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Influence of Culture Conditions and Virulence Plasmids on Expression of Immunoglobulin-Binding Proteins of *Yersinia pseudotuberculosis*

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Abstract—The influence of culture conditions and plasmids on immunoglobulin (Ig)-binding activity of two isogenic strains of *Yersinia pseudotuberculosis* (plasmid-free strain $48^{-}82^{-}$ and strain $48^{+}82^{+}$ bearing plasmids pYV48 and pVM82) was studied. The highest activity was observed in the bacteria grown on glucose-containing liquid medium in the stationary growth phase. The Ig-binding activity of the bacteria cultured on the liquid medium at pH 6.0 was about 1.5-fold higher than that of the bacteria grown at pH 7.2. Expression of the Ig-binding proteins (IBPs) was most influenced by temperature of cultivation. The IBP biosynthesis was activated in the bacteria grown at 4°C and markedly decreased in those grown at 37°C. The Ig-binding activity of lysates from the bacteria was caused by proteins with molecular weights of 7-20 kD. The activities of the plasmid-free and plasmid-bearing *Y. pseudotuberculosis* strains ($48^{-}82^{-}$ and $48^{+}82^{+}$, respectively) were analyzed, and the plasmids were shown to have no effect on the IBP expression and biosynthesis, which seemed to be determined by chromosomal genes.

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Key words: *Yersinia pseudotuberculosis*, immunoglobulin-binding proteins, culture conditions, plasmids

Pathogenic bacteria of the *Yersinia* genus (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) cause various diseases in humans and animals and are capable of withstanding the host's systems of nonspecific defense [1, 2]. Some bacterial proteins have been discovered that are considered to be pathogenicity factors of these bacteria. Adhesin A (Yad) and the outer membrane proteins (Yops) (their biosynthesis is encoded by the virulence plasmids pYV) can inhibit phagocytosis, opsonization, and complement activation [3, 4]. The pH6-antigen (PsaA) and invasins (Inv, Ail) encoded by chromosomal genes are associated with adhesion and invasiveness of *Yersinia* [5, 6]. Expression of the chromosomal and plasmid genes encoding the biosynthesis of pathogenicity factors of *Yersinia* depends on the environmental factors, especially on the temperature of growth [7, 8]. The proteins (pathogenicity factors of yersinias) are shown to be polyfunctional and capable of interacting with some proteins of mammals [9]. Adhesin (the pH6-antigen) from *Y. pestis* binds a 500-kD apolipoprotein from human and animal blood serum [10] and causes agglutination of erythrocytes [5]. This antigen is thought to produce adhesion pili of *Y. pestis* and form large oligomers consisting of subunits with molecular weights of 15-16 kD [5].

It is now established that in the *Yersinia* genus only the pH6-antigen from *Y. pestis* reacts with the Fc-subunits of human immunoglobulins of the G1, G2, and G3 subclasses [1, 11]. From *Y. pseudotuberculosis* we have isolated a high-molecular-weight immunoglobulin-binding protein (IBP) [12] and also a low-molecular-weight 14.3-kD IBP (for results of its investigation see Sidorin et al., pp. 1278-1283, this issue). These IBPs interacted with the Fc-fragments of human and animal immunoglobulins.

IBPs are heterogeneous proteins different in location in bacteria, molecular structure, and Ig-binding activity. These proteins are believed to be important pathogenicity factors of the bacteria. Their interaction with immunoglobulins is thought to withstand complement

Abbreviations: IBP) immunoglobulin-binding protein; IgG–HRP) immunoglobulin G conjugate with horseradish peroxidase.

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binding and decrease opsonization and phagocytosis of the bacteria, which finally allows the microorganisms to escape the influence of the host's immune system [13, 14].

