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Secrening Producer of Truncated *Bacillus anthracis* Protective Antigen in *Bacillus subtilis*

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The protective antigen (PA) of *B. anthracis* is a subunit of a complex toxin that mediates recognition of the eukaryotic cell receptors. Once bound to the receptor, PA recruits effector subunits (lethal and edema factors) and ensures their transportation to the cytoplasm of a target cell. The PA-neutralizing antibodies provide protection against the anthrax infection. Therefore PA is the main antigen used in vaccines. The 83 kDa PA consists of four domains. Domains I and II carry the major epitopes recognized by the protective IgGs from immunized humans and animals. We developed an integrative recombinant construct WPag201-SW that contains a fragment of PA gene encoding domain I (PA20). This construct allows production of this polypeptide in *B. subtilis*. Expression of PA20 is controlled by the *Bacillus*-specific promoter of cell wall bound subitlisin WprA. The secretion is mediated by the natural PA leader peptide. Due to the optimal position of the truncation point, we achieved an efficient secretion of a soluble protease-resistant protein to the medium. This protein was shown to react with the immune sera risen against full-length PA. The B *subtilis* strain carrying WPag201-SW construct is suitable for the large-scale production of the PA domain I fragment that can be used in test systems for serological detection of anti-PA antibodies. The live spore culture, producing PA domain I peptide, can be considered as a candidate to attenuated veterinary vaccine. Such a strain is of an urgent necessity for anthrax prophylaxis in cattle.

Keywords: Anthrax, vaccine, protective antigen, *Bacillus anthracis*, *Bacillus subtilis*.

The vaccination of the farm animals against anthrax was historically the first example of design and commercialization of a vaccine based on a live attenuated bacterial strain that was originally isolated in L. Pasteur's lab[1]. The live anti-anthrax vaccines are in use so far. In Russia, the human vaccine is based on *B. anthracis* STI strain and the veterinary vaccine contains the viable spore culture of 55-VNIIVV-M strain (deficient in capsule forming).

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The anti-anthrax vaccination is a part of a mandatory annual animal prophylaxis in Russia. The adult cattle is vaccinated once a year, preferably in spring before the pasture period, the calves and the lambs are vaccinated in 3-month age[2].

The main pathogenicity factors of *B. anthrax* are the tripartite exotoxin and the capsular exopolysaccharide conferring a phagocytosis resistance to the bacterium. The synthesis of these factors is determined by two large pathogenicity plasmids pX01 and pX02, respectively[3].

The attenuated vaccine strain 55-VNIIVV-M has lost pXO2 plasmid and by this reason is not

able to produce the capsule. So the protective effect of the immunization by the live vaccine based on this strain is mediated mainly by the toxin-neutralising antibodies[2].

The tripartite anthrax toxin consists of PA – protective antigen, EF – edema factor and LF – lethal factor subunits. However, PA is the most important polypeptide for the protective immunity induction[3]. This 83 kDa protein acts as a receptor recognition subunit specifically targeting two known toxin receptors on the eukaryotic cell surface: the tumor endothelial marker (TEM8) and the capillary morphogenesis factor 2 (CMG2).

After the binding to one of these receptors PA undergoes a proteolytical processing by the furine class membrane protease which removes PA N-terminal 20 kDa domain (domain I, PA20). The remaining 63 kDa polypeptide (PA63) forms heptameric or octameric complexes recruiting the active subunits EF and/or LF[4].

The phagocytosis of the toxin-receptor complex is followed by the transportation of the catalytic subunits to the cell cytoplasm where they exert their enzymatic activities that lead to the cytopathic effects[5]. In absence of PA the catalytic subunits are not able to penetrate to the cell and do not exhibit any toxicity. Hence the PA blockage by the antibodies provides complete resistance to the toxin and creates high level of protection against *B. anthracis* infection in animals.

