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EXPERIMENTAL ARTICLES

Effect of Secreted Rpf Protein on Intracellular Contacts in *Micrococcus luteus* and *Mycobacterium smegmatis* Cultures

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Abstract—The effect of Resuscitation promoting factor (Rpf) on intercellular contacts in the cultures of *Micrococcus luteus* and *Mycobacterium smegmatis* was investigated using dynamic light scattering (DLS, photon correlation spectroscopy). During the stationary growth phase, the cells of the tested cultures formed extensive aggregates 100 and 300 μ in size for *M. smegmatis* and *M. luteus*, respectively. The number of solitary cells was insignificant. Addition of the recombinant Rpf protein (15 μg/ml) resulted dispersion of cell aggregates and emergence of solitary cells. This effect of Rpf decreased in the presence of nitrophenylthiocyanates (NPTs), specific Rpf inhibitors. Presumably, Rpf is involved in the regulation of intracellular interactions and in biofilm formation.

Keywords: Micrococcus luteus, Mycobacterium smegmatis, biofilms, resuscitation promoting factor (Rpf), nitrophenylthiocyanate.

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It is presently widely accepted that chemical-signal-mediated intercellular interactions exist in bacterial cultures and are involved in the development and differentiation of their cells [1]. In the 1970s, chemical factors of bacterial communication were discovered, including low-molecular weight products of secondary metabolism, as well as peptides, lipids, and secreted proteins. These molecules perform important functions in such bacterial physiological activities as cell division, conjugation, sporulation, and virulence [2].

Considerably less attention has been given to physical interactions among the cells in a population and their involvement in the cellular physiological activity. Aggregation of bacterial cells is understood as a response to stressful growth conditions [3]. However, the issue of the biological sense of such aggregation has not yet been finally resolved.

Despite the progress in research on biofilms and bacterial colonies, the mechanisms controlling cell aggregation/desaggregation during growth of bacterial cultures have not been elucidated.

The only protein (enzyme) implicated in bacterial biofilm dispersal that has been identified and characterized is dispersin B (EC 3.2.1.52) produced by *Aggregatibacter actinomycetemcomitans*. This enzyme (molecular mass of 42 kDa) belongs to hydrolases. It causes hydrolysis of poly-N-acetylglucosamine, the polysaccharide responsible for intercellular interactions [4].

Earlier, we reported a factor released by *Micrococcus luteus* growing cells, which stimulates resuscitation of the dormant forms of micrococci [5]. The factor was denoted as Rpf (resuscitation-promoting factor). It was established that Rpf is a protein with a molecular mass of ~19 kDa. Its picomolar concentrations induce resuscitation of the *M. luteus* dormant cells. It was revealed that Rpf also stimulates division of physiologically active cells, which enabled us to consider this factor as a bacterial cytokine [6]. The genes homologous to *rpf* were detected in a number of G + C-rich gram-positive bacteria. According to the relevant databases, homologous genes are present in *Mycobacterium tuberculosis* (five genes) and *M. smegmatis* (four genes). Presumably, *rpf*-homologue-encoded proteins possess Rpf-similar properties and have the same stimulatory effect on cell division [7].

The techniques of NMR and X-ray diffraction analysis were used to establish that the conserved domain of Rpf is structurally similar to the analogous domain of lysozyme [8]. This finding suggested the existence of the hydrolytic activity of Rpf, which was subsequently detected experimentally. A relationship between the enzymatic and the biological activities of Rpf was revealed [9]. In all likelihood, Rpf is closely related to the class of lytic transhydrolases, the hydrolytic enzymes involved in hydrolysis of the glycoside–glycose 1–4 bond between N-acetylglucosamine and

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N-acetylmuramic acid residues with the formation of 1,6-anhydromuramic acid residues [8].

Hence, the proteins of this family can exert an influence on bacterial cell walls, resulting in their partial hydrolysis and release of the compounds that may be required for resuscitation [10]. Nevertheless, we cannot rule out the possibility that Rpf, like dispersin, causes alterations in the cell wall. This may result in disrupting the intercellular contacts and dispersing cell aggregates.

