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EXPERIMENTAL ARTICLES

Analysis of Congo Red-Induced Changes in the Cell Surface and Macrocolony Structure of the Bacterium *Azospirillum brasilense*

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Abstract—Adsorption of the vital dye Congo red suppresses swarming of *Azospirillum brasilense* in a semiliquid medium, and the bacteria become able to spread with the formation of microcolonies. By using direct and stereoscopic light microscopy, the patterns of the front of *Azospirillum* spreading in a semiliquid medium containing the dye were analyzed. It was found that in a medium with Congo red, small motile colonies were formed among the individual cells, and once formed, they left the boundaries of the swarming front. The microcolonies produced by azospirilla in the presence of the dye were ordered bacterial structures, rather than random cell aggregates. Transmission electron microscopy revealed that the cells grown without the dye had polar flagella, whereas the cells from the medium with Congo red had no flagella and were covered with a layer of fibrillike material. Immunochemical data for the cell surface changes resulting from interaction with the dye make it possible to consider *Azospirillum* lipopolysaccharide as a probable Congo red receptor.

Keywords: Azospirillum brasilense, cell surface, lipopolysaccharides
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It is known that the vital dye Congo red can be adsorbed on bacterial cells its interaction with carbohydrate-containing polymers of bacteria of the genus *Azospirillum* (Konnova et al., 1994; Skvortsov and Ignatov, 1998) and with protein components of the *Escherichia coli* surface (Hammar et al., 1995) was shown. Arnold and Shimkets (1988) showed the interaction of cell surface structures with Congo red in the implementation of social behavior of *Myxococcus xanthus*, a soil bacterium with a primitive form of multicellular development. Congo red inhibited agglutination of *M. xanthus* cells and their ability to form fruiting bodies, decreased the level of myxospore formation and the rate of cell swarming. The authors of the cited work suggested that extracellular material surrounding the bacteria and fruiting bodies was probably the receptor of the dye.

Shelud'ko et al. (2006) found that adsorption of Congo red suppresses the swarming of azospirilla and emergence of a stable ability to spread in semiliquid medium with formation of microcolonies resembling granules included in the medium. This phenotype of *Azospirillum* was previously termed Gri^+ (granular inclusions) (Shelud'ko and Katsy, 2001). To date there have been no published results of detailed analysis of the changes in the cells and on the cell surface of the bacteria grown on media containing Congo red.

The relevance of such studies is determined by the significant role of the preexisting protein surface structures involved in the process of microcolonial spreading of *Azospirillum* and act as antigenic markers of the Gri-phenotype of these bacteria, in the interactions of *Azospirillum* with the roots of wheat (Sheludko et al., 2010). Moreover, the vital dye Congo red, which gives the cells the ability to spread with the formation of microcolonies, can mimic the effect of certain components of the plant root surface (Katsy, 2007).

The goal of the present work was to evaluate microscopically the influence of Congo red on the spreading of *Azospirillum* in semiliquid media and on the bacterial surface structures, as well as to carry out carry out immunochemical analysis of changes in the components of the cell surface occurring as a result of their interaction with the dye.

MATERIALS AND METHODS

Bacterial strains *Azospirillum brasilense* Sp7 (IBPPM 150) and Sp245 (IBPPM 219) from the Collection of Rhizosphere Microorganisms (Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (IBPPM RAS)) were used. Bacteria were grown at 30°C on a synthetic malate medium (Döbereiner and Day, 1976) supplemented with NH_4Cl (1 g/L). The semiliquid

medium contained 0.4% agar. To suppress swarming of bacteria and to facilitate emergence of their Gri$^+$ phenotype, Congo red was added to the culture medium at 37.5 μg/mL. When growing *A. brasilense* Sp245, in some cases strain-specific antibodies to the lipopolysaccharides (LPS) at concentrations of 5 and 7 μg/mL and genus-specific antibodies to the flagellin of the polar flagellum at a concentration of 100 μg/mL were added. Semiliquid medium was stab-inoculated.

To study the characteristics of the front of *Azospirillum* spreading in semiliquid medium containing the dye, bacteria were plated and after two days the front of bacterial propagation was examined with a Leica DM 2500 upright light microscope and a Leica M 165 C stereomicroscope (Leica-microsystems, Germany).

