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Organization of Mesosomes in Fixed and Unfixed Cells

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After the addition of glutaraldehyde (GA) to cells incubated at 3 or 37°C, mesosomes were observed with increasing frequencies in freeze fractures of cells. These increases were related to the kinetics with which GA cross-linked adjacent amino acids. Upon the addition of GA, mesosomes were first observed in the periphery of freeze-fractured cells usually attached to septal membranes. However, with time, while the septal attachment sites were maintained, the "bodies" of the mesosomes were observed to move toward the center of the cytoplasm. This centralization process was much more rapid at 37 than at 3°C. It is hypothesized that upon fixation, or receipt of some physical insult, mesosome precursors found in undisturbed cells undergo a change in state that results in their visibility in freeze fractures.

Bacterial mesosomes are bag-shaped membranous structures that are thought to be involved in several fundamental processes, including chromosome replication and segregation, cell division, endospore formation, and exoenzyme transport (5, 8). In *Streptococcus faecalis* (ATCC 9790), a role in cell division and/or chromosome replication was suggested from observations of thin sections in that: (i) mesosomes appeared to be preferentially attached to the cell's division apparatus (i.e., the cross wall) (4); and (ii) the size of mesosomes appeared to be predictably altered by selectively uncoupling DNA synthesis from the other aspects of macromolecular synthesis before fixation (1). In 1971, Nanninga showed that mesosomes were present in fixed cells, but were virtually absent from freeze fractures of unfixed cells)6). Similar observations were made with cells of *S. faecalis* where mesosomes were rarely seen in freeze fractures unless the cells had first been fixed or treated with high concentrations of glycerol (2).

Here we investigate the transition of mesosomes to the "visible state" in freeze fractures of *S. faecalis* after addition of glutaraldehyde (GA) to exponential-phase cultures. In addition, we have asked whether this transition is related to the formation of covalent cross-linkages by the fixative.

MATERIALS AND METHODS

Cells used in these experiments were first allowed to go through seven exponential mass doublings in a chemically defined medium (9) at 37°C. For the cross-linking studies (Fig. 1A), a culture was divided into two portions. One portion was kept in a 37°C water bath, whereas the other was chilled to ice bath temperature. [^{3}H]leucine (0.02 μCi/ml, 0.00625 μCi/μg) was then added to both portions, and GA (vacuum distilled) was immediately added (zero time) to a final concentration of 2.5%. Samples of 0.5 ml were removed from the incubation mixtures, and the cellular material was precipitated in cold 10% trichloroacetic acid. The cold-acid precipitates were captured on glass-fiber filters and washed three times with 5.0 ml of cold trichloroacetic acid, and the amount of radioactive label found in these precipitates was determined by liquid scintillation counting procedures as previously described (7).

For morphological observations, cells were concentrated before the addition of GA by membrane (Millipore Corp.) filtration or by centrifugation. For observations made at 37°C, the cells were quickly transferred to a 37°C room in a water bath at the same temperature. The culture was rapidly filtered through a Millipore apparatus (0.65-μm pore size), and the cells were resuspended by vortexing in 0.5 ml of the original medium. A portion of the concentrated culture was frozen for freeze fracture before the addition of GA (zero-time control sample). Prewarmed GA (37°C) was then added to a final concentration of 2.5%, and samples were removed and frozen for freeze fracture.

For observations made at 3°C, cells grown at 37°C were chilled by swirling a 1,000-ml flask, containing 150 ml of a culture, in an ice bath. In the experiment shown in Fig. 1, the cells were concentrated by centrifugation (1,500 × *g*; 10 min, 3°C) and then resuspended in 0.5 ml of the original medium (3°C). However, very similar results were obtained when cells were concentrated by membrane filtration and resuspended in a small volume of medium in a 3°C room (data not given). Regardless of the method used to concentrate the cells, all operations were carried out in a 3°C room, where a zero-time control sample was frozen for freeze fracture without fixative, and subsequent samples were frozen after the