The goal of the present work was to test the above suggestions using dynamic light scattering for investigation of the influence of Rpf on the intercellular interactions in *M. luteus* and *M. smegmatis* grown in liquid media. In addition, the impact of specific Rpf inhibitors on cell deaggregation was of particular interest. Since Rpf is involved in resuscitation of the dormant forms of mycobacteria, potential inhibitors of Rpf could make it possible to develop anti-TB drug preparations that prevent the resuscitation of latent TB pathogen forms.

MATERIALS AND METHODS

The *M. luteus* culture (NCIMB 13267) was grown in the rich Nutrient Broth medium (Himedia) for 24 h to attain an optical density (OD_{600}) of 1.5–2.0. This culture was used for inoculation. The culture employed in the experiments was grown in flasks with 30 ml of the medium for 16 h at 30°C with intense stirring (200 rpm) up to the early stationary phase.

The *M. smegmatis* culture (strain mc^{2155}) was grown in the modified Hartman de Bont medium [11] with stirring (200 rpm) at 37°C for 72 h.

Optical density was measured with a BioPhotometer spectrophotometer (Eppendorf) at 600 nm in 1-cm cuvettes.

In studies concerned with the effects of Rpf, bacterial cultures were grown as described above. Cell samples (500 μl) were washed three times with 500 μl of freshly prepared sterile cultivation medium and resuspended in the initial volume (500 μl). The samples were supplemented with various concentrations of recombinant protein RpfSm (a truncated form of Rpf from *M. luteus*).

The cells were incubated for 1 day at 37°C. Thereupon, we examined the distribution of cell aggregates in the samples and conducted the microscopic studies.

The recombinant Rpf protein was obtained as described in [12].

The distribution of cell aggregates was monitored at 37°C using a photon spectrometer (PhotoCor Instruments). A helium–neon laser emitting a monochromatic beam of light with a wavelength of 633 nm was used as the light source. Bacterial cultures were centrifuged, washed three times with sterile cultivation medium, resuspended in the initial volume, and placed in a quartz cuvette. The signal was recorded with a PhotoCor-PC photon-counting system. The data obtained were processed using the DynaLS software package.

Microscopic studies were conducted using a phase-contrast EclipseE 400 microscope (Nikon, Japan).

RESULTS

Several techniques exist (flow cytometry, capillary electrophoresis, etc.) for assessing the aggregation degree in bacterial cultures. One of the quickest and simplest techniques involves dynamic light scattering (DLS, photon correlation spectroscopy or laser correlation spectroscopy). The technique is based on determining the temporal pattern of light scattering on a moving microscopic particle. This method makes it possible to detect particles with the sizes varying within a wide range (from several nanometers to several millimeters) at a concentration as low as 100000 particles/ml.

In our studies, we investigated the cultures of *M. smegmatis* and *M. luteus* under conditions promoting cell aggregation. We established earlier that dormant and nonculturable *M. smegmatis* cells form extensive aggregates during the poststationary phase, which distinguishes them from the exponential-phase cells [11].

In Fig. 1, a bar chart diagram of the distribution of *M. smegmatis* cells under these conditions is shown. The diagram demonstrates that the initial culture consisted of aggregates differing in size. Most of them were particles with Stokes radii of 10^5–10^4 nm (the average size was ~7.3×10^4 nm).

Addition of the recombinant Rpf protein to the culture resulted in a decrease in the content of large particles and formation of two particle subpopulations with the sizes of 5.5×10^2 and 2.4×10^3 nm (0.55 and 2.4 μm), respectively. Obviously, the particles of one subpopulation represented individual *M. smegmatis* cells (with an average size of ~2 μm). The other subpopulation consisting of smaller particles (0.5 μm) plausibly contained the fragments of cell walls resulting from their partial hydrolysis by Rpf. The dispersing activity of Rpf was shown to be concentration-dependent (Fig. 2).

