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Ultrastructural Study of Crossbands Occurring in the Stalks of *Caulobacter crescentus*

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An ultrastructural study of crossbands isolated from *Caulobacter crescentus* Sk1 418 stalks permitted a view of these structures in a plane other than the perpendicular view normally presented in the electron microscope. These crossbands were composed of concentrically arranged, alternating light and dark bands when stained with phosphotungstic acid. Evidence supports the hypothesis that crossbands appear to compose a structural barrier extending the width of the stalk and may function to support unit membrane extending the length of the stalk.

Stalks of *Caulobacter* sp. contain structures known as crossbands which divide the stalks at intervals. Investigations of these and other similar structures have indicated that crossbands are annular structures contained within the outer cell wall material of the stalk (4), rigid and electron dense when stained with 1% OsO_4, (3), and composed partly of murein (7). Pate and Ordal (3) have suggested that such structures are hollow and occur in the space between the outer envelope membrane and the inner unit membrane. This suggestion is due to the observation that membrane material appears to be continuous the length of the stalk through the crossband (3, 5).

Recent evidence from thin sections of stalk material (7) has resulted in reevaluation of the above interpretation, and it has been suggested that the crossband provides a rigid barrier across the stalk. It may act as a septum to prevent disruption of components within the stalk.

Our present investigation permits a view of these crossbands in a plane ordinarily occurring perpendicular to the length of the stalk. Ultrastructural study reveals that the crossband is composed of concentrically occurring layers of material formed in continuous rings. This study supports the contention that these structures are not hollow and may, in part, function as barriers across the stalk and, in addition, may function in membrane support.

MATERIALS AND METHODS

The long stalk mutant of *C. crescentus* strain Skl 418 (6) was used for this study. This mutant forms stalks which are considerably longer (10–20 μm) than the wild type.

The cultures were grown in broth medium containing 0.2% peptone, 0.1% yeast extract, and 1% Hutner's Base as modified by Cohen-Bazire et al. (1).

Bacterial cultures were grown initially in 20 ml of the broth medium contained in a 125-ml Erlenmeyer flask and incubated at 30 C on a shaker water bath. After 24 h the content of the 125-ml flask was introduced aseptically into a 1-liter Erlenmeyer flask containing 500 ml of the broth medium. This flask was then incubated on a rotary shaker at 27 C for approximately 36 h, i.e., until early stationary phase of growth.

Approximately 400 ml of the broth containing early stationary-phase Skl 418 cells was placed in a Waring blender (model 1120) for 3 min at full speed. This procedure effectively sheared the stalk from the *Caulobacter* cell.

Differential centrifugation was used to concentrate and purify stalk material. Cellular debris and any whole cells remaining after blending were sedimented at 2,500 $\times$ *g* for 15 min, leaving cellular stalks in the supernatant fluid. Supernatant fluid was then centrifuged at 10,000 $\times$ *g* for 20 min to concentrate stalk material. The sedimented stalks were suspended in 0.1 M Tris(hydroxymethyl)aminomethane-hydrochloride (Sigma), pH 8.0, at one-tenth the original volume, the sediments were pooled, and the two centrifugation procedures were repeated twice more.

Concentrated stalks (5 ml) were placed in a 50-ml centrifuge tube and sedimented at 10,000 $\times$ *g*. The supernatant fraction was decanted, and a 5 ml-volume of 2% Triton X-100 (Calbiochem) in 10 mM *N*-2-hydroxyethyl piperazine-*N*-2-ethane sulfonic acid (Nutritional Biochemical Corp.) plus 5 mM sodium ethylenediaminetetraacetate was added. This mixture was allowed to react at room temperature for 10 min, during which time it was mixed thoroughly in a Vari-Whirl mixer. After incubation, a 5-ml volume of 0.1 M $MgSO_4$ was added. The partially digested stalks were then divided into 5-ml samples and sonically treated for 30 s to 10 min with a Branson Sonifier (model S75). The treated stalks were then

centrifuged at 35,000 × *g* for 1 h. No true residue was evident although a flocculent streak appeared on the side of the centrifuge tube, approximately one half the distance from supernatant fluid meniscus to the bottom of the tube. The supernatant fraction was decanted, and the flocculent material was saved for electron microscopy.