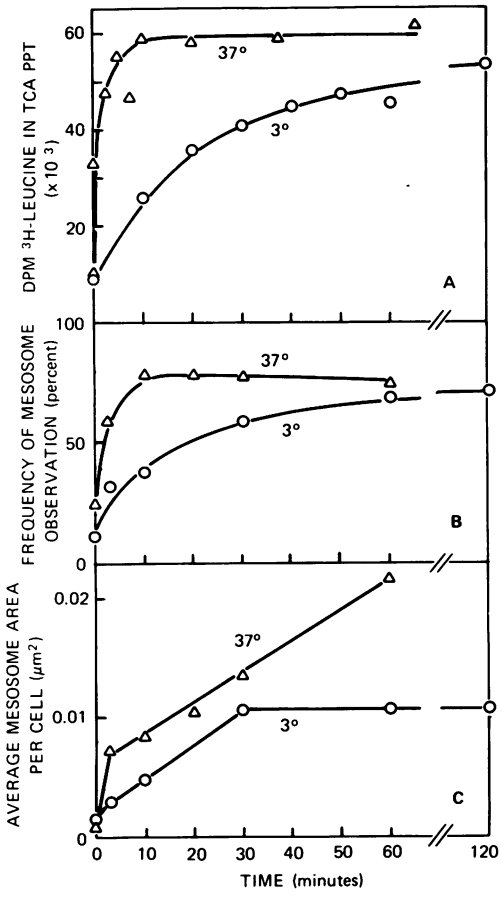

FIG. 1. *Comparison of kinetics by which GA cross-links adjacent amino groups (A) with the frequency (B) and area (C) of mesosomes seen in freeze fractures of cells after the addition of GA. The GA cross-linking reaction was quantitated by measuring the binding of [³H]leucine to unlabeled, unconcentrated cells (A) incubated at 3 or 37°C in the presence of 2.5% GA. The frequency (B) and area (C) of mesosomal membranes was studied from longitudinal freeze-fractured cells, prepared in the following manner: cells maintained at 3 or 37°C were concentrated by centrifugation or filtration, a sample was removed and frozen for freeze fracture before addition of GA (zero-time control sample), prechilled or prewarmed GA was then added to a final concentration of 2.5% at zero time, and samples were subsequently frozen at the times shown. See Materials and Methods for further experimental details.*

addition of prechilled GA (final concentration of 2.5%). The freeze-fracture procedures used have been previously described (2). Fractures were etched 1 min before being replicated. For quantitative studies, at least 50 longitudinally cross-fractured cells having diameters of about 1.0 μm were randomly selected from each sample (such fractures are assumed to approximate longitudinally axial views of cells [2]). The average area of the mesosomal membranes observed in these cells was determined by planimetry (Numonics Digitizer, North Wales, Pa.).

RESULTS AND DISCUSSION

Figure 1A shows the time course of the GA cross-linking reaction at 37 and 3°C, which was quantitated by measuring the binding of [³H]leucine to unlabeled cells in the presence of GA (2.5% final concentration). The binding of the label was due to the formation of covalent linkages in the presence of the fixative and was not the result of protein synthesis, for the radioactivity measured in trichloroacetic acid precipitates was: (i) resistant to hot-acid hydrolysis (10% trichloroacetic acid at 90°C for 30 min), and (ii) not affected by the addition of chloramphenicol (50 μg/ml) to cells before fixation. In addition, the extent of the reaction at 15 min was linearly dependent upon the final GA concentration over a concentration range of 0.1 to 2.5%. At every concentration of GA, the label was bound more rapidly at 37 than at 3°C.

Figure 1B shows the effect of GA fixation on the frequency of mesosomes as seen in longitudinal freeze fractures of cells. At zero time, before GA had been added, about 15 to 20% of the cells concentrated for study by centrifugation or filtration showed mesosomes regardless of whether they were incubated at 37 or 3°C. When unfixed cells were frozen directly in a 37°C room without concentration, only 2% (or one out of the 50 cells counted) contained mesosomes. This low frequency is consistent with similar measurements made in the past (2) and suggests that the frequency of cells showing mesosomes can be increased to 15 to 20% without fixation simply by filtering or centrifuging cells. However, the number of mesosomes observed after "gentle" centrifugation or filtration was rather low when compared with the number of mesosomes observed after the addition of GA (Fig. 1B). These increases upon fixation

FIG. 2. *Selected freeze fractures showing "typical" views of S. faecalis incubated at 3 and 37°C before and after the addition of GA. Before the addition of GA, most freeze fractures of cells (i.e., 85 to 90%) showed no mesosomes (A) and (F); however, after the addition of GA, mesosomes were seen with increasing frequency, first at the periphery of the cell (B, G) and later in the central portion of the cytoplasm (E, H). Regardless of whether the body of the mesosome was found at a peripheral or central location, the mesosomes of S. faecalis appeared to be preferentially attached to the septal region of cells (B–E and H, I) or under cell wall protuberances (G and J) which mark future sites of septum formation in this organism (3, 5). The bar in (A) equals 0.1 μm and applies to all other micrographs.*

