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Electron Microscopic Studies on Opaque Colony Variants of Group A Streptococci

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Opaque colony variants of two strains of group A streptococci have been compared with blue colonies of the same strains by electron microscopy. In opaque colonies, the cocci are joined into elongated chains by exaggerated intercellular septa that often occupy the major portion of each cell's circumference. The thickness and lamination of cell walls in opaque colony variants are identical to those aspects of cell walls in blue colony forms. The similarity in cell wall architecture is found between opaque and blue forms whether or not M protein (and M associated surface fimbriae) is present. Extensive, direct contact between the nucleoid and the cytoplasmic membrane beneath intercellular septa is seen in opaque colony variants. The relationship of this marked nucleoid-cytoplasmic membrane association to the unusual chain forms in the opaque colony variants is unclear.

Opaque colony variants are frequently found in agar cultures of group A streptococci of several serological types. Griffith described the occurrence of the opaque variants and made use of these colonies in studying streptococcal protein antigens (2). Gooder and Maxted suggested that a high content of M protein on the streptococcal cell wall might account for the variation in colony form (1). Later studies, however, showed that the opaque colony variants could be found both in cultures of M-containing streptococci and in cultures in which no M antigen was detectable (6, 9). Further studies revealed no differences between blue and opaque colonies with respect to their content of several chemical and antigenic moieties that were assayed (6). It was found that the chains of streptococci in variant opaque colonies were strikingly longer than those in the more usual, blue colonies. Also, it was demonstrated that the long chain forms persisted after extraction of the opaque colony-derived streptococci with hot formamide. These observations suggested that the long chains resulted from exaggerated cross-bridges or septa which held individual cocci together because of greater continuity between adjacent cell walls following division. The current study was designed to provide morphologic evidence for the nature of the long chain effect and to determine whether greater wall thickness in the vicinity of the cross septa is related to the opaque colony form. We have examined opaque colony variants and blue colonies from two strains of group A streptococci by electron microscopy to define further the differences that account for the occurrence of the two colony forms.

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MATERIALS AND METHODS

Organisms. Streptococci were obtained from the collection of Rebecca L. Lancefield and were assayed for M antigen by established microprecipitin methods (5). Morphological studies were carried out on strain S 43 and on strain S 23.

Selection of variants. Organisms were grown on a clear agar medium that facilitates detection of opaque colony variants, as previously described (6). Cultures were grown on the agar at 37 C overnight and examined with a dissecting microscope by using obliquely transmitted light. Blue and opaque colonies were selected and were repeatedly transferred until pure lines were established.

Electron microscopy. Agar cultures of blue or opaque colonies were flooded with fixative (2% glutaraldehyde in 0.1 M sodium cacodylate, *p*H 6.8) and were allowed to stand for 2 to 3 min. At the end of that time, colonies could be freed easily from the agar surface by directing a gentle stream of fixative from a Pasteur pipette toward the colony surface. The intact, suspended colonies were aspirated into the pipette, transferred to fresh fixative, and allowed to stand at room temperature for 2 to 4 hr. They were rinsed in 0.1 M cacodylate, treated for 1 hr with veronal acetate-buffered osmium tetroxide (4), and were either dehydrated at this point or were first soaked in 1% uranyl acetate prior to dehydration in graded alcohol solutions. The whole colonies were embedded in Epon and

thin sections were stained with lead citrate (8). Micrographs were obtained with a Philips 200 microscope (Philips Electronic and Pharmaceutical Industries Corp., New York, N.Y.) that was used through the courtesy of Councilman Morgan.

RESULTS

Sections were taken from all segments of the epoxy-embedded colonies. The part of the colony examined was related to the degree, but not to the type, of differences (described later) which were found between blue and opaque colonies. In general, the characteristic form of opaque colony cell chains was most marked in sections taken through the centers of the colonies. En bloc treatment with uranyl acetate, prior to dehydration, did not affect the overall morphology of cells or of their cell walls. The only difference which is attributable to uranyl acetate exposure prior to dehydration is in the morphology of the nucleoid region. This is described later.

