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Isolation and Characterization of Autoagglutination Factor of *Yersinia pestis* Hms⁻ Cells

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Abstract—An approach for isolation of an autoagglutination factor (AF) from Hms⁻ cells of the plague agent has been developed. Purified AF has been obtained and characterized in physicochemical properties. The AF is found to be a complex of a 17.5-kD protein with a low molecular weight peptide component, which binds iron ions and shows siderophore activity. This low molecular weight component is responsible for hydrophobic properties and immunochemical activity of the AF, as well as for its ability to interact with the plague diagnosticum L-413c bacteriophage.

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The ability of bacteria for autoagglutination (AA) manifesting itself in a spontaneous aggregation of cells in salt solutions and liquid nutrient media is usually associated with the presence of hydrophobic proteins on the cell surface. These proteins are responsible for the attachment of the bacteria to different surfaces, which can induce formation of biofilms, and this is a serious medical and industrial problem [1]. In various pathogenic bacteria, hydrophobic surface proteins play a significant role in their virulence, because they provide for attachment of the microorganisms to the host's cells and protect them against phagocytosis and/or complement [2-4]. Therefore, studies on hydrophobic surface components of bacteria are promising for interpretation of molecular mechanisms and prospects for control of such unfavorable phenomena as formation of biofilms and virulence of bacterial infection agents.

The ability for AA is also a characteristic feature of *Yersinia pestis*, which is an agent of plague, one of the most dangerous human diseases. So far a factor responsible for AA (AF) of the plague agent has not been identified. The AA of *Y. pestis* cells was supposed to be associated with hydrophobicity of their lipopolysaccharide lacking O-polysaccharide chains [5]. But other authors [6] thought AA to be associated with a pigmentation phenotype (Hms) characteristic of the wild type *Y. pestis* strains and displayed by the ability of cells to absorb at 26°C neutral dyes (hemin, Congo Red) from the medium. What surface components of the pigment-sorbing cells were responsible for AA was not established. We have found that both Hms⁺ and Hms⁻ strains of the plague agent display AA, but this property is determined in them by different mechanisms [7]. In the Hms⁺ cells, AA is not associated with hydrophobic interactions but is due to the presence on their surface of a pigment receptor (its isolation is described in [8]). In the Hms⁻ cells, AA is determined by hydrophobic interactions [7] and is associated with the presence on the cell surface of a protein with a supposed molecular weight of 17 kD [9]. The absence of this protein in AA-lacking mutants was accompanied by a decrease in their surface hydrophobicity and also the absence of sensitivity to the plague diagnostic L-413c bacteriophage and the ability to interact with the plague polyclonal immunoglobulin diagnosticum. Based on the available data, it is suggested that the hydrophobic 17-kD protein antigen, which could be a receptor of the L-413c bacteriophage, can be the AF of *Y. pestis* Hms⁻ cells.

The purpose of present work was to isolate this antigen and characterize its physicochemical properties.

Abbreviations: AA) autoagglutination; AF) autoagglutination factor; LMWF) low-molecular-weight fraction of the autoagglutination factor; PHAR) passive hemagglutination reaction.

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MATERIALS AND METHODS

Strains. *Yersinia pestis* EV76 strain (Phe⁻, Met⁻, Glp⁻, Hms⁻, Ybt⁻, Caf1⁺, Cad⁺, Pst⁺) was obtained from H. Mollaret (Pasteur Institute, France) in 1988 and stored in the culture collection of the Rostov-on-Don Plague Control Institute. The plasmid-free variant of *Y. pestis* EV76 strain not bearing the three plasmid replicons specific for the plague agent was obtained by successive elimination of plasmids detected by screening electrophoresis [10]. A clone with the spontaneously lost plasmid encoding a protein capsular antigen Caf1 was selected from the culture, which had been many times replated on nutritional media. This clone did not produce the Caf1 antigen detectable in the reaction of neutralization of antibodies to Caf1 [11] and had no *caf1* gene detectable by PCR [12]. The plasmid, which determined the calcium dependence of the cell growth at 37°C (Cad), was eliminated by growing the culture on a calcium-free medium at 37°C. To remove the plasmid encoding a bacteriocin pesticin (Pst), the cells were grown at 4°C. The absence of expression of the Cad and Pst was confirmed by methods described in the *Guidebook for Laboratory Diagnosis of Plague* [11].

