



Transmission and Scanning Electron Microscopy of Contacts between Bacterial and Yeast Cells in Biofilms on Different Surfaces

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Abstract

The mechanism formation of colonies and biofilms of bacteria and yeasts are studied always of great interest. The aim of the presented work was transmission and scanning electron microscopic analysis contacts between cells of bacteria and yeast in biomofilms on natural structures and inorganic surface, as a result of formation of close contacts between a cellular wall, a fringe, cross-pieces, symplasts and cells of *Escherichia coli*, *Shigella flexnerii* *Salmonella* of *typhi*, *Salmonella typhimurium* and also some probiotic lactic acid on nutritious agar surfaces. Intercellular contacts in yeast biomofilms on plates of zirconium were scanning electron microscopic visualized by *Candida guilliermondii*.

Keywords

Biofilms, Intercellular Contacts, Bacteria, Yeast, Transmission and Scanning Electronic Microscopy, Morphology Image Analysis

Subject Areas: Biochemistry, Cell Biology

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1. Introduction

Bacterial and yeast colonies and biofilms are well known to present populations of cells on different surfaces as a result of their duplications from one or several ones [1]-[5]. Peculiarities of colonies and biofilms are studied always of great interest; however the mechanisms for formation of intercellular contacts remain unclear.

The colonies and biofilms of microorganisms are offered to consider also as society where planktonic forms are interconnected in the various ways allowing them to get new properties of resistance in biofilms *in vitro* and *in vivo* against various stress factors of the microenvironment [6]-[9]. And only using modern high-resolution electronic microscopy of different types has opened the possibility of detailed study on structured components in bacterial cells in plankton and colonies.

Moreover, microorganisms can form biofilms attached to the surfaces of different prostheses and may play a biodegradation role [10] [11].

In the present work, in order to install the intercellular contacts between cells *in vitro* in biofilms of bacteria and yeast on different surfaces including the plates, structures of zirconia have been studied by transmission and scanning electronic microscopy. In addition, some cytochemical analysis was done to make clear interpretations of walls contacts of bacteria.

2. Materials and Methods

2.1. Bacteria and Yeast

2.1.1. Bacterial Strains, Culture Media

Different strains of bacteria and yeast were used in the study (Table 1).

The *E. coli* wild type strain was grown in peptone medium. Components grown medium: 0.2% peptone, 0.5% NaCl, and 0.2% K₂HPO₄, pH 7.5, in anaerobic conditions by fermenting glucose (0.2%) at 37°C, till stationary growth phase (18 - 20 h). The cultures of *Salmonella typhi*, *Salmonella typhimurium* and *Shigella flexnerii* were grown up in the synthetic medium nutrient-enriched agar slants (NEA, containing: nutrient-enriched broth (NEB), 1.5% agar, final pH 7.1 ± 0.2 at 37°C) were grown in NEB, containing: peptone 15 g/l, sodium chloride 6.0 g/l, yeast extracts 3.0 g/l, final pH 7.5 ± 0.2 at 37°C in thermostat for overnight (18 h) at 37°C till 18 - 24 h. Then, bacterial cultures were transferred and grown on the nutrient agar-based miliporous filters (pore size 0.22 µm) at 37°C during 24 - 48 h.

2.1.2. Yeast Strain, Culture Media

C. guilliermondii NP-4M (Cg) cells were grown on 2% wort agar, then the liquid nutrient medium. For obtaining culture, an optimized synthetic growth medium containing 3.1 g (NH₄)₂SO₄, 1.23 g KH₂PO₄, 0.625 g

Table 1. The bacterial and yeast strains used in the study.