Toxin-neutralizing IgGs are found in human or animal blood during 6-12 months period after the vaccination. However, the immunological memory cells were shown to persist at least for several years. That provides a certain level of protection from infection by low dosage of *B. anthracis* cells[6].

The PA X-ray structures have been solved for monomeric PA83 (5) as well as for heptameric and octameric form of PA63 and for its complex with the receptor[5, 7]. PA polypeptide chain has the cleavable N-terminal leader peptide targeting the protein to the secretion *via* Sec pathway. The mature PA83 formed after the leader peptide removal has 4 structural domains[5]. It has been shown that the domains of PA have different immunogenic properties. More than 62% of all antibodies appeared in mice after immunization with PA83 or circulating in the blood of humans vaccinated by absorbed AVA vaccine (contains the filtrate of vaccine strain Stern culture) recognized the N-terminal domain (PA20). The biological significance of the high immunogenicity of PA20 is not clear. This domain is removed after the recognition with the receptor and is not involved to the toxin internalization. So the massive response to PA20 may cause worse protective effect of immunization with PA83 versus immunization with PA63. The antibodies binding to domain I may inhibit the interaction of the PA with the receptors and with the furin-like protease, thus creating a sufficient protective effect. Being relatively small and stable even in absence of the other PA domains, PA20 protein appears to be an attractive candidate antigen for developing of the recombinant subunit vaccines against anthrax[8].

Here we present the engineering a non-pathogenic *B. subtilis* strain producing N-terminal domain of PA (PA20) in a soluble form, secreted to the cultivation medium. The expression of PA20 is achieved under the control of a medium-strength promotor of WprA gene (cell wall bound subtilisin)[9]. The secretion was driven by the natural PA leader sequence. The main challenge was to select an optimal translation termination point that would allow producing a stable, soluble protein that retains its antigenic properties *in vitro*.

MATERIALS AND METHODS

Design of the integrative construct

The 935 b.p. DNA fragment containing gene *wprA* promotor was amplified from *B. subtilis* AJ73 genomic DNA using Wpr141 and WPR201 primers. The PCR fragment was cloned into pQE30 (Invitrogen) using XhoI and SphI restriction sites to create pQ-Wpr plasmid.

The DNA fragment coding the PA20 protein was obtained by PCR with PAG201 and PAG3 primers using the genomic DNA of *B. anthracis* 55-VNIIVV-M as a template. The nucleotide sequence of PAG gene in this strain corresponds to NC_001496.1 GenBank entry. The 611 b.p. fragment was digested by Eco32.I and KpnI restriction enzymes and ligated with pQ-Wpr vector DNA digested by the same restrictases to obtain pQE30-WprA-PAG vector.

The transcription terminator of *B. subtilis wprA* gene was amplified by PCR with WPR1.1 and Wpr2 primers from *B. subtilis* AJ73 DNA. The

150 b.p. fragment was cloned by PstI and SacI restriction sites to pBlueScript-KS+ (Stratagene, USA) vector to obtain pBS-WprA vector. This vector was used for cloning 770 b.p. fragment excised from the plasmid pSG1154 (12) by PstI digestion. This fragment contained spectinomycin resistance marker. The resulting construct was named pBS-Spc-WprA. The 2060 b.p. fragment containing the gene *wprA* terminator and spectinomycin-resistance marker Spc was excised from pBS-Spc-WprA plasmid by EcoR32.1 and Ecl136.II digestion. The fragment was gel-purified and cloned into pQE30-WprA-PAG construct by a single PvuII site. The colonies resistant simultaneously to ampicillin (100 mg/ml) and spectinomycin (75 mg/ml) were picked. The variant with the insertion of Spc marker in the orientation opposite to WprA-PAG gene was than selected and this plasmid was named pWPag201-SW (Fig.1). The sequence of the insert was confirmed by an automated Sanger sequencing.