Blue and opaque colonies of strain S 43. Streptococci in blue colonies of strain S 43 are usually seen as single or diplococcal forms (Fig. 1). Short chains composed of four or five cocci are sometimes observed. In all these configurations, the individual cocci have roughly circular outlines. Incomplete septa in varying stages of maturity indent the outline of the streptococci slightly. Complete cross septa are associated with more marked constriction of the outer surfaces of the divided cells so that figure-of-eight diplococcal forms are present. At this stage of separation, the individual sister cells have overall round contours. The cells are joined by a completed intercellular septum the length of which is shorter than the *maximal* diameter of each cell. Division and maturation progresses until the point of contact between the outer surfaces of completely spherical sister cells is small and does not flatten the outline of either cell (arrows, Fig. 2). The cell wall of each streptococcus has a smooth exterior and is composed of two laminae, as shown in the inset of Fig. 1.

Streptococci in opaque colonies of strain S 43 (Fig. 2) are markedly different in their arrangement as compared to that of blue colonies of streptococci from the same strain. The majority of cells form chains in which the individual "cocci" have distorted, flattened appearances. The overall width of each cell in these chains is greater than the diameter of individual cells from blue colonies. The opaque colony streptococci are joined by prominent complete intercellular septa that are nearly as long as the maximal width (or diameter) of the divided cells. Occasional cells along the chains have rounded contours. These cells are the points at which the streptococcal chain bends and continues as another series of flattened, joined streptococci.

No differences in the cell wall architecture of streptococci from blue and opaque colonies have been observed. The two laminae and the overall thickness of the cell wall appear identical in streptococci from both colony forms (*compare* the inset of Fig. 1 with the inset of Fig. 2). The external surfaces of these cells in both opaque and blue colonies are smooth and bare of fimbriae. This suggests that M protein is absent (J. Swanson, K. Hsu, and E. Gotschlich, *unpublished data*). Microprecipitin assays that were carried out on both types of colony forms were negative for M protein.

Blue and opaque colonies of strain S 23. The morphological differences between blue and opaque colonies of strain S 23 are similar to, but less marked than, those shown for strain S 43. The chains from S 23 opaque colonies are composed of cells joined by long complete intercellular septa. The streptococci from these opaque colonies (Fig. 4) are less oval or circular and are somewhat flattened as compared to the round streptococci in blue colonies (Fig. 3). The walls of streptococci from blue colonies (inset, Fig. 3) are identical in thickness and lamination to those of streptococci in opaque colonies (inset, Fig. 4). Both colony types contain cells whose walls are covered by hair-like fimbriae (Fig. 3 and 4) which suggest the presence of M antigen (Swanson, Hsu, Gotschlich). Microprecipitin assays done on strain S 23 demonstrated that type 14 M protein is present both on the blue and on the opaque colony forms.

Nucleoid regions in opaque variant streptococci. Opaque variant streptococci often seem to contain several nucleoid regions (Fig. 5). One gains the impression that not only is the nucleoid material multilocular in location, but that it is increased in amount as compared to the nucleoid of blue colony cells. Observations on serial sections have shown that the nucleoid material of opaque colony cells has an arborized rather than a multilocular distribution. The appearance of the nucleoid most frequently observed is due to the limited portion of the total arborized nucleoid region that is present in a single thin section.