Bacteria or yeast strains	Characteristics, source or reference
<i>Escherichia coli</i> (serotype O124)	Enteroinvasive strain, isolated from the cattle-breeding farm, Institute of Epidemiology, Virology and Medical Parasitology, Ministry of Health of the Republic of Armenia, Yerevan.
<i>Escherichia coli</i> K-12	Laboratory stock [13] [14]
<i>Lactobacillus acidophilus</i> 317/402	Laboratory stock [23]
<i>Oenococcus oeni</i>	Isolated from grape wine "Meghrabjur", Department of Food Safety and Biotechnology, Armenian National Agrarian University, Yerevan
<i>Salmonella enterica</i> ATCC700931	Laboratory stock
<i>Salmonella typhi</i> 925	Isolated from the patient of belly typhus, Institute of Epidemiology, Virology and Medical Parasitology, Ministry of Health of the Republic of Armenia, Yerevan
<i>Salmonella typhimurium</i> 546	Laboratory stock [14]
<i>Shigella flexnerii</i> 130	Isolated from the patient of bacterial dysentery, Institute of Epidemiology, Virology and Medical Parasitology, Ministry of Health of the Republic of Armenia, Yerevan
<i>Candida guilliermondii</i> NP-4	Laboratory stock [13] [14]

MgSO₄·7H₂O, 0.125 g CaCl₂·2H₂O, 0.125 g NaCl, 0.1 g ZnSO₄, 8×10^{-5} g biotin, 10 g glucose and 10 g yeast extract in a total volume of 1 L was used; pH was adjusted to 5.5 by 0.1 N HCl. The grown-up biomass of *C. guilliermondii* was subjected to formation of biofilms on different surfaces including solid nutrient agars and porous zirconium and incubated at 30°C during 24 h.

2.2. Electronmicroscopy, Preparations and Image Analysis

Transmission electron microscopic (TEM) methods with negative staining by means of 2% phosphotungstic acids at pH 6.8 - 7.0 [12] as well as positive staining by 1% uranyl acetate were used. For electron microscopy of the ultrathin sections, bacterial colonies were fixed in 2.5%-glutaraldehyde on 0.1 M cacodylate buffer (pH 6.8 - 7.0). Then, after fixation by means of 1% osmium tetroxide on 0.1 M cacodylate buffer (pH 6.8 - 7.0), the dehydrations and soaks in araldyte cuts were flooded with araldytes. As biosamples polymerization poured in capsules of gelatin was performed at 37°C and 60°C during 48 h, and ultrathin sections obtained on ultramicrotome (Reichert-Yung, Austria) were contrasted by aqueous solution of uranyl acetate and citric acid lead. Transmission electron microscopes Tesla-500 (Tesla, Czech Republic) or JEM-100B (JEOL, Japan) were employed. For scanning electron microscopy (SEM) of *C. guilliermondii*, samples were installed after fixing on metallic substrates and evaporations by particles of silver in a vacuum-evaporator and studied in scanning electronic microscopes of TeslaBS-301 and Tescan (Czech Republic). Morphometric and stereo-metric computer analysis of electronmicroscopic images was performed by the programs "Video-test, structure-5. Nanotechnology" and "Morphology" [13] [14]. Microanalysis of yeasts biosamples was performed using the program "Tescan".

2.3. Cytochemical Assays

Localization of mucopolisaccharides was determined by the method of Luft [15]. After centrifugation of *Sh. flexnerii* culture, a pellet was fixed in 1% OsO₄ in the cacodylate buffer (pH 7.4), after washing fixing was continued in 2.5% solution glutaraldehyde in cacodylate buffer, then it was incubated in 1% ruthenium red in the cacodylate buffer. Dehydration and impregnation of a biosample were carried out with mixture of araldytes. Positive reaction was considered as establishing electron-dense layer on an outer membrane of cell wall of bacteria by a microscopy.

3. Results

Ultrastructural analysis of bacterial colonies *in vitro* has shown that they have fine structures which are typical for gram-negative [14]. The clarification of the structured particularities of the zone of intercellular contacts was realized with more detailed presentation of the surface structures and cell walls.

The study of the surface structures of gram-negative bacteria of *E. coli* (Figure 1(a)), *Sh. flexnerii* (Figure 2(a), Figure 3) and *S. typhimurium* (Figure 2(d)) in colonies has revealed the different forms of intercellular contacts. Beside enteropathogens strains of *E. coli* with adhesive characteristics there were fimbria, which take part both to delivering plasmid, and in fastening to the other subjects and substrates, forming three-dimensional (3D) imaging fimbriae (pilli). Stereo-metric computer analysis of transmission electronmicroscopic images was performed by the programs "Video-test, structure-5. Nanotechnology" and "Morphology" (Figure 1(b)) and of peritrichial orientation of flagellas. The sizes of the pilli varied of 100 nm to 200 nm, but diameter was ~8 nm (Figure 1(a), Figure 1(b)).

