ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 7, pp. 735-743. © Pleiades Publishing, Ltd., 2007.
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Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-090, June 3, 2007.

Cloning and Expression of Protective Antigens of *Mycobacterium tuberculosis* Ag85B and ESAT-6 in *Francisella tularensis* 15/10

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Received April 3, 2007

Abstract—The possibility of expression of genes encoding mycobacterial antigens in *Francisella tularensis* 15/10 vaccine strain cells has been shown for the first time. To obtain stable and effective expression of mycobacterial antigens in the *F. tularensis* cells, the plasmid vector pPMC1 and hybrid genes consisting of the leader part FL of the *F. tularensis* membrane protein FopA and structural moieties of the mature protein Ag85B or the fused protein Ag85B–ESAT-6 were constructed. Recombinant strains *F. tularensis* RVp17 and RVp18 expressing protective mycobacterial antigens in the fused proteins FL–Ag85B and FL–Ag85B–ESAT-6, respectively, were obtained. Expression of the protective mycobacterial antigens in *F. tularensis* was analyzed using specific antisera to the recombinant proteins Ag85-$(His)_6$ and ESAT-6-$(His)_6$ isolated from *Escherichia coli* producer strains created on the basis of the pET23b(+) and pET24b(+) vectors. The expression of heterologous protective antigens in *F. tularensis* 15/10 is promising for creation of live recombinant anti-tuberculosis vaccines on the basis of the tularemia vaccine strain.

DOI: 10.1134/S0006297907070073

Key words: expression, Ag85B, ESAT-6, *Mycobacterium tuberculosis*, *Francisella tularensis*

Every year about two million people die worldwide from tuberculosis [1]. A decreased immunity of some people to the tuberculosis agent *Mycobacterium tuberculosis* and emergence of multi-antibiotic resistant clinical strains of *M. tuberculosis* make it urgent to improve the strategy of diagnosis, treatment, and prevention of tuberculosis infection. The search for key antigens of *M. tuberculosis* required for the development of immunity against tuberculosis is a current problem in the elaboration of modern vaccines and diagnostics. Among tuberculosis antigens, extracellular proteins of the *M. tuberculosis* antigen complex 85 (Ag85) and the protein family ESAT-6 are especially interesting [2].

Antigen 85B (Ag85B, MPT59, molecular weight 30 kD) is one of three mycolyl transferases of the Ag85 complex which can be detected rather early in the filtrate of *M. tuberculosis* culture. The mycolyl transferase activity is necessary for maintaining the integrity of the bacterial cell wall, but the role of the complex in pathogenesis of tuberculosis is not clear. Immunization of mice and guinea pigs with the Ag85B protein with adjuvant formed partial protective immunity against tuberculosis [3, 4].

The other mycobacterial protein ESAT-6 (molecular weight 6 kD), which is also secreted during the early stage of the mycobacterial growth, contains epitopes recognizable by protective T-cells in experimental tuberculosis. This protein was detected on comparing the genomes of the virulent strain *M. tuberculosis* H37Rv and vaccine strain *M. bovis* BCG [5, 6]. ESAT-6 is an immune dominant protein because it is recognized by sera of the majority of patients with tuberculosis [7].

Prospects of the Ag85B and ESAT-6 proteins as constituents of subunit and DNA-vaccines as individual substances or fused proteins are estimated by many researchers [8-13]. The crucial problem in creation of live recombinant vaccines is expression of a heterologous antigen in the selected bacterial vector, which acts as an adjuvant providing for the adequate presentation of protective antigens to the immune system. Live attenuated strains of *M. tuberculosis* and *Salmonella* are most often

Abbreviations: BS) buffered saline; DTT) dithiothreitol; IPTG) isopropyl-β-D-thiogalactopyranoside.

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used as carriers of heterologous protective antigens; closely related mycobacteria, some viruses, and some other microorganisms are also used [8, 14-17].

Live tularemia vaccine on the basis of the vaccine strain *F. tularensis* 15 was prepared in Russia in the middle of the XX century. This vaccine mainly induces the cellular component of the immune system and ensures a lasting expressing immunity along with moderate reactogenicity [18]. Overcoming the interspecies barrier for expression of mycobacterial antigens in the vaccinating strain of the tularemia microbe is promising to enlarge the list of bacterial vectors for creation a live recombinant vaccine against tuberculosis, more completely understand mechanisms of protective immunity formation, and investigate factors which influence this process.

The purpose of the present work was to construct recombinant genes encoding the Ag85B antigen and the hybrid antigen Ag85B–ESAT-6 and study their expression in the vaccine strain *F. tularensis* 15/10. The data would allow us to develop methods for constructing recombinant vaccine strains on the basis of *F. tularensis* 15/10 and use it for creation of live tuberculosis recombinant vaccines.

MATERIALS AND METHODS

Bacterial strains and plasmids. The expressing vectors pET23b(+) and pET24b(+) and the plasmid pBlu2SKM (Novagen, USA) were used. The strains *F. tularensis* 15/10, *M. tuberculosis* H37Rv, *M. bovis* BCG, *E. coli* BL21(DE3), and *E. coli* JM83, the plasmids pFNL10, pC194, and pUC57 were obtained from the Live Culture Museum, State Research Center of Applied Microbiology and Biotechnology (Obolensk, Moscow Region).

Culture conditions. The *E. coli* BL21pET23b(+)85B and *E. coli* BL21pET24b(+)ESAT-6 cells were incubated with stirring overnight in LB medium in the presence of ampicillin (100 μg/ml) and kanamycin (40 μg/ml), respectively, at 37°C and then re-inoculated into fresh medium. Upon cultivation for 2 h, synthesis of cloned proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 2 mM, and the incubation was continued for 2.5 h.