For electron microscopy, the bacterial cells were applied to formvar-coated nickel grids and the sample was left to stand in a dry heat oven for 1 min at 50°C. This was followed by negative contrasting in a 1% solution of uranyl acetate in double-distilled water. The samples were rinsed with distilled water and dried in a thermostat at 37°C. Analysis of the samples was performed with a LIBRA 120 electron microscope (Carl Zeiss, Germany). All microscopic studies were performed using the resources of the Simbioz Center of Collective Use, IBPPM RAS.

Linear immunoelectrophoresis was performed by using a Multiphor II setup (LKB, Sweden) in 1% agarose gels prepared in a barbital-glycine-Tris buffer with an ionic strength of 0.02 and pH 8.8. Immunoelectrophoresis was performed for 30 min at 10 V/cm. At the end of electrophoresis, each trench formed by removing strips of agarose gel was supplemented with 100 μL of antibodies to the LPS of *A. brasilense* Sp245, which were obtained as described by Matora et al. (1998); antibody concentration was 50 mg/mL. After pressing and repeated washing, the gels were stained with Coomassie R-250.

For agglutination, the cells of an 18-hour culture were washed by centrifugation and resuspended in phosphate-buffered saline (PBS) to $A_{660} = 1–1.2$ ($l =$ 1 cm). Samples of the cells (50 μL) were added to 96-well plates for immunological reactions with an equal volume of anti-LPS antibodies (initial concentration 80 μg/mL) titrated in PBS. The reaction was considered positive in the case of formation of a fluffy precipitate of irregular shape at the bottom of the relevant wells.

The method of LPS extraction and the preparation of genus-specific antibodies to electrophoreticaly purified and eluted from the gel flagellin preparation of the type strain *A. brasilense* Sp7 were described by Burygin et al. (2007).

RESULTS AND DISCUSSION

Light microscopy was used to study the front of spreading of the cells of two *A. brasilense* model strains (Sp245 and Sp7) from the point of inoculation into the semiliquid medium. To suppress the swarming and facilitate emergence of the Gri$^+$ phenotype, Congo red was added to the culture medium. After two days the front of swarming on the medium without the dye and the front of microcolonial spreading in the medium with Congo red formed by the cells of the studied strains were compared.

Addition of the dye led to a change in the visual structure of bacterial macrocolonies, which consisted of swarming cells. In the semiliquid medium containing Congo red, small motile colonies arose among the individual cells of both strains and, when formed, left the border of the swarming front (Figs. 1.1 a, 1.1b). The cells of Sp7 on the medium without the dye spread exclusively by swarming (Fig. 1.1c), while in the case of Sp245 single migrating microcolonies were revealed in front of the swarming cells (Fig. 1.1d). Such observation is in good agreement with the data of Sheludko and Katsy (2001) on identifying approximately 0.3–1.0% of clones with an unstable Gri$^+$ phenotype among the swarming populations of Sp245 in the standard semiliquid medium. It also demonstrates strain differences of the studied bacteria in their capacity for spontaneous manifestation of the Gri$^+$ phenotype. The results of stereomicroscopy (Fig. 1.2) show that microcolonies formed by *Azospirillum* strains in the presence of the dye are not chaotic aggregations of cells but ordered bacterial structures.

Alterations in the surface of bacteria grown for 48 h in a semiliquid medium in the presence of Congo red were investigated using transmission electron microscopy. It was shown that the cells of both strains grown in the medium without the dye had polar flagella (Fig. 2a), whereas the cells from the medium with Congo red did not have flagella and were covered with a layer of extracellular material (Fig. 2b), which probably mediated microcolonial spreading. It should be noted that the thickness of the polar flagella of the studied strains exceeded the normal thickness of bacterial flagella and was approximately 30 nm due to the presence of a polysaccharide sheath on the surface of the polar flagella in *A. brasilense* (Burygin et al., 2007; Shirokov et al., 2017).