Determination of immunochemical activity. The immunochemical activity of preparations was determined using a conventional micro method of passive hemagglutination reaction (PHAR) [11] with the plague erythrocyte immunoglobulin diagnosticum (Central Asian Antiplague Institute, Kazakhstan). This diagnosticum is tannin coated sheep erythrocytes sensitized by polyclonal serum to a preparation of a capsular substance, the so-called fraction 1 (F1) isolated from *Y. pestis* cells as described in [13]. The F1 preparation is known to contain the Caf1 antigen as a principal component, but it also contains unidentified antigens of *Y. pestis* [14-16]. Therefore, the plague immunoglobulin diagnosticum, in addition to the plasmid-encoded capsular antigen Caf1, also detects other antigens of the plague agent. And one of these antigens is the AF [9] characterized in the present work. To monitor the specificity of the AF reaction with the diagnosticum, a hyperimmune antiplague horse serum was used (Russian Antiplague Institute, Saratov).

Determination of phage-neutralizing activity. To determine the ability of AF to neutralize the plague diagnostic L-413c bacteriophage (Central Asian Antiplague Institute), pH of solutions was adjusted to 7.0. Preparations of the AF (0.5 ml) twofold different in concentration (beginning from 1 mg/ml) were added to 1 ml of the phage suspension (100 plaque forming units) and incubated for 30 min at 26°C. The phage suspension containing 0.5 ml of the solvent instead of the AF was used as the control. After the incubation, the specimens were centrifuged for 10 min at 10,000 rpm, and the supernatant together with the semi-liquid agar containing in 1 ml 10^8 microbial cells (m.c.) of the parental strain *Y. pestis* EV76 was poured onto Hottinger agar plates (pH 7.0). After growth for 48 h at 26°C, the plaque-forming units were counted in the experimental specimens and in the control. The retention of the plaque-forming units in the control and the inverse dependence of their number on the AF content in the experimental specimens were evaluated as an indicator of the phage neutralizing activity of the AF.

Physicochemical analysis of the AF. Protein content was determined by the Lowry method [17] with BSA as a standard. Neutral carbohydrates were determined by colorimetric method [18] using glucose as a standard. A low molecular weight fraction resulting after gel filtration of the heated AF preparation and dissolved in ethyl acetate was subjected to UV spectroscopy using a Shimadzu UV1601A spectrophotometer (Shimadzu, Japan). SDS-PAGE was performed by the Laemmli method [19], with staining with Coomassie R-250 (Sigma, USA) or silver [20]. Mass spectroscopy of the AF was performed using a Vision 2000 mass spectrometer (Vision, USA) by measuring the flight time of positive ions produced by laser desorption from the matrix (MALDI-TOF). The mass spectrum was obtained in 52 accumulation cycles in the linear regimen using a nitrogen laser with wavelength of 437 nm and pulse frequency of 3 Hz. The delay time of the analyzer was 1500 nsec, and voltage on the accelerator electrode was 20, on the accumulating electrode 16.5, and on the focusing lens 9.8 kV.

For reverse-phase HPLC, the AF preparation (30 μg AF dissolved in 10 μl of 0.1% TFA) was placed onto a Nucleosil C-4 column (150 × 4.6 mm) (Macherey-Nagel, USA). The AF was eluted with acetonitrile solution with a linear concentration gradient (0-60%) from solution A (0.1% TFA in water) to solution B (0.1% TFA in acetonitrile) at the rate of 1 ml/min within 30 min. Products of the separation were detected at 280 nm. The fractions obtained by chromatography were dried and dissolved in 10 mM NaOH for determination of the immunochemical activity and in 5.7 M HCl for analysis of the amino acid composition. The amino acid composition was determined with a Hitachi 835 amino acid analyzer (Hitachi, Japan) and the MultiChrom for Windows program (Ampersand Ltd., USA) for processing the results.

Gel filtration was performed on Biogel TSK-20 columns (150 × 4.6 mm) (LKB, Sweden), loaded with the AF preparation (100 μg) dissolved in 100 μl of 30 mM HCl and heated at 80°C for 5 min. The specimens were eluted with 50 mM acetic acid at the rate of 1 ml/min and detected at 220 nm.

Reverse-phase TLC was performed on silica gel plates with the C3 phase (Plasmokhrom RP3, Manometr, Russia). For ascending chromatography, 60% ethanol was used as the mobile phase. Chromatograms were developed by spraying with 0.2% ninhydrin in acetone with subsequent heating at 110°C for 10 min.

Analysis of siderophore activity. The siderophore activity of the AF was determined on an indicator medium [21] containing a chromogenic chelator of iron, chrome azurol S. At 30% saturation with iron, this chelator is green, and this color changes to yellow after removal of iron and to blue after the binding of additional iron ions.