The *M. bovis* BCG strain was cultured on glycerol–albumin medium supplemented with bovine serum albumin to 0.1% without Tween 20 for 20 days at 37°C in an atmosphere with 0.5% CO_2 until OD_{600} ~0.5.

The *F. tularensis* strains were cultured at 37°C on a solid erythritol–agar medium supplemented with bovine dry blood (State Research Center of Applied Microbiology and Biotechnology, Obolensk) and polymyxin to 50 μg per ml of the medium and chloramphenicol to 3 μg per ml of the medium.

Gene engineering approaches. Isolation of DNA and transformation of the *E. coli* cells were performed as described in the guidebook [19]. The *F. tularensis* cells were transformed with plasmid DNA by electroporation [20]. Restriction, ligation, and isolation of fragments from agarose gel were performed as described in [19] and according to the producers' instructions.

Primers. The following primers were synthesized by a Sintol Biotechnology Company (Russia):

RepB-Pst, 5′-GAAGCTGCAGCAGTCAACTACTTA-GGCAG-3′;

RepB-Sph, 5′-GAGTTAAGTTATGAAAGTTCTTACT-GGCATGC-3′;

Cm-Nhe, 5′-AAGCTAGCTCGAGAATAACTTAAGG-GTAACTAGCC-3′;

Cm-Xho, 5′-CCGACTCGAGAAAGTACAGTCGGC-ATTATC-3′;

groES-Xho, 5′-CCCCCTCGAGTGTATGGATTAGTC-GAGC-3′;

groES-Sac, 5′-AAACGAGCTCATCATTTTTCTCCTT-CTGCACGACG-3′;

E85-Nde, 5′-GATCATATGTTCTCCCGTCCGGGTC-TG-3′;

85-Bam, 5′-GGTGGATCCTGACAGCCTGCGCC-3′;

E85CF, 5′-GTCGCAAACAACACTCGTCTGTGGG-3′;

E85CR, 5′-CCGCAATAAACCCACAGACGAGTGTT-G-3′;

EES-Nde, 5′-GGACATATGACAGACCAGCAGTGG-AAT-3′;

EES-Xho, 5′-TTTCTCGAGTCCGAACATCCCAGTC-AC-3′;

FL-ATG, 5′-ATGAGATTAAAAAGTATTGTTATAGC-3′;

FL-H3, 5′-ATTAGTTAAGCTTGCAGCGATAGATGC-3′;

F85-H3, 5′-GATCAAAGCTTCTCCCGTCCGGGT-CTG-3′;

F85-Bam, 5′-GCTCGAATTCGGATCCTGTCAGCC-TG-3′;

F85ES-Bam, 5′-ATGGATCCTGCGCCTAACG-3′;

FES-Bgl, 5′-TCTAGATCTATGACCGAGCAGCAGT-GG-3′;

FES-Bam, 5′-TATGGATCCTTATGCGAACATACCA-GTGAC-3′.

Creation of plasmids for expression of the recombinant proteins Ag85B-$(His)_6$ and ESAT-6-$(His)_6$ in *E. coli*. Two fragments of the gene *fbpB* encoding the Ag85B protein gene were obtained by PCR using two primer pairs E85-Nde/E85CR and E85CF/E85-Bam and DNA of the *M. tuberculosis* H37Rv strain as a template. The fragments were purified and used as a template for PCR with the primers E85-Nde and E85-Bam. The PCR product with the recombinant gene *85b* and the pET23b(+) vector were hydrolyzed with restrictases NdeI and BamHI, mixed, and treated with DNA ligase. The resulting recombinant plasmid pET-85B was transformed in *E. coli* BL21(DE3). The selection and analysis of the nucleotide sequence of the fragment cloned in the pET23b(+) vector resulted in the *E. coli* BL21(pET23-85B) strain.

The *esat-6* gene was cloned using the primer pair EES-Nde/EES-Xho and DNA of the *M. tuberculosis* H37Rv strain as a template. By PCR, a product was obtained with the recombinant *esat-6* gene. The PCR product and the vector plasmid pET24b(+) (Novagen) were hydrolyzed with restrictases NdeI and XhoI. The restricts were ligated using DNA ligase, and the resulting recombinant plasmid pET24b(+)-ESAT-6 was transformed in the *E. coli* BL21(DE3) cells. The selection and analysis of the nucleotide sequence of the fragment cloned in pET24b(+) resulted in the *E. coli* BL21(pET24-ES) strain.

