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CONFERENCE PROCEEDINGS

The Electron Microscopic Study of Cell-to-Cell Interactions between Antagonistic Microorganisms

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Abstract—The electron microscopic study of thin sections and positively stained specimens of cells taken from particular cocultures of *Lactobacillus acidophilus* D75, *Lactobacillus casei* YIT 9018, *Shigella flexnery* 2a, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 25923 (some of these bacteria are antagonistic to others) showed the presence of specific ultrastructural elements indicating cell specialization and cooperation. The responses of antagonistic bacteria manifested themselves at the cellular and population levels.

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Key words: microbial community, antagonism, cooperation, specialization, intercellular communication, ultrastructure of microbial colonies

There is presently no doubt that microorganisms possess complex social organization and behavior [1, 2]. A population of microbial cells can be considered a cooperative system with particular cells performing different functional roles; it can even be thought of as a multicellular organism [1].

The phenomenon of polymorphism is typical of any living system and reflects the heterogeneity of its elements [1, 3, 4]. The coexistence of cells with different properties in a microbial community provides for the stability of the community [1, 4, 5]. Cells in microbial populations differ in morphological, biochemical, serological, and other properties [1]. The heterogeneity of microorganisms grown on solid media manifests itself in a complex ultrastructure of microbial colonies, which differ in size, shape, color, consistency, and growth rate. Moreover, various regions of colonies may have a different ultrastructure. All this implies a complex social organization of microbial communities [6, 7].

The electron microscopic study of monoculture communities (colonies and biofilms) has allowed us to reveal some specific features of their organization; such features include the formation of films on the colony surface, the intercellular matrix, and specific cell-to-cell interactions [8, 9]. The basic structural element of the surface film is a trilaminar membrane whose inner and outer sides are made up of electron-opaque amorphous polysaccharide layers. The dynamics of film formation on the colony surface has been described at length for *Escherichia coli* [10]. The formation of the electron-opaque intercellular matrix is mainly due to cell lysis. Contacts between cells are due to adherence of their walls and the formation of cytoplasmic tubes, through which bacterial cells produce a branched network in the microbial mass [8]. These structural features of monoculture colonies suggest the existence of cell specialization and cooperation in microbial communities.

It should be noted that little is known about the ultrastructure of heteroculture colonies and about cell specialization and cooperation in communities of antagonistic microorganisms, although antagonistic interactions are widespread among bacteria, in particular, lactobacilli [11]. These bacilli are able to inhibit or slow down the growth of gram-positive and gram-negative bacteria, as well as yeasts. The antagonism of lactobacilli is due to nonspecific metabolites (lactic acid, hydrogen peroxide, and lysozyme) and specific lactobacillar products, lactocins. The production of these substances of protein and peptide origin is an example of quorum-sensing processes [12]. The nature, physicochemical properties, and mechanism of action of lactocins are the subject of intensive research [12, 13].

The antagonism of lactobacilli has not yet been studied at the morphophysiological level. This work was undertaken to investigate the ultrastructural organization of populations and individual cells of the sensitive cultures *Shigella flexnery* 2a, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 25923 and their antagonists *Lactobacillus acidophilus* D75

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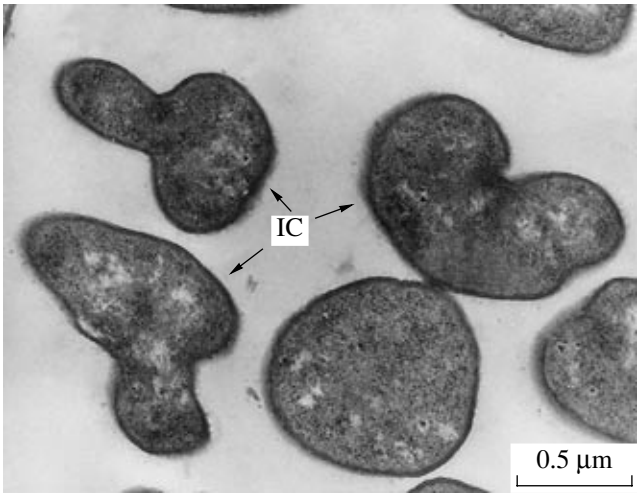

Fig. 1. Thin section of involuted cells (IC) of *S. flexnery* 2a.