To detect the siderophore activity of the low molecular weight fraction (LMWF) of the AF preparation obtained by gel filtration, 5 μl of LMWF solution in water and in 50 mM HCl (pH 1) were placed onto the indicating medium surface and incubated for 3 h at 26°C. As controls, 1 mM aqueous solution of the hydroxamate siderophore desferrioxamine (Desferal; CIBA-GEIGY, Switzerland), 50 mM HCl, and 1 mM solution of $FeCl_3$ in 50 mM HCl were used. The siderophore-containing specimens turned the indicator color to yellow, whereas the specimen containing free iron ions turned it to blue.

To reveal the siderophore activity of the LMWF, its aqueous solution was supplemented with HCl to the concentration of 50 mM, kept for 30 min, and then the specimen was subjected to chromatography on silica gel plates with the C3 phase. The siderophore Desferal was used as a control. After the chromatography, the plates were placed over the indicator medium and incubated for 24 h at 26°C. The siderophore activity of the preparations was manifested by appearance of yellow spots on the surface of the medium.

RESULTS

Isolation of AF. For isolation of the AF, bacterial mass of a plasmidless variant of the *Y. pestis* strain EV76 dried with acetone was used, but this method could be used for isolation of the AF from living cells of this strain. The strain displayed the ability for AA and did not express the plasmid-encoded surface antigens, including the capsular antigen Caf1, the principal antigen detected with the polyclonal immunoglobulin plague diagnosticum. The antigen associated with AA ability, reacting with the immunoglobulin plague diagnosticum and neutralizing the plague bacteriophage L-413c, could be washed off the cell surface with a weak alkaline solution (10 mM NaOH) [9]. Therefore, the immunochemical and phage-neutralizing activities of the preparations were used as tests on searching for an approach for isolation of the AF from the alkaline extract of undestroyed cells of *Y. pestis*. By gel filtration, the neutralized alkaline extract was shown to contain components with different molecular weights and possessing the immunochemical and phage-neutralizing activities. Thus, the antigen was heterogeneous in molecular weight, and, consequently, it was impossible to use gel filtration to isolate it.

For isolation of the AF, selective precipitation from the solution of components with the immunochemical and phage-neutralizing activities was used. As a result, the following stages were proposed for isolation of the AF from *Y. pestis* Hms^- cells:

– cultivation at 26°C on agar LB for 48 h;
– preparation of acetone-dried bacterial mass;
– double washing of the cells with saline;
– extraction of the cells with 10 mM NaOH;
– filtration of the extract through a filter with pores 0.22 μm in diameter;
– precipitation with neutralized 25% ammonium sulfate solution;
– dialysis of the precipitate against 10 mM NaOH;
– triple isoelectric precipitation at pH 4.6;
– precipitation with three volumes of cold acetone;
– lyophilization of the preparation.

The purification of the preparation from other proteins was monitored by SDS-PAGE (Fig. 1a). In the electrophoregram, the fractions at different stages of the purification presented multiple protein bands, which correspond in mobility to proteins with different molecular weight including high molecular weight proteins. This seemed to be caused by the presence in the preparation of different proteins with the same isoelectric point (4.6) and precipitated from the solution at the low concentration of ammonium sulfate (25%). However, after the treatment of the preparation with guanidine hydrochloride and precipitation with ethanol, only one intense band appeared on the electrophoregram, which corresponded by mobility to a 17-kD protein (Fig. 1b). The guanidine hydrochloride causing disappearance of the bands corresponding to high molecular weight proteins suggested that they could appear because of protein aggregation with involvement of hydrogen bonds. This seems to explain the retention of such bands after the heating of the preparations in lysis buffer containing 2-mercaptoethanol and SDS. The protein aggregation also manifested itself by formation in the preparation stored in solution at 4°C in solution for 3-5 days of a sediment, which was dissolved on addition of guanidine hydrochloride. Therefore, it was suggested that the preparation should contain only the 17-kD protein, and the heterogeneity in SDS-PAGE could be associated with the ability of the protein for autoaggregation and/or complexing with nonprotein components. Further studies on the preparation confirmed these hypotheses.