Construction of the plasmid vector pPMC1. The plasmid pFNL10 DNA was hydrolyzed with restrictases SphI and NheI, and upon separation of the fragments by electrophoresis in agarose gel a 2361-bp fragment was isolated which contained the *RepA* gene. Then, using the primer pair RepB-Pst/RepB-Sph and the plasmid pFNL10 DNA as a template, an amplicon was prepared with the *RepB* gene of the pFNL10 plasmid of 537 bp size, which was flanked with the PstI and SphI sites. The resulting PCR product RepB and the plasmid pUC57 DNA were treated with restrictases PstI and SphI, mixed, and ligated with DNA ligase. The resulting ligate was used to transform the *E. coli* JM83 cells. The clones were analyzed by PCR using the RepB-Pst and RepB-Sph primers, and the *E. coli* JM83 clone with the pUC57-RepB plasmid was selected. The absence of errors in the nucleotide sequence of the *RepB* gene was checked by sequencing of the cloned fragment. The plasmid DNA isolated from the *E. coli* JM83(pUC57-RepB) strain was treated with restrictases SphI and SacI, and upon separation of the fragments by electrophoresis in agarose gel a fragment RepB-SphI–SacI of 552 bp size was isolated. An amplicon containing the *cat* gene of the plasmid pC194 of 1013 bp size and flanked with NheI and XhoI sites was prepared using the primer pair Cm-NheI/Cm-XhoI and the pC194 plasmid DNA as a template. The resulting amplicon was purified by electrophoresis in agarose gel and then treated with restrictases NheI and XhoI. An amplicon containing the groES promoter of *F. tularensis* of 367 bp size flanked with the XhoI and SacI sites was prepared using the primer pair groES-Xho/groES-Sac and *F. tularensis* 15/10 DNA as a template. The resulting amplicon and the pBlu2SKM plasmid DNA were treated with restrictases SacI and XhoI, mixed, and ligated using DNA ligase. With the resulting ligate, the *E. coli* JM83 cells were transformed. Upon selecting the clone by PCR with the primers groES-Xho and groES-Sac the pBluescript II SK-groES plasmid DNA was isolated. The absence of errors in the nucleotide sequence of the groES promoter was checked by sequencing of the cloned fragment. The pBluescript II SK-groES was treated with restrictases SphI and SacI, the fragments were separated by electrophoresis, and a fragment groES-XhoI–SacI of 357 bp size was isolated. DNA fragments RepA-SphI–NheI, cat-NheI–XhoI, groES-XhoI–SacI, and RepB-SphI–SacI were mixed and ligated using DNA ligase. With the resulting ligate the *F. tularensis* 15/10 cells were transformed by electroporation [20]. Clones containing the vector plasmid pPMC1 were selected on chloramphenicol-containing medium.

Construction of the fused protein FL–85B gene. A DNA fragment with the *fbpB* gene was obtained by PCR with the primer pair F85-H3/F85-Bam and the plasmid pET-85B DNA as a template. The resulting amplicon was purified by electrophoresis in agarose gel and then treated with restrictases HindIII and BamHI. The PCR product containing the sequence encoding the FL leader peptide of the FopA protein was obtained using the primer pair FL-ATG/FL-H3 and DNA of the *F. tularensis* 15/10 strain as a template. The DNA preparation was purified by electrophoresis in agarose gel and then treated with restrictase HindIII.

The pPMC1 vector was successively treated with restrictase SacI, T4-DNA polymerase, and then restrictase BamHI.

The three DNA fragments were ligated using DNA ligase. The resulting plasmid pPMC-FL–85B was transformed in the *F. tularensis* 15/10 cells by electroporation. Upon selection of the plasmid-containing clones, PCR analysis of the recombinant plasmids containing the FL-ATG and F85-Bam primers, and analysis of the nucleotide sequence of the DNA fragment cloned into pPMC1, the strain *F. tularensis* RVp17 was obtained.

Construction of the fused protein FL–85B–ESAT-6 gene. Using DNA of the *M. tuberculosis* H37Rv strain as a template and the primer pair FES-Bg1/FES-Bam, a PCR product ES-BglII-BamHI with the *esat-6* gene was obtained by PCR. The resulting amplicon was cleaved with restrictases BglII and BamHI. The PCR product FL–Ag85B-BamHI containing the *fl-fbpB* gene was obtained using the primer pair FL-ATG and F85ES-Bam and the plasmid pPMC1-FL–85B DNA as a template.

The resulting amplicon was purified by electrophoresis in agarose gel and then treated with restrictase BamHI.

The pPMC1 vector was successively treated with SacI, T4-DNA polymerase, and then BamHI. The DNA fragments of ESAT-6-BglII–BamHI, FL-Ag85B–BamHI, and pPMC1-SacI-BamHI were co-precipitated with ethanol and ligated. DNA of the resulting plasmid pPMC1-FL–85B–ESAT-6 was transformed in the *F. tularensis* 15/10 cells by electroporation. Upon selection of the plasmid-containing clones, PCR analysis of the recombinant plasmids with the FL-ATG and FES-Bam primers, and analysis of the nucleotide sequence of the DNA fragment cloned into pPMC1, the *F. tularensis* RVp18 strain was obtained.

Expression and purification of the proteins Ag85B-$(His)_6$ and ESAT-6-$(His)_6$. The *E. coli* BL21(pET23-85B) was centrifuged at 6000 rpm for 20 min at 4°C, and the bacterial cells were frozen. Then the cell precipitate was suspended in 20 mM Tris-HCl buffer (pH 7.9) supplemented with lysozyme (1 mg/ml), and after incubation for 1 h at room temperature DNase was added to 10 μg/ml and $MgSO_4$ to 5 mM. The suspension was incubated until the high viscosity disappeared. Then the inclusion bodies were washed twice in 20 mM Tris-HCl buffer (pH 7.9) supplemented with 2 M urea. The resulting precipitate was for 1 h dissolved in 20 mM Tris-HCl buffer (pH 7.9) supplemented with 20 mM dithiothreitol (DTT) and 6 mM guanidine chloride. Supernatant resulting by ultracentrifugation at 23,000 rpm for 1 h was filtered through a 0.45-μm membrane filter and applied onto a chelating carrier Ni^{2+}NTAHisBind® Superflow™. Chromatography was performed according to the modified protocol of the producer (Novagen). Upon absorption of the protein and washing of the column with 20 mM Tris-HCl buffer (pH 7.9) supplemented with 5 mM imidazole and 8 M urea, the recombinant protein was eluted with linear gradient of imidazole (5 mM-0.5 M). The resulting fractions were analyzed by electrophoresis in 12.5% SDS-polyacrylamide gel. The fractions containing the Ag85B-$(His)_6$ protein were combined and renatured by dialysis against 20 mM Tris-HCl buffer (pH 7.2) in the presence of 5 mM DTT.