The arborized nucleoid region of opaque variant cells is sometimes seen extending into the immediate vicinity of the cytoplasmic membrane that is located beneath the intercellular septum (arrows, Fig. 5 and 6a). Closer study of this region has revealed that there is clearly direct contact between the nucleoid and the inner aspect of the cytoplasmic membrane. In some instances, the direct contact, suggested by low magnification micrographs, is not borne out at higher magnification (Fig. 6b, left side). Many examples have been found, however, in which the direct contact between nucleoid material and cytoplasmic membrane is unequivocal (Fig. 6b, right side, and Fig. 7–10). In cells which have not been treated with uranyl acetate prior to alcohol dehydration, the condensed, intensely opaque, "coagulated" appearing nucleoid is seen to impinge directly on the inner electron-dense lamina of the cytoplasmic membrane (Fig. 7 and 8). The extent of contact may be quite large (Fig. 7). In other instances (Fig. 6) a single cell has several points of nucleoid contact (or near contact) on the cytoplasmic membrane of one of its intercellular septa. Micrographs taken on opaque variant streptococci which have been stained en bloc with uranyl acetate exhibit contact of the fibrillar nucleoid with the cytoplasmic membrane (Fig. 9 and 10). We have been unable, however, to resolve the precise morphology of attachment between nucleoid and membrane.

Examples of nucleoid-cytoplasmic membrane contact are most readily observed in strain S 43 opaque