The presented scheme for preparation of AF allowed us to obtain 80-100 mg of freeze-dried AF from 1 g dry bacterial mass of the plasmidless variant of the *Y. pestis* EV76 strain. The preparation was capable of neutralizing the plague bacteriophage L-413c and at dose higher than 100 ng displayed immunochemical activity in the PHAR with the plague polyclonal immunoglobulin diagnosticum. The preparation increased the hydrophobicity and provided for AA of the cells of the earlier described [9] mutant of *Y. pestis* EV76 strain lacking AA. Thus, after incubation of the mutant (10^{10} m.c./ml) with the isolated

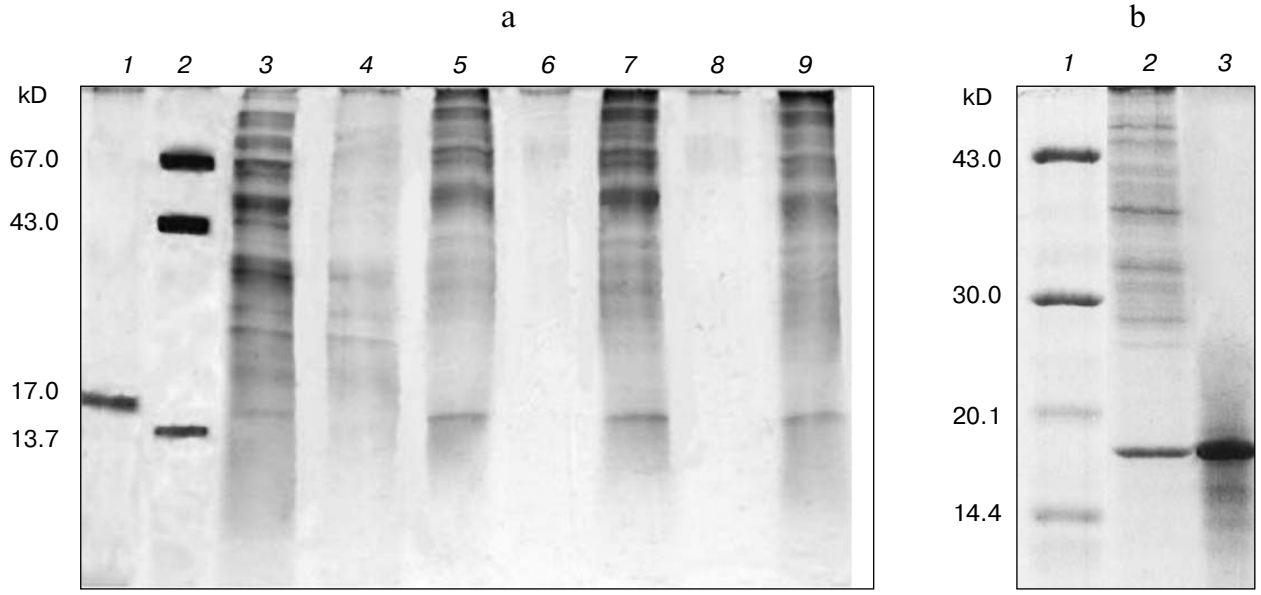

Fig. 1. Isolation of the AF from cells of the *Y. pestis* EV76 plasmidless variant. a) SDS-PAGE in 12.5% gel of proteins obtained at different stages of the AF purification. Staining with Coomassie R-250. *1*) The F1 preparation (5 μg) isolated from *Y. pestis* EV76; *2*) molecular weight marker proteins; *3*) cell extract in 10 mM NaOH; *4*) supernatant after precipitation of the extract with ammonium sulfate; *5*) precipitate after precipitation of the extract with ammonium sulfate; *6*) supernatant at pH 4.6; *7*) precipitate at pH 4.6; *8*) supernatant after precipitation with three volumes of acetone; *9*) preparation precipitated with three volumes of acetone. b) SDS-PAGE in 15% gel of the AF preparation after the treatment with guanidine hydrochloride. Staining with Coomassie R-250. *1*) Protein molecular weight markers; *2*) AF preparation, 50 μg; *3*) AF preparation after treatment with 6 M guanidine hydrochloride and precipitation with three volumes of acetone, 50 μg.

preparation (200 μg) at 26°C for 1 h the cells increased from 22 to 42% their surface hydrophobicity measured by the phase separation method as described in [9]. After the incubation, the mutant *Y. pestis* EV76 strain displayed AA in 0.25 M ammonium sulfate solution, similarly to the parental strain. Thus, these results indicate that the developed method provides AF preparation of the Hms⁻ cells of the plague agent.

Studies on physicochemical properties of AF. The AF preparation after the freeze-drying was insoluble in water, but could be dissolved in alkaline medium and manifested a clearly pronounced capability of foaming inherent in amphipathic compounds. On PAGE without SDS (data not presented), the preparation moved to the anode and gave multiple Coomassie-stainable bands, which indicated the negative charge of the protein.

Analyses by various physicochemical methods showed that the preparation also contained nonprotein components. Thus, it was found to contain 80% protein and 10% neutral carbohydrates. The presence of carbohydrates suggested that the AF could be a glycoprotein. But this is a subject requiring further study.