The ESAT-6-$(His)_6$ protein was isolated as follows: precipitate of the *E. coli* BL21(pET24-ES) cells was suspended in two volumes of buffered saline (BS, pH 7.4) and supplemented with nine volumes of 20 mM sodium phosphate lysing buffer supplemented with 0.5 M NaCl and 7 M guanidine chloride (pH 7.8). The resulting mixture was treated twice for 5 sec on a CP501 ultrasonic cell disintegrator (Cole-Parmer, USA). The suspension was clarified by centrifugation at 12,000 rpm for 5 min before loading onto a column with Ni^{2+}NTAHisBind® Superflow™ sorbent. The column was pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.8) supplemented with 0.5 M NaCl and 8 M urea. After the sample had been loaded, the column was washed with the same buffer, and the protein was eluted by a stepwise decreasing in the buffer pH to 6.0, 5.3, 4.0, 3.5, and 3.0. The resulting fractions were analyzed by electrophoresis in 15% SDS-polyacrylamide gel. The fractions containing the ESAT-6-$(His)_6$ protein were combined and stored in buffer containing 20 mM sodium phosphate, 0.5 M NaCl, 2 M urea (pH 6.0) at 10°C. The protein amount was determined by the Lowry method with bovine serum albumin as a standard [21].

Preparation of monospecific polyclonal mouse antisera to the recombinant proteins Ag85B-$(His)_6$ and ESAT-6-$(His)_6$. The protein 85B-$(His)_6$ (20 μg) in the volume of 0.1 ml with Freund's complete adjuvant at the ratio of 1 : 1 was injected subcutaneously into white outbreed mice (Swiss Webster CFW, Pushchino nursery). Twenty-one days later the mice were treated with 20 μg protein with Freund's incomplete adjuvant at the ratio of 1 : 1. The immune serum was collected seven days after the second immunization. Titers of specific antibodies were determined in the animals' sera by a solid phase enzyme immunoassay, with the protein Ag85B-$(His)_6$ as an adsorbed antigen.

The protein ESAT-6-$(His)_6$ (30 μg) in the volume of 0.1 ml in the mixture with Freund's complete adjuvant at the ratio of 1 : 1 was injected subcutaneously into white outbreed mice (Swiss Webster CFW, Pushchino nursery). The second and third immunizations with 30 μg protein with Freund's incomplete adjuvant at the ratio of 1 : 1 were performed at the interval of 14 days. The immune serum was collected 14 days after the third immunization. Titers of specific antibodies were determined in the animals' sera by a solid phase enzyme immunoassay, with the protein ESAT-6-$(His)_6$ as an adsorbed antigen.

Concentration of culture fluid from the strain *M. bovis* BCG. The bacterial cells were precipitated by centrifugation at 10,000 rpm for 30 min, and the supernatant was supplemented with Merthiolate to the final concentration of 0.05% and kept for 24 h at room temperature. Then the supernatant proteins were salted out by addition of $(NH_4)_2SO_4$ to the final concentration of 60%. The precipitate resulting by centrifugation at 6000 rpm for 30 min was dissolved in 20 mM Tris-HCl buffer (pH 7.2) and dialyzed overnight against the same buffer. After the dialysis, the solution was additionally clarified by centrifugation at 6000 rpm for 30 min.

Electrophoresis in SDS-polyacrylamide gel. The proteins were separated by electrophoresis in 12.5% or 15% SDS-polyacrylamide gel by the Laemmli method [22]. Samples of the recombinant strains were prepared for electrophoresis as follows: the bacterial cells were suspended in BS; suspension containing $5 \cdot 10^9$ cell/ml was centrifuged at 12,000 rpm for 5 min, the precipitate was resuspended in 50 μl of BS, with 50 μl of buffer as a sample-buffer, and boiled for 5 min. The samples were clarified by centrifugation under the same conditions, and 10-μl samples were placed onto the gel.

Western blotting. From the acrylamide gel, the proteins were transferred for 30 min onto a Hybond P membrane (PVDF; Amersham, Great Britain) in a semi-dry blotter system at the current of 150 mA. The membrane was blocked with 1% solution of bovine serum albumin for 30 min and incubated for 1 h with operating dilutions of polyclonal mouse sera to the proteins Ag85B-$(His)_6$ and ESAT-6-$(His)_6$ at 37°C. Antigen–antibody complex was detected using sheep antimouse antibodies conjugated with horseradish peroxidase (Amersham, USA) in the operating dilution. The complex was incubated with the conjugate for 1 h at 37°C. After washing, the membrane was stained in solution of a horseradish peroxidase substrate, 3,3′-diaminobenzidine tetrahydrochloride.

Enzyme immunoassay. Titers of antibodies to Ag85B-$(His)_6$ and ESAT-6-$(His)_6$ in the mouse sera were determined by solid phase enzyme immunoassay, with the adsorbed protein Ag85B-$(His)_6$ or ESAT-6-$(His)_6$ in the concentration of 1 μg/ml. Specimens of the sera were initially diluted 1 : 20 and then titrated at the step of 1 : 2. The sera were incubated with the antigens for 1 h at 37°C. Antigen–antibody complex was detected using sheep antimouse antibodies conjugated with horseradish peroxidase (Amersham, Great Britain) in operating dilutions. The complex was incubated with the conjugate for 1 h at 37°C. *o*-Phenylenediamine was used as a substrate for horseradish peroxidase. The reaction was stopped with 0.1 M HCl. Absorption in the wells of the plate was determined at 492 nm with a Multiscan apparatus (Labsystem, Finland).

