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Analysis of the Features of the Genomic Organization of Plague Microbe Strains Suitable for the Creation of a New Attenuated Vaccine

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Abstract

Relevance. *To date, the specific prevention of plague remains the most effective measure to prevent to prevent the deterioration of the epidemiological situation for this especially dangerous infection in the territory of the Russian Federation. The development of new vaccine preparations created on the basis of avirulent strains of Yersinia pestis with specific genetic defects, can play a crucial importance in the issues of plague immunoprophylaxis.* ***Aim.*** *Determination and comparative analysis of the genomic organization of avirulent strains Y. pestis I-1, Y. pestis I-3536 and vaccine strain Y. pestis EV line NIIEG.* ***Materials and methods.*** *The features of the genetic structure of three Y. pestis strains were studied using plasmid screening and whole genome sequencing.* ***Results and discussion.*** *It was found that the Y. pestis I-1 and Y. pestis I-3536 strains, like Y. pestis EV, lack the chromosomal pgm region. Analysis the genome sequence of Y. pestis I-1 showed that this strain is devoid of the plasmid pMT1, while its individual genes were found in the chromosome structure and the unique 340 kb plasmid. It was revealed that the Y. pestis strain I-3536 lost the plasmid pCD1, however, fragments homologous to this plasmid were found in the structure of its genome.* ***Conclusion.*** *The data obtained allow us to recommend Y. pestis I-1 and Y. pestis I-3536 as the basis for the development of a modern live attenuated plague vaccine.*

Keywords: *Yersinia pestis, vaccine strain, live attenuated vaccine, analysis of plasmids, whole genome sequencing, plague*

No conflict of interest to declare.

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Introduction

Vaccination is a major measure that reduces the risk of sporadic cases and outbreaks of plague in enzootic territories, as well as preventing imported cases of the disease that may occur when people return from countries that are unfavorable for this infection [1,2]. Two preparations are available for the prevention of plague in the Russian Federation: a live plague vaccine developed on the basis of the *Yersinia pestis* EV strain of the NIIEG line, and a molecular microencapsulated plague vaccine, which consists of recombinant F1-antigen and V-antigen (LcrV) [3].

In our country, the live plague vaccine (LPV) has been actively used since 1942 to immunize people living in the territories of natural plague foci [1]. The loss of virulence by the *Y. pestis* EV strain is based on a spontaneous deletion of the chromosomal pgm region of 102 thousand b.p. in size [4], which includes the hmsHFRS operon, as well as the HPI genomic island, whose gene products

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play an important role in the virulence of the plague microbe [5]. The long-term practice of using LPV clearly demonstrates its immunological effectiveness [6]. However, the vaccine strain is not without its drawbacks. Thus, despite its more pronounced protective properties compared to the killed vaccine, rodents and primates immunized with *Y. pestis* EV frequently experienced local and systemic adverse reactions [7]. In addition, this strain exhibits selective virulence against certain species of non-human primates [7], and its tests on humans have shown high reactogenicity [8]. Due to these features, LPV does not meet the requirements of the safety profile recommended by WHO [9]. It should be noted that pgm-negative isolates are able to restore their virulence in the presence of an external source of iron. It has been established that the addition of a small amount of iron chloride during intravenous or intranasal administration to *Y. pestis* EV mice leads to the development of systemic and pneumonic plague in them [10]. Thus, LPV can be dangerous for people with iron metabolism disorders, such as hemochromatosis. In the USA two lethal cases of laboratory infection of researchers with hemochromatosis by *Y. pestis* strains with a deletion of the pgm$^-$ region were recorded [11]. Later, the ability of pgm isolates to cause disease against the background of hemochromatosis was proven in mice with a knockout for the hemojewelin gene [12]. The russian subunit vaccine was registered by the Ministry of Health in 2018 [3]. It consists of capsular and V antigens produced by recombinant *Yersinia pseudotuberculosis* EV11M/pFSK3/9 and E. coli BL21(DE3)/pETV-I-3455 strains. This vaccine is recommended for the immunoprophylaxis of servicemen operating in emergencies [2]. The components used in its composition have the most pronounced immunogenicity among the antigens of the plague microbe, in particular, the analysis of blood serum samples from individuals who had previously suffered the plague showed the possibility of maintaining antibodies to F1 and LcrV after more than 10 years from the moment of infection [13]. The using of a antigen combination can provide protection against infection by *Y. pestis* strains that carry a deletion of the caf1 operon or have a structurally different LcrV variant [14]. The safety and immunogenicity of F1-V vaccines have been confirmed in a number of clinical trials [15], and their protective properties have been repeatedly evaluated in experiments on various animal models. It has been shown that two-component vaccines are able to protect rodents from subcutaneous and aerosol infection with *Y. pestis* [16, 17], their protective effect is comparable to the effect of immunization of animals with the *Y. pestis* EV strain [17] and far exceeds the effectiveness of the killed vaccine. At the same time, experiments conducted on non-human primates gave controversial results: vaccines based on F1 and V ensured the survival of cynomolgus monkeys after aerosol infection and rhesus monkeys after subcutaneous injection of *Y. pestis* [18], but failed to provide adequate protection for green monkeys [19], which introduces uncertainty into the question of the ability of such drugs to protect people. The negative side of F1-V vaccines is their ability to predominantly stimulate the immune response along the Th2 pathway, which was shown in experiments on rhesus monkeys [18] and in clinical trials. It has also been found that immunization of mice with *Y. pestis* KIM5 stimulates the formation of CD4 and CD8 T cells that recognize antigens other than F1 and LcrV [20], which dictates the need to revise the composition of developed subunit vaccines. In this regard, there remains a need both to improve the available means of immunoprophylaxis of plague and to develop fundamentally new drugs, including a new generation of attenuated vaccines based on avirulent *Y. pestis* strains with clearly controlled genetic defects [21], which will increase effectiveness and safety of the developed prophylactic agents. This task requires a detailed analysis of the characteristics of the genetic structure of perspective strains of the plague microbe.