Rpf had a similar effect on the *M. luteus* culture grown in a rich nutrient medium (Figs. 3 and 4). During 16 h of growth, the culture contained large cell aggregates with an average size of 3×10^5 nm (300 μm). Treating the aggregates with Rpf resulted in their destruction with formation of particles with sizes of

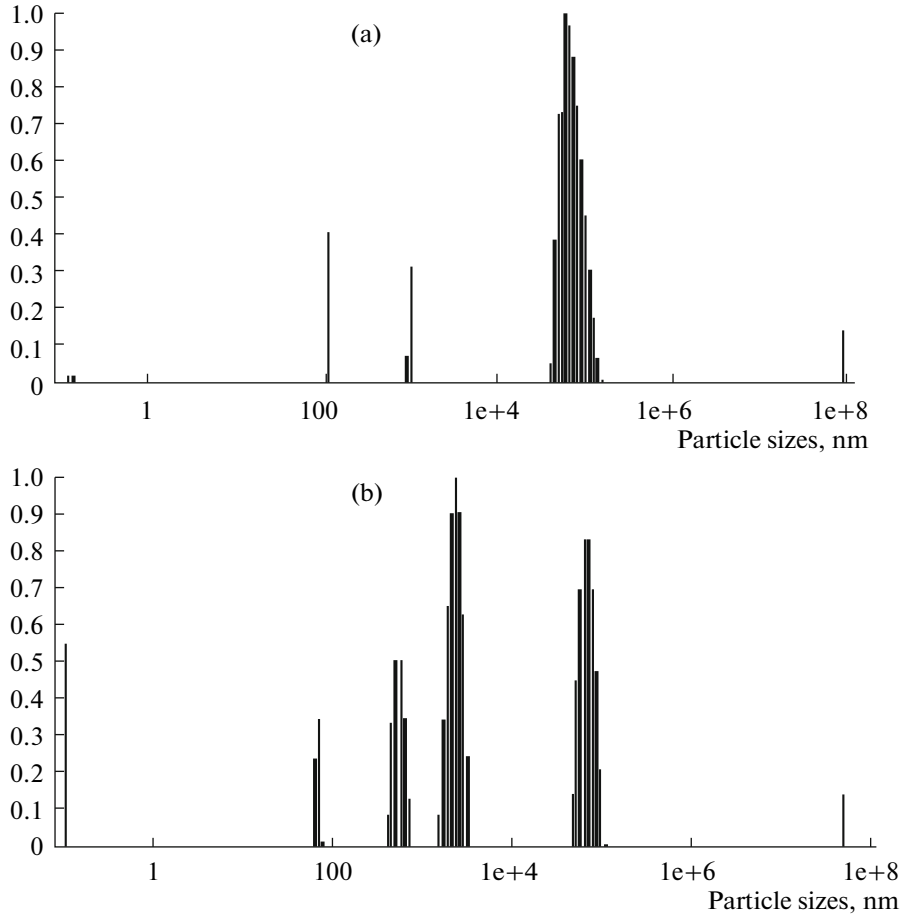

Fig. 1. Diagram of the distribution of *M. smegmatis* cell aggregates in the original culture (a) and in the sample incubated with RpfSm (15 μg/ml) (b). The bar chart represents a typical result of three measurements.

0.7 (individual bacteria) and 8–10 μm that represented oligomeric aggregates containing up to ten cells.

The data concerning the effect of Rpf on both cultures were confirmed by light microscopy that revealed the capacity of Rpf to disperse extensive cell aggregates completely, resulting in the formation of solitary cells (Fig. 5) under the same conditions as in the DLS experiments. Interestingly, *M. luteus* cells with an inactivated chromosomal *rpf* gene and a supplementary *rpf* gene on a plasmid exhibited an extraordinarily high degree of aggregation during virtually all growth phases (unpublished data).

The specificity of the effects observed in the presence of the recombinant Rpf protein was tested in studies with inhibitors of the enzymatic and biological activities of the proteins of this kind. Recently, we synthesized nitrophenylthiocyanates, a new class of chemical compounds. They are capable of inhibiting the peptidoglycan hydrolase activity of Rpf and suppressing its biological effect, i.e., resuscitation of the dormant forms of mycobacteria [12]. For this purpose, protein Rpf (15 μg/ml) and the inhibitor (15 μg/ml) were added to washed bacterial cultures. The cells were incubated for 24 h at 37°C. The inhibitor used was (3-Nitro-4-thiocyanato-phenyl)-phenylmethanone, and 3-nitro-4-thiocyanatobenzoic acid was used as the negative control. In the presence of (3-nitro-4-thiocyanato-phenyl)-phenylmethanone, deaggregation of bacterial aggregates was inhibited, whereas 3-nitro-4-thiocyanatobenzoic acid did not affect this process (Fig. 5). A lack of the inhibitory effect in the case of the second compound may be due to the presence of a negative charge in its molecule. Since the catalytic domain of Rpf is also charged negatively, based on the data of [13], this is a serious obstacle that may prevent the inhibitor's binding to the catalytic site of the protein. Accordingly, 3-nitro-4-thiocyanatobenzoic acid did not influence the enzymatic and biological activities of Rpf [9].