By means of comparative computer programs to manage reconstruction, stereometrical orientation of fimbria was determined to reconstruct [6].

The other varieties for intercellular closed contacts of cell walls, crosspieces and symplasts between cells were established in the colonies of with gram-negative bacteria used. At the sites of close adhesion the fusion of cytoplasmic and outer membranes of these bacterial cells have been found to occur as shown by Bayer and Bayer [16]. The length of the bridge emergence leaves the impression of complete division of bacteria cells (Figure 2(a) and Figure 2(b)). Biofilms from colonies of bacteria and positive manner painting revealed the existence of three-dimensional surfaces bridges (see Figure 2(c)). Then, formation of *S. typhimurium* cells symplasts has been also visualized (see Figure 2(d)). In the last century a number of researchers distinguished R, S and L colony types in bacterial populations. Heteromorphical and L-transformations at bacteria were established both during their growth in culture and under the influence of different antibiotics and enzymes. This seems to be

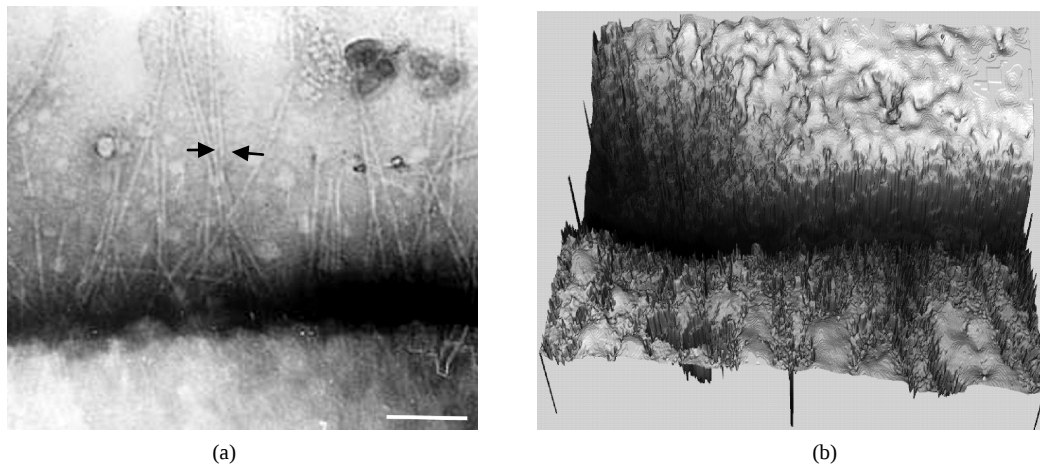


Figure 1. The fimbrii (arrow) of *E.coli* (serogroup O124). TEM, negative contrasting (a) and 3D imaging analysis with program “Video-test, structura-5, nanotechnology”; (b) A-Scale bar: 80 nm.

