Invasive growth of *Actinobacillus actinomycetemcomitans* on solid medium (TSBV)

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When grown on agar, most *Actinobacillus actinomycetemcomitans* form circular and convex colonies with an internal star-shaped morphology. Such colonies adhere firmly to the agar, and when removed, a star-shaped imprint similar to that of the intact colony remains. This study was undertaken to determine the nature of this in vitro growth. Stereo-microscopy and scanning and transmission electron microscopy showed that the star-shaped imprint of *A. actinomycetemcomitans* colonies reflected pseudopod-like extensions of the bacterial colony which penetrated deep into the TSBV agar. The center of the colonies consisted primarily of ghost-like cells, while the dense border of each colony, including the pseudopods, expressed characteristics of vital cells. The latter were embedded in vast amounts of extracellular vesicles, and the outer aspect of the pseudopods was lined with a border of such vesicles. □ *Electron microscopy; gram-negative bacteria; scanning microscopy; stereo-microscopy*

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When wild strains of *Actinobacillus actinomycetemcomitans* are cultured on solid medium, most of them form small, rough or smooth, circular, and translucent colonies with an internal morphology described as 'star-shaped' (1) or 'crossed cigars' (2). These colonies adhere firmly to the agar, and when removed, an imprint similar to that of the intact colony remains (2). This study investigated the nature of these central star-like formations on TSBV agar (3) by microscopy.

Materials and methods

A. actinomycetemcomitans were recovered from patients with rapidly destructive periodontitis by culturing dispersed and diluted dental plaque samples on TSBV medium. *A. actinomycetemcomitans* colonies were recognized by colony morphology, Gram staining, and a positive catalase test. Diagnosis was confirmed by fermentation of selected carbohydrates (4) and DNA restriction endonuclease mapping (5). *A. actinomycetemcomitans* colonies expressing various forms of central star or crossed cigar-like, internal morphology were selected for the experiment.

Several TSBV agar cubes, approximately 5 mm^3, each containing a single *A. actinomycetemcomitans* colony, were cut from the agar plate. The cubes were fixed for 24 h in 2.5% glutaraldehyde buffered with Sorensen phosphate buffer, pH 7.4, at 4°C and then rinsed in buffer. The same fixation was used throughout this study, unless otherwise stated.

Stereo-microscopy

Fixed cubes were turned upside down and transectioned vertically through the star-shaped, central part of the colony. The cut surfaces of the agar blocks were then examined by stereo-microscopy (Stereomicroscope Mod. M 400, Wild Heerbrugg Ltd, Switzerland). Unfixed intact colonies were also examined in top view under the same stereo-microscope.

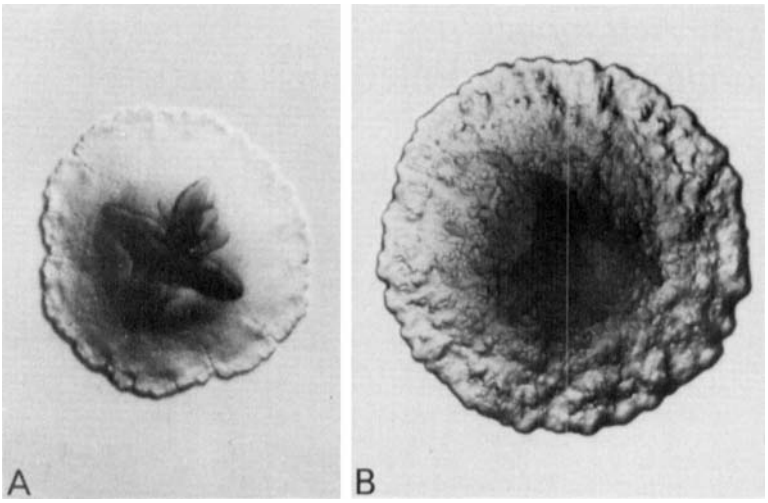

Fig. 1. Stereo-micrographs (top view) of a smooth *Actinobacillus actinomycetemcomitans* colony showing the typical internal, star-shaped morphology (A), and a rough colony showing randomly distributed circular or elipsoidal structures grown on TSBV medium (B).

Scanning electron microscopy

Fixed and transectioned blocks similar to those examined by stereo-microscope were dehydrated, critical-point-dried with carbon dioxide, and sputter-coated with 300 Å gold palladium alloy. The cut surfaces were then examined in a Philips SEM 515 (Eindhoven, The Netherlands).