PAG201 (EcoRV)
GGGATATCatatgaaaaaacgaaaagtgtt
PAG3 (KpnI)
GGGGTAccagcacttgtacttcgcttt
Wpr141 (BglII/XhoI)
GGAGATCTCGagcttggatacgacatgtcc
WPR201 (SphI/EcoRV)
GGGCATGCGATATCcctcctgcaaaataatgaat
Wpr1.1 (XhoI)
ggctcgagctggtttttttatatttatc
Wpr2 (SacI)
gggagñtcgtccgacaaaattgtgtccga

The integration of the construct into *B. subtilis* chromosome and analysis of its stability

The chemical transformation of *B. subtilis* AJ73 cells by pWPag201-SW was done according to Spizizen protocol[10]. As far as pQE30 replicon (MC16) is not functional in *B. subtilis*, the rescue of the Spc marker was possible only due to integration of pWPag201-SW to the host chromosome. The double recombination event directed by the shoulders flanking the gene of the interest that are homologous to the *B. subtilis* chromosome locus leads to the removal of the vector part. Only the insertion sequence remains in the bacterial chromosome. This integrated construct was named WPag201-SW.

The transformed *B. subtilis* AJ73:WPag201-SW strain was cultured in 20 ml tubes containing 3 ml of LB liquid medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, deionized water up to 1 L) without antibiotic. The cultures were propagated for 16-20 h at 37°C under an agitation at 200 rpm. In order to estimate the genetic stability of the strain, two sequential passages in the same conditions were done using 3 ml of the previous culture as the inoculum. After the last passage the aliquots of the appropriate dilutions were plated in triplicate on the plates LB agar (LB supplemented with 15 g L^{-1} of bacto-agar) with 75 mg/ml of spectinomycin and without antibiotic to compare the titres of the cells containing the plasmid and the cells that had lost the construct.

For the analysis of the recombinant protein production, the cells were pelleted by centrifugation at 9000 g for 3 min. The proteins from 1 ml of the supernatant were precipitated by addition of 100 ml of saturated trichloracetic acid (TCA) solution. The cultural liquid of the parental plasmid–free strain *B. subtilis* AJ73 was processed in the same way and served as the control.

The pelleted cells were used for analysis of the intracellular accumulation of the recombinant protein. For this purposes the cells were resuspended in 500 ml of the ice-cold physiological saline supplemented with 10 mM of EDTA and 10 mM of PMSF used as the protease inhibitors. The cells were disintegrated by vortexing with 50 mg of 100 mm glass beads (Sigma-Aldrich cat. # G4649) for 5 min. The 50 ml aliquots were taken and the total protein was precipitated by TCA added up to 10%.

The samples were incubated with TCA at 4°C for 1 h and proteins were pelleted by centrifugation at 12 000 g for 10 min. The pellets were washed by 70% ethanol, air-dried and diluted in 20 µl of 1× Laemmli sample buffer. The samples were denatured for 5 min at 96°C and analysed by SDS-PAGE.

SDS-PAGE was carried out in two equivalent gels one of which was stained by 1% Coomassie R-250 following a standard protocol and the second one was used for Western-blot analysis. The proteins were blotted onto a nitrocellulose membrane Hybond-C with a semi-dry electro-transfer protocol. The products were detected with commercial horse polyclonal

antibodies manufactured by Orel Biofactory after three subsequent immunizations with a dry attenuated anthrax vaccine. The whole hyper-immune serum was 5,000-fold diluted in PBS (pH 7.2). The horse Ig's bound at the filter were visualized with protein A – HRP conjugate (Imtek, Moscow). 0.02% 3,3' diaminobenzidene (Sigma) supplemented with 0.01% H_2O_2 was used as a chromogenic substrate. PageRuler (Thermo Scientific) was used as a molecular mass standard.