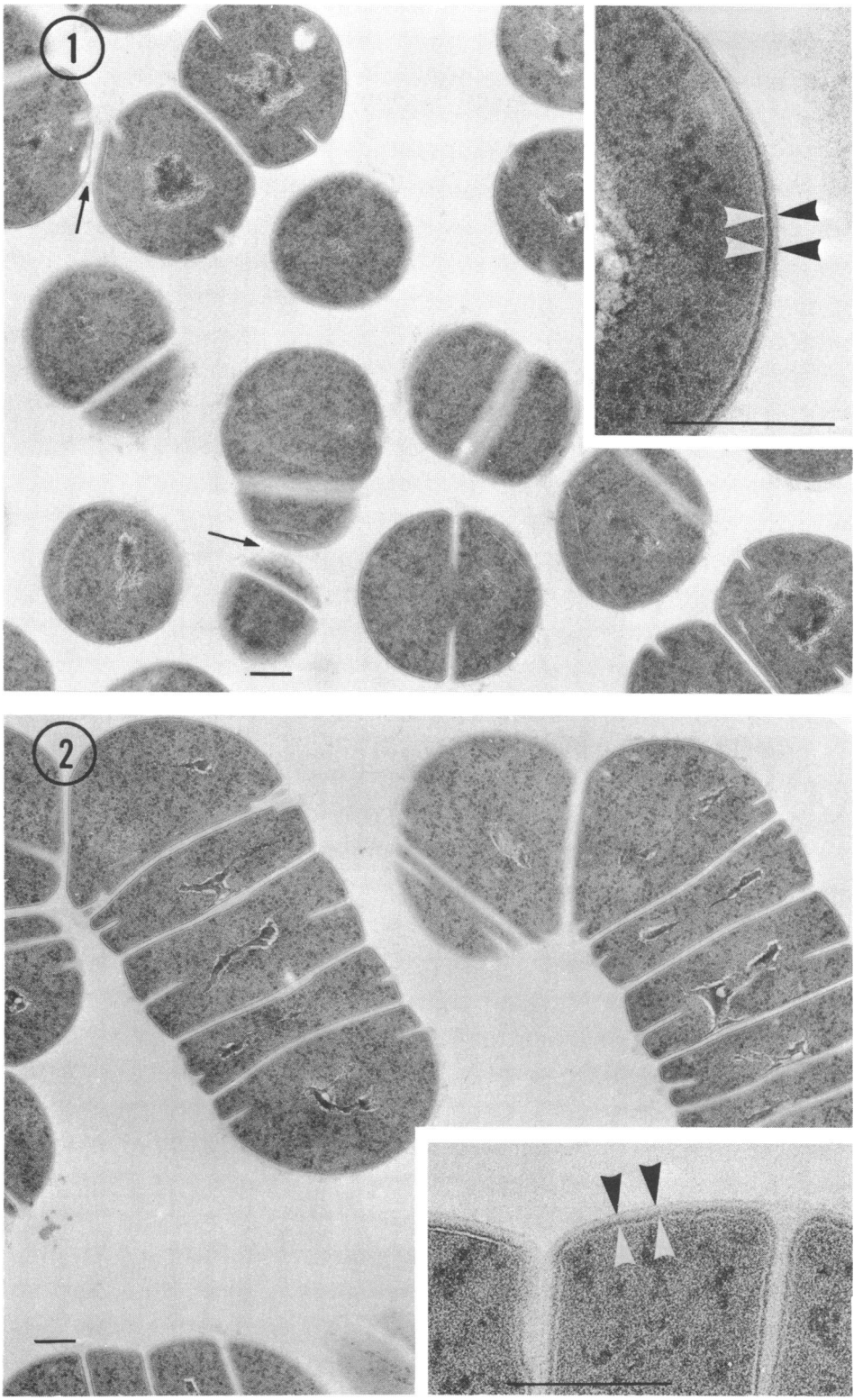
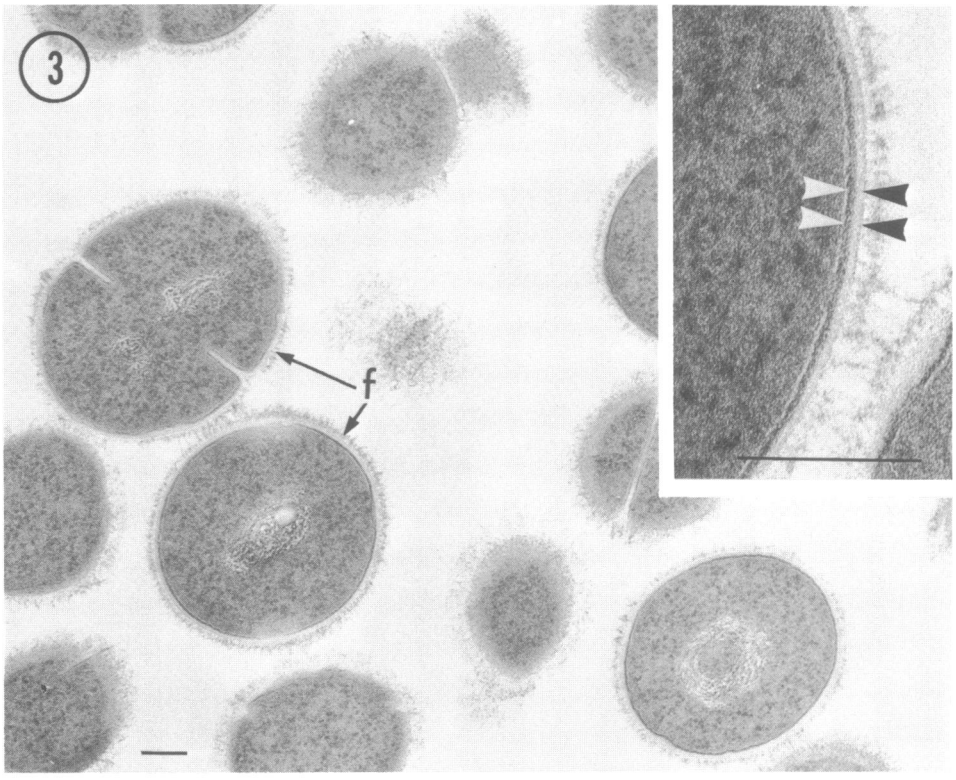


FIG. 1. *Strain S 43 streptococci in blue colony. Cells appear singly or as diplococcal forms. The spherical cells along a chain may contact one another along a short segment of their walls (arrows) that hardly indent the round outline of the adjoined cells. The thickness (between white and black arrows) and the almination of cell walls of blue colony streptocci is clearly seen in the inset. Marker on all figures represents 200 mn.*