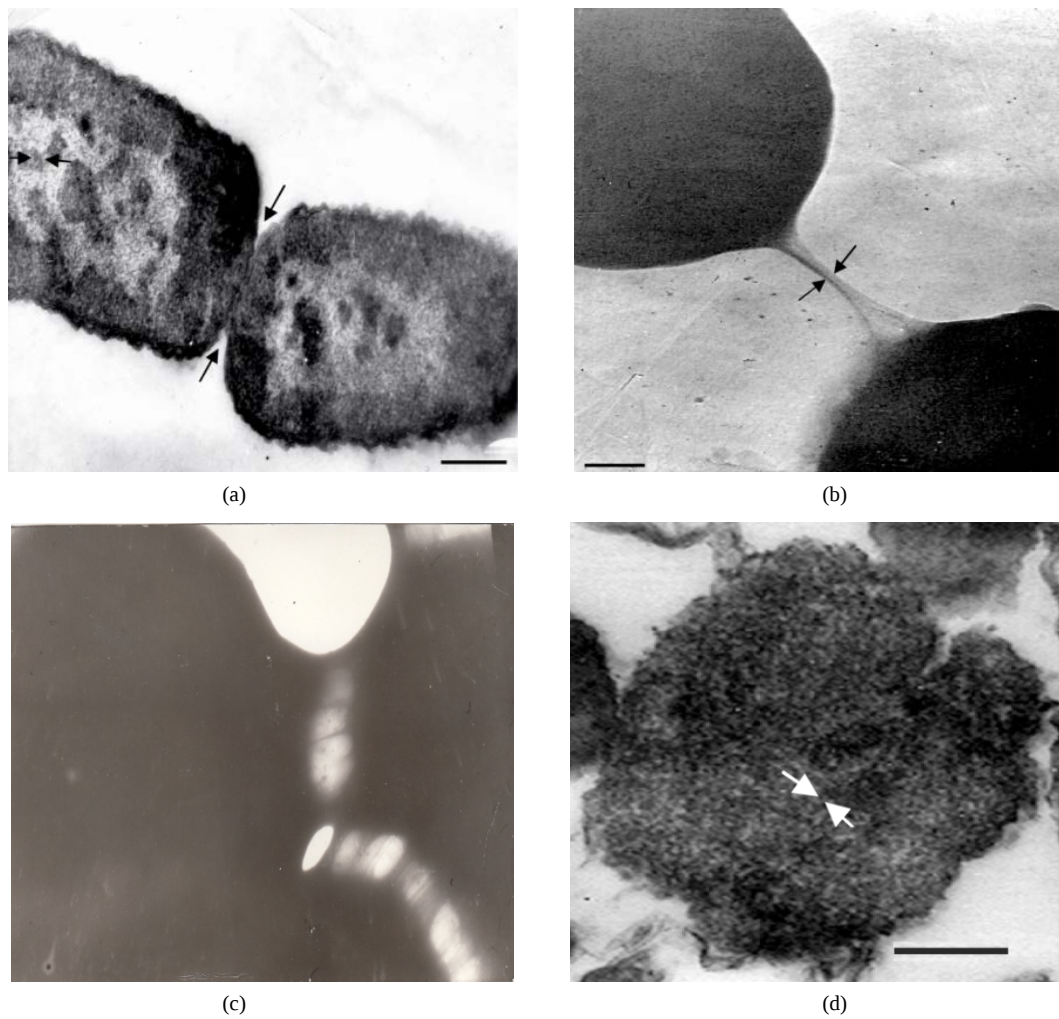


Figure 2. The contacts between cells (arrow) of *Sh. flexnerii*130 (a) TEM., ultrathin section. Cross-piece of a cellular wall between cells (arrow) of *E. coli* K-12; (b) and positive staining by uranylacetat revealed the existence of surfaces bridges (arrow); (c) TEM. Simplast cells (arrows) of *S. typhimurium*546; (d) TEM, ultra-thin section. Bars: 0.15 μ m.

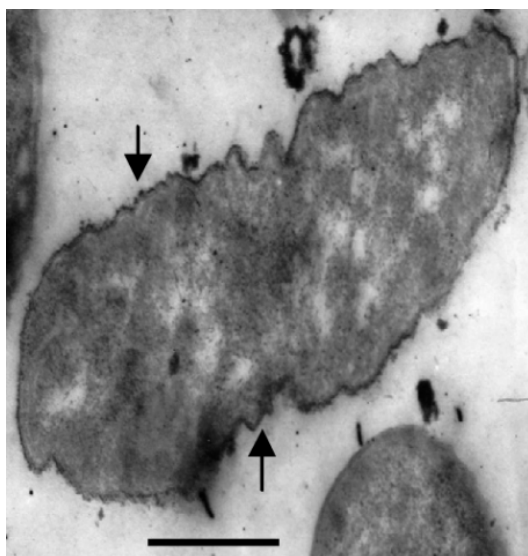


Figure 3. Localization of mucopolysaccharides electron-dense layer (arrow) of a microcapsule on the outer membrane of *Sh. flexneri* 130. TEM, ultrathin section. Scale bar: 0.15 μm .

likely to data reporting fusion of protoplasts and formation of symplasts for growing bacterial cells and under the influence of antibiotics during L-transformation in culture of *Salmonella* as suggested [17] [18]. These have practical interest, giving a possibility to use them for crossbreeding and constructions of strains with useful characteristics.

The contacts between gram-negative bacterial cells were also found with the help electron cytochemistry reaction. Probably mucopolysaccharid layers of a microcapsule play important cytoprotective role in those contacts between bacteria [19] [20]. Localization of mucopolisaccharides positive reaction by cytochemistry was considered as establishing electron-dense layer on outer membrane of *Sh. flexnerii* was obtained (see **Figure 3**).

Capsule-like layer has been visualized for gram-negative *S. typhi* under interaction with different eukaryotic cells, for instance macrophages. Interestingly, duplication and formation of micro-colonies in phagosomes of eukaryotic cells were installed at electron microscopic study of interactions of *S. typhi* with peritoneal macrophages [21]. It is likely that intercellular contacts were formed depending on the degree of hydrophobic cellular surface of bacteria and on their antiopsoninecy to protective action [22].

Besides, together with sinergetic interrelations between studied and other type of bacteria in mixed culture of *S. enterica* and *O. oenii* there are the antagonistic interactions (**Figure 4**). The latters were observed with different bacteria resulted as structural changes in *Salmonella* cells walls. These findings can point out changing interrelations in mixed cultures. A change in the nature and forms of the relations between bacteria in mixed culture confirms the possibility of the manifestation of new functions in their community in nature. This finding might be applied for prevention and treatment in intestine microbiota of different strepto-staphylococcal infections [23] [24].

SEM of intact yeast cultures *C. guilliermondii* NP-4 has shown typical structural images for yeasts colonies (**Figure 5(a)**); the clarification of the structured particularities of the different forms of intercellular contacts zones is cell wall and is fastening to the inorganic plate substrate of zirconia. Measurement of *C. guilliermondii* sizes by means of the program “Morphology” has shown the following: diameter was 1.15 - 2.71 μm , length—3.22 μm and buds—0.318 μm . In addition, adhesion of yeast on the plate surface with porous of zirconia and multiform division of cells and multitude of buds were established. SEM analysis of yeasts colonies showed that the clarification of the structured particularities of the different forms of intercellular contacts zones is with cell wall and in fastening to the plate substrate of zirconia (**Figure 5(a)** and **Figure 5(b)**).

4. Discussion

Ultrastructural analysis of bacterial colonies and biofilms has shown that they have surface structures and cell

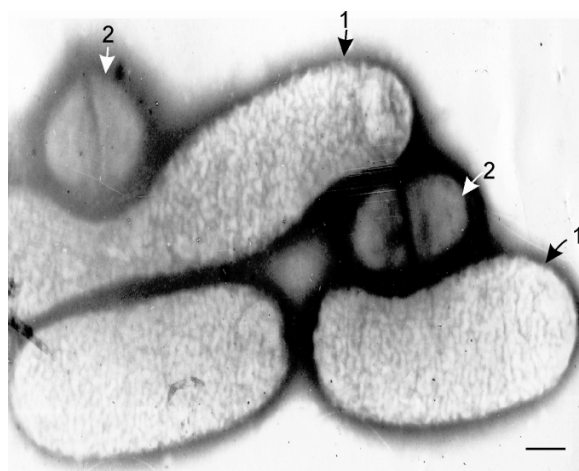


Figure 4. Interactions between *S. enterica* ATCC700931 (arrow—1) and *O. oenii* (arrow—2) cells. TEM. Scale bar: 0.15 μm .

