. These walls looked intact in ultrastructural experiments (Fig. 4). Consequently, factor PG-I could traverse relatively intact walls. Of course, it was possible that the walls were damaged during the toluene treatment, LiCl extraction, and reconstitution steps in ways not visible by light or electron microscopy. Nevertheless, the passage of a membrane protein through the walls of toluene-treated cells suggests the possibility of isolating mutants deficient in factor PG-I, mutants for which the wild-type phenotype can be recovered by the external addition of the factor. The factor should be able to penetrate to the appropriate

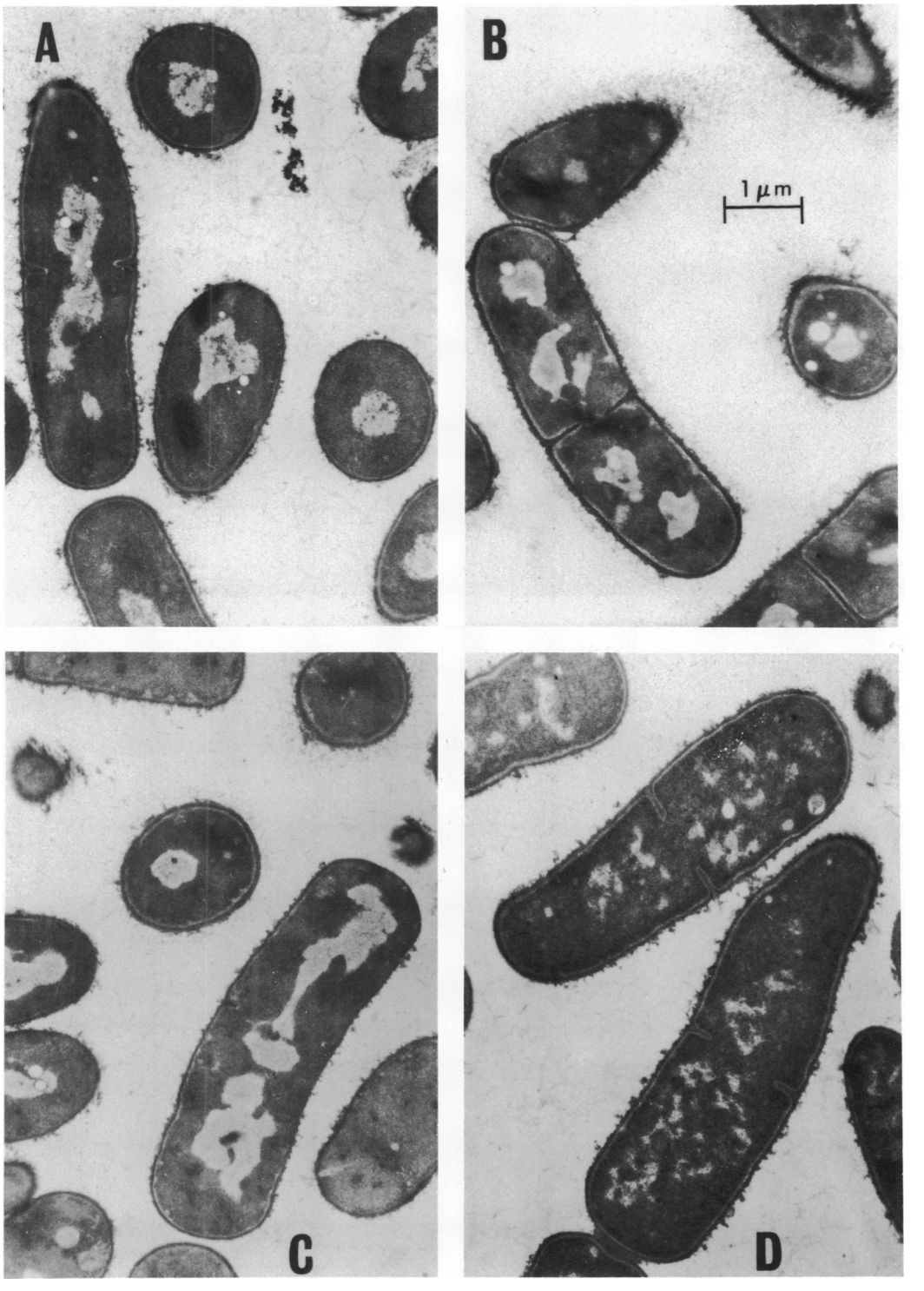

FIG. 4. *Electron micrographs of B. megaterium cells before the incubation for peptidoglycan synthesis. (A) Toluene-treated cells; (B) LiCl-extracted cells; (C) reconstituted cells; (D) untreated cells.*