Based on the data of Konnova et al. (1994) on the complexation of Congo red with carbohydrate-containing surface structures of a number of *A. brasilense* strains, the ability of the LPS of these bacteria to act as a dye receptor was assessed. Interest in LPS was caused by its significant contribution to the composition of the *Azospirillum* outer membrane,as well as by the fact that the previously revealed polysaccharide sheath, which isolates the polar flagellum filament of

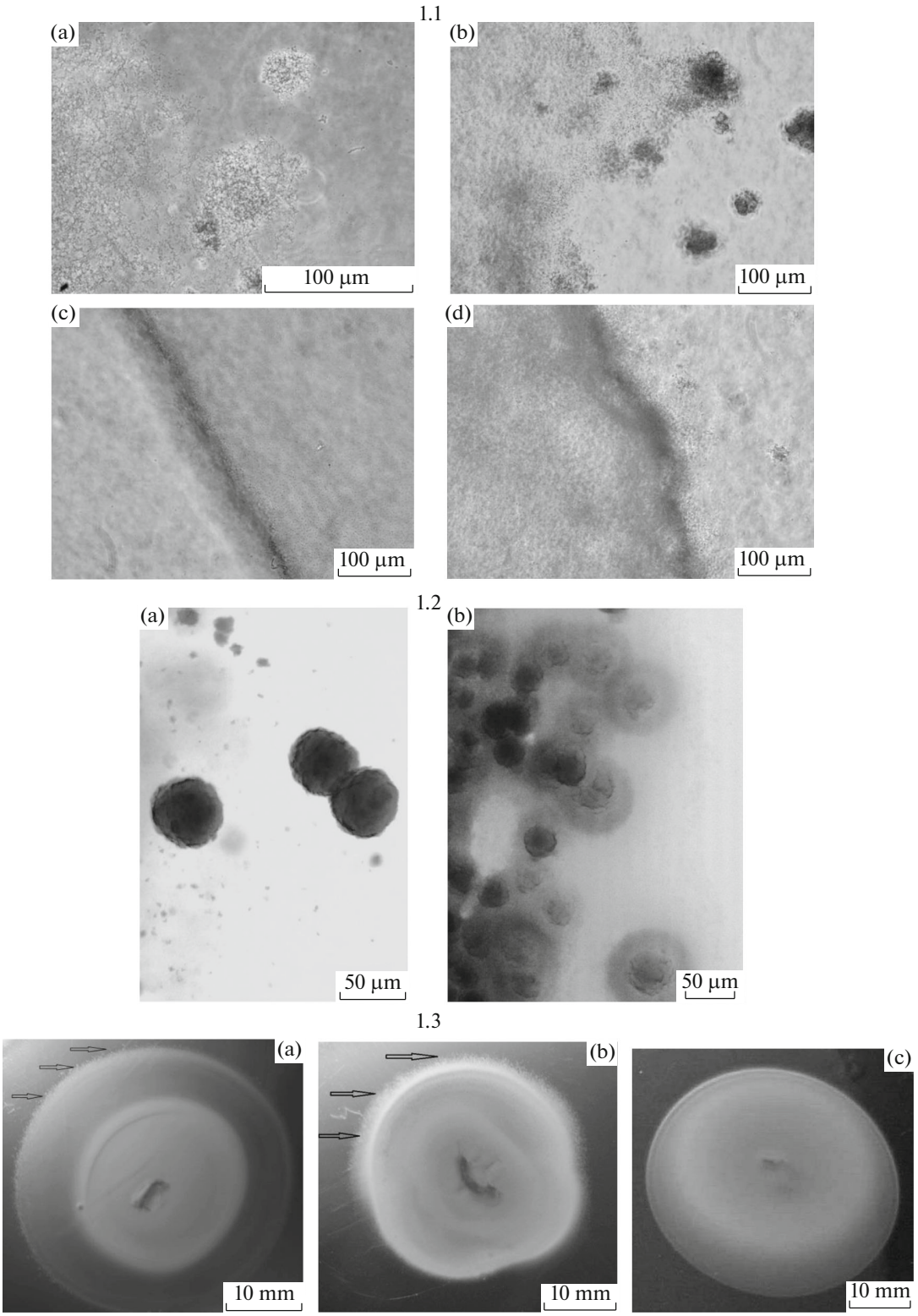

Fig. 1. Features of the front of spreading of *A. brasilense* strains Sp7 and Sp245 in semiliquid medium containing Congo red (1.1 and 1.2) and strain-specific antibodies to lipopolysaccharides (1.3). 1.1, results of upright light microscopy of the front of spreading of *A. brasilense* Sp7 (a, c) and Sp245 (b, d) in semiliquid medium with Congo red (a, b) in comparison with the control (bacterial swarming in the medium without the dye: c, d). 