The IBPs from the cell walls of gram-positive bacteria, such as group A staphylococci, C and G group streptococci, and peptococci are now rather well studied [14]. Some gram-negative bacteria are also found to have Ig-binding activity [15-17]. IBP expression has been shown to depend on conditions of cultivation, especially composition of the nutrient medium and its temperature and pH [15, 18]. Thus, protein A production by the gram-positive bacterium *Staphylococcus aureus* is strongly inhibited on cultivation on peptone and mannitol-containing salt agar [13]. To provide for the biosynthesis by *Y. pestis* of the pH6-antigen capable of binding with human IgG, special culture conditions are required: temperature of 37°C and pH < 6.5 [5, 11].

In the present work, the influence of culture conditions (growth phase of bacteria, temperature, and medium composition) and virulence plasmids on IBP expression in *Y. pseudotuberculosis* was studied.

MATERIALS AND METHODS

Bacterial cultures. Two isogenic strains of *Y. pseudotuberculosis* IB serovar constructed in Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences (Moscow) [19] were used: the plasmid-free strain $48^{-}82^{-}$ and the strain $48^{+}82^{+}$ bearing the plasmids pYV48 and pVM82. Some strains of pseudotuberculosis bacteria isolated from different sources were obtained from the Museum of the All-Russian Center of Yersinioses and Pseudotuberculosis (Institute of Epidemiology and Microbiology, Siberian Division, Russian Academy of Medical Sciences, Vladivostok). The microorganism strains were typical in their cultural, morphological, biochemical, and antigenic properties. The bacteria were cultured on nutrient agar (Makhachkala, Russia), nutrient broth (NB) (Makhachkala), NB supplemented with 0.5% glucose, and NB supplemented with 0.5% galactose. The cells were grown under aerobic conditions without intermixing for 6, 4, and 1 day at 4, 20, and 37°C, respectively, to the stationary growth phase.

Location of IBP in *Y. pseudotuberculosis* cells. The bacterial cells were washed thrice with 20 mM Tris-HCl buffer (pH 7.2) (buffer A) and precipitated by centrifugation. For transmission electron microscopy, the bacterial suspension was placed on a tungsten grid covered with a Formvar film substrate and treated with a blocking solution (20 mM Tris-HCl, 150 mM NaCl, 0.02% Tween-20 (pH 7.2)) for 30 min. The cells were incubated for 2 h with commercial rabbit IgG (50 μg/ml) labeled with colloidal gold in the blocking buffer supplemented with 0.02 M $CaCl_2$. The cells were washed off the conjugate with the blocking buffer thrice for 15 min. The excess moisture was removed with a filter paper. All operations were performed in a moist chamber. *Escherichia coli* cells strain M55 lacking Ig-binding ability were used as the control. The resulting preparations were viewed with a JEM-7A transmission electron microscope (Jeol, Japan) at the accelerating voltage of 80 kV.

Methods of determination of Ig-binding activity. Enzyme immunoassay (EIA), dot-analysis, and electroblotting were performed as described in [15-17]. The bacterial cells were washed thrice in buffer A and precipitated by centrifugation. The cell precipitate was suspended in buffer A, and concentrations of the different strain microorganisms were equalized based on the absorption at 610 nm. PolySorp polystyrene plates (Nunc, USA) and nitrocellulose membranes (Sartorius, Germany) were used for analysis as solid phase carriers. The bacteria were fixed from the volume on a plate for 16 h at 37°C, and Ig-binding activity was detected using commercial immunoglobulins conjugated with horseradish peroxidase (IgG–HRP). The binding was performed in buffer A supplemented with 0.25% Triton X-100 and 20 mM $CaCl_2$. Results of EIA were recorded with a μQuant spectrophotometer (Bio-Tek Instruments, Inc., USA) at 492 nm. As a chromogen, 0.04% solution of *o*-phenylenediamine was used. The *E. coli* strain M55 cells were used as a negative control. Each determination of the Ig-binding activity was performed in three replicates, and the findings were processed using Microsoft Excel statistical analysis standard packet, version 7.0.