The purpose of this work is to determine and comparatively analyze the genomic organization of avirulent *Y. pestis* I-1 and *Y. pestis* I-3536 strains and the *Y. pestis* EV vaccine strain of the NIIEG line.

Materials and methods

In the course of the work, three strains of *Y. pestis* subsp. pestis: *Y. pestis* I-1, isolated from a human in 1923 (Chita), *Y. pestis* I-3536, selected on the basis of the Irkutsk Antiplague Research Institute of Rospotrebnadzor, and the *Y. pestis* EV vaccine strain of the NIIEG line. Plague microbe strains were cultivated on Hottinger agar (pH 7.2) at 28 °C for 48 hours.

In order to assess the possibility of reversing the initial virulence of *Y. pestis* I-1 and I-3536 strains, a number of passages were carried out on bioassay animals, which were infected with a microbial suspension prepared at a concentration of 1 10^7 mc/ml. As an experimental model, 15 guinea pigs weighing 200-250 g and 10 outbred white mice with standard conditions of detention and weight (18-20 g) were obtained from the nursery of the Irkutsk Research Anti-Plague Institute of Rospotrebnadzor (RD 42- 26-3… 3738, NPO «Vector», Novosibirsk). Animals were taken out of the experiment under anesthesia in accordance with the Rules of Good Laboratory Practice and Directive No. 2010/63/EU «On the Protection of Animals Used for Scientific Purposes» [22]. The study was approved by the local ethical committee of the institute (protocol No. 5 dated November 1, 2021). Plasmid screening of plague microbe strains was carried out according to the method proposed by T. Kieser [23], modified by S.V. Balakhonov [24]. Plasmid sizes were determined by electrophoresis in 0.7% agarose gel. The plasmid profiles of both initial cultures and passaged through laboratory animals were studied. A four-plasmid reference *Y. pestis* strain I-2638 (pMT1, pCD1, pPCP1, and pTP33) was used as a molecular marker. Analysis of electrophoretic separation was carried out using a Bio-Rad Gel Doc XR+ transilluminator (USA), a «DNA Analyzer» video recording system, and the «Quantity One ver. 4.6.0». Isolation of the total DNA of bacterial strains was performed by the method of phenol-chloroform extraction, as well as using the «DNAeasy Blood & Tissue Kit» (Germany).

Preparation of DNA libraries for nanopore sequencing was carried out according to the Native barcoding genomic DNA protocol using the «Legation Sequencing Kit» SQK-LSK109 (Great Britain) and the «Native Barcoding Expansion» EXP-NBD104 and EXP sets of barcodes for double-stranded DNA multiplexing. -NBD114 (UK). Sequencing was performed on an Oxford Nanopore MinION instrument (Great Britain).