1.2, result of stereomicroscopy of microcolonies formed by *A. brasilense* Sp7 (a) and Sp245 (b) in semiliquid medium containing Congo red. 1.3, appearance of microcolonies formed by the cells of *A. brasilense* Sp245 in semiliquid medium in the presence of antibodies to LPS at a concentration of 5 μg/mL (a) and 7 μg/mL (b) compared to the control (c) (swarming bacteria in the medium without antibodies). The arrows indicate the change in collective motility (from swarming to microcolonial spreading) of Sp245 cells in the presence of antibodies.

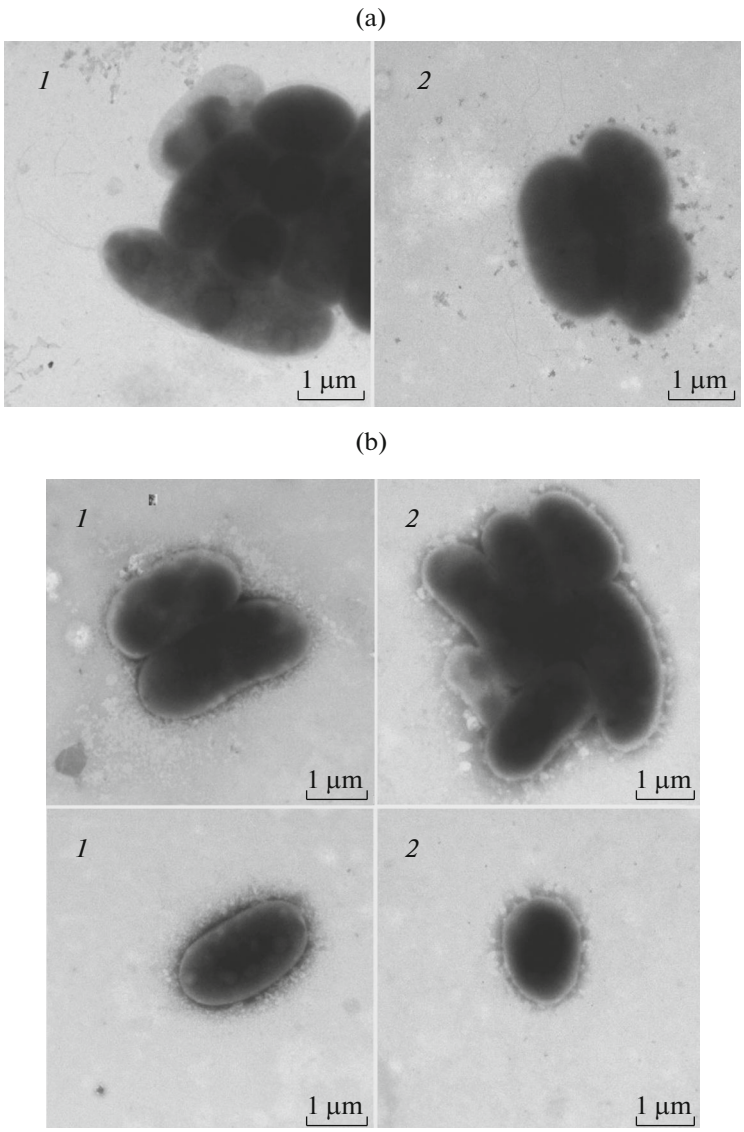

Fig. 2. Electron microscopy of the cells of *A. brasilense* Sp7 (*1*) and Sp245 (*2*) grown in the medium without Congo red (a) and with the dye (b).