To prepare lysates from different cultures of *Y. pseudotuberculosis*, equal weights of dry microbial mass (20 mg) were treated with 2% SDS solution for 2 h at 37°C. The cell residues were precipitated by centrifugation, and the supernatant passed through a filter with pores of 0.22 μm in diameter. The Ig-binding activities of the lysates were compared by electroblotting. Electrophoresis was performed by the Laemmli method [20] in 12-25% gradient SDS-polyacrylamide gels. All specimens for electrophoresis were prepared in the sample buffer without heating at 100°C. After the transfer onto nitrocellulose, the IBP was detected using commercial IgG–HRP.

RESULTS AND DISCUSSION

Intact *Y. pseudotuberculosis* and proteins of the bacterial outer membrane were earlier shown to have an ability to bind human and animal immunoglobulin molecules [12]. By electron microscopy, rabbit IgG labeled with colloidal gold was shown to interact with the external surface of the bacterial cell (Fig. 1). These data indicating the surface location of the Ig-binding receptors are consistent with our previous findings obtained with fluorescent IgG [12].

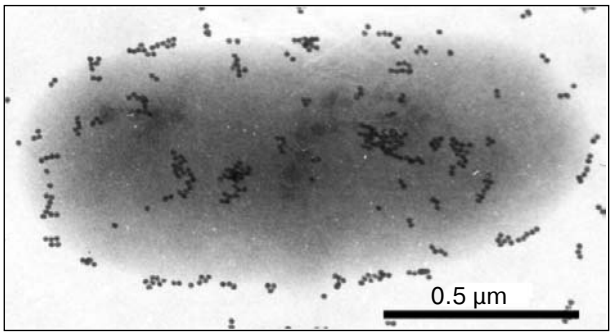

Fig. 1. Interaction of rabbit IgG labeled with colloidal gold with structures on the cell surface of *Y. pseudotuberculosis*.

Figure 1 shows that a rather large number of IgG molecules bind to the bacterium, but they are nonuniformly distributed on the cell surface. It should also be noted that not all *Y. pseudotuberculosis* cells even of the same population could bind IgG molecules (data not presented). A similar heterogeneity of cultures in the expression of immunoglobulin-binding receptors was earlier shown for some *E. coli* strains [15]. At present, it is not clear whether this is associated with the absence of the corresponding genes or caused by an insufficient expression of IBPs by the bacteria.

The influence of some external and internal factors on expression of the Ig-binding receptors in pseudotuberculosis bacteria was studied by EIA and dot-analysis. These approaches were successfully used to monitor IBP expression in some gram-positive and gram-negative bacteria [16-18]. For comparative studies on the Ig-binding activity of different strains of bacteria, it is important to adequately choose the microorganism concentrations and experimental conditions. The optimal dilution of *Y. pseudotuberculosis* cells was determined in preliminary experiments. It was shown that addition into the reaction medium of calcium ions (20 mM $CaCl_2$) increased threefold the immunoglobulin binding with *Y. pseudotuberculosis* cells.

The activity of bacterial pathogenicity factors, including the IBP, was earlier shown to depend on the cell growth phase [14, 21]. Analysis of IgG–HRP conjugate binding with pseudotuberculosis bacteria from cultures of different age revealed that the Ig-binding activity of the bacteria during the stationary growth phase was approximately 1.5-fold higher than during the logarithmic phase. Based on these data, in subsequent experiments *Y. pseudotuberculosis* cells in stationary growth were used.

The culture conditions and composition of the nutrient medium are known to significantly influence cell morphology, surface hydrophobicity, and chemical composition of the cell envelope of *Y. pseudotuberculosis* [22]. In our experiments, the binding efficiency with immunoglobulins significantly varied depending on cultivation conditions (solid or liquid medium) and the culture medium composition (Fig. 2). Figure 2 shows that the bacteria attached to agar bind with immunoglobulins less actively than the free-living bacteria. Addition into the liquid medium of carbohydrates (galactose and glucose) as carbon sources increased the expression of Ig-binding receptors. This effect was especially pronounced in cells grown on glucose-containing medium (Fig. 2, column *3*). The addition into the nutrient medium of glucose or galactose differently influenced the properties (morphology and invasiveness) of pseudotuberculosis bacteria. Glucose decreased and galactose increased the invasion of *Y. pseudotuberculosis* into the epithelial cells of mammals [22]. The presence of glucose in the nutrient medium of *Listeria monocytogenes* decreased the expression of proteins responsible for adhesion and invasion [23].