Notwithstanding the presence of many protein bands revealed in the preparation by SDS-PAGE (Fig. 1), the reverse-phase HPLC revealed only two fractions (Fig. 2a). They were immunochemically active in PHAR with the plague immunoglobulin diagnosticum and could neutralize the bacteriophage L-413c. Determination of the amino acid composition of the two fractions showed the presence of the same protein. Moreover, the presence in the preparation of many high molecular weight proteins was not supported by mass spectrometry (Fig. 2b). The maximal molecular weight recorded by mass spectrometry was 17.485 kD.

Gel filtration of the preheated preparation showed (Fig. 2c) that the AF, in addition to the protein, also contains a low molecular weight component noncovalently bound with the protein. By gel filtration two fractions were revealed in the preparation: a high molecular weight fraction containing a protein component with the mobility during SDS-PAGE at the level of a 17-kD protein, and a low molecular weight fraction lacking Coomassie-stainable substances. As judged by the peak areas in the chromatogram, the low molecular weight component was ~40%, but because its molecular absorption coefficients have not been determined its true content in the AF preparation is unknown.

The properties of the protein component were different from those of the initial preparation: the protein component was soluble in water, did not foam, and had no immunochemical activity and capability of neutralizing the plague bacteriophage L-413c. However, the LMWF, dried in air and dissolved in water, displayed the lost properties: thus, the LMWF resulting by gel filtration of the

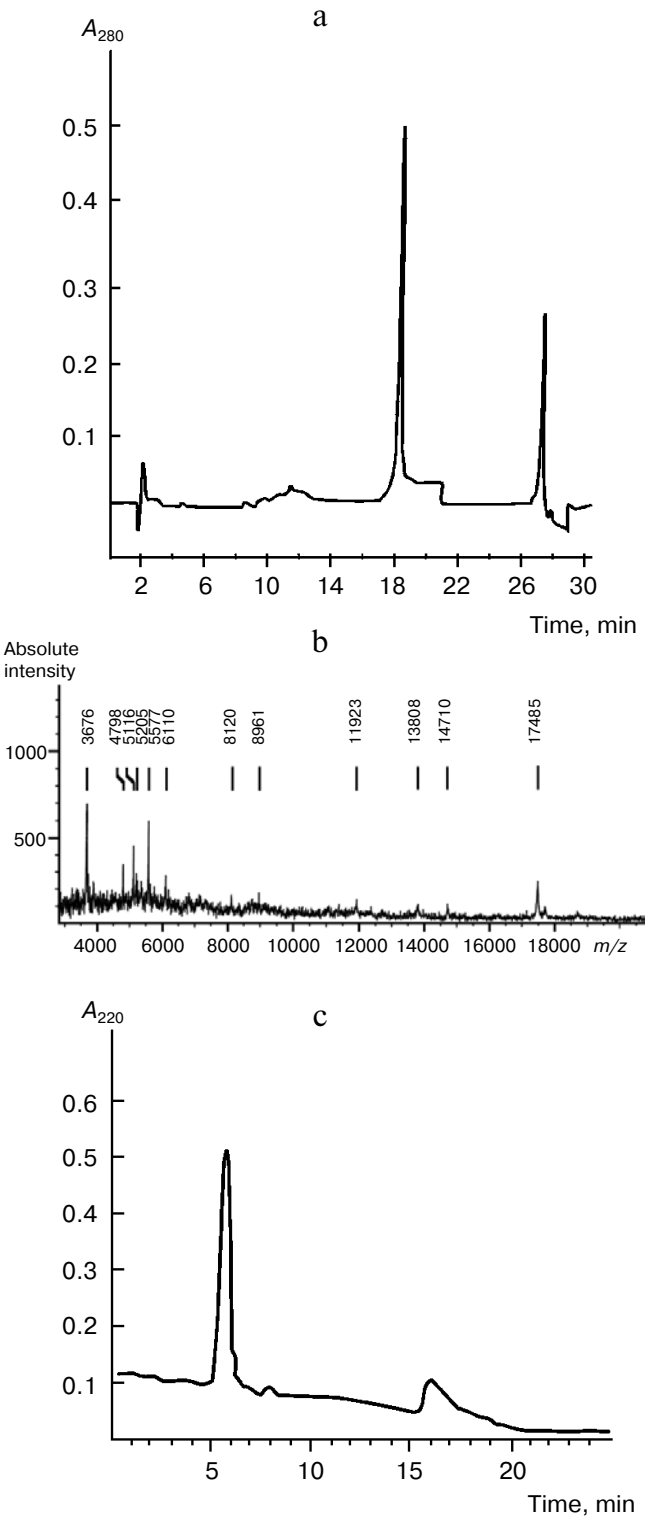

Fig. 2. Physicochemical characteristics of the AF preparation. a) Elution profile during HPLC on a C-4 Nucleosil ODS column with a linear acetonitrile gradient (0-60%) for 30 min. b) MALDI-TOF mass spectrum of the AF preparation. The spectrum is recorded in the linear positive ion mode. Scanning was performed at $m/z = (3\text{-}70)\cdot 10^3$. c) Elution profile during gel filtration of the AF preparation heated at 80°C for 5 min. The column contained TSK-20 Biogel and 50 mM acetic acid was the eluent.