FIG. 2. *Strain 43 streptococci in opaque colony. The cells form long chains and are joined together by exaggerated, long intercellular septa. The appearance of the nucleoid is due to lack of uranyl acetate treatment prior to dehydration. It can be observed in the inset that the thickness and lamination of the cell wall of opaque variant streptococci are identical to those in blue colony streptococci (as shown in the inset of Fig. 1).*



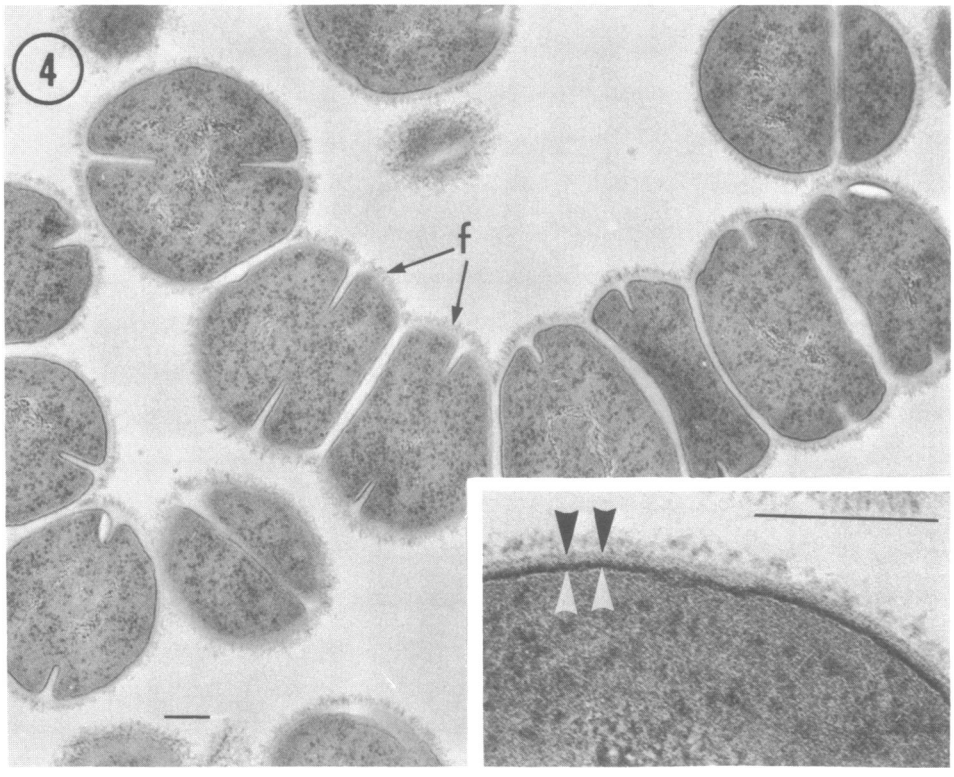


FIG. 3. *Strain S 23 streptococci in blue colony fimbriae (f) cover the exterior of cells that appear as single or diplococcal forms. Detail of the cell wall is shown in the inset.*

FIG. 4. *Strain S 23 opaque colony variant streptococci. Exaggerated intercellular septa and elongated chains are present in these streptococci whose surfaces are coated by fimbriae. Detail of the cell wall (inset) appears identical with that of cell wall from blue colony streptococci.*