As mentioned, adhesin from *Y. pestis*, or pH6-antigen, can bind with the Fc-fragments of immunoglobulins, but for expression needs temperature of 37°C and pH < 6.5 [11]. It was of interest to investigate how these abiotic factors influence the Ig-binding activity of *Y. pestis*.

Cultivation of pseudotuberculosis bacteria at pH 6.0 increased the binding of the IgG–HRP conjugate as com-

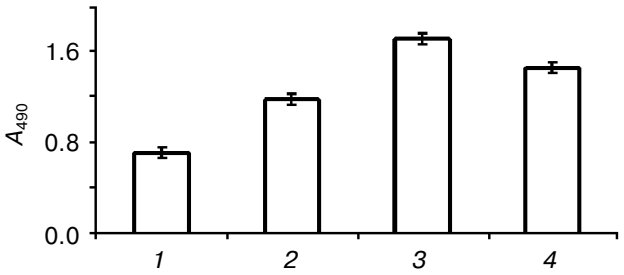

Fig. 2. Influence of culture conditions and nutrient medium composition on Ig-binding activity of *Y. pseudotuberculosis*. The cells were grown on nutrient agar (*1*), nutrient broth (*2*), nutrient broth supplemented with glucose (*3*), and nutrient broth supplemented with galactose (*4*).

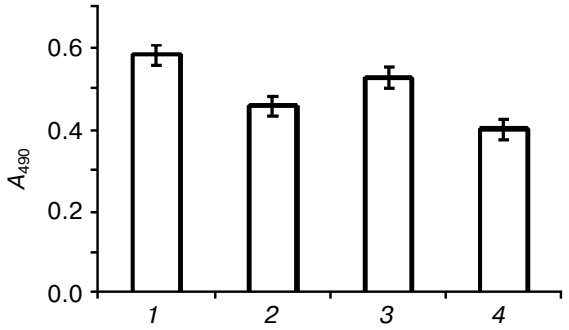

Fig. 3. Influence of medium pH on expression of Ig-binding factors of *Y. pseudotuberculosis* in cells (*1*, *2*) and into the culture medium (*3*, *4*). The bacteria were grown at 4°C and pH 6.0 (*1*, *3*) and 7.2 (*2*, *4*).

pared to its binding with the bacteria grown at pH 7.2 (Fig. 3, columns *1* and *2*), but the difference was not significant. Note, that no biosynthesis of the Fc-receptor (pH6-antigen) was observed in the *Y. pestis* [5, 11] cultured at pH 7.2. At pH values of 6.0 and 7.2, the Ig-binding factors were also expressed into the culture medium (Fig. 3, columns *3* and *4*).

The IBP biosynthesis was most strongly influenced by temperature of *Y. pseudotuberculosis* cultivation. The Ig-binding activity was high at low cultivation temperature (4°C) (Fig. 4). The binding activity was the lowest when the cells were grown at 37°C.

It is known that many yersinia pathogenicity factors, which are proteins of the bacterial outer membrane, are encoded by the virulence plasmid pYV48 and the plasmid pVM82. The Ig-binding activities of two isogenic strains of the pseudotuberculosis bacteria bearing the pYV48 and pVM82 plasmids and of the plasmid-free strain varied insignificantly. Consequently, the presence of the plasmid does not influence IBP expression (Fig. 4). It seems that the biosynthesis of these proteins is encoded by chromosomal genes. It should be noted that the temperature factor manifested itself for both isogenic strains, independently of the presence or absence of plasmids.

In addition to the isogenic strains, the ability to bind with immunoglobulins was also tested in five other strains of pseudotuberculosis bacteria. The Ig-binding activities of these strains also differed insignificantly. Thus, the ability to express the Ig-binding receptors on the surface seems to be a common feature of pseudotuberculosis bacteria.