Several intact TSBV agar cubes containing *A. actinomycetemcomitans* colonies were fixed and then boiled in a waterbath until the agar was completely melted. Fixed and thus released colonies were dehydrated, critical-point-dried, sputter-coated, and examined by scanning electron microscopy. Several other intact and fixed agar cubes were rinsed in buffer and dehydrated successively in 30%, 50%, 60%, 70%, 80%, 90%, and finally, in absolute alcohol. The cubes were, while still immersed in absolute alcohol, enclosed in gelatin capsules. The capsules were dropped into liquid nitrogen and thereafter cracked. The debris was then thawed in absolute alcohol and critical-point-dried. Selected specimens were subsequently sputter-coated and examined under the same scanning electron microscope.

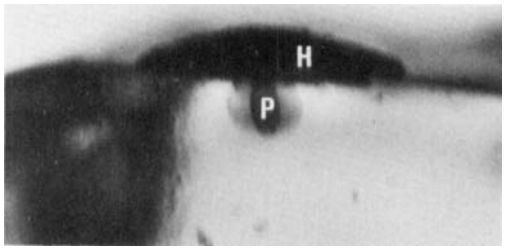

Fig. 2. Stereo-micrograph of a vertically transectioned agar cube containing one single centrally cut *Actinobacillus actinomycetemcomitans* colony, showing a mushroom- or jellyfish-like appearance, with a convex 'hat' (H) above the agar surface and 'pseudopods' (P) penetrating into the medium.

Transmission electron microscopy

Blocks were fixed for 24 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, subsequently rinsed for 10 min in 0.15 M phosphate buffer, pH 7.3, and postfixed in 1% osmium tetroxide at 4°C for 2 h. After fixation the blocks were rapidly dehydrated in graded series of acetone and embedded in Vestopal-W. Ultrathin sections were cut on an LKB Ultratome III. The sections were treated with uranyl acetate for 20 min and lead citrate for 3 min (6). Sections were examined in a Philips 400T (Eindhoven, The Netherlands) electron microscope.

Results and discussion

A. actinomycetemcomitans colonies, characterized macroscopically by an internal star (1), crossed cigars (2), and/or by randomly

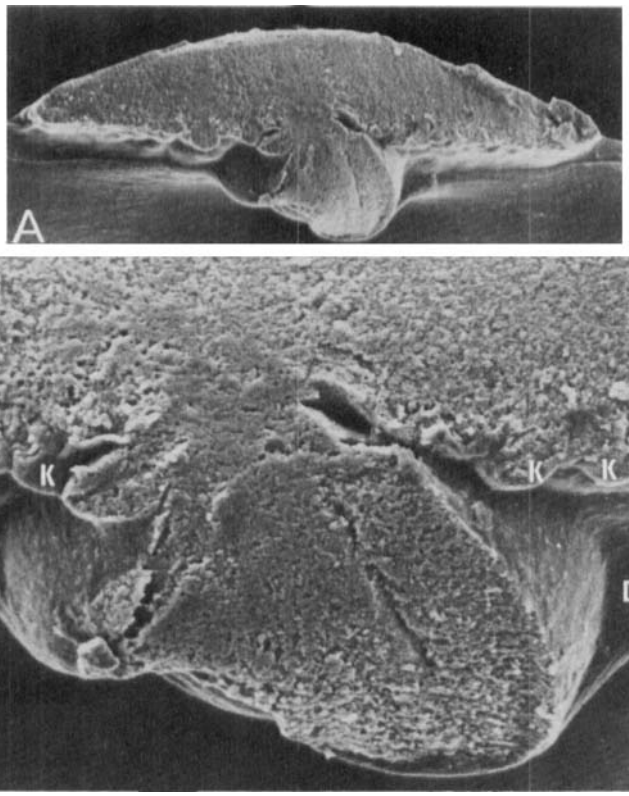

Fig. 3A. Scanning electron micrograph of a vertically sectioned *Actinobacillus actinomycetemcomitans* colony, showing protrusions ('pseudopods') penetrating into the agar. (Magnification, ×126.) 3B. Higher magnification of the pseudopod area, showing small knobs (K) penetrating the medium and a narrow zone of apparently dissolved agar (DAG) adjacent to the colony. (Magnification, ×3880.)

distributed ellipsoidal structures, penetrate deep into Slots medium on which they grow (Fig. 1A, B). Cross-sectioned colonies had a mushroom or jellyfish-like appearance, with a convex 'hat' on the surface and 'pseudopods' and small knobs penetrating