RESULTS

Expression of the Ag85B-$(His)_6$ protein in *E. coli*. When creating the hybrid gene encoding the mature protein Ag85B, to increase the protein synthesis in the *E. coli* cells we substituted nucleotides by PCR-mutagenesis in the rare for *E. coli* codons of five amino acids: Arg43 (cgg by act), Gly45 (ggg by ggt), Thr245 (acc by acg), Arg246 (cgg by cgt), and Leu247 (cta by ctg). Insertion of the structural moiety of the gene encoding the mature protein Ag85B into the plasmid vector pET23b(+) resulted in a hybrid gene encoding a protein with the calculated molecular weight of 33,428 daltons (308 amino acids), which consisted of 17 amino acids from the pET23b(+) vector, 285 amino acids from the mature protein Ag85B, and a sequence of six His on the C-end of the molecule. By SDS-PAGE of the cell lysate of *E. coli* BL21(pET-85B) culture after IPTG induction, the major band was revealed of a protein with the molecular weight of 32.7 ± 0.6 kD, which corresponded to the recombinant protein Ag85B-$(His)_6$. The protein Ag85B-$(His)_6$ was in the *E. coli* cells as inclusion bodies, and its relative amount in the cells was ~60% of the total protein (data not presented).

Purification of the recombinant protein Ag85B-$(His)_6$. The sequence of six His on the C-end of the molecule allowed us to purify the recombinant protein Ag85B-$(His)_6$ by affinity chromatography. By washing the inclusion bodies, dissolving them in guanidine chloride, and affinity chromatography on Ni^{2+}NTAHisBind® Superflow™, an electrophoretically homogenous protein preparation was obtained of about 80% purity (Fig. 1a, lane *2*). By SDS-PAGE, a band was revealed which corresponded to the protein with the apparent molecular weight of 32.7 ± 0.6 kD, close to the calculated value of 33,428 daltons. For renaturation of the protein, the preparation was dialyzed against 20 mM Tris-HCl buffer (pH 7.2).

Expression of the protein ESAT-6-$(His)_6$. The gene encoding the mature protein ESAT-6 was synthesized by PCR. Insertion of the product into the plasmid vector pET24b(+) resulted in a hybrid gene encoding the protein with the calculated molecular weight of 10,940 daltons (103 amino acids) which consisted of 95 amino acids of the mature protein ESAT-6, two additional amino acids Leu and Glu, and a sequence of six His on the C-

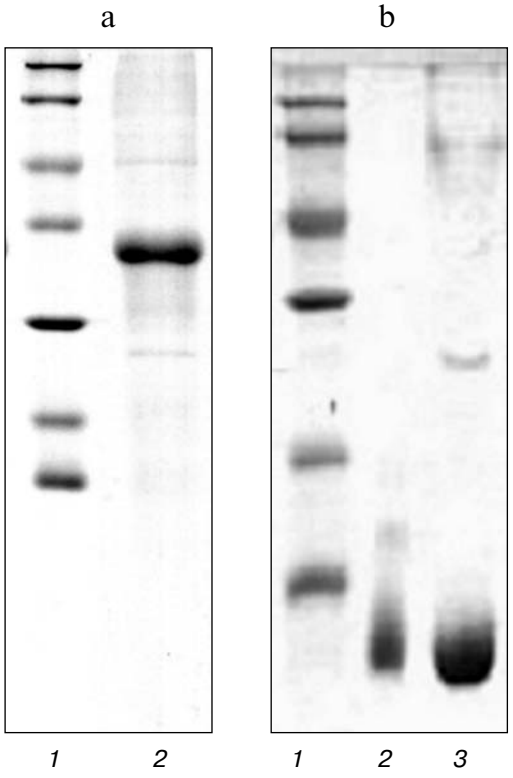

Fig. 1. Purification of the recombinant proteins: a) Ag85B-$(His)_6$ (12.5% polyacrylamide gel); b) ESAT-6-$(His)_6$ (15% polyacrylamide gel). a) Lanes: *1*) set of standard proteins from Fermentas (Lithuania) (116.0, 66.0, 45.0, 35.0, 25.0, 18.0, and 14.2 kD); *2*) the renatured protein Ag85B-$(His)_6$. b) Lanes: *1*) set of standard low-molecular-weight proteins from Amersham (Great Britain) (97.0, 66.0, 45.0, 30.0, 20.1, and 14.4 kD); *2*) aprotinin (6.5 kD); *3*) the renatured protein ESAT-6-$(His)_6$.

end of the molecule. In the electrophoregram (15% SDS-PAGE) in the cell lysate of the *E. coli* BL21(pET24-ES) culture upon induction with IPTG, a band was detected which corresponded to the recombinant protein ESAT-6-$(His)_6$ (data not presented).

Purification of the recombinant protein ESAT-6-$(His)_6$. The electrophoretically homogenous protein (Fig. 1b, lane *3*) was prepared in urea upon dissolving the biomass in guanidine chloride, decreasing the viscosity of the solution by ultrasonication, and the subsequent affinity chromatography on Ni^{2+}NTAHisBind® Superflow™. Aprotinin with the major band corresponding to 6.5 kD was used as an additional marker (Fig. 1b, lane *2*). The purity of the protein in the fraction with pH 3.75 was no less than 80%. The apparent molecular weight of the recombinant protein ESAT-6-$(His)_6$ was 6.0 ± 0.5 kD, which was nearly 4 kD less than the calculated value of 10,940 daltons. The protein was renatured in two stages: in the first stage the solution was diluted to the final concentration of urea 2 M and then diluted in 10 mM Tris-HCl buffer (pH 7.5) for 24 h at 10°C. The protein ESAT-6 was stored in buffer (pH 6.0) supplemented with 2 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 6.0, at 10°C.