A few drops of water were added to each centrifuge tube to resuspend the partially pelleted material. Portions of each suspension were deposited on 200-mesh electron microscope grids which had been previously coated with a film of parlodion and carbon stabilized. Grids with thin carbon films were also used. The deposited material was then stained with 1.5% phosphotungstic acid (PTA; pH 7.2) or 1% uranyl acetate (pH 4.6). Grids were examined in a Philips 300 electron microscope operated at 60 kV. Density traces were obtained from positive electron micrographs using a Photovolt model 425 densitometer.

RESULTS

The usual appearance of crossbands in intact stalks of *C. crescentus*, prepared with the negative contrast method, is shown in Fig. 1. The final sedimented material treated with Triton X and sonically treated for 1 min appeared amorphous when viewed with the electron microscope. A few undisintegrated stalks were present. Close examination of the amorphous material revealed evidence of stalk wall material empty of crossbands. Circularly arranged material was observed frequently in these preparations. These circles, 0.10 to 0.15 μm in diameter, were approximately the same size as crossbands and gave the appearance of target "bull's-eyes." Concentrically layered material appeared from the center of these organelles outward. The presence of these organelles in partially disrupted stalk material can be seen in Fig. 2, 3, and 4.

Released crossbands appear as 9 or 10 alternating electron-dense and less-dense circles arranged concentrically (Fig. 2, 3, and 5). This appearance coincides well with a vertical or edge-on view of crossband material in a partially disintegrated stalk (Fig. 3, arrow). The electron-dense rings of the crossband represent the penetration of PTA stain. These areas then show a relative lack of substructure as compared to the electron-transparent circles of the crossband. The electron-dense regions could possibly be areas for attachment of membrane material.

Crossbands stained with uranyl acetate, viewed from the edge (Fig. 4a, b), reveal little if any distinctive substructure, in contrast to the appearance of the vertical PTA-stained crossband (Fig. 3, arrow). Released crossbands, stained with uranyl acetate (Fig. 4c, d), possess

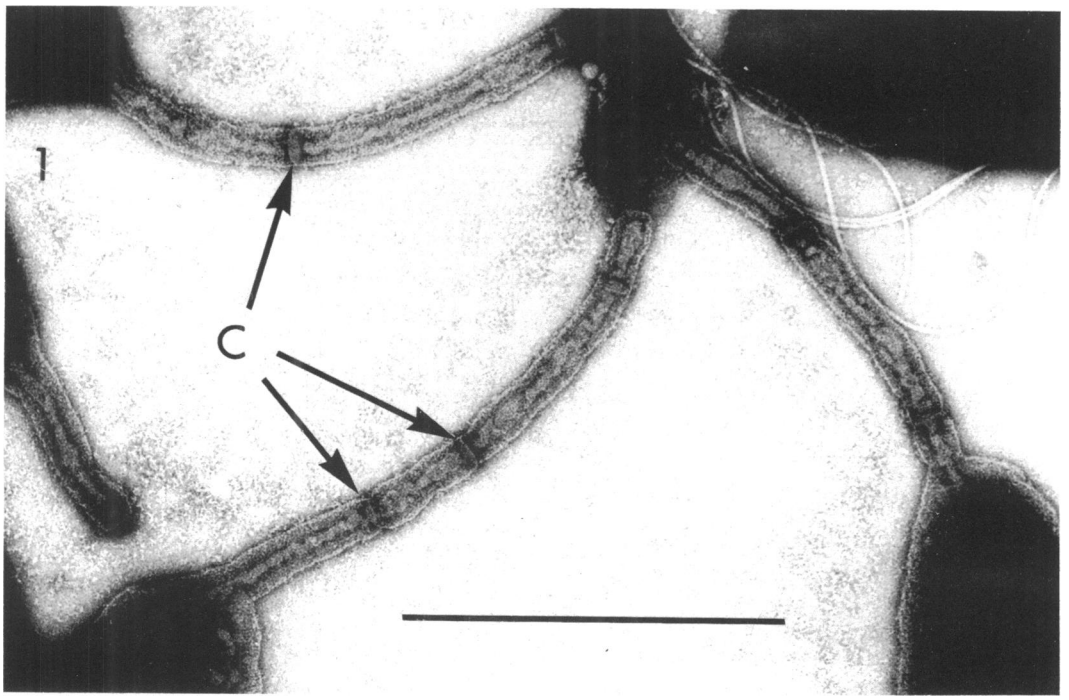

FIG. 1. *Crossbands (C) in intact stalks of C. crescentus Skl 418 which have been negatively stained with phosphotungstic acid. The marker represents 1 μm.*