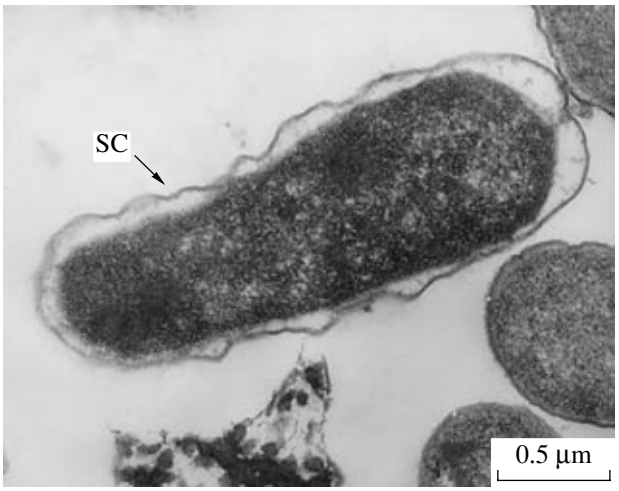

Fig. 2. Thin section of a scalloped cell (SC) of *S. flexnery* 2a.

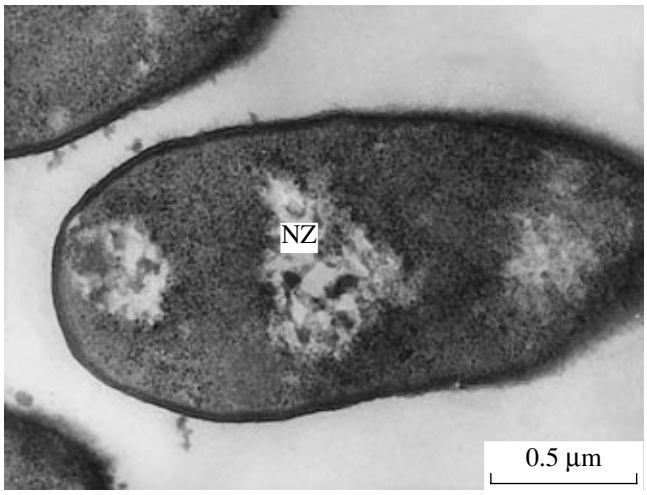

Fig. 3. Thin section of an *S. flexnery* 2a cell with an altered structure of the nucleoid zone (NZ).

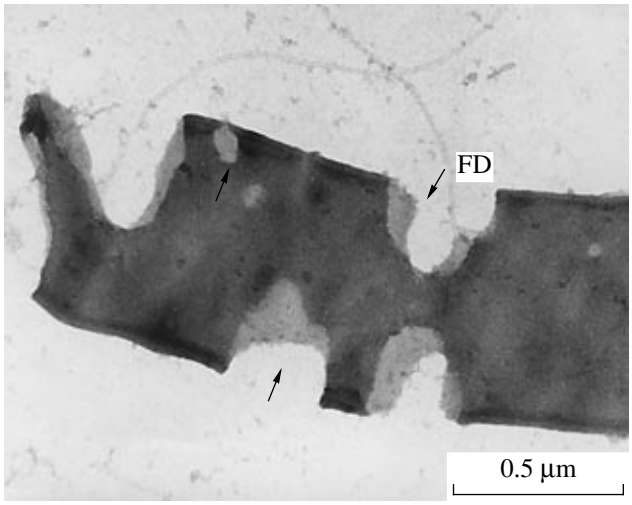

Fig. 4. Positively stained thin section showing a focal destruction (FD) of the cell wall of *B. subtilis* ATCC 6633.

and *Lactobacillus casei* YIT 9018 when these bacteria were cocultured on agar media.

Morphophysiological Changes in Sensitive Bacterial Cells Grown in Cocultures with L. acidophilus D75 and L. casei YIT 901

Gram-negative bacteria. The electron microscopic analysis of thin sections of *S. flexnery* 2a cells revealed destructive processes, which manifested themselves in the appearance of involuted cells (Fig. 1), lysing scalloped cells (Fig. 2), resting electron-opaque cells, and nucleoids with impaired structural organization (Fig. 3). Changes in the nucleoid were associated with the formation of electron-opaque globular inclusions and rough fibrillar DNA threads in the distinct nucleoid zone, while the cell wall remained intact.

Thus, the gram-negative *S. flexnery* 2a cells responded to antagonistic lactobacilli at both the population and cellular levels.

