Ultrastructure of the surface film of bacterial colonies

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The structure of the surface of colonies of various Gram-negative and Gram-positive bacteria was examined by transmission electron microscopy. The results indicate that bacterial colonies in the course of their development produce a film which becomes thicker with increased duration of growth. The basic part of the film is an elementary membrane, which is a stable structure with a large surface area. The inner and outer surfaces of the film membrane are covered by amorphous layers. These layers are thicker in the surface film of Gram-negative bacterial colonies than in those of Gram-positive bacteria. Membrane vesicles from the bacterial colonies take part in the formation of the surface film. The presence of the film on the surface of the colonies of different bacteria suggests that this structure may play an important role.

Introduction

The ultrastructure of bacterial colonies has been extensively investigated in recent years (Shapiro & Higgins, 1988; Tetz *et al.*, 1990; Todd *et al.*, 1984; Vysotsky *et al.*, 1985). As a result of these studies, new data on the process of colony development and on bacterial interactions within colonies have been obtained. It has been shown that the bacterial cells in colonies are linked by different types of intercellular contacts. These contacts contribute to the formation of a three-dimensional system (Tetz *et al.*, 1990*a,b*, 1991; Todd *et al.*, 1984). Elements of cell specialization and differentiation in bacterial colonies have also been demonstrated (Tetz *et al.*, 1990*b*). Bacterial cells exhibit different patterns of genetic expression in different colony zones (Shapiro & Higgins, 1988). As whole integral systems, bacterial colonies separate themselves from the external environment by a surface film. Recently, we have shown that these surface films have a complex organization and consist of amorphous and membranous layers (Tetz *et al.*, 1990*b*, 1991). The structure of surface colonial films is of great interest because this is a rare example of a single common membrane covering many cells.

The aim of this work was to examine the ultrastructural organization and formation of colonial surface films of different Gram-negative and Gram-positive bacteria.

Methods

Organisms. The standard *Escherichia coli* strains K12 and M17 were used. *Brevibacterium flavum* E531 (a lysine-producing strain) was obtained from the Russian Collection of Industrial Micro-organisms

(Moscow). *Shigella flexneri* 2a (strain VT100), *Salmonella typhimurium* (strain VT40) and *Staphylococcus aureus* (strain VT30) were isolated from patients in the hospitals of St Petersburg. These bacteria had typical morphological, staining, biochemical and serological characteristics of the corresponding species and serogroups. The media used in this study were as described previously (Tetz *et al.*, 1990*a,b*).

Ultrathin section preparation. Colonies were fixed *in situ* for 24 h at 4 °C in 2.5% (v/v) glutaraldehyde in 0.05 M cacodylate buffer pH 7.2. The fixing fluids were introduced at the bases of agar plates, underneath the colonies. The colonies were fixed by diffusion of the fixing fluid through the agar as well as by its vaporization. This method prevented damage to colonies and the appearance of artifacts. Fixed colonies were washed in the same buffer and then postfixed for 24 h in 1% (w/v) osmium tetroxide. Specimens were dehydrated in a graded ethanol series and embedded in Spurr medium (Spurr, 1969). Ultrathin sections were prepared using an LKB-8800 ultramicrotome and were stained as described by Reynolds (1963). The specimens were examined in a JEM-100C electron microscope at 80 kV.

Positive staining. Cells were removed from colonies by touching the colony surface with Formvar-coated grids and positively stained with 0.1% aqueous uranyl acetate (pH 7.0).

Results and Discussion

We have studied the material from more than 500 colonies of Gram-positive and Gram-negative bacteria at different stages of growth. One- or two-day-old colonies were the main objects of our investigations, because after 6–18 h of growth the surface film was rarely seen, and in colonies examined after 72–96 h, processes of destruction were enhanced. Although these alterations are more pronounced in the deeper parts of the colony, they can also affect the structural organization of the surface film.