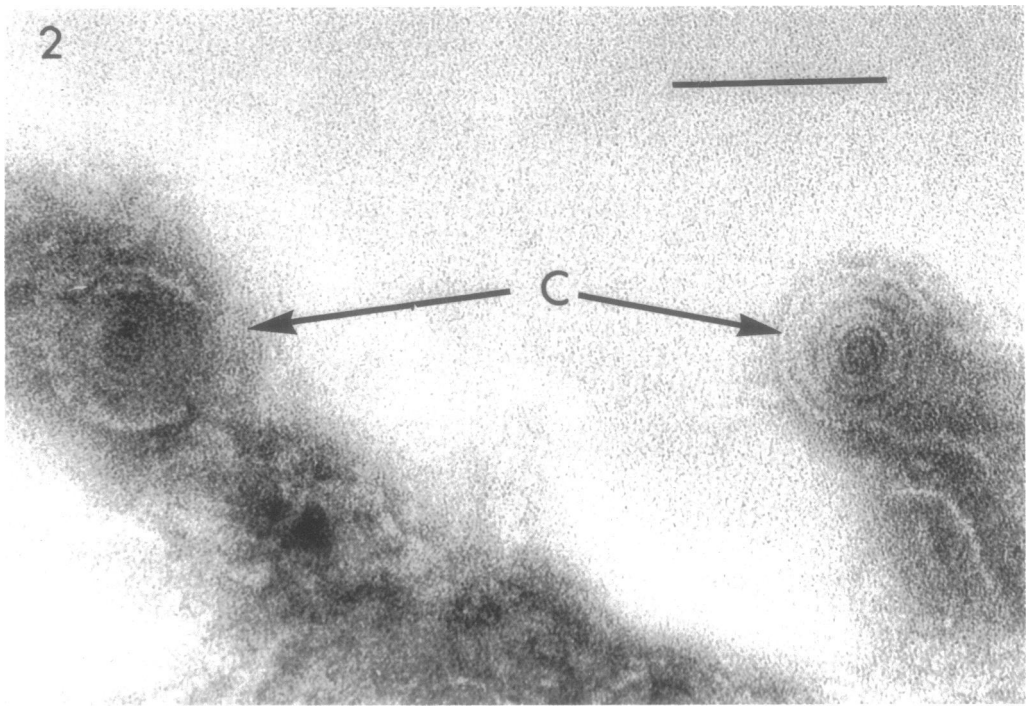

FIG. 2. *Crossbands appearing flat to the plane of view permitting resolution of concentric circles composing each organelle. This and all subsequent markers represent 0.1 μm.*