Gram-positive bacteria. Ultrastructural changes in cells of the spore-forming bacterium *B. subtilis* ATCC 6633 manifested themselves in the appearance of multiple zones of focal destruction on the cell wall (Fig. 4) and in the formation of resting electron-opaque cell forms and spores.

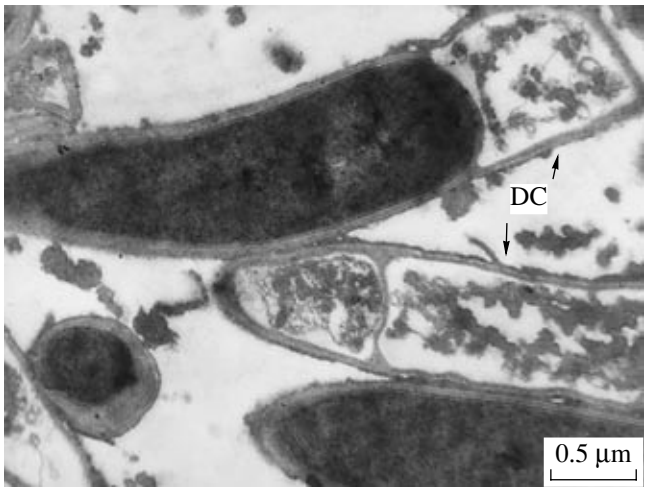

Fig. 5. Thin section of degraded cells (DC) of *L. acidophilus* D75.

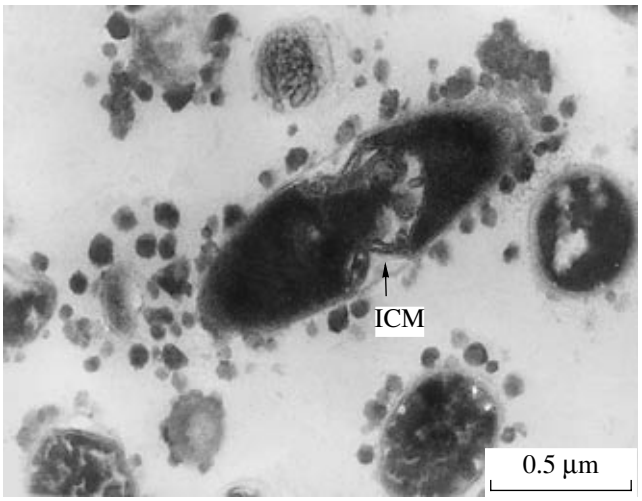

Fig. 7. Thin section of an *L. casei* YIT 9018 cell with the intracytoplasmic membranes (ICM).

Morphophysiological Changes in the Cells of L. acidophilus D75 and L. Casei YIT 9018 Grown in Cocultures with Other Microorganisms

The ultrastructural organization of *L. acidophilus* D75 colonies grown in association with *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 significantly differed from that observed in monocultures. Namely, *L. acidophilus* D75 colonies contained three boundary layers: upper, middle, and lower (near the agar surface). The upper and lower layers showed the presence of a large amount of degraded *L. acidophilus* D75 cells (Fig. 5), which were surrounded by resting cells and an intercellular matrix. Ultrastructural changes in *L. acidophilus* D75 cells showed up as a considerable increase in the thickness of the peptidoglycan layer (from 60 to 130 nm) and the appearance of an additional amorphous layer (which then detached from the cells) (Fig. 6). Furthermore, one could observe a large number of cells with destructive changes in the cytoplasm, which tended to lyse with the formation of cell ghosts. Such ultrastructural changes have never been observed in *L. acidophilus* D75 monocultures.

Ultrastructural changes in the other antagonistic bacterium *L. casei YIT 9018* were characterized by the appearance of multiple membrane structures in the cytoplasm (Fig. 7), followed by a fragmentation of the protein–ribosomal particles with the formation of specific spherical granules inside the cells and then their excretion into the medium (Fig. 8).