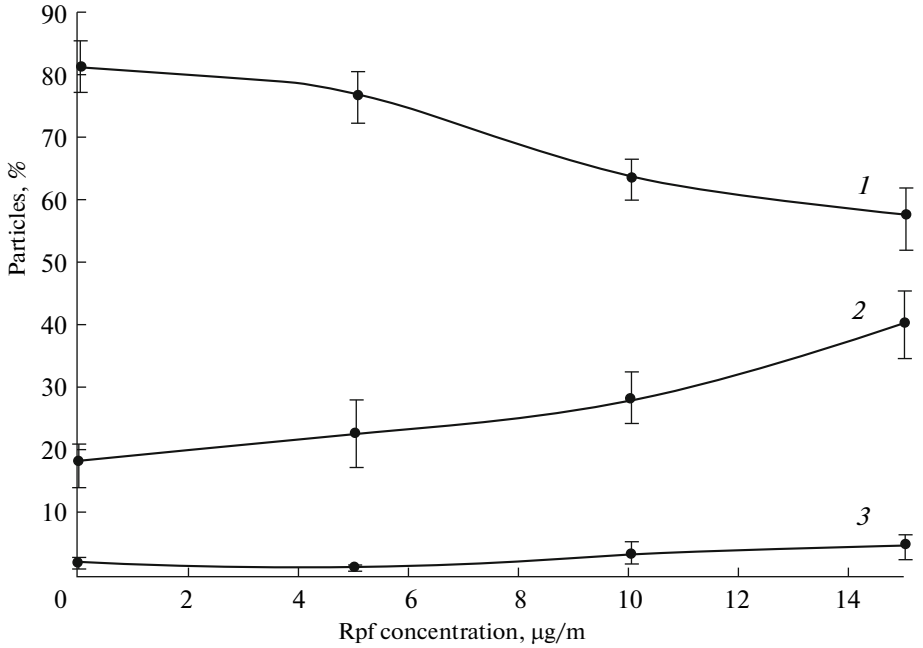

Fig. 2. Effect of RpfSm on the content of particles with various sizes in the culture of *M. smegmatis*: particles with sizes of 10 μm and above (*1*), particles with sizes of 1–10 μm (*2*), and particles less than 1 μm in size (*3*).

DISCUSSION

Voloshin et al. have shown [14] that aggregation of *M. luteus* cells plays an important role in initiating the growth of bacterial cultures during the lag phase under nonoptimal growth conditions. In this case, the cells form aggregates that enable them to start dividing, probably due to their cryptic growth in the microvolume. The onset of exponential growth results in the dispersal of these aggregates, so that the cells reproduce autonomously. According to the results of our study, we suggest a molecular mechanism of aggregate dispersal that involves proteins of the Rpf family. Rpf is known to stimulate bacterial growth [9]. It is, however, still unclear whether this effect is associated with deaggregation. Importantly, the Rpf concentrations used in this study to disperse the aggregates were several orders of magnitude higher than those that stimulate cell division (dormant cell resuscitation) and are present in the culture fluid as bacterial secretions. However, taking into account the microvolumes enclosed by the aggregates during the lag phase, such local concentrations may presumably result from secretion of Rpf proteins into the growth medium. We cannot rule out the possibility that cell desaggregation is the factor that underlies the effects produced by Rpf as a bacterial cytokine. Alternatively, this may be an important additional factor enabling the stimulatory activity of Rpf to manifest itself [6]. There are other hypothetical mechanisms of action of these proteins on the target cells involving the signal effect of products of hydrolysis of bacterial peptidoglycan, which are formed under the influence of Rpf [15].