For N-terminal protein sequencing, the proteins were electro-blotted to Amersham Hybond SEQ 0.2 PVDF membrane (GE Health Care) by the protocol described above. The membrane was stained in 1% Amide Black stain (Reanal, Hungary) solution in 10% acetic acid. The band of interest was excised, destained by 5 washes in 10% acetic acid, washed 3 times in deionized water and air-dried. The samples were submitted for commercial N-terminal protein sequencing using ABI Procise Model 492 Edman Micro Sequencer to Innotech-21 company (Moscow, Russia).

RESULTS

The artificially designed PA20 gene encoded a protein which contained 29 a.a. leader peptide and 172 a.a. corresponding to the N-terminal domain of PAG. The protein has a 10 a.a. extension at the C-terminus (TPGRPAAKLN) corresponding to the fragment of the pQE30 plasmid polylinker.

The expression construct pWPag201-SW allowed PA20 gene expression under the control of WprA promoter. It bears two arms flanking the main expression unit and the antibiotic resistance marker from two sides and directing them to the chromosomal copy of *B. subtilis* genome using "two arm homology recombination mechanism". The pWPag201-SW construct carries spectinomycine resistance maker from pSG1154 plasmid with its natural promoter and terminator located in an opposite orientation to pWprA-PA20 expression unit.

The integration of WPag201-SW to *B. subtilis* chromosome was achieved by homologous recombination between the sequences flanking PA20 genes that were homologous to the sequences surrounding the *wprA* gene in *B. subtilis* genome. As the result, our construct replaced the natural *wprA* gene on the chromosome. Since the plasmid replicon of pQE30 vector is not functional in *B. subtilis*, only the cells with integrated WPag201-SW survived the spectinomycine selection following the

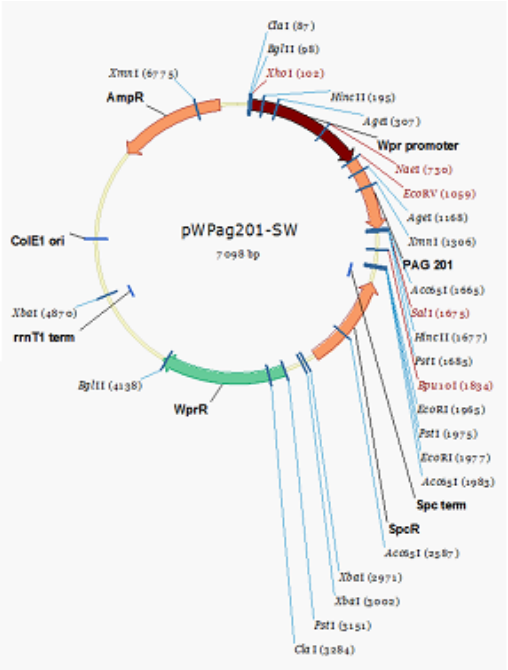
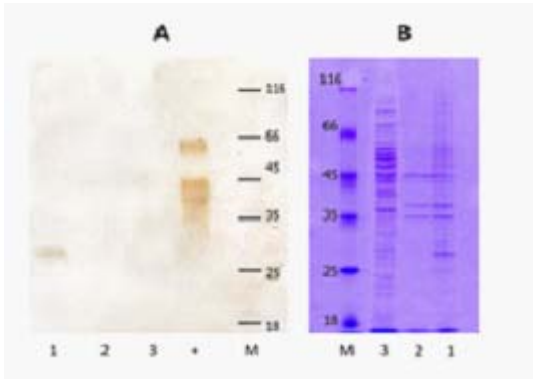


Fig. 1. The schematic map of the pWPag201-SW construct integrated to the chromosome of *B. subtilis*



A. Coomassie-stained gel. The lanes: M – molecular mass standard 1 - *B. subtilis* AJ73: WPag201-SW extracellular fraction 2 - non-recombinant *B. subtilis* AJ73 strain extracellular fraction, 3. *B. subtilis* AJ73 WPag201-SW homogenized cells, "+" – *B. anthracis* 55-VNIIVV-M strain extracellular fraction. B. Western-blot analysis. The lanes are identical to the panel A.