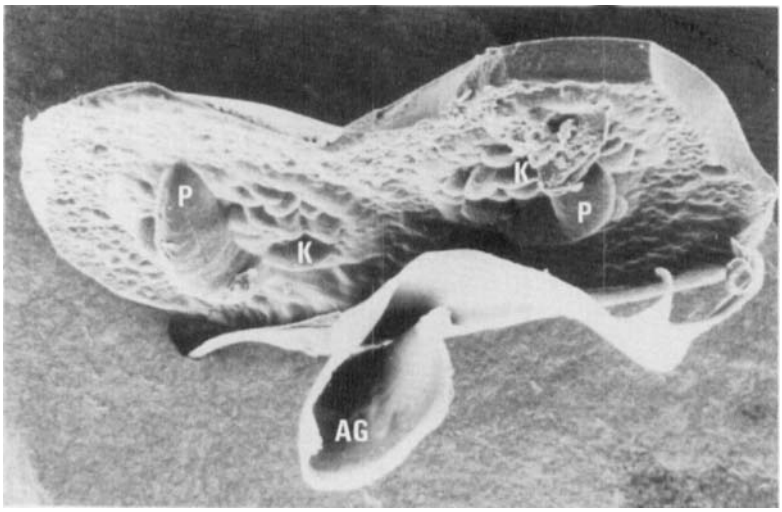

Fig. 4. Scanning electron micrograph of the 'bottom' (adjacent to agar) of the *Actinobacillus actinomycetemcomitans* colony exposed by freeze-fracturing, showing both major protrusions (P) and small knobs (K) originally penetrating the agar (AG). (Magnification, ×130.)

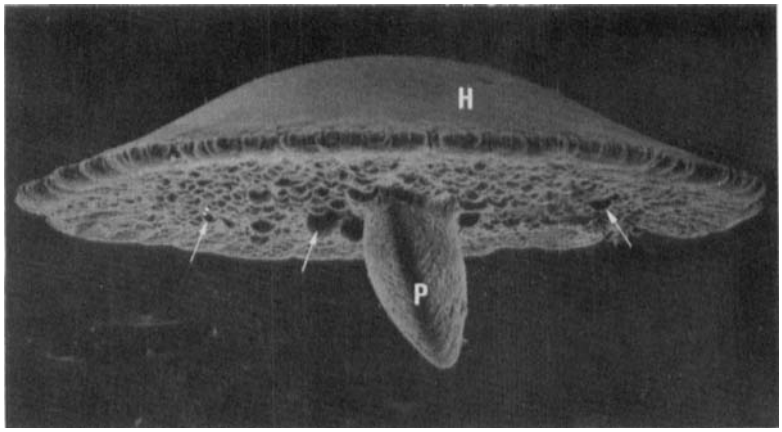

Fig. 5. De-agarized colony showing the smooth hat (H), the major protrusion (P), and small knobs (arrows) originally embedded in the agar. (Magnification, ×110.)

into the agar (Fig. 2). These pseudopods are most likely identical with the structures seen when the transilluminated colonies are viewed from the top through a stereo-microscope.

Scanning electron microscopy of fixed and sectioned colonies confirmed the image of protrusions into the agar (Fig. 3A, B). A narrow cleft of apparently dissolved agar was recognized next to the colony. Moreover, both freeze-fractured (Fig. 4) and de-agarized colonies (Fig. 5) showed major protrusions and small knobs that penetrated into the agar.

Transmission electron microscopy showed that the center of the colonies, including the center of the major protrusions, consisted almost entirely of 'ghost' cells. A denser border, lining the colony on all aspects, contained seemingly intact cells (Fig. 6). Vast amounts of extracellular vesicles were seen among the border cells. Such vesicles were abundant in the interphase between the agar and the pseudopods at all aspects. We sug-

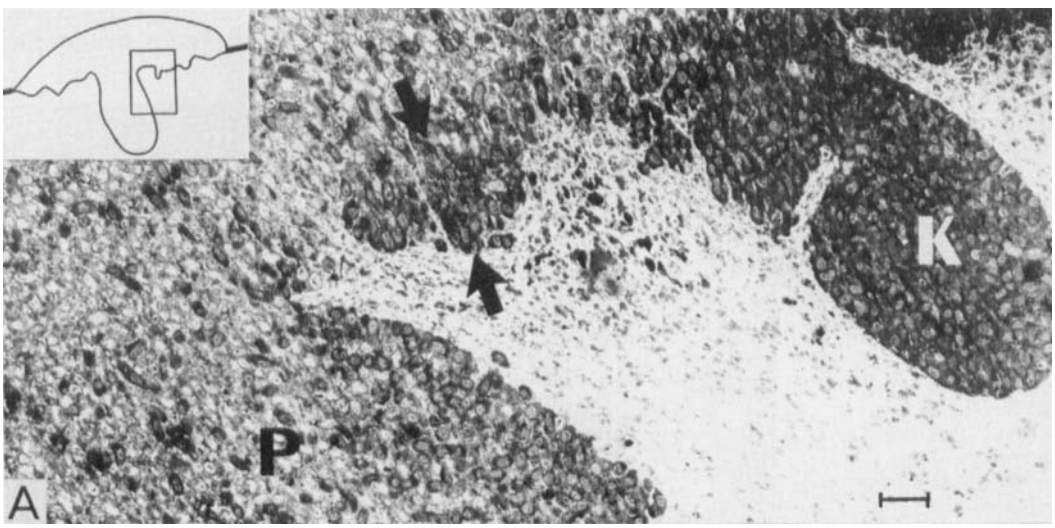

Fig. 6A. Framed area: Transmission electron micrograph of the vertically sectioned *Aa* colony, showing mainly lysed or empty cells, although the border area (arrow) seems to consist of vital cells. A major protrusion (P) and a small knob (K) are indicated (length of bar, 1 μm).

gest that it is these pseudopods and the small knobs that mainly cause *A. actinomycetemcomitans* colonies to stick so firmly to agar.