Genomic libraries for sequencing on the Illumina MiSeq™ System (initial data of nanopore sequencing, as well as demultiplexing of raw reads were carried out using the MinKNOW program and the Guppy ver. 3.6.0 instrument. Paired reads obtained on the Illumina MiSeq instrument were qualitatively FastQC version 0.11.9 and pre-processed using the Trimmomatic tool version 0.40 [25].The coverage and number of reads for each sequenced genome were estimated using the Proch::N50 version 1.3.0 package. Hybrid assembly of sequencing data obtained on both platforms was performed using the SPAdes ver. assembler. 3.13.0 [26]. The resulting sequences were annotated using Prokka ver. 1.12 [27]. With the tool Roary ver. 3.13.0 [28], these annotations were examined to find core, group-specific, and unique genes. An alternative approach to genome assembly was to map reads to chromosome sequences and plasmids of the *Y. pestis* CO92 reference strain. The analysis of large genetic rearrangements was carried out using the multiple alignment method in the Mauve ver. 2.4.0 [29]. In order to search for biosynthetic gene clusters, the software tool antiSMASH ver. 5.0 [30].

Results and discussion

Analysis of the plasmid profile of the *Y. pestis* I-1 strain showed the presence of 45 and 6 MDa plasmids in its composition, which corresponds to pCD1 and pPCP1. In the structure of the *Y. pestis* I-3536 strain, two plasmids with molecular weights of 61 and 6 MDa (pMT1 and pPCP1) were found. The *Y. pestis* EV NIIEG strain taken into the work contained pMT1, pCD1, and pPCP1 plasmids typical for it with molecular weights of 61, 45, and 6 MDa. It was shown that the plasmid composition of *Y. pestis* strains I-1

and I-3536 did not change during passage through bioassay animals.

Whole genome sequencing

According to the results of de novo hybrid assembly of the genomes of three *Y. pestis* strains, the number of contigs in the obtained sequences varied from 3 to 4.

When using the method based on mapping of reads to the genome of the reference *Y. pestis* CO92 strain (Table 1), it was shown that the length of the *Y. pestis* I-3536 circular chromosome is 4.5 Mb, this strain is characterized by the presence of two plasmids, pMT1 95.7 kb and pPCP1 7.8 kb, as confirmed by plasmid analysis. The genome of the *Y. pestis* EV NIIEG strain is represented by a ring chromosome about 4.5 Mb in size and three plasmids pMT1, pCD1 and pPCP1, having a length of 94.5; 70 and 7.8 kb respectively.

Unlike the other two strains, *Y. pestis* I-1 is characterized by a smaller chromosome size (about 4.3 Mb), and its plasmid profile is represented by three plasmids: pCD1 and pPCP1, 68.5 and 7.8 kb long, and an additional plasmid with the size 340 kb, which was not previously determined from screening data, which may be due to the higher resolution of genome-wide analysis. The detected plasmid was not detected in the genome of other previously studied *Y. pestis* strains, and is probably the result of genetic rearrangements that occurred during laboratory passages of the *Y. pestis* I-1 culture. The presence of 4140-4227 protein-coding sequences, 70-73 tRNA-coding loci, from 19 to 22 rRNA sequences, and one tmRNA gene was predicted in the genomes of strains of the studied sample (Table 1). By aligning the annotated sequences of Y. pestis, it was found that their pangenome includes 4391 loci, while 3957 genes are cow genes. Among the identified group-specific genes, 98 loci were common for *Y. pestis* EV and *Y. pestis* I-1, and 123 genes were characteristic for plague microbes EV and I-3536. The number of unique loci varied depending on the strain of the microbe and was maximum for *Y. pestis* I-1 (123 genes).

The search for genes encoding microbial pathogenicity factors (Table 2) showed that

Table 1. Features of the genomic organization of the studied strains

Показатель Index *Y. pestis EV*		**Наименование штамма Name strain**		
		***Y. pestis* И-1**	***Y. pestis* И-3536**	
Структура генома, размер п.н. Genome structure, bp size	Хромосома Chromosome	4 553 041	4 296 984	4 553 148
	pMT1	94 506	–	95 741
	pCD1	70 336	68 592	–
	pPCP1	7897	7897	7897
Число контигов Number of contigs		4	4	3
GC-состав, % GC composition, %		47,6	47,6	47,6
Общее число ORF Total number		4317	4286	4230
Белок-кодирующие последовательности Protein coding sequences		4227	4190	4140
тРНК		70	73	70
рРНК		19	22	19
тмРНК		1	1	1