Fig. 2. Identification of the recombinant protein PA20 in the liquid of the culture *B. subtilis* AJ73:WPag201-SW strain

transformation by the p WPag201-SW plasmid.

The integration into the desired site was confirmed by PCR with Wpr141 and Pag3 primers with subsequent Sanger sequencing. This location was chosen because the *wprA* deletion does not affect the growth of *B. subtilis* under the laboratory conditions[9] thus ensuring the stability of the construct. Nevertheless the stability was confirmed by serial passaging in non-selective conditions. The comparative plating of the cultures after the third passage with and without spectinomycin revealed no significant differences of the cell titres, thus indicating that almost all the cells still contained the construct.

The expression of the construct yielded 27 kDa soluble secreted protein that comprised about 20% of the total secreted proteins of the expressing culture (Fig. 2. A). The Western-blot analysis revealed no positive signal in the control cells or culture liquid. At the same time, a 27 kDa recombinant protein was clearly highlighted (Fig. 2B). The electrophoretic mobility of this protein corresponded to 27 kDa, slightly higher than the expected molecular weight of 22 kDa.

In order to obtain further confirmation of the identity of this protein we performed direct Edman sequencing of its 7 N-terminal a.a. residues. The sequenced obtained was EVKEEXR while the published[4] sequence of the mature (after the removal of the leader peptide) PAG protein contains on its N-terminus EVKQENR residues. Taking into account the expected deamination of the Gln4 and Asn6 residues under the conditions of Edman sequencing, this result perfectly corresponds to the expected N-terminal sequence of the recombinant product. Taking together the results of the Western-blot analysis and of the N-terminal protein sequencing we conclude that the observed band corresponds to the recombinant product and its appeared higher molecular weight is due to the anomalous gel mobility of the protein.

DISCUSSION

B. subtilis is an attractive platform for the recombinant PA production since it is biologically safe, has a high capacity to extracellular protein secretion, is easy to culture and produces endospores. The existing anthrax vaccine manufactured by Orel Biofactory, the only veterinary anthrax vaccine produced in Russia, is designed to inoculate the animals by ca. 10^6 c.f.u. dose of the live spores. The vaccine substance, being free of the vegetative cells, does not contain any PA or other antigens inducing the protective anti-anthrax immunity. All these antigens are synthetized *in vivo* once the spores germinate. This mechanism allows using very low doses of the vaccine that gives an opportunity to keep down the costs of the substance purification and, as the result, reduces the final prices for the vaccine. However, if we use this approach in combination with recombinant antigen producing strain instead of the attenuated natural strain, it will require a high stability of the genetic system that can be expressed only during the relatively short period of transitory growth *in vivo*, in the conditions that are out of the control of the vaccine producer.

These limitations may be a cause of the failure to use in commercial vaccine production the PA producing strains that carry the PA gene on the plasmid[11-13].

In this work, we demonstrate that the usage of the *wprA* promotor allows achieving the production of the recombinant PA20 protein at the level of ~20% of the total bacterial secretome despite the fact that the integrative construct is present only in a single copy per chromosome. At the same time the presence of the integrative construct on the chromosome does not impair the strain's viability and allows highly stable inheritance of the construct.

The adequate choice of the translation termination point and the usage of the moderate strength promoter allowed us to achieve effective production of the protein in soluble secreted form. The resistance of the protein to proteases indicates that it adopts the compact native conformation. This fact is relevant since parental *B. subtilis* AJ73 strain exhibits significant activity of an extracellular alkaline protease (~800 Folp units per ml)[14].

Taking together all these properties suggests that *B. subtilis* AJ73:WPag201-SW strain can be tested as a candidate recombinant vaccine strain. The advantage of this organism is that the vaccine based on *B. subtilis* AJ73:WPag201-SW strain may be produced using the same technology as currently applied for *B. anthracis* 55-VNIIVV-M based vaccine. At the same time the recombinant PA20 protein can be used for immunological

diagnostic kits production, anthrax antisera production and for other purposes.