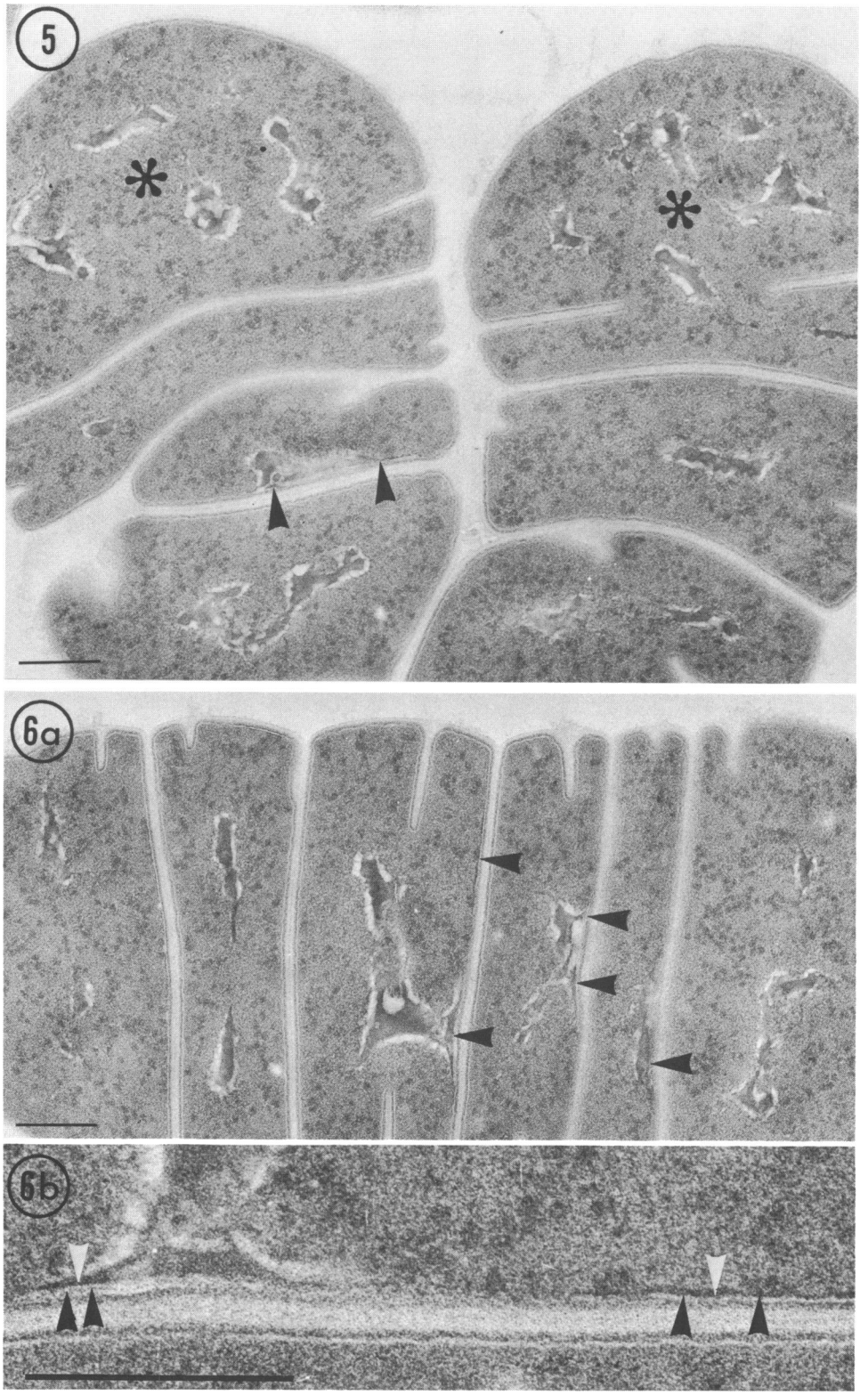


FIG. 5 and 6. *Opaque colony variant of strain S 43 streptococci. The multilocular appearance of nucleoids (*) and apparent associations of nucleoid and cytoplasmic membrane (arrows) are seen. (6b) Higher magnification of the cells in Fig. 6a are seen in Fig. 6b. Two areas of close approximation between nucleoid and cytoplasmic membrane are present. In one area (left side), the outermost limit of the nucleoid (white arrow) does not contact the innermost electron-dense lamina of the cytoplasmic membrane (black arrows). In the other area (right side), there is direct contact between the nucleoid and the cytoplasmic membrane.*