preheated AF preparation (100 μg) and dissolved in 1 ml of water positively reacted in PHAR with the plague immunoglobulin diagnosticum in the titer of 1 : 32. The inhibition of PHAR after incubation of the LMWF solution with the antiplague hyperimmune horse serum indicated specificity of the reaction. The LMWF dissolved in water gave a positive reaction (titer 1 : 8) when tested for the presence of the phage-neutralizing activity (see "Materials and Methods"), and this reaction disappeared after incubation of the LMWF with the antiplague hyperimmune horse serum.

Based on the physicochemical analysis, hydrophobic properties, as well as the immunochemical and phage-neutralizing activities of the AF are found to be associated with the low molecular weight component noncovalently bound with the protein.

Analysis of the low molecular weight fraction of the AF preparation. The LMWF prepared by gel filtration of the AF and dried in air was a white-to-yellowish powder. Analysis of the LMWF solubility indicated the presence of amphipathic substances. The dry preparation of the LMWF was soluble in methanol, ethanol, ethyl acetate, and also in hot deionized distilled water. On cooling, aqueous solution of the LMWF formed a film on the surface and acquired a pronounced opalescence, possibly due to production of micelles.

By UV spectroscopy of the LMWF prepared by gel filtration from 100 μg of the AF and dissolved in 1 ml of ethyl acetate, four absorption maxima were revealed: at 246, 253, 259, and 270 nm (Fig. 3a). SDS-PAGE with subsequent silver staining (data not presented) revealed in the LMWF a band, which migrated markedly faster than the smallest of the marker peptides (2.6 kD). By TLC, aqueous solution of the LMWF was shown to have a component stainable with ninhydrin (Fig. 3b). The staining with ninhydrin suggested that the LMWF could contain substances of peptide nature. In fact, amino acid analysis revealed serine, glycine, alanine, valine, and hydroxylysine in the LMWF. These amino acids have no optical activity in the middle UV region; thus, the LMWF seems also to contain chromophores of unknown nature. The amino acids found in the LMWF occur in siderophores, which are low molecular weight peptide chelators of iron, different in structure and responsible for iron assimilation in bacteria [22-24]. Many siderophores, in addition to amino acid residues, also contain hydroxybenzoic acid residues optically active in the middle UV region.

On the suggestion that the LMWF could contain a siderophore, it was tested with the indicator medium used to detect the siderophore activity of the bacteria. In this test, no such activity was detected in the LMWF, which did not change the green color of the indicator within the observation period of five days. However, on lowering pH of the LMWF solution to 1, which promoted dissociation of the bound iron of some siderophores, free iron ions