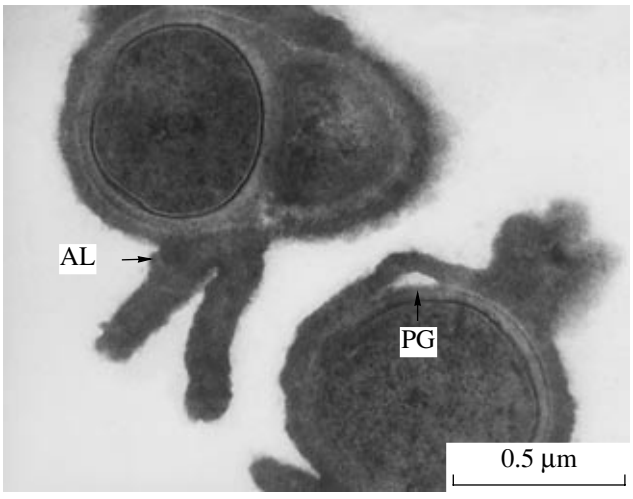

Fig. 6. Thin section of *L. acidophilus* D75 cells with thickened cell walls. PG, peptidoglycan; AL, amorphous layer.

A Comparative Morphometric Analysis of L. acidophilus D75 Clones with Different Degrees of Antagonism

A comparative electron microscopic study of two types of *L. acidophilus* D75 clones (colonies) with different degrees of antagonism (low and high) was performed with account for the main morphometric parameters: the physiological activity of cells, the character of cell degradation, the production of metabolites, and their excretion into the intercellular space.

Estimation of the heterogeneity of *L. acidophilus* D75 populations with account for the degree of cell degradation. The morphometric analysis of the population heterogeneity of *L. acidophilus* D75 showed that the degree of cell degradation in a population correlates with the level of its antagonism. For example, slightly antagonistic colonies contained 90% physiologically active cells and 10% degraded cells, whereas heavily antagonistic colonies had up to 35% degraded cells.

Destructive changes in *L. acidophilus* D75 cells. The ultrastructural changes observed in *L. acidophilus*

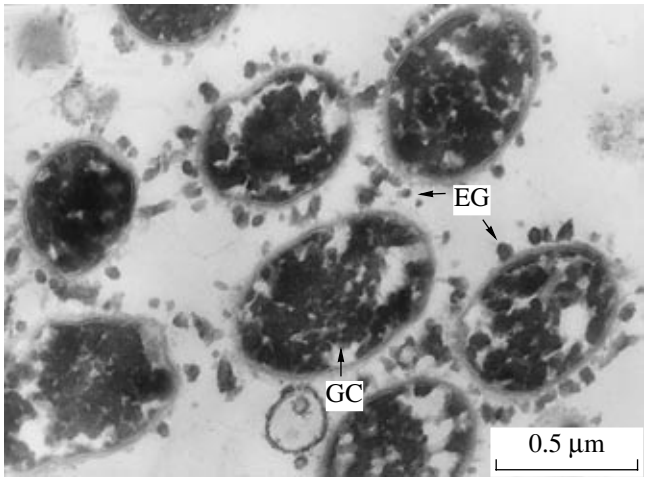

Fig. 8. Thin section of *L. casei* YIT 9018 cells with a granulated cytoplasm (GC) and excreted granules (EG).

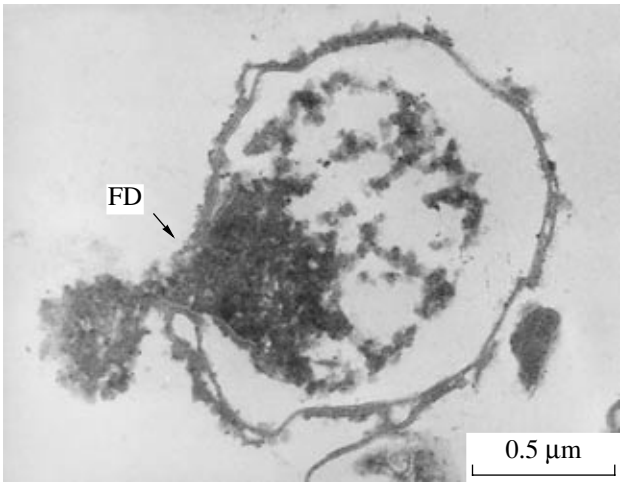

Fig. 9. Thin section of a degraded cell of *L. acidophilus* D75.

D75 cells involved the formation of additional mono- and multilayer membranes on the surface of the cell wall, a thickening of the peptidoglycan layer, the detachment of the surface amorphous layers from the cell wall (Fig. 6), and a specific thinning of the cytoplasm (Fig. 5). The appearance of membrane vesicles on the surface of bacterial cells is usually associated with the excretion of metabolic products (such as exoenzymes and exotoxins) from cells [14]. By analogy, it can be suggested that *L. acidophilus* D75 cells excrete lactocins (Figs. 7, 8).