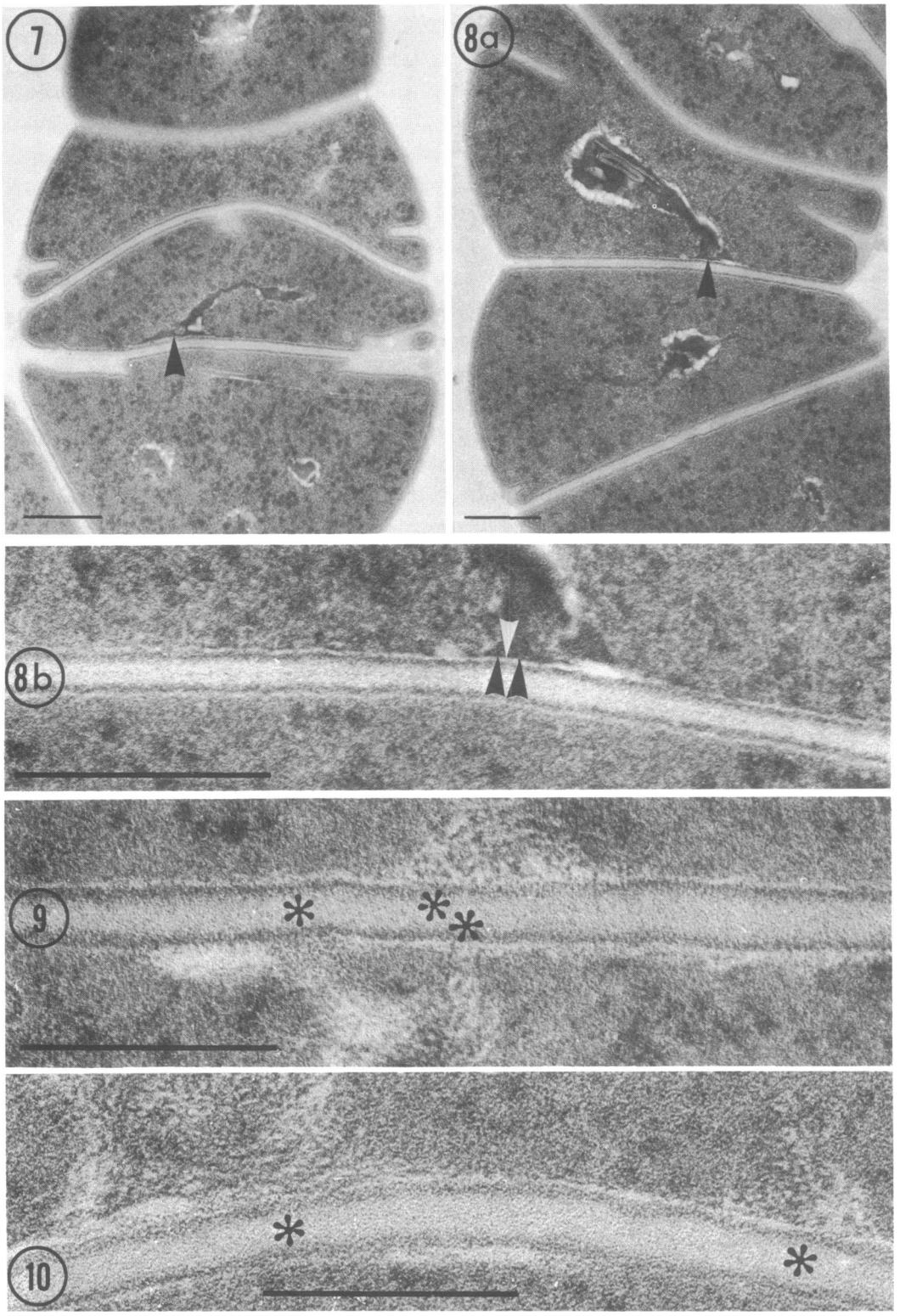


FIG. 7 and 8. *Strain S 43 streptococci in opaque colonies. Direct contact between the nucleoid and cytoplasmic membrane are present (arrows). Higher magnification (Fig. 8b) of cells shown in Fig. 8a reveals direct impingement of the outermost portion of the nucleoid (white arrow) on the innermost lamina of the cytoplasmic membrane (black arrows).*

FIG. 9 and 10. *Strain S 43 streptococci from opaque colonies. Specimens were treated with uranyl acetate prior to dehydration as was also done in specimens of Fig. 1, 3, and 4. Fibrillar nucleoid material impinges on cytoplasmic membranes at several points (*). Two foci of contact are present in the bottom cell of Fig. 9 and in the top cell in Fig. 10.*

variants, and the contacts are most numerous in cells which also exhibit the most marked exaggeration in chaining. The nucleoid-cytoplasmic membrane contact was observed rarely in the strain S 23 opaque variant colonies. Foci of nucleoid-cytoplasmic membrane contact were not seen in cells of blue colonies in either strain S 43 or strain S 23.

DISCUSSION

The results of this electron microscopic study support the concept that opaque variants of group A streptococci owe their colonial form to the presence of exaggerated intercellular bridges that lead to persistent chaining of progeny cells during their growth on agar. These intercellular septa represent the major portion of the circumference of each cell which is thereby firmly linked to adjacent cells. The cell walls of opaque variant streptococci are not unusual in morphology, except for the extensive contribution they make to the intercellular septa. Both the thickness and the lamination of cell walls are identical in streptococci of the same strain whether they are found in opaque or blue colonies. This cell wall similarity is present whether M antigen is present or absent from the organisms that form the two types of colonies. These findings support previous studies (6) which revealed no differences in the major chemical and antigenic constituents of the cell wall between paired blue and opaque variants that could account for the colony variations.

Opaque variant streptococci exhibit extensive, direct contact between their nucleoids and those portions of the cytoplasmic membrane that lie beneath the exaggerated intercellular septa. This arrangement is somewhat unusual in gram-positive bacteria in which contact between nucleoid and cytoplasmic membrane is usually indirectly mediated through mesosomes (7). Suboptimal conditions for growth (e.g., incubation at 0 C under anaerobic conditions) have been reported to lead to mesosomal destruction and subsequent direct contact between the nucleoid and the cytoplasmic membrane of *Bacillus subtilis* (7). Those observations are not similar to ours in that the direct nucleoid-cytoplasmic membrane contact of opaque streptococcal variants is found under conditions that support a reasonable rate of growth in the culture.

Connection between nucleoid material and cytoplasmic membranes of bacteria has been hypothesized by genetic studies (3) and supported, usually, by finding communication of nucleoid with mesosomes and continuity of mesosomes with the cytoplasmic membrane. Our findings further support the hypothesis of such a connection. The concept of nucleoid-cytoplasmic membrane associations has been concerned primarily with separation of daughter chromosomes following their replication. The separation is thought to proceed because of synthesis of new cytoplasmic membrane at the point where nucleoid (or chromosome) attaches to the membrane. This portion of the hypothesis may have a bearing on the opaque colony variants of streptococci which we have studied. Although the streptococci from opaque colonies exhibit cell wall and cytoplasmic membrane formation that is quantitatively unusual, we are unable to correlate definitively this phenomenon with the unusually extensive contact between nucleoid and cytoplasmic membrane that is present. It seems plausible that, for reasons which are not understood, chromosome replication (nucleoid duplication) is followed by persistent attachment of incompletely divided daughter chromosomes to the cytoplasmic membrane. This would account for the finding of more than one attachment or contact site of nucleoid to membrane that was observed in several cells. This aberration might then lead to absence of the usual attainment of a spherical shape of the cell wall because the nucleoid does not assume its usual central position in the cell. However, the influence of the position of nuclear material to "maturation" or "differentiation" of the cell wall and cytoplasmic membrane is poorly understood. Furthermore, we have little knowledge as to whether opaque variants exist because of defects in chromosome replication, chromosome separation, or some primary alteration in cell wall synthesis.

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