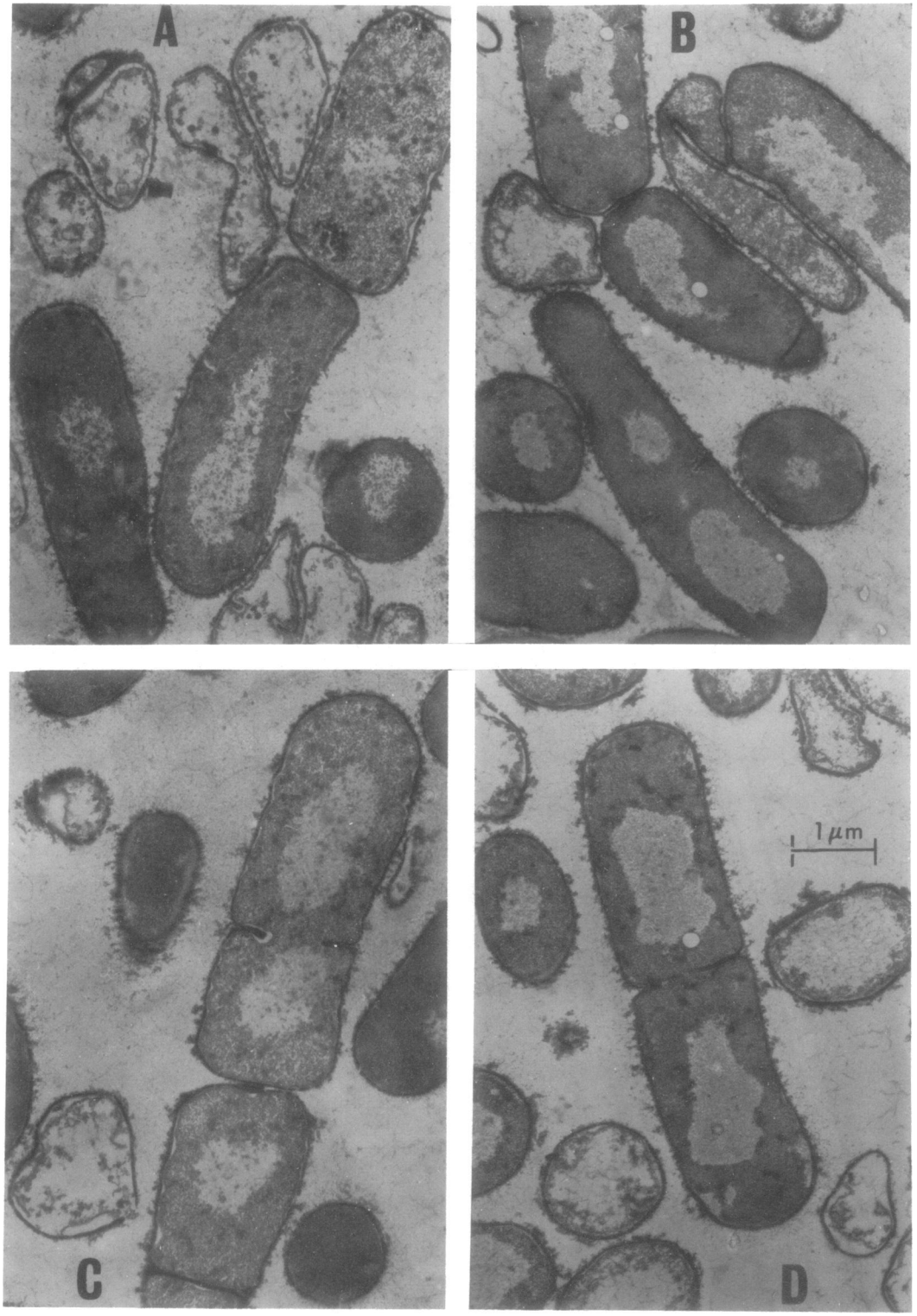

FIG. 5. *Electron micrographs of B. megaterium cells after incubation for peptidoglycan synthesis. The cells used for preparing the autoradiograms of Fig. 1 to 3 were examined in thin sections. (A) Toluene-treated cells; (B) LiCl-extracted cells; (C) reconstituted cells; (D) control sample of toluene-treated cells incubated without added UDP-[^3H]GlcNAc.*

site in the membrane to function properly in wall synthesis if the walls of toluene-treated cells are like those of intact cells. The study of such a mutant could give valuable information about the physiological role of a membrane protein essential for wall synthesis. Attempts to isolate such a mutant are now in progress. The successful isolation of a mutant with a membrane protein replacable by the external addition of the protein would suggest that membrane proteins involved in other cell processes should also be amenable to study by the removal and readdition of the protein to mutant cells.

The synthesis of peptidoglycan from externally added precursors over the entire length of the toluene-treated cells was consistent with in vivo experiments performed with a large variety of rod-shaped bacteria, both gram positive and gram negative. The general finding has been that wall synthesis occurs all along the length of the bacteria although some investigators have reported wall synthesis only in localized regions (3, 4).

The observation of lysis during the incubation for peptidoglycan synthesis suggested that significant amounts of autolysins capable of degrading cell wall were retained by the bacteria. The same enzymes were also present during the toluene treatment and LiCl extraction both performed over significant time intervals at the same temperature as the incubation for peptidoglycan synthesis. However, there was no appreciable lysis during those steps (Fig. 4) because the continued presence of toluene or LiCl-inhibited autolysin activity.

ACKNOWLEDGMENTS

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