Evidently, the ability of mycobacterial cells to aggregate promotes the formation of biofilms, i.e., cell assemblies attached to various biotic or abiotic surfaces. Research on the mechanisms of biofilm structure and formation revealed that biofilm formation is essential for mycobacterial pathogenicity and development of the phenotypic resistance of bacteria to antibiotics [16, 17]. Studies aimed at determining the substances that prevent the formation of cell aggregates (biofilms) or disperse them can help us cope with the issue of the resistance of pathogenic mycobacteria to antibiotics and, therefore, accelerate the treatment of relevant diseases.

The data obtained in this work provide evidence that proteins of the Rpf family are involved in controlling the intercellular contacts in two gram-positive bacterial species. Presumably, it is the mechanism that is implicated in dispersing the biofilms including those formed by mycobacteria.

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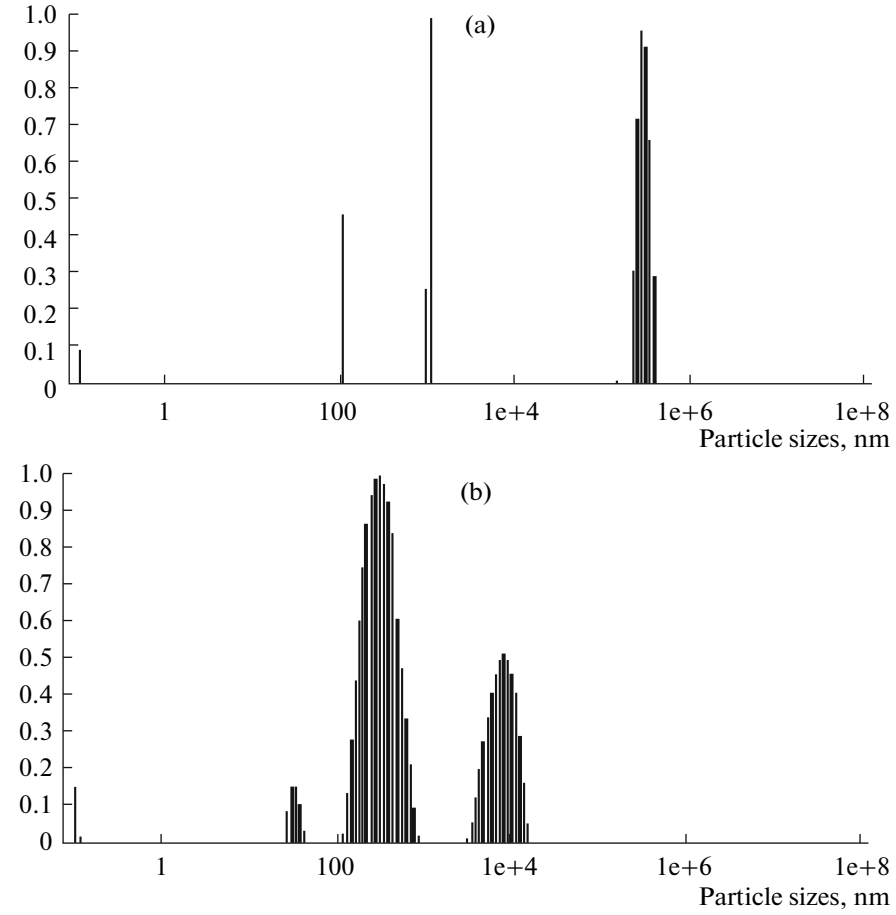

Fig. 3. Diagram of the distribution of *M. luteus* cell aggregates in the original culture (a) and in the sample incubated with RpfSm (15 μg/ml) (b). The bar chart represents a typical result of three measurements.