In ultrathin sections of whole colonies, studied by transmission electron microscopy, the surface film of 1-day-old colonies of both Gram-positive and Gram-negative

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Fig. 1. Surface film (arrows) of 1-d-old colonies as seen in ultrathin sections. (a) *E. coli* K12 (bar 0.5 µm), (b) *B. flavum* (bar 0.1 µm).

bacteria was represented by a thin elementary membrane (Fig. 1). This membrane isolated the surfaces of colonies from the air. In sections of different parts of the colony (central, peripheral and intermediate), we found no bacterial cells outside the surface film. In the film covering 2-d-old colonies of Gram-positive bacteria, it was possible to distinguish three layers – the elementary membrane, and amorphous layers covering the inner and outer surfaces (Fig. 2b). The surface films of 2-d-old colonies of Gram-negative bacteria also had a more complicated organization than those after 1 day of growth (compare Figs 1a and 2a).

Using transmission electron microscopy, we observed two variants of surface film. In the first variant, the inner

Fig. 2. Surface film (arrows) of 2-d-old colonies as seen in ultrathin sections. (a) *E. coli* K12 (bar 0.5 µm), (b) *Staphylococcus aureus* VT30 (bar 0.1 µm).

and the outer surfaces of the film membrane were covered by an amorphous substance. Similar material was observed between the bacterial cells within the

Fig. 3. Surface film with one layer of the amorphous substance of 2-d-old colonies as seen in ultrathin sections. The arrows show the inner layer of the amorphous substance. (a) *E. coli* K12 (bar 0.5 μ m), (b) *Shigella flexneri* VT100 (bar 0.1 μ m).

colony (Fig. 2). The thickness of the amorphous substance of the surface film of Gram-negative bacteria was greater than that of the Gram-positive bacteria tested. In the second variant, only the inner part of the film membrane was covered by amorphous material, while the outer surface of the film appeared almost smooth (Fig. 3). It may be suggested that *in vivo*, both surfaces of the colony film are covered by amorphous material, which is partly removed from the outer surfaces during the preparation of the samples for electron microscopy. These data indicate that the membranous part of the film is more stable than its amorphous component and that it maintains its structure. This observation was confirmed in sections of film folds. Many of these film structures had large surface areas; we also observed similar folds in ultrathin sections of the 1- and 2-d-old Gram-negative bacterial colonies (not shown). It seems likely that the formation of these folds is the result of the 'slipping down' of the surface film during the preparation of colonies for electron microscopy.

The formation of the membranous component of the surface film is very intriguing because of its 'gigantic' dimensions and extracellular position. Our experimental results indicate that membrane vesicles play an important role in this process. The intercellular matrix of the colonies of Gram-negative and Gram-positive bacteria contains great numbers of membrane vesicles, many of which adhere to the inner surface of the colonial film. Fusion of the membrane vesicles and surface film may be observed. Similar vesicles are located on the surface of bacterial cells and on the inner membrane of some Gram-negative micro-organisms. The existence of membrane vesicles has been observed previously in different bacteria (Chatterjee & Das, 1967; Chen *et al.*, 1985; Mulks *et al.*, 1980; Rothfield & Pearlman-Kothencz, 1969; Smith, 1980). Evidently, vesicles of Gram-positive bacteria are formed by the cytoplasmic membrane. In Gram-negative bacteria we also observed vesicles on the cytoplasmic membrane surface. In this respect our results are similar to the observation of the presence of membrane vesicles in the periplasmic space of *Salmonella typhimurium* (Lindsay *et al.*, 1973). However, we cannot exclude the possibility of the formation of membrane vesicles of Gram-negative bacteria from the outer membrane of the cell wall. It may be suggested that membrane vesicles are involved in the transport of materials for formation of the surface film membrane and amorphous layers. This would be in agreement with data on the transport of endo- and exotoxin, alkaline phosphatase (Lindsay *et al.*, 1973), and proteins (Chatterjee & Das, 1967; Smith, 1980) by membrane vesicles.

The results presented here indicate that colonies of different Gram-positive and Gram-negative bacteria,

whether pathogenic or non-pathogenic, are isolated from their external environment by a surface film which has a complex structure. The basic part of this film comprises an elementary membrane, in which the inner and outer surfaces after 48 h of cultivation are covered by an amorphous layer. The membrane component of the films is a stable structure occupying a large surface area. Membrane vesicles of the bacterial colony contribute to the formation of the surface film. The presence of the film on the surface of different micro-organisms indicates that this structure plays an important role, although its function needs further investigation.

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