A. brasilense Sp7 and Sp245 from the environment (Burygin et al. 2007; Shirokov et al., 2017), had antigenic determinants identical to the determinants of somatic LPS of these strains. It was found that antigenic determinants of the LPS affect the social behavior of azospirilla, as evident from the results of cultivation of bacteria in a semiliquid medium containing anti-LPS antibodies. Fig. 1.3 shows that the addition of such antibodies to the semiliquid medium to final concentrations of 5 (Fig. 1.3a) and 7 μg/mL (Fig. 1.3b) resulted in microcolony formation (granular cell formations marked by arrows) in the spreading front of strain Sp245 and, accordingly, in changes of the appearance of bacterial macrocolonies. At the same time, swarming of bacteria in a semiliquid medium without antibodies led to formation of macrocolonies with a smooth edge (Fig. 1.3c).

Addition of the antiflagellin antibodies to the semiliquid medium even at a concentration of 100 μg/mL did not change the spreading pattern of the tested strains and did not decrease the diameter of the ring of bacterial swarming (data not shown).

Changes in the lipopolysaccharides of *A. brasilense* Sp245 growing in the medium containing Congo red were analyzed by linear immunoelectrophoresis with specific antibodies. It was found that cultivation of Sp245 cells in the presence of Congo red changed the immunoprecipitation of their LPS. Fig. 3 shows that

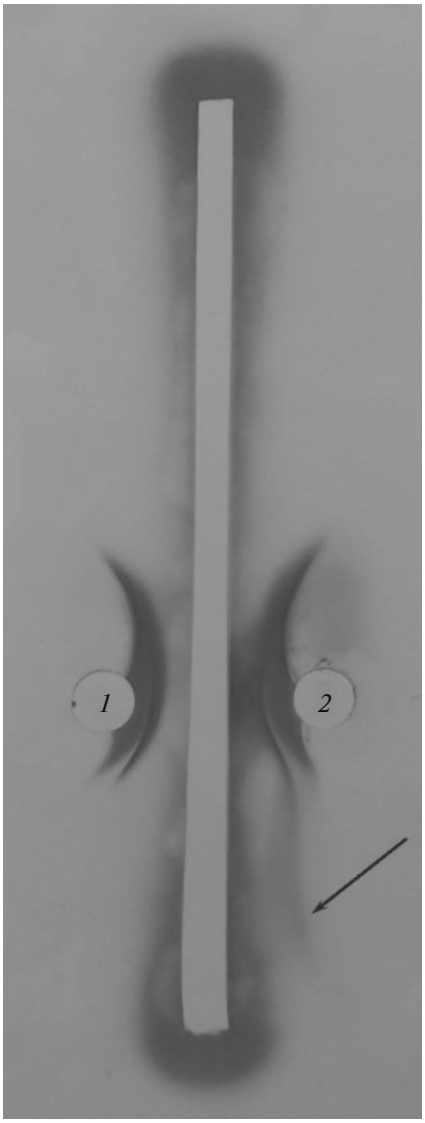

Fig. 3. Change in immunoprecipitation of LPS preparations of *A. brasilense* Sp245 in semiliquid medium on the addition of Congo red: *1*, LPS of bacteria grown in the medium without Congo red; *2*, LPS of bacteria grown in the presence of the dye.

these preparations form additional immune complexes (marked by an arrow), apparently owing to the dye binding with some determinants in the LPS.

The results of agglutination of *Azospirillum* cells with strain-specific anti-LPS antibodies also indicate an interaction of Congo red with LPS. It was shown that the addition of the dye to the culture medium led to partial inhibition of this reaction: agglutination of a suspension of Sp245 native cells occurred when the concentration of the antibodies was 5 ug/mL while for agglutination of the cells of this strain grown in the presence of the dye the required concentration was 20 μg/mL.

Thus, in the present work, by using various methods of microscopy, it was established that in the medium with Congo red motile microcolonies able to leave the boundaries of the front of swarming are formed among *Azospirillum* single cells. Cells grown in the medium without the dye have polar flagella, while cells from the medium with Congo red do not have flagella and are covered with a layer of fibrilllike material, which probably mediates the spread of microcolonies. The results obtained with the use of specific antibodies made it possible to consider the *Azospirillum* LPS as a possible receptor of Congo red. Probably the dye blocks certain determinants in the LPS, which makes swarming of the cells in semiliquid medium impossible (or difficult), resulting in induction of the Gri^+ phenotype.

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