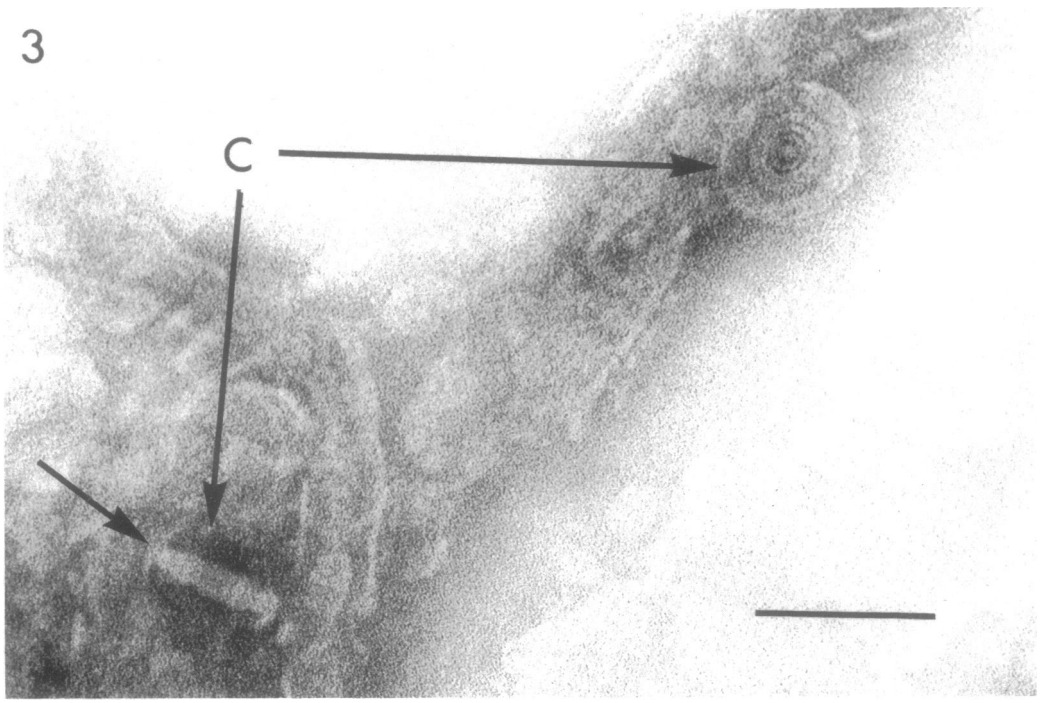

FIG. 3. *A PTA-stained crossband viewed in a flattened plane (upper right) can be compared with another crossband as viewed edge-on (arrow).*

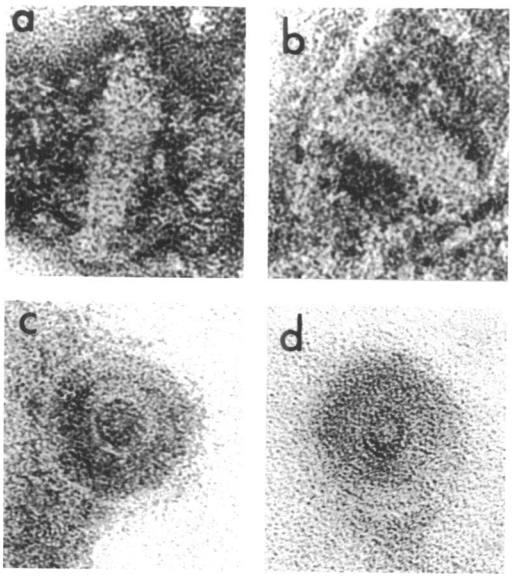

FIG. 4. *(a, b) Edge-on view of uranyl acetate-stained crossbands. (c, d) View of uranyl acetate-stained crossbands in a flattened plane.*

the concentric circle arrangement also found in PTA stains.

DISCUSSION

The effect of Triton-X treatment on membranous material in gram-negative bacteria (2, 8) has led to the conclusion that cell membrane is dissociated, while little damage occurs to cell wall material. This detergent was selected as the method of choice to dissolve membrane material from within isolated *Caulobacter* stalk fragments. The remaining stalk structure was postulated to be sonically fragile, and this proved to be the case.

Crossbands which have been separated from their stalk (Fig. 2, 3, and 4) are interpreted to be composed of concentric layers of material. Schmidt (7) has shown that the principal constituent of crossbands is murein. The concentrically circular structures found here have not been observed in lysozyme-treated preparations. Therefore, we believe that these distinctive structures are the crossbands and that their rings are composed of alternating thick and