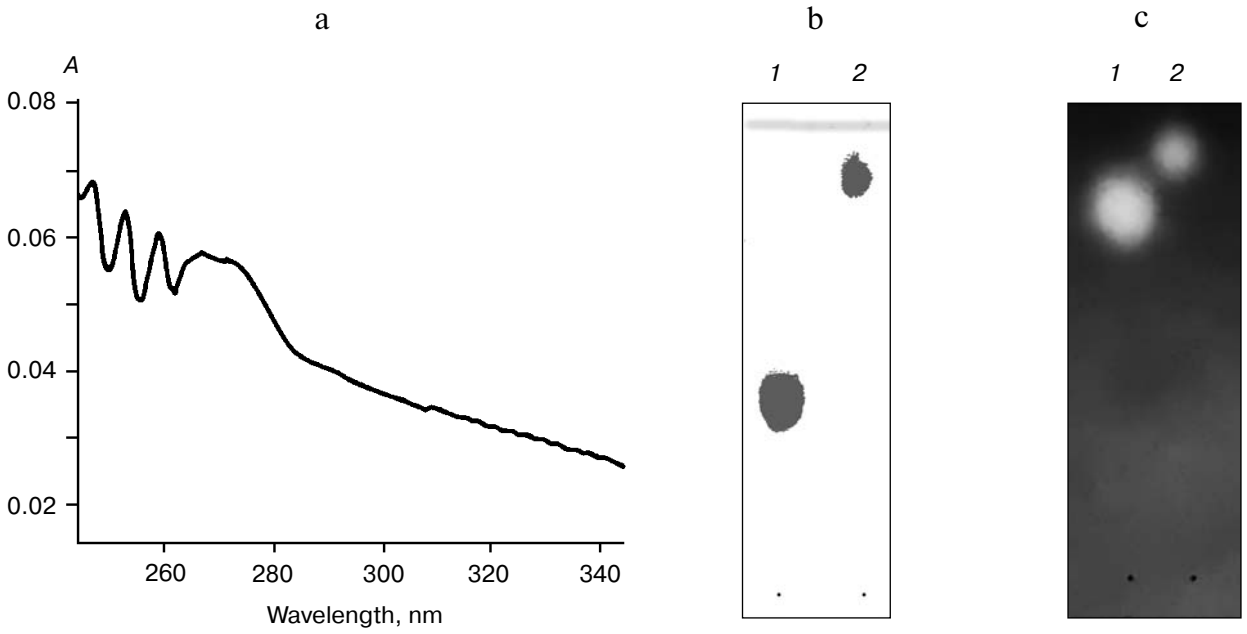

Fig. 3. Analysis of the low molecular weight fraction (LMWF) obtained after gel filtration of the AF preparation preheated at 80°C for 5 min. a) UV spectrum of the LMWF obtained after gel filtration of the preheated AF preparation (100 μg) dissolved in 1 ml of ethyl acetate. b) Thin-layer chromatogram on silica gel plates with the phase C3, elution with 60% ethanol, and development with ninhydrin. *1*) The LMWF dissolved in water; *2*) aqueous solution of the siderophore Desferal (10 μg). c) Thin-layer chromatogram blot on the indicator medium for determination of the siderophore activity. *1*) LMWF dissolved in 50 mM HCl; *2*) aqueous solution of the siderophore Desferal (1 μg).

were revealed, which turned the indicator to blue color. Note that the siderophore Desferal used in the test as a positive control lost the activity after the preliminary binding of an equimolar quantity of $FeCl_3$. After acidification of the solution, ferri-Desferal similarly to the LMWF stained the indicator blue.

We could reveal the siderophore activity of the LMWF only after TLC of the preparation pretreated with 50 mM HCl. After this treatment, a yellow-stained spot appeared on the blots of thin-layer chromatograms on the indicator medium (Fig. 3c) of both the LMWF and the control Desferal-containing preparation. These results suggest that the LMWF of the AF preparation could contain an iron-bound siderophore which could manifest activity only after dissociation of iron ions in acidic medium. But to prove this, further studies are needed directed for isolation of the siderophore and determination of its structure.

DISCUSSION

In this work, a method was elaborated for isolation of a factor responsible for AA of *Y. pestis* Hms⁻ cells, and physicochemical properties of the resulting preparation were characterized. It was really a preparation of this factor that was confirmed by its ability to enhance the surface hydrophobicity and provide for AA of *Y. pestis* EV76 mutants deficient in this character. Comparison of these mutants with the parental strain [9] allowed us to suggest that the AF of the Hms⁻ cells of the plague agent should be a 17-kD protein, which positively reacts with the plague immunoglobulin diagnosticum, and is able to neutralize the plague diagnostic bacteriophage L-413c. The present investigation of the AF isolated from the plague agent cells confirmed these suggestions.

AF interacted with the plague diagnosticum that indicated its being an antigen, antibodies to which are present in the plague diagnosticum preparation.

Comprehensive immunochemical characterization and diagnostic significance of the AF were subjects of our separate study, and its results will be published in another paper. The AF preparation displayed phage-neutralizing activity with respect to the bacteriophage L-413c. Therefore, the AF might be supposed to be a receptor of the L-413c phage, but to solve the problem of the role of AF in the phage reception additional studies are necessary. By different approaches, the AF preparation was shown to contain a 17.5-kD protein. This protein forms a complex with a low molecular weight component bound with iron ions and responsible for the main features of the AF (high hydrophobicity and immunochemical and phage-neutralizing activities).

The high yield of the AF (8-10%) on its isolation from the dry bacterial mass of the plasmidless variant of the *Y.*

pestis EV76 Hms$^-$ strain indicated its significant amount on the cells of this strain. Studies on different *Y. pestis* strains revealed the presence of large amounts of an AF-like antigen on the surface of the Hms$^-$ and Hms$^+$ strains grown at 26 and 37°C. The findings of the present work were compared with the earlier data on the pigment receptor isolated from *Y. pestis* Hms$^+$ cells [8], and the AF was shown to have some features similar to those of the pigment receptor. The two preparations contain the 17-kD protein, have the same p*I* values, and contain a protein-bound low molecular weight component. Moreover, both the AF and pigment receptor possess similar immunochemical and phage-neutralizing activities and contain bound iron. However, as discriminated from the pigment receptor, the AF does not bind pigments. The reason for this difference is a subject of a special investigation.