Table 2. Structure of genome elements of the studied Y. pestis strains

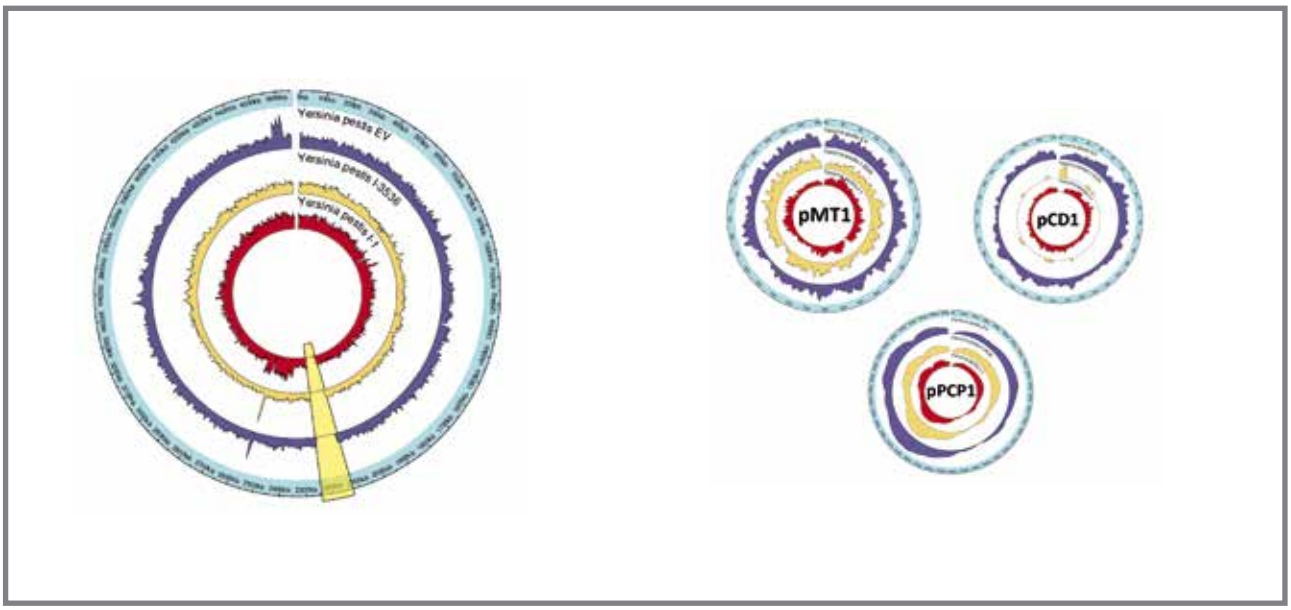
Наименование штамма Name strain	Количество выявленных генов факторов вирулентности Number of identified virulence factor genes				
	Хромосома Chromosome	pMT1	pCD1	pPCP1	NN, 340 kb
Y. pestis EV	95	5	42	1	–
Y. pestis И-1	104	–	42	1	2
Y. pestis И-3536	95	5	–	1	–

the chromosome structure of the studied strains includes waaI, waaII, and wab clusters associated with lipopolysaccharide synthesis, a number of loci that determine lipid A biosynthesis including genes for late acyltransferases LpxL, LpxP, and MsbB, genes for lipoprotein Lpp and Ail protein, and psa operon loci encoding factors for the synthesis and assembly of the pH6 antigen.

It was found that the pMT1 plasmid found in *Y. pestis* EV and *Y. pestis* I-3536 contains the caf1 operon genes, which determine the synthesis and assembly of the F1 antigen, and the mouse toxin Ymt gene. The organization of the pPCP1 plasmid was the same for all strains and included the Pla plasminogen activator gene. In turn, the plasmid pCD1, present in the genome of *Y. pestis* I-1 and *Y. pestis* EV, included 42 loci, including the pseudogene of the YadA adhesin protein, genes for the components of the secretion apparatus of the third type (Ysc), effector proteins Yops, and regulatory T3SS factors. It was determined that the genome of the *Y. pestis* I-1 strain contains genes of the caf1 operon, which have chromosomal rather than plasmid localization. Also, in the structure of the chromosomes of this isolate, nine copies of the InvA invasin pseudogene were found, which was present in the genome of two other strains in the amount of two copies.

It was shown that the genome structure of all strains contained a deletion of the chromosome locus pgm with a size

Figure 1. Map of the depth of reed coverage of the studied strains relative to the genome of the reference strain Y. pestis CO92. The segment highlighted in Figