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Metrology, Standartization, and Quality Control

A Selection Method for the Recombinant Clones Bearing the Plague Surface-localized Proteins Encoded by the pFra Plasmid

V. N. Korsukov*,[#] Yu. I. Yashechkin, A. K. Nikiforov*, A. P. Anisimov**, S. A. Yeremin*, and Yu. A. Popov***

**Microbe Russian Antiplague Research Institute, Saratov, 410005 Russia*
***State Scientific Center of Applied Microbiology, Obolensk, Serpukhov region, Moscow oblast, 142279 Russia*

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Abstract—The approaches to the selection of recombinant *Escherichia coli* and *Salmonella minnesota* cells containing the genetic determinants of the plasmid pFra from the plague microbe *Yersinia pestis* were developed. The products of the plasmid affect the cell surface charge. The inheritance stability of the recombinant plasmids with the *fra* operon was determined. The screening of the gene library of pFra plasmid was carried out, and the method of direct selection of transformants and superproducers of the antigen of fraction I was suggested.

Key words: electrophoresis in free liquid flow, Escherichia coli, pFra plasmid, Salmonella minnesota, superproducers, Yersinia pestis

INTRODUCTION

The screening and subsequent selection of recombinant clones are some of the first stages in experiments on genetic engineering. The lack of selection methods impedes the search for cloned genes encoding unknown compounds.[1]

It was previously shown [1–4] that the biopolymers situated on cell surface could change the overall charge of cell and, hence, affect the electrophoretic mobility of the bacteria in which the antigens that form surface structures have varying composition. The use of FFE, which allows the separation of cells according to their surface charges, makes it possible to select the bacterial cells that acquired with the new genetic information an ability to synthesize the compounds changing their surface properties.

The goal of this work was the development of selection methods for the recombinant *Escherichia coli* and *Salmonella* strains containing the pFra plasmid genes from the *Yersinia pestis* strains, whose products affect the cell surface charge, and the determination of the inheritance stability of the recombinant plasmids containing the *fra* operon.

EXPERIMENTAL

We performed the experiments with the strains of plague microbe, *E. coli,* and *S. minnesota* (Table 1).

The *E. coli* and *S. minnesota* bacteria were grown on the agar LB and Hottinger nutrient media, pH 7.2, at 37°C for 18–20 h; the culturing time of 48 h was used in the case of the *Yersinia pestis* strains. For FFE, bacterial cells were washed out from the nutrient medium on a Sigma 202 MK centrifuge (Germany) for 10 min at 4000 *g* and suspended in 0.033 M Tris buffer (2 ml), pH 8.6. The suspension was electrophoretically separated on an Elphor-VAP5 device (Bender Hobein, Germany) for 30 min under the following conditions: the flow rates of the separating buffer and the injected sample were 500 and 2 ml/h, respectively, voltage 500 V, and current 50 mA. A 0.033 M Tris buffer, pH 8.6 or 9.4, was used as a separating buffer and a 0.15 M Tris buffer, pH 8.6, as an electrode buffer. To analyze the electric properties of cell surfaces of the recombinant clones, *E. coli* L87 strain was grown on an LB medium, pH 7.2, at 37°C for 18–20 h. A suspension of the bacterial cells was disinfected by the addition of sodium merthiolate at 1 : 10 000 dilution

[#]To whom correspondence should be addressed; e-mail: microbe@san.ru.

[1]Abbreviations: EM, electrophoretic mobility; FFE, electrophoresis in free flow; IHAR, the indirect hemagglutination reaction; and MRARI, Microbe Russian Antiplague Research Institute.

followed by heating at 56°C for 30 min. The cells were washed out from the nutrient medium and subjected to electrophoresis using 0.033 M Tris buffer, pH 8.6, or 9.4. When FFE was used for the direct selection of transformants, the chamber and Teflon hoses of Elphor-VAP5 device were sterilized for 30 min with 70% ethanol. The electrophoretic buffer was twice sterilized by heating at 70°C for 60 min. Aliquots (0.2 ml) of the resulting fractions were seeded on the corresponding nutrient media and incubated at 37°C for 24 h. Then the numbers of grown colonies were calculated. The cells of the clones that differed in their EM values from the reference (recipient) bacterial strain were used for the determination of the plague capsular antigen. The presence of the F1 antigen was determined by IHAR with plague erythrocyte immunoglobulin diagnostic kit (Central Asian Antiplaque Institute) [8]. Optical density (D_{540}) of electrophoretic fractions was determined using an SF-26 spectrophotometer.