In the literature, we found no reports about the presence on the microbial cell surface of complexes similar to the AF characterized in this work. Nevertheless, the data obtained in 1989 [25] indicate the presence in the outer membrane of the plague agent cells of a protein presumably associated with iron storage. By two-dimensional electrophoresis of the outer membrane proteins of *Y. pestis* cells, the authors found a 17.9-kD protein and specified dependence of its p*I* value and amount on the presence of iron ions in the medium. In the presence of iron, this protein was ~11% of all proteins of the outer membrane and had p*I* 4.60-4.67. The resemblance of this protein and the AF studied in the present work in molecular weight, p*I*, and high content in the *Y. pestis* cells suggests their identity. The AF preparation also has much in common with the preparation of an iron-binding ferritin-like protein isolated from broken *Y. pestis* cells in [26]. Both proteins are negatively charged, can aggregate, and have similar subunit molecular weight (17.5-19 kD), contain bound iron, and are represented by heterogeneous bands after SDS-PAGE.

Our study has shown that the known features of the AF (high hydrophobicity, ability to bind iron, immunochemical activity with the plague immunoglobulin diagnosticum, and ability to interact with the plague bacteriophage L-413c) are associated with the low molecular weight component. At present, it is unclear whether this component is an individual substance or a mixture of several substances. Although the structure of this low molecular weight component is not yet determined, the data available so far allow us to hypothesize about its nature. The low molecular weight, peptide nature, amino acid composition specific for hydroxamate siderophores, optical activity in the middle UV region characteristic of phenolate siderophores, presence of iron ions, as well as the ability to extract iron from its complexes with chrome azurol S indicate that it contains a siderophore (siderophores) bound with iron.

It is commonly believed that bacteria synthesize siderophores only under conditions of iron insufficiency [24]. The presence of siderophores on the surface of cells grown under iron-rich conditions is now considered an unusual phenomenon in the world of microorganisms. This phenomenon is exemplified by the siderophore mycobactin of the tuberculosis agent, which is abundant in the cell wall of the mycobacteria. It is suggested [27] that mycobactin acts as an ionophore transporting iron across the hydrophobic wall into the bacteria. In work [24] mycobactin is considered to be responsible for short-term storage of iron for its transfer into the cell where it can be required for synthesis of iron-containing enzymes or for long-term storage within iron–ferritin complexes. The presence of siderophores on the surface of bacteria grown under iron-rich conditions is also supported by adsorption by *Escherichia coli* cells of antibodies to enterobactin, which is a widely distributed siderophore of enterobacteria [28]. At present, the physiological role of the siderophore immobilized in the cell wall of enterobacteria is unclear. It is suggested [28] that this phenomenon can determine the place of the bacteria inhabitance in the host organism. The presence of siderophore on the cell surface and finding of antibodies to enterobactin in normal blood sera of mammals allowed the authors to consider this phenomenon as an additional mechanism of the animal defense against enterobacteremia.

In pathogenic bacteria iron bound with the cell surface, in addition to formation of storages, which can be used by the cell under conditions of iron insufficiency, can also perform another biological function [29, 30]. The involvement of iron in reactions providing for generation of superoxides can promote damage to the host's cells by bacteria carrying the bound iron on their surface. Moreover, iron ions immobilized on the microbial cell surface and capable of preventing effects of the phagocyte-generated radicals can also protect the bacteria against phagocytosis.

No doubt, it is interesting to determine the role of AF in the physiology of the plague agent. So far the AF is known to be an abundant surface antigen endowing *Y. pestis* cells with hydrophobicity and responsible for their AA. Based on the findings of the present work, the AF is suggested to perform different functions. On one hand, due to its hydrophobicity, the AF can provide for the attachment of the *Y. pestis* cells to various hydrophobic surfaces, including hydrophobic membranes of phagocytes. In the Hms$^+$ strains of *Y. pestis* capable of producing an extracellular polysaccharide matrix, the attachment to the surface can initiate the formation of biofilms, which is considered to be important for plague transmission by fleas [31]. On the other hand, the ability to bind iron allows the AF to be involved in the storage and neutralization of the excess iron toxic for all living cells. Such stores can provide for the survival of the plague agent under iron-deficient conditions of the mammalian organism. Whether the AF really performs these functions, further studies will show.

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