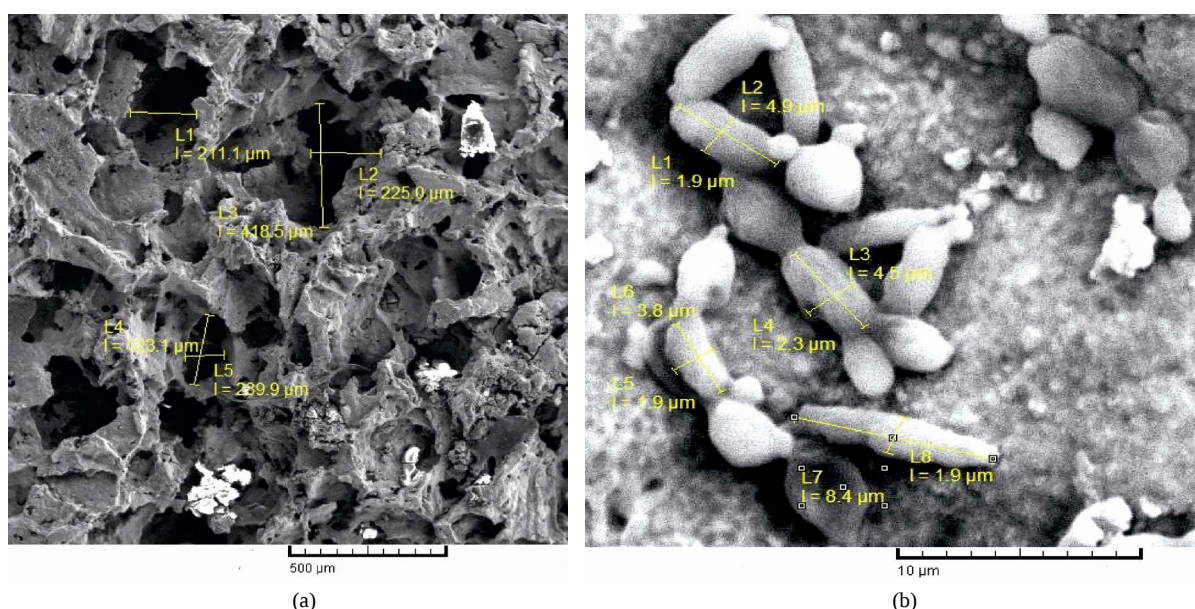


Figure 5. The ceramic porous of zirconia plate (a) and biofilms of *C. guilliermondii* NP-4 cells; (b) adhesion. SEM.

walls which are typically Gram-negative [14]. In the last century a number of researchers distinguished R, S and L colony types in bacterial populations. Heteromorphic and L-transformations at bacteria were established both during their growth in culture and under the influence of different antibiotics and enzymes. This seems to be likely to data reporting fusion of protoplasts and formation of symplasts for growing bacterial cells and under the influence of antibiotics during L-transformation in culture of *Salmonella* [17] [18]. These have practical interest, giving a possibility to use them for crossbreeding and constructions of strains with useful characteristics.

Capsule-like layer has been visualized for gram-negative *S. typhi* under interaction with different eukaryotic cells, for instance macrophages. Interestingly, duplication and formation of micro-colonies in phagosomes of eukaryotic cells were installed at electron microscopic study of interactions of *S. typhi* with peritoneal macrophages [21]. It is likely that intercellular contacts were formed depending on the degree of hydrophobic cellular surface of bacteria and on their antiopsoninecy to protective action [22]. Adhesion and immobilization of bacteria on porous ceramic surfaces create prerequisites for the prevention of microbial contamination with prosthesis [25] and, on the other hand, for wastewater treatment [26] [27].

TEM, SEM and cytochemistry electron microscopy of bacteria and yeast forming biofilms have clearly visua-

lized the intercellular contacts caused by the presence of fimbrias, flagellas, polysaccharides of microcapsules, and intracytoplasmic polyphosphates. These structures promote Bayer-like dense contacts, membranes fusion, crosspieces of cell walls and, at the last, merge protoplasts with formation of simplasts. Moreover, the results of this study indicate that in colonies bacterial cells are not completely isolated: their interaction leads to the formation of cooperative cell systems.

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Conflict of Interest

The authors have no conflict of interest.

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