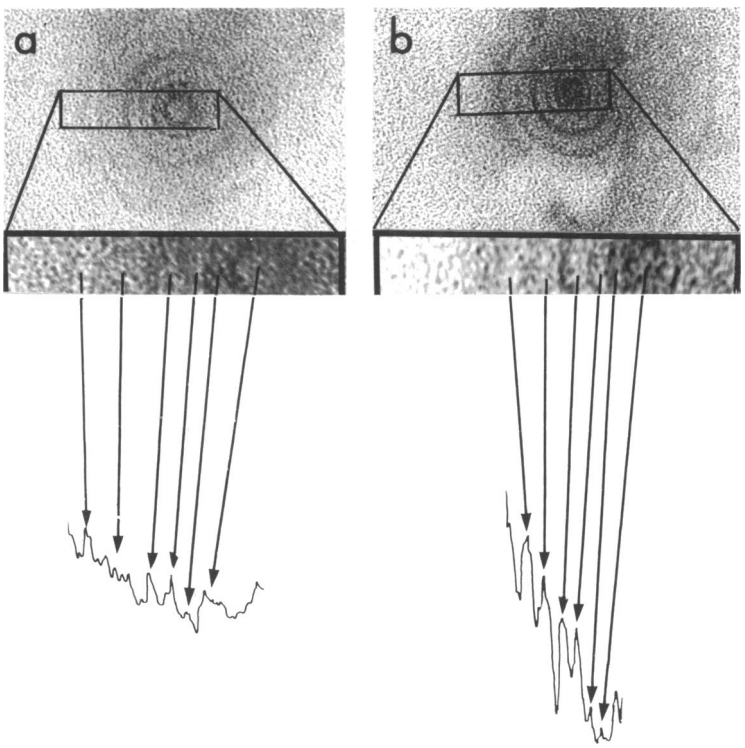

FIG. 5. *(a) Densitometer trace of flattened crossband correlating the relatively unstained center with the peak shown by the right arrow. The first two arrows at the left correspond to the outer ring or band 1 of the model (Fig. 6). (b) Densitometer trace of crossband with darkly staining center. Inset magnified 2× original above.*

diffuse bands of murein. The dark rings may represent a site for attachment of annular membrane structures (removed by the Triton-X treatment) and provide a virtual continuance of membranes throughout the length of the intact stalk.

In Fig. 3, a readily recognizble crossband on edge (arrow) can be compared with the concentric circular structure, interpreted to be a flattened crossband . Of importance is the appearance of striations which mediate the structure in a regularly arranged pattern. These striations compare exactly in size and arrangement with the rings of flattened crossbands. An exaggerated structure has been drawn (Fig. 6) to demonstrate the probable correspondence of these concentric rings and striations.

A peculiar staining characteristic of the flattened crossbands contrasted with PTA is the variability of electron density in their center (band 5 in the model, Fig. 6). In some (Fig. 3, 5a) the centers are quite electron transparent; in others (Fig. 2, 5b) the centers are densely stained. In a negative-contrast preparation, one could expect an area of increased electron density to represent an accumulation of PTA, and a corresponding lack of structural material. A previous suggestion (3) that the crossband is a hollow annular structure would predict that the centers should be densely stained. However, many of the crossbands observed here have electron-transparent centers, and this suggests that these crossbands occur as solid barriers without hollow centers. The densities of radial sections of variably stained crossbands have been evaluated (Fig. 5). The peaks of the traces shown correspond to the more transparent regions of the crossbands. A crossband with a relatively electron-transparent center (Fig. 5a) shows a substantial transmission peak at the right side of the trace, corresponding to that center. The outer bands of Fig. 5a (corresponding to bands 1 and 2 of Fig. 6) are diffuse and give rather indistinct traces. A crossband with a densely stained center (Fig. 5b) shows a minimal peak corresponding to the center, suggesting that even in those having densely stained centers, the center is not as dense as the dark ring which surrounds it.

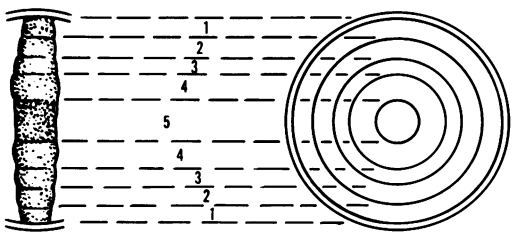

FIG. 6. *Model of crossband structure as based on correlation of bands with concentric circles noted in Fig. 3.*

An assignment of function for the crossbands is not so readily accomplished as the definition of their structure. Perhaps the crossband acts to support membranes arranged in annular structures and which traverse the stalk from proximal to distal end (3). Preliminary studies in this laboratory suggest crossbands may prove to have an integral relationship intimately associated with events taking place during cell division. If so, this would provide a unique method for determining the age and observing the natural history of certain stalked bacteria.

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