The renaturation of the isolated proteins Ag85B-$(His)_6$ and ESAT-6-$(His)_6$ by the preliminary decrease in the urea concentration to 2 M and subsequent dialysis caused considerable losses of the proteins because of their aggregation and changing of some protein molecules into an insoluble form; therefore, we could isolate from 1 g biomass no more than 2 mg of the renatured protein Ag85B and 10 mg the renatured protein ESAT-6. The resulting renatured proteins Ag85B-$(His)_6$ and ESAT-6-$(His)_6$ were used for preparation of specific mouse antibodies.

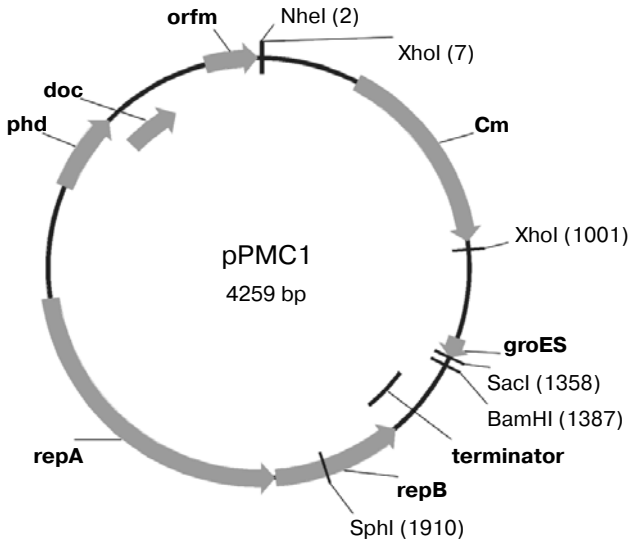

Fig. 2. Structural map of the plasmid pPMC1.

Immunogenicity of the recombinant proteins Ag85B-$(His)_6$ and ESAT-6-$(His)_6$. Double immunization with the recombinant protein Ag85B-$(His)_6$ (in total 40 μg) in the presence of Freund's adjuvant induced high titers of antibodies to the Ag85B-$(His)_6$ antigen in the mouse antisera. The antibody titers determined by enzyme immunoassay were in the range from 1 : 10,240 to 1 : 20,480, which suggested a high immunogenicity of this protein.

Triple immunization with a greater amount of the protein ESAT-6-$(His)_6$ (in total 90 μg) induced low titers of antibodies (in the range from 1 : 640 to 1 : 1280), which suggested a poor immunogenicity of the recombinant protein ESAT-6-$(His)_6$.

Construction of the plasmid vector pPMC1. We have earlier shown that the pFNL10 plasmid isolated from the *F. novicida*-like strain F6168 can constantly replicate in *F. tularensis* cells [23]. The stable inheritance of the plasmid pFNL10 by the bacterial cells seems to depend on the presence in the plasmid of a phd/doc-like operon [20]. The plasmid pFNL10 was used as a basis for creation of the plasmid vector pPMC1 for cloning and expression of recombinant proteins in the tularemia microbe cells. The *cat* gene of the plasmid pC194 was inserted into the pPMC1 vector to provide for selection of plasmid-containing clones, because this gene could express in *F. tularensis* [24]. The promoter and site of translation initiation of the *F. tularensis* groES operon were used as a structure for initiation of transcription–translation of the recombinant genes.

The pPMC1 plasmid is a TGATG vector, with the translational fusion used for effective expression of the cloned genes [25]. The structural moiety of the cloned gene deprived of the ATG codon is joined to the ATG codon located on the vector, and this forms a hybrid operon under control of the groES promoter of *F. tularensis*. The structural map of the plasmid pPMC1 is presented in Fig. 2.

Expression of the protein FL–Ag85B in *F. tularensis* cells. Fusion of the leader sequence of the outer membrane proteins of gram-negative bacteria with the structural moiety of the cloned protein results, as a rule, in the protein transport into the periplasm with the subsequent detachment of this leader sequence. In particular, attachment of the cloned gene DNA fragments to the leader part FL of the outer membrane protein FopA of *F. tularensis* markedly increased the expression of recombinant genes [26]; therefore, on creating the recombinant genes we adjoined the leader part FL of the outer membrane protein FopA of *F. tularensis* to the N-terminal part of the mature polypeptide Ag85B.

The recombinant gene consisted of the DNA fragment encoding FL (25 amino acids, the calculated molecular weight being 2488 daltons) and a fragment, which included the structural moiety of the gene encoding the mature protein Ag85B (285 amino acids, the cal-

culated molecular weight being 30,659 daltons). The hybrid gene was inserted into the vector pPMC1 and transformed in *F. tularensis* 15/10. Finally, the clone *F. tularensis* RVp17 with the plasmid pPMC-FL–85B was obtained. The calculated molecular weight of the recombinant gene product FL–Ag85B was 33,146 daltons.

By immunoblotting with a specific antiserum to the recombinant protein Ag85B-$(His)_6$ of the lysate from the *F. tularensis* RVp17 clone, two bands were detected (Fig. 3a, lane *1*), the lesser of which corresponded to the molecular weight of the cloned fused protein FL–Ag85B (33.1 kD), whereas the larger corresponded to its processed form with the detached leader sequence FL (~30.6 kD). Consequently, in the cells of *F. tularensis* bacteria the major part of the FL–Ag85B protein was represented by the mature mycobacterial protein Ag85B.