It was recently demonstrated that wild strains of *A. actinomycetemcomitans* carry a considerable amount of fimbriae (7, 8), which may well contribute further to the ability of the colonies to adhere to agar. Both the pseudopods and the fimbriae disappear after several generations in the laboratory. It is not known whether this is due to changes in phenotypic character or to genetic changes.

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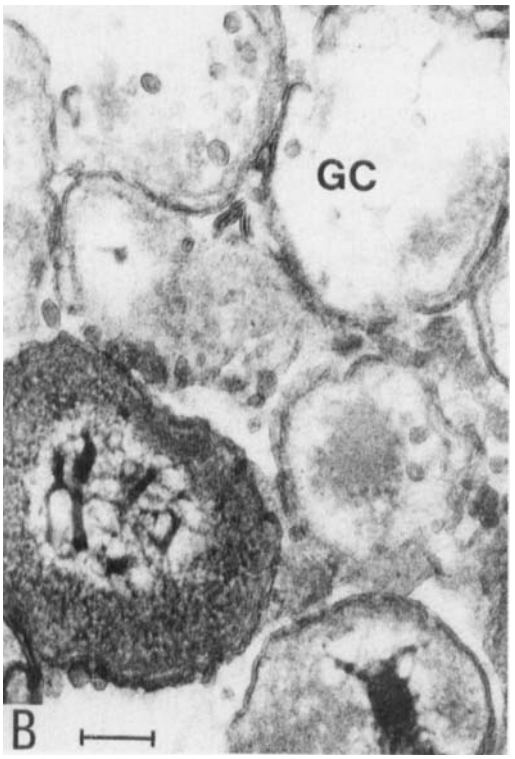
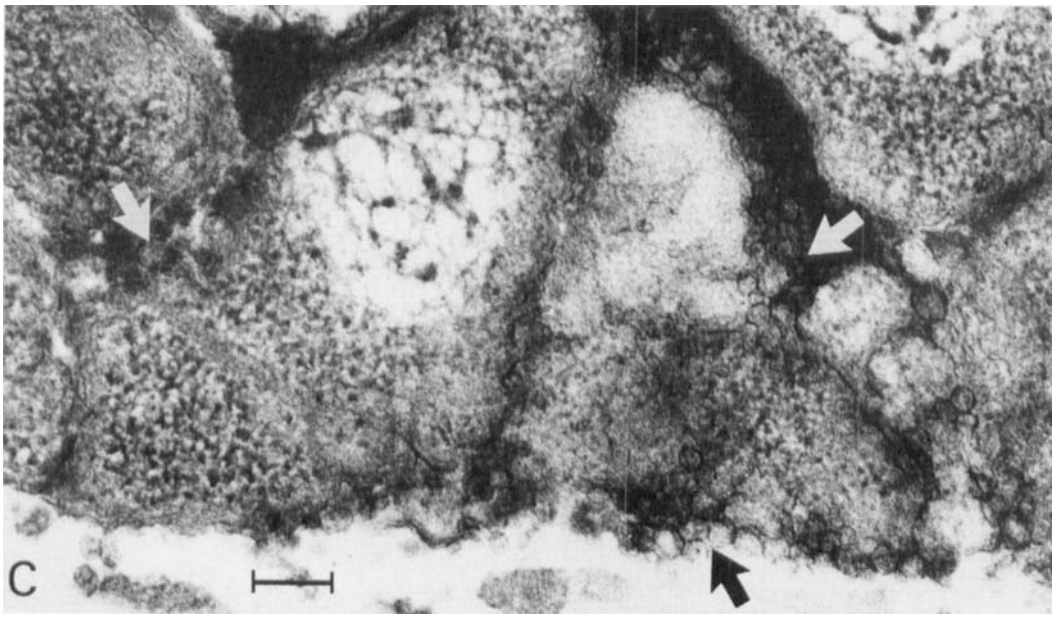


Fig. 6B. Higher magnification of the central 'ghost' cells (GC) of 6A (length of bar, 0.1 μm). 6C. Higher magnification of the border cells of 6A, which were surrounded by numerous extracellular vesicles (arrows) (length of bar, 0.1 μm).



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