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REFERENCES

1. Berche, P. Louis Pasteur, from crystals of life to vaccination. *Clin. Microbiol. Infect.*, 2012; **18**(5): 1-6.
2. Sadovoy, N.V., Kravets, D.I., Selivanenko, G.M., Kharechko, G.S., Sadovaya, E.A., Vasilyev, P.G., Litasov, N.In., Elagin, G.D., Supotnitsky, M.V. Anthrax combined vaccine. *Patent of Russian Federation*, 1998; # 2115433**,** A61K39/07, A61K39/40.
3. Zakowska, D., Bartoszcze, M., Niemcewicz, M., Bielawska-Drózd, A., Kocik, J. New aspects of the infection mechanisms of *Bacillus anthracis*. *Ann. Agric. Environ*., 2012; **19**(4): 613-8.
4. Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H., Liddington, R.C. Crystal structure of the anthrax toxin protective antigen. *Nature*, 1997; **385**(6619): 833-8.
5. Santelli, E., Bankston, L.A., Leppla, S.H., Liddington, R.C. Crystal structure of a complex between anthrax toxin and its host cell receptor. *Nature*, 2004; **430**(7002): 905-8.
6. Reason, D.C., Ullal, A., Liberato, J., Sun, J., Keitel, W., Zhou, J. Domain specificity of the human antibody response to *Bacillus anthracis* protective antigen. *Vaccine*, 2008; **26**(32): 4041-7.
7. Feld, G.K., Thoren K.L., Kintzer, A.F., Sterling, H.J., Tang, I.I., Greenberg, S.G., Williams, E.R., Krantz, B.A. Structural basis for the unfolding of anthrax lethal factor by protective antigen oligomers. *Nat. Struct. Mol. Biol*., 2007; **17**(11): 1383-90.
8. Zeltins, A. Construction and characterization of virus-like particles: a review. *Mol. Biotechnol*., 2013; **53**(1): 92-107.
9. Belyakova, A.V., Epova, E.Y., Gra, O.A., Zylkova, M.V., Plaksina, A.G., Smirnova, M.S., Elagina, E.M., Filimonova, N.A., Hasanova, A.R., Smirnov, A.V., Kazeeva, T.N., Shibaeva, A.V., Shevelev, A.B., Aleshin, V.V. Recombinant producer of chicken somatoliberin in bacilli. *Fundamental researches*, 2012; **5**(2): 401-406. [Article in Russian].
10. Anagnostopoulos, C., Spizizen, J. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.*, 1961; **81**: 741-6.
11. Baillie, L., Moir, A., Manchee, R. The expression of the protective antigen of *Bacillus anthracis* in *Bacillus subtilis*. *J Appl Microbiol*., 1998; **84**(5): 741-6.
12. Barnard, J.P., Friedlander, A.M. Vaccination against anthrax with attenuated recombinant strains of *Bacillus anthracis* that produce protective antigen. *Infect Immun*., 1999; **67**(2): 562-7.
13. Mohamadzadeh, M., Durmaz, E., Zadeh, M., Pakanati, K.C., Gramarossa, M., Cohran, V., Klaenhammer, T.R. Targeted expression of anthrax protective antigen by *Lactobacillus gasseri* as an anthrax vaccine. *Future Microbiol*., 2010; **5**(8):1289-96.
14. Sharipova, M.R., Shagimardanova, E.I., Chestukhina, I.B., Shamsutdinov, T.R., Balaban, N.P., Mardanova, A.M., Rudenskaya, G.N., Demidyuk, I.V., Kostrov, S.V. The expression of Bacillus intermedius glutamyl endopeptidase gene in Bacillus subtilis recombinant strains. *Mol Biol Rep*., 2007; **34**(2): 79-87.