Expression of the fused protein FL–Ag85B–ESAT-6 in *F. tularensis* 15/10. To enlarge the spectrum of mycobacterial antigens expressed in *F. tularensis*, a recombinant gene was created which included the DNA fragment encoding the FL leader moiety of the *F. tularensis* outer membrane protein FopA (25 amino acids, calculated molecular weight 2488 daltons), the fragment with the structural moiety of the gene encoding the mature Ag85B protein (285 amino acids, calculated molecular weight 30,659 daltons), and the DNA fragment encoding the mature protein ESAT-6 (95 amino acids including the N-terminal Met, calculated molecular weight 9841 daltons). An additional amino acid Ser was inserted between the genes of the proteins Ag85B and ESAT-6. The hybrid gene was inserted into the pPMC1 vector and transformed in *F. tularensis* 15/10. As a result, the *F. tularensis* RVp18 clone was selected with the plasmid pPMC-FL–85B–ESAT-6. The calculated molecular weight of the protein FL–Ag85B–ESAT-6 (405 amino acids) was 42,988 daltons. Immunoblotting of the resulting PCR-positive clones of *F. tularensis* RVp18 (Fig. 3b, lane *1*) a band was detected which corresponded to a protein with the molecular weight of 40 ± 3 kD. The immunoblotting was performed using the mouse antiserum to the recombinant protein Ag85B-$(His)_6$ because this antigen was a constituent of the protein FL–Ag85B–ESAT-6 and the antiserum to the protein ESAT-6 badly recognized linear epitopes in the ESAT-6-$(His)_6$ molecule (data not presented). In the cells, the protein FL–Ag85B–ESAT-6 seemed to be unprocessed and retain the leader peptide FL.

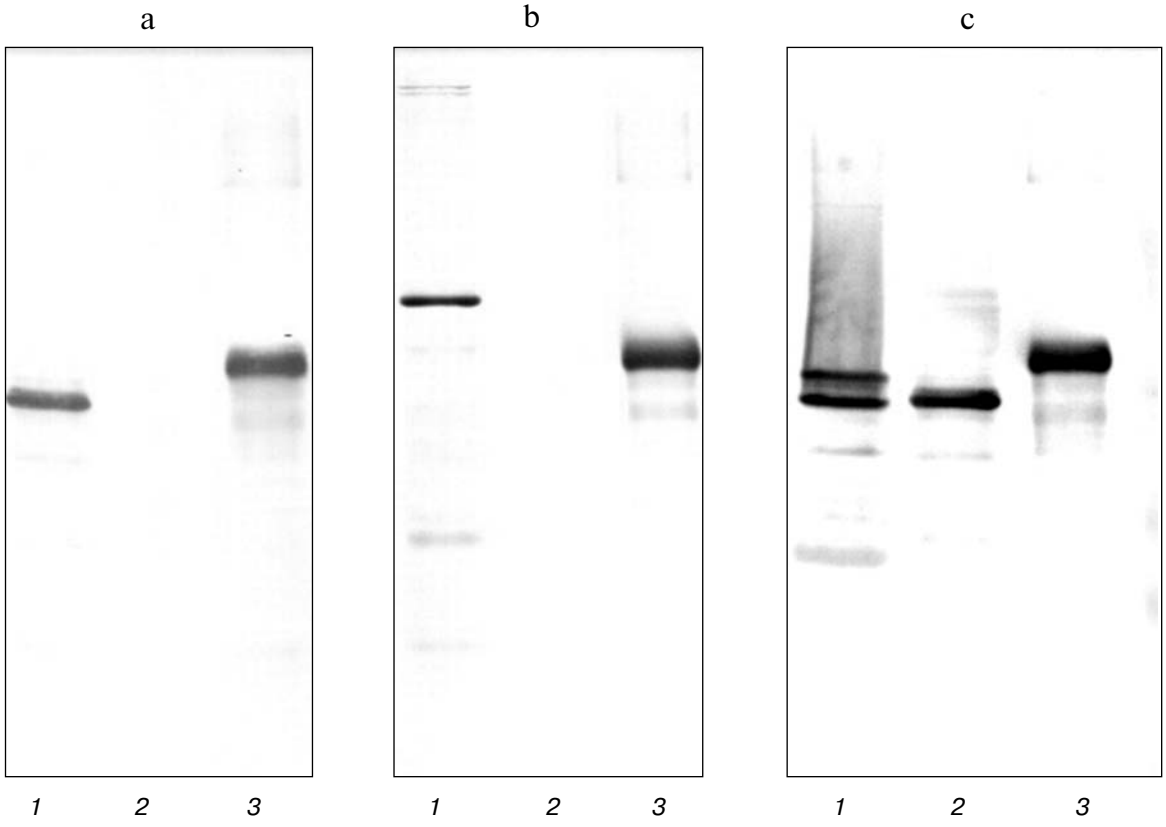

Fig. 3. Immunoblotting of the *F. tularensis* clones RVp17 (a, c) and RVp18 (b) with the antiserum to the recombinant protein Ag85B-$(His)_6$. a) Lanes: *1*) *F. tularensis* RVp17; *2*) *F. tularensis* 15/10(pPMC1); *3*) the recombinant protein Ag85B-$(His)_6$. b) Lanes: *1*) *F. tularensis* RVp18; *2*) *F. tularensis* 15/10(pPMC1); *3*) the recombinant protein Ag85B-$(His)_6$. c) Lanes: *1*) concentrated cultural medium of *M. bovis* BCG; *2*) *F. tularensis* RVp17; *3*) the recombinant protein Ag85B-$(His)_6$.