Other specific morphological changes included point ruptures (focal degradation) of the cell wall (Fig. 9), which are not typical of normal processes of cell aging and death induced by autolysis. These lesions in the cell wall of *L. acidophilus* D75 gave rise to specific mushroom-shaped structures on the cell wall surface. These structures represented protrusions of the cytoplasm with protein–ribosomal particles into the intercellular space. The number of cells with the focal degradation of the cell wall correlated with the level of clone antagonism. For example, slightly antagonistic clones contained few cells with destructive changes in the cell wall, whereas the percentage of such cells in heavily antagonistic clones reached 10%.

Electron microscopic examination of the formation and excretion of metabolic products from *L. acidophilus* D75 cells. As mentioned above, the antagonistic activity of *L. acidophilus* D75 cells correlated with the excretion of metabolic products into the intercellular space. This excretion was accompanied by the appearance of electron-opaque spherical granules on the surface of these cells (Fig. 10). The granules were from 40 to 75 nm in diameter and contained no membranes either on their surface or inside. Later, the granules detached from the cell wall, leaving specific cavities in it, and passed to the intercellular space. This observation suggests that the composition of these granules and the cell wall is the same.

Cells in the slightly antagonistic *L. acidophilus* D75 colonies were found to excrete into the intercellular space only a few granules, no greater than 40 nm in diameter. However, cells in the heavily antagonistic *L. acidophilus* D75 colonies produced a greater number of larger granules (from 60 to 75 nm in diameter). Granules as large as these were also observed when *L. acidophilus* D75 cells were grown in cocultures with sensitive bacteria.

Thus, the antagonistic activity of *L. acidophilus* D75 was accompanied by the following morphological changes in the bacterium: an increased percentage of degraded cells (Fig. 5), specific focal destruction of the

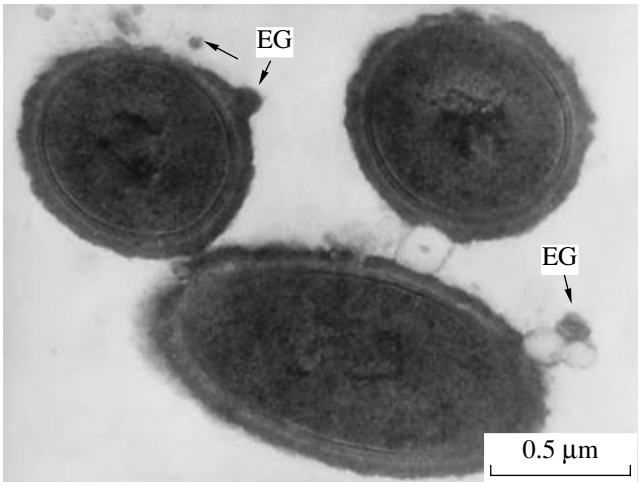

Fig. 10. Thin section of *L. acidophilus* D75 cells with thickened cell walls and excreted electron-opaque granules (EG).

cell wall (Fig. 9), and enhanced excretion of electron-opaque granules (Fig. 10). The degree of antagonistic activity correlates with the degree of these morphological alterations.

In conclusion, the morphometric analysis of populations of *S. flexnery* 2a, *B. subtilis* ATCC 6633, and *S. aureus* ATCC 25923 cells grown on agar media in cocultures with their antagonists *L. acidophilus* D75 and *L. casei* YIT 9018 made it possible to reveal specific cell responses at the population and cellular levels. At the population level, cell-to-cell interactions in the mixed populations change the proportion of different morphotypes of cells and increase the relative number of involuted, lysed, and resting forms. The intensity of ultrastructural alterations in the cells correlates with the degree of bacterial antagonism. The ultrastructural changes in the antagonistic lactobacillar cells indicate that they possess specific protecting mechanisms, due to which some lactobacillar cells transform into resting forms and produce additional protecting layers on the cell surface. Furthermore, the specific differentiation of cells leads to the death of a fraction of the population. The data presented in this paper show a specific character of the cell-to-cell interactions between antagonists and other microorganisms at the cellular and population levels, and suggest the existence of a special form of social behavior of microorganisms in mixed microbial communities with unidirectional antagonistic interactions.

The morphophysiological changes observed by electron microscopy are indicative of complex communicative relations between microorganisms in mixed communities.

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