Table 1. Strains of the microorganisms used

No.	Name and some strain characteristics	Source
1	*Yersinis pestis* 231 (pFra)	A gift of S.V. Samoilova, MRARI
2	*Yersinis pestis* EV (pFra, pCad, pPst) (NIIEG lines)	SCIB of MRARI
3	*Escherichia coli* L87 (pfs)	Amersham, UK
4	*E. coli* χ 925 (pfs)	SCIB of MRARI
5	*E. coli* L87 (pACYC184)	Obtained by Yu.I. Yashechkin [5]
6	*E. coli* L87 (pYS)*	Obtained by Yu.I. Yashechkin [5]
7	*E. coli* χ 925 (pYS)*	Obtained by Yu.I. Yashechkin [5]
8	*E. coli* DH1 (pYS)*	Obtained by Yu.I. Yashechkin [5]
9	*E. coli* DH1 (pfs)	SCIB of MRARI
10	*E. coli* DH1pFSK3**	Obtained by A.P. Anisimov *et al.* [6]
11	*E. coli* DH1pFSK4***	Obtained by A.P. Anisimov *et al.* [6]
12	*Salmonella minnesota* R 9 pAE1****	Obtained by S.A. Yeremin *et al.* [7]

Notes: *A series of strains containing recombinant plasmids with cloned pFra DNA fragments of *Y. pestis*.
**Superproducer of the FI antigen of plaque microbe (plasmid pFSK3 bears the *fra* operon of the plaque microbe encoding the FI antigen).
***Plasmid pFSK4 differs from pFSK3 in the direction of the cloned fragment that includes the *fra* operon of the plaque microbe and produces no capsule antigen.
****The producer of the FI antigen of *Y. pestis*.
Abbreviations: MRARI, Microbe Russian Antiplague Research Institute; pfs, plasmid-free strain; and SCIB, State Collection of Industrial Bacteria.

RESULTS ANS DISCUSSION

The presence of three plasmids, pPst (9.5 kbp), pCad (~ 70 kbp), and pFra (~ 100 kbp), is characteristic of most *Y. pestis* strains. Some of the known genes encoding classical antigens of plague microbe are localized in these replicons, including the largest pFra plasmid. It was found that the determinants that control the synthesis of the "mouse" toxin and the capsular antigen "fraction I" (FI) were localized in this plasmid. The region of disposal of these genes has been comprehensively studied by now [9–12]. Moreover, the pFra plasmids from several plague strains were sequenced [13], although the majority of the products revealed by the computational analysis of open reading frames were not characterized.

Analysis of Gene Library of pFra Plasmid Clones from the Y. pestis 231 Strain

We had previously obtained a series of recombinant strains containing hybrid plasmids with the inserts of SalGI fragments more than 14 kbp in length from the pFra plasmid of *Y. pestis* 231 strain [5]. The products of the marker gene of the chloramphenicol resistance were found not to change the charge of cell surface. Therefore, the plasmid-free *E. coli* L87 strain was used as the control.

The library of DNA fragments of pFra plasmid from *Y. pestis* 231 strain comprised 34 clones of *E. coli* L87 strain, which contained recombinant plasmids with 15–20-kbp DNA inserts, was analyzed by FFE. The EM values for most of them coincided with those in the reference *E. coli* L87 strain. For further experiments, we selected seven clones with hybrid pYS plasmids that differed in their EM values from the EM of the control strain. In addition, the strain with plasmid pYS29 was selected; it contained the largest (9.1 kbp) of the cloned pFra231 DNA fragments, and its EM coincided with that of intact cells (Table 2). One can presume that the products of genes situated in the plasmid do not affect the overall charge of the cell surface.

Table 2. Electrophoretic mobility of the cells containing recombinant plasmids of type pYS and some characteristics of the proteins encoded by them in the systems of conjugated transcription–translation

Plasmid	Insert size, kbp	EM, relative units, at pH 8.6	EM, relative units, at pH 9.4	Molecular masses of proteins, kDa
pYS2	2.9	+2	–5	41.2 and 20.6
pYS3/17	3.6	+2	–0.5	16.5
pYS6	4.3	–	–1.5	23.5 and 40.5
pYS7	1.4	–	+1	17.0 and 11.3
pYS1	3.8	–	+1	21.6
pYS22	4.3, 1.0, and 0.4	+4	+0.5	30.0 and 40.5
pYS26	3.8	–	+2	62.4
pYS29	9.1	0	0	105.0 and 28.0

Note: EMs of the recombinant clone cells were compared with those of the corresponding reference strains; they are given in relative units, which represents the differences between the fraction numbers of these strains with the maximal optical density.

The results of expression of the cloned pFra231 DNA fragments in *E. coli* maxi- and minicells demonstrated that the major proteins are synthesized in the cells of eight recombinant clones (Table 2). It is not improbable that 41.2-kDa (plasmid pYS2) and 105-kDa (plasmid pYS29) proteins are the oligomers of 20.6-kDa (plasmid pYS2) and 28-kDa (plasmid pYS29) proteins, respectively. Since both plasmids pYS6 and pYS22 contain the same SalG1 fragment of pFra231 DNA (4.3 kbp), their expression results in an identical protein with a molecular mass of 40.5 kDa.