For immunoblotting, samples of the recombinant *F. tularensis* 15/10 strains cellular lysates were used from a standardized number of cells. This allowed us to predetermine the expression level of the fused proteins in both strains upon application of varied amounts of the recombinant protein Ag85B, which was a constituent of both fused protein constructions (data not presented). In particular, a protein band with the intensity corresponding to 50 ng of the recombinant protein Ag85B-$(His)_6$ was revealed by immunoblotting in the sample of the cellular lysate from the *F. tularensis* RVp17 strain which contained $5 \cdot 10^8$ CFU.

Evaluation of specificity of antibodies to the recombinant protein Ag85B-$(His)_6$. The immunological correspondence of the protein Ag85B-$(His)_6$ cloned and expressed in the *E. coli* cells to Ag85B mycolyl transferase from *M. tuberculosis* was evaluated by immunoblotting with the monospecific polyclonal mouse antiserum to the purified protein Ag85B-$(His)_6$. The concentrated culture fluid prepared from the growing bacterial cells *M. bovis* BCG was used as a source of the mycobacterial protein Ag85B. The resulting immunoblotting is shown in Fig. 3c.

The antibodies to Ag85B-$(His)_6$ interacted with both the recombinant protein Ag85B expressed by the *F. tularensis* RVp17 strain and the mycobacterial Ag85B protein in the concentrated culture medium of the *M. bovis* BCG strain. With these antibodies two protein bands were revealed, the upper band having molecular weight close to that of the recombinant protein Ag85B-$(His)_6$, and the molecular weight of the lower band was 1-2 kD less. The calculated molecular weight of the mature protein Ag85B was 30.6 kD; therefore, the two equally intense bands of the *M. bovis* BCG strain seemed to correspond to the unprocessed and processed forms of Ag85B mycolyl transferase.

Based on the immunoblotting data, it was concluded that the recombinant protein Ag85B-$(His)_6$ isolated from *E. coli* and the protein cloned in the *F. tularensis* strains RVp17 and RVp18 were immunologically similar to the Ag85B mycobacterial mycolyl transferase.

DISCUSSION

In the present work, expression of the *M. tuberculosis* major extracellular proteins Ag85B and ESAT-6 in the vaccine strain *F. tularensis* 15/10 has been shown for the first time. The plasmid vector pPMC1 created by us included all elements of the plasmid pFNL10 necessary for the stable replication in the *F. tularensis* 15/10 cells. Consistent with findings of the high expression of tularemia proteins controlled by the groE-promoter [27], the recombinant mycobacterial proteins cloned by us also actively synthesized in the *F. tularensis* cells. But the G + C genome content of *M. tuberculosis* is ~70%, whereas the content of this genome in *F. tularensis* is 37%, and this is a significant barrier for expression of tuberculosis genes in *F. tularensis* [28, 29]. We have partially overcome this barrier due to starting the translation of the hybrid genes from the homologous for *F. tularensis* matrix RNA encoding the leader FL peptide of the *F. tularensis* outer membrane FopA protein.

The expression system chosen by us using the homologous for *F. tularensis* leader FL peptide of the *F. tularensis* outer membrane FopA protein transported the expressed mycobacterial protein into the periplasm. Although we did not isolate the cloned proteins from the periplasm, the protein transport with the subsequent detachment of the leader sequence was indirectly confirmed by the correspondence of the molecular weight of the Ag85B protein expressed in the *F. tularensis* RVp17 strain cells to the molecular weight of the mature protein Ag85B found in the culture fluid of the *M. bovis* BCG culture.

The used system of expression of mycobacterial antigens in the *E. coli* BL21 cells as components of the expressing vectors pET23b(+) and pET24b(+) allowed us to perform a one-step purification of the secreted mycobacterial proteins Ag85B and ESAT-6 by affinity chromatography and to obtain electrophoretically homogenous preparations of the recombinant proteins with the purity of no less than 80%.

As differentiated from the purified protein Ag85B-$(His)_6$ the molecular weight of which occurred to be close to the value calculated from the nucleotide sequence, the apparent and calculated molecular weights of the ESAT-6-$(His)_6$ protein displayed a pronounced difference. This is in agreement with data of Sorensen et al. [30] who had shown that the native protein ESAT-6 (calculated molecular weight equal to 9975 daltons) isolated from the *M. tuberculosis* H37Rv strain at SDS-PAGE gave the major band corresponding to 6 kD and two weak bands between 6 and 4 kD. The authors think that the existence of the protein forms different in charge, size, and p*I* is explained by a possible acetylation of the protein. Our analysis of the recombinant protein ESAT-6-$(His)_6$ by immunoblotting with the mouse antiserum to the ESAT-6-$(His)_6$ protein (data not presented) has shown a very weak recognition of the purified protein by the antibodies and also revealed an additional band with the lower molecular weight. Therefore, as we did not expect with confidence a super-expression of the cloned mycobacterial proteins, we used the antiserum to Ag85B-$(His)_6$ to monitor expression of the recombinant proteins by the cells of both *F. tularensis* 15/10 strains, although later we found that the antiserum to ESAT-6-$(His)_6$ also could recognize this antigen in the fused protein in the lysate of the *F. tularensis* RVp18 strain.

The preliminary evaluation by immunoblotting of the Ag85B expression in the *F. tularensis* RVp17 cells indicated that the bacterial cells could express no less

than 1000 molecules of the cloned protein. Such an expression level is thought to be sufficient for induction of a specific immune response to cloned antigens [31].

In further work, we will study induction of the specific immune response in experimental animals and assess the protectivity of the obtained recombinant strains *F. tularensis* RVp17 and *F. tularensis* RVp18 using the model of experimental lung tuberculosis in mice.

This work was supported by the International Science and Technology Center (project No. 2237) and the Biotechnology Engagement Program of the US Public Health Service (project No. 7).

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