In cell lysates from different cultures of *Y. pseudotuberculosis*, active proteins with molecular weights of 7-20 kD were detected by electroblotting (Fig. 5). The expression of these proteins strongly depended on temperature of cultivation, and at the growth temperature of 37°C no IBP could be detected in the blot (Fig. 5, lanes *3* and *6*). Cultivation at pH 6.0 increased the IBP content in the cell lysates as compared with that in the lysates from the bacteria grown at pH 7.2 (Fig. 5, lanes *1* and *2*). These regularities were observed for both the strain bearing the plasmids pYV48 and pVM82 and the plasmid-free strain (Fig. 5). Thus, these findings show a good correlation of the electroblotting results and EIA data.

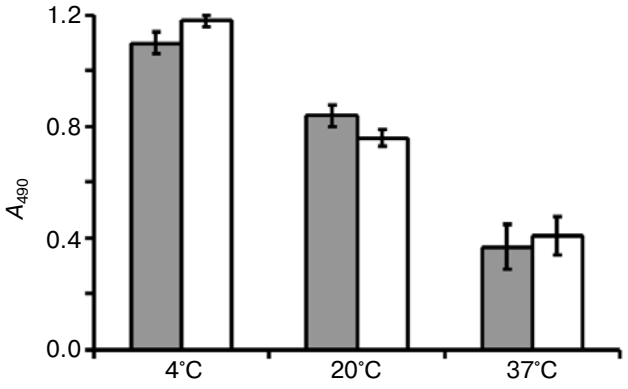

Fig. 4. Ig-binding activity of *Y. pseudotuberculosis* strains bearing plasmids pYV48 and pVM82 (dark columns) and of plasmid-free strain (light columns) grown at different temperatures.

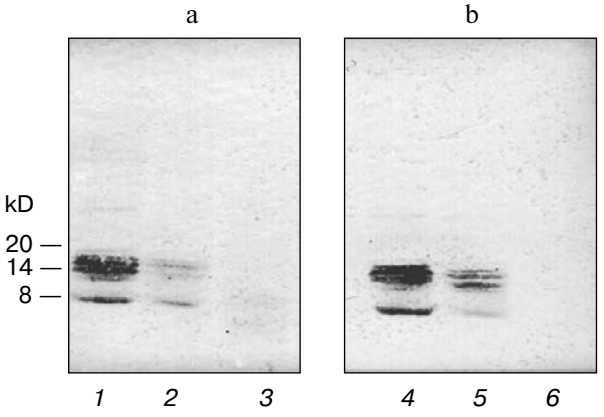

Fig. 5. Electroblot of cell lysates from different cultures of *Y. pseudotuberculosis*. a) Strain bearing the pYV48 and pVM82 plasmids grown at 4°C and pH 6.0 (*1*), 4°C and pH 7.2 (*2*), 37°C and pH 7.2 (*3*). b) Plasmid-free strain grown at 4°C and pH 6.0 (*4*), 4°C and pH 7.2 (*5*), 37°C and pH 7.2 (*6*). Molecular weights of the protein markers are shown on the left.

Thus, comparative analysis of the Ig-binding activity of *Y. pseudotuberculosis* cells cultured on media with different composition and at different temperatures has revealed a strong dependence of the IBP expression on growth conditions. The IBP synthesis most strongly depended on the temperature. Data on IBP expression at pH 6.0 (independence of the biosynthesis of the plasmid profile, the low molecular weight) suggest a similarity between the IBP biosynthesis in *Y. pseudotuberculosis* and the biosynthesis of the Ig-binding pH6-antigen in *Y. pestis*. However, the IBPs are expressed at the low temperature (4°C) and pH 7.2, whereas there are no such observations for the pH6-antigens of the plague and pseudotuberculosis yersinia. The IBP expression at the low temperature (4°C) is likely to contribute to the increase in virulence of the pseudotuberculosis bacteria cultured at decreased temperatures.

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