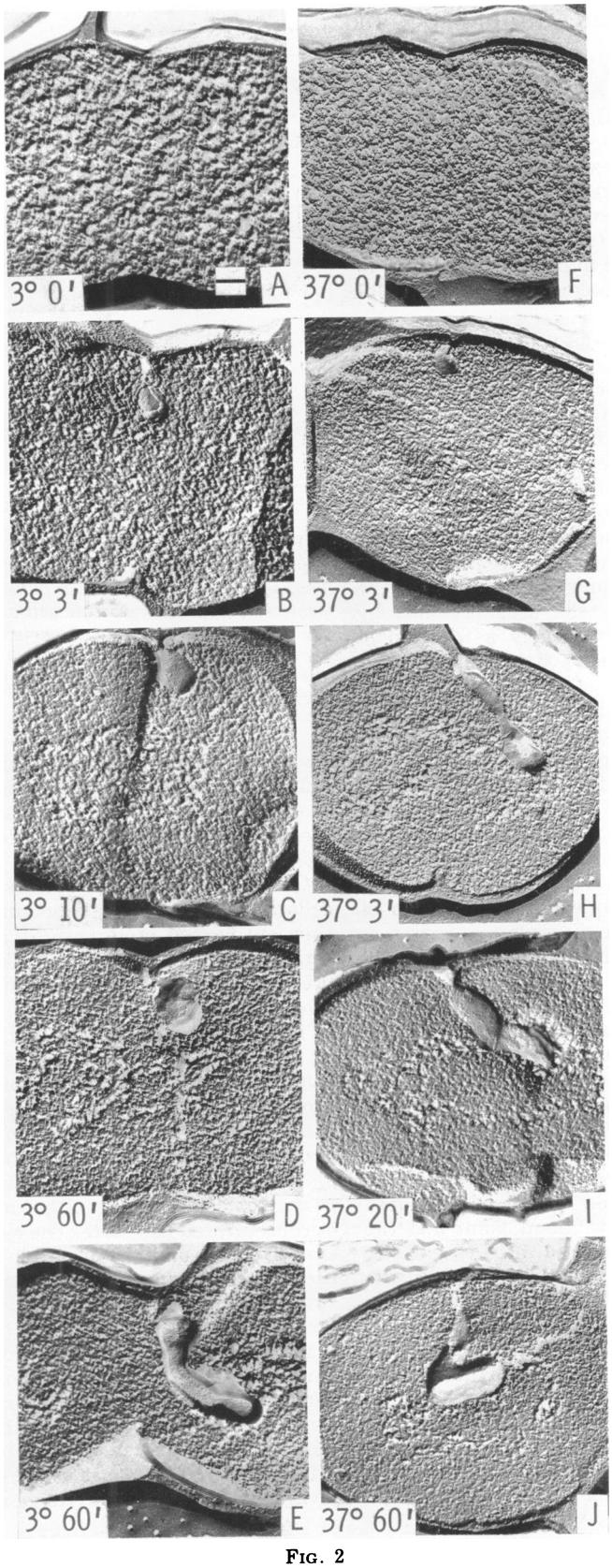

FIG. 2

were large in both the 3 and 37°C cultures, but the process was more rapid at the higher incubation temperature. Comparison of the cross-linking and mesosome frequency kinetics in Fig. 1A and B indicates that the observation of mesosomes in freeze fractures of GA-fixed cells is closely related to the covalent cross-linking of amino groups by GA (as exemplified by covalent linking of leucine to cellular material), or to some process that occurs in parallel to this event.

By contrast, there was poor correlation between the kinetics of cross-linking (Fig. 1A) and the average area occupied by mesosomes in freeze fractures (Fig. 1C). The increase in mesosome area during the first 30 min appeared to be temperature independent, whereas the increase that occurred after 30 min at 37°C continued long after the cross-linking reaction had reached the saturation point. This indicates that although the frequency of freeze fractures showing mesosomes may be related to the cross-linkage reaction, the area occupied by mesosomes is probably largely a function of structural rearrangements that occur after GA has been added.