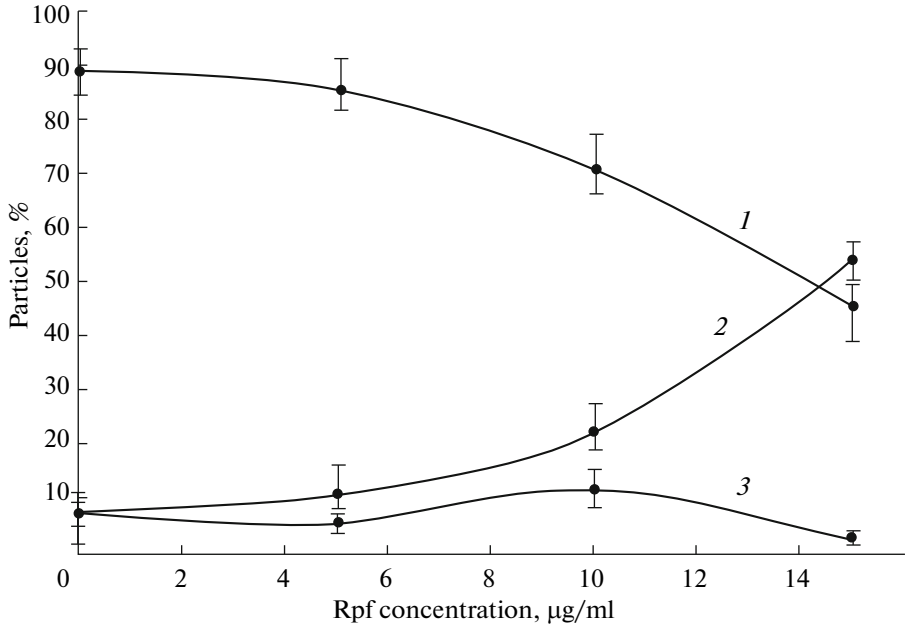

Fig. 4. Effect of RpfSm on the content of particles with various sizes in the culture of *M. smegmatis*: particles with sizes of 10 μm and above (*1*), particles with sizes of 1–10 μm (*2*), and particles less than 1 μm in size (*3*).

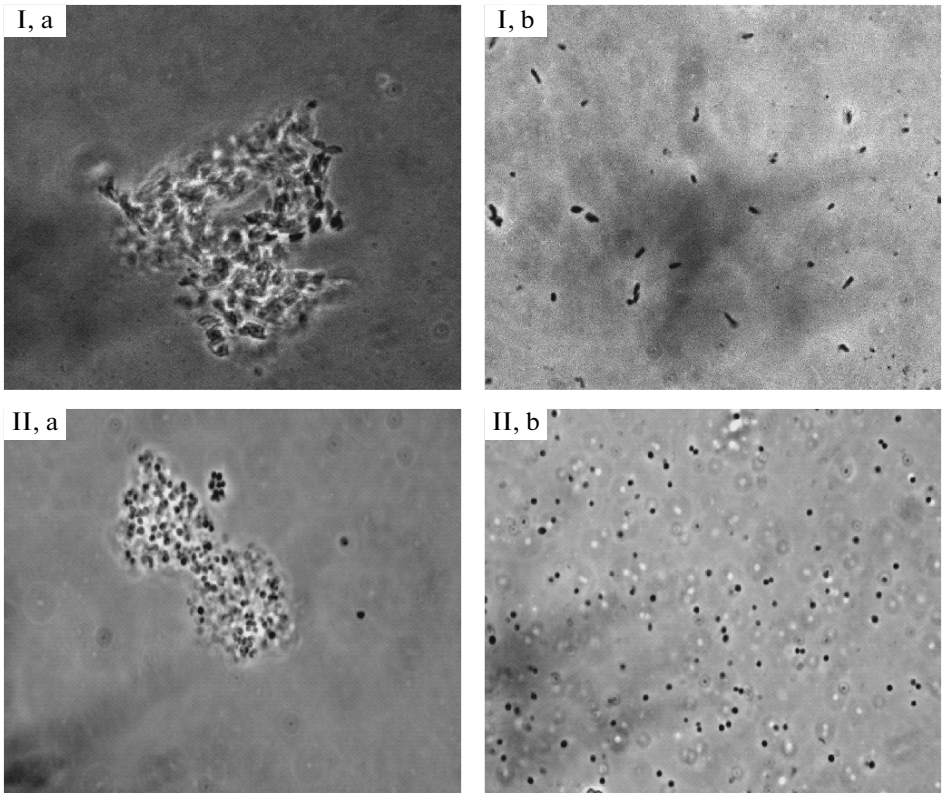

Fig. 5. Light microscope images of aggregated *M. smegmatis* (I) and *M. luteus* (II) cultures without RpfSm (a) and after incubating with 15 μg/ml RpfSm for 36 h (b). Magnification, 1000.