Therefore, FFE allowed the analysis of gene library of pFra plasmid and the determination of clones whose recombinant plasmids include genes encoding the synthesis of biopolymers with still unknown functions.

A Direct Selection of the E. coli Recombinant Clones that Produce the Y. Pestis FI Antigen

The electrophoretically heterogeneous suspension containing both the transformed and intact cells was studied in model experiments using the *E. coli* DH1 cells transformed with hybrid plasmids pFSK3 and pFSK4. These differed in the direction of insert into the vector pUC4K of the cloned pFra plasmid DNA fragment and in the ability to form the *Y. pestis* protein capsule, whose major component is FI antigen [6] encoded by the *fra* operon.

The experiments with the killed cells showed that the FI antigen-free bacteria were in fractions 29–39, whereas the FI producers possessing a lower surface charge were in fractions 48–54.

At the next stage, the live cells of the *E. coli* DH1pFSK3 strain, a superproducer of plague FI antigen, and the *E. coli* DH1pFSK4 strain that does not produce the capsular antigen were mixed at various proportions. After FFE of the inactivated cells, the bacteria from fractions 50–56 were selected for the further analysis on expression of the FI antigen products.

The experiments showed a possibility to isolate from the mixture of transformants $5 \cdot 10^7$ cells that have capsules. This quantity was sufficient for the selection of hybrid clones of microorganisms with the necessary properties from the gene libraries.

The Selection of E. coli and S. minnesota R595 Recombinant Clones Producing the FI Antigen of Plague Microbe

The level of the capsular antigen production by *E coli* DH1pFSK3 strain, a superproducer of plague FI antigen, was very high immediately after transformation (the titer of 1 : 8192 according to IHAR) [6]. After several generations, the antigen secretion level decreased up to the titer of 1 : 512, the markers of antibiotic resistance being preserved. Because of the reduction in the production of plague capsular antigen, a periodic selection of the clones out of the heterogeneous population of the microorganism that steadily inherit and express the plague *fra* operon genes encoding the FI antigen synthesis is necessary.

Some authors mentioned an electrophoretic heterogeneity of individual subpopulations within a strain [14–17]. Using the benefits of the FFE technique that enables not only obtaining of analytical information, but also a fractionation of studied cells, we screened the plague FI antigen producers according to the FFE results in order to isolate the clones with an increased production of the capsular antigen.

It was found that the inactivated *E. coli* DH1pFSK3 cells unable to produce the FI antigen were in fractions 29–39, whereas the FI antigen producers possessing a lower surface charge were in fractions 48–54. The ability to produce the plague capsular antigen caused therefore a decrease in the surface charge; the bacteria with the lowest EM should hence be selected in the experiments with heterogeneous populations.

The suspension of live *E. coli* DH1pFSK3 cells whose population reduced the ability to produce a capsular antigen in comparison with the initial culture of transformants was fractioned by FFE. As a result, the subpopulation cells selected from fractions 50–56 were shown to secrete as much capsular antigen as the initial *E. coli* DH1pFSK3 transformants did, and no further decrease in the antigen FI production was observed during 50 generations of them.

The cells of *S. minnesota* R595pAE1 strain, a superproducer of *Y. pestis* FI antigen, were fractioned in order to select the clones with an enhanced secretion level of the target product. In the experiments with inactivated cells we found that this strain was heterogeneous with respect to the production of FI antigen. During electrophoresis, the largest number of cells was found in fraction 31, whereas the maximal titer according to IHAR was in fraction 46. This means that the cells with a lower EM produced a larger amount of the capsular antigen. The clones obtained upon the fractioning of live cells were analyzed for their ability to produce FI antigen. The analysis of fractions 54–63 revealed that the clone isolated from fraction 59 has the production level of fraction I corresponding to the titer of 1 : 1024 (note that the titer of initial strain was 1 : 128), and this titer was retained upon the subsequent passages.

Thus, the FFE method showed its efficiency in the screening of the recombinant *E. coli* strains containing cloned DNA fragments of the pFra plasmid of plague microbe and can be recommended as one of the approaches for the analysis of bacterial gene libraries.

To summarize, we separated heterogeneous cell populations using FFE and obtained the fractions that differ in the ability to biosynthesize the target product. We found that the lower EM, the higher the level of FI biosynthesis. Electrophoresis makes the isolation of active producers less laborious and time-consuming in comparison with the standard method; it can therefore be used for the direct selection of recombinant clones.

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