The complexity of these rearrangements is probably best illustrated with micrographs, as shown in the two reconstructed sequences in Fig. 2. Regardless of time or temperature of fixation, mesosomes were seen preferentially attached to the septal regions of cells (i.e., in better than 85% of the micrographs analyzed). However, although a septal attachment was the predominant observation, the position of the body, or bag, of the mesosome appeared to depend on the time and temperature of fixation.

In the first 60 min of fixation at 3°C, the bag appeared in most cells to be peripherally located (Fig. 2B). However, after 60 min, although a few peripheral mesosomes were seen (Fig. 2D), the bag of most mesosomes seemed to be dislocated toward the center of the cell associated with central nucleoid fibrils (Fig. 2E). As the mesosomal bag moved from the periphery of the cytoplasm, a tail-like structure developed between the septal attachment point and the bag. Centralization appeared to be essentially completed by 120 min in the 3°C cells. In the samples fixed at 37°C, the centralization process and tail formation occurred much earlier, being observable in some cells after only 3 min of fixation (Fig. 2H), and was virtually completed after 20 min (Fig. 2I).

Presumably, centralization is more rapid at the higher temperature due to the increased fluidity of the membrane lipids at this temperature (i.e., the transition temperature of the membrane lipids of this organism is about 10°C [10]). This increased fluidity is also possibly responsible for the continued enlargement of mesosomes observed at 37°C after 30 min (Fig. 1C).

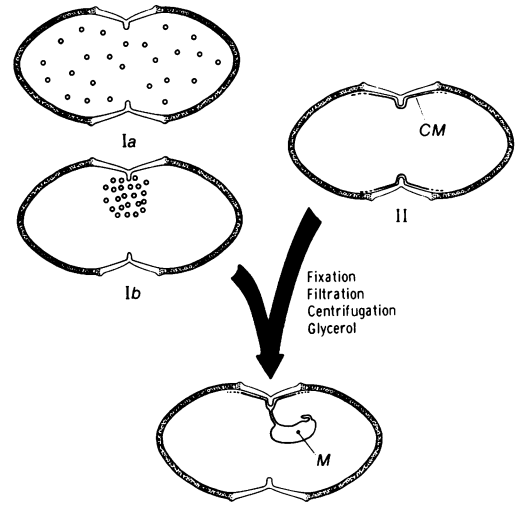

FIG. 3. *Diagrammatic representation of possible explanations of the results. The "stalk" or "tail" extending between the septum and the body of the mesosome (M) appears to be an artifact of preparation resulting from the rearrangement of mesosomal membranes upon fixation. (I) and (II) suggest possible distributions of mesosomal precursors in undisturbed, unfixed cells.*

Our view is that mesosomes seen in fixed cells have physiological meaning in that: (i) they are formed preferentially at site-specific, septal locations (1, 4), and (ii) their size can be predictably altered by uncoupling macromolecular synthesis (1). However, the structure seen in fixed cells, including the tail process shown in Fig. 2 which connects the bag of the mesosome to the septum, is apparently an artifact produced by fixation. Regarding the location of the precursors of mesosomes in unfixed cells, two of several possible models are shown in Fig. 3. In model I, these precursors would be found in a cytoplasmic pool in either a dispersed (Ia) or concentrated (Ib) state. The cytoplasmic precursor pool would be invisible in cross-fractures, possibly due to organization of lipids in small micelles or some other undetectable form. Upon receipt of an insult (e.g., fixation, filtration, centrifugation, or the addition of large amounts of glycerol [2]), this pool would aggregate into some form that is observable in cross-fractures, such as a tight trilaminar configuration.

In model II, mesosomes would result from an invagination of the septal membrane. One pos-

sible mechanism could be that the septal membrane invaginates due to a contraction of the cell wall during fixation. This invagination could be assisted by some prior attachment of the septal membrane to the bacterial chromosome. Although the invagination hypothesis remains open, the wall contraction aspect of the model seems inconsistent with the observed increase in the frequency of mesosomes seen when cells are filtered or centrifuged. In either model, dislocation of the mesosomal bag from the cell's periphery would accompany the central appearance of nucleoid fibers. This process may involve a partition of the cytoplasm upon fixation which could result in a central enrichment or contraction of the nuclear fiber that carries the mesosome to the center of the cell.

At present, both models appear equally difficult to test, for an analysis of the location of mesosomal precursors in unfixed cells would supposedly require that at some point cells be concentrated, broken, or fixed. Unfortunately, it seems that all of these procedures could alter the state of the precursors, resulting in the formation of the structure known as a mesosome.

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