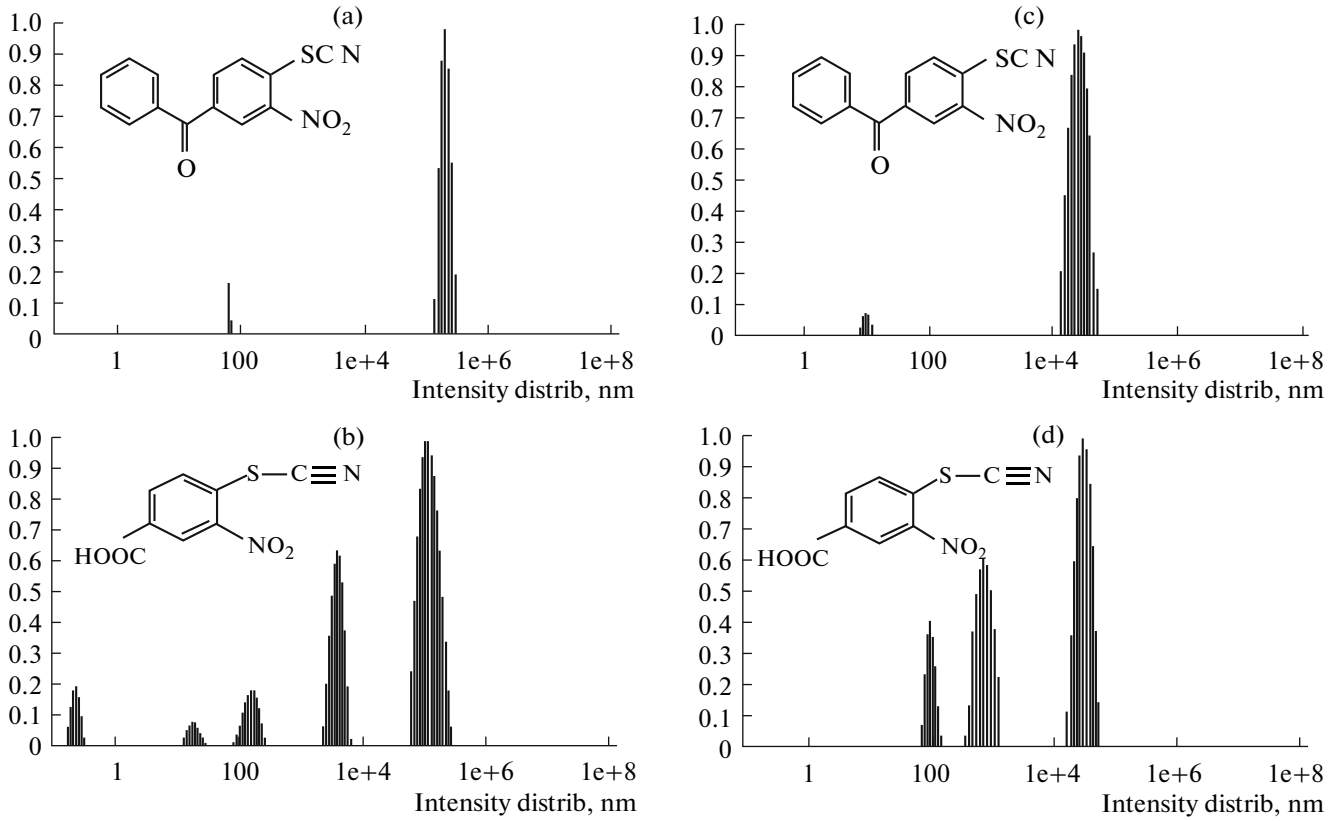

Fig. 6. Influence of nitrophenylthiocyanates on the effect of the protein RpfSm (15 μg/ml) on bacterial aggregates: (A) and (C), effect of (3-nitro-4-thiocyanato-phenyl)-phenylmethanone on *M. smegmatis* and *M. luteus* aggregates, respectively; (B) and (D), effect of 3-nitro-4-thiocyanatobenzoic acid on *M. smegmatis* and *M. luteus* aggregates, respectively. The inhibitor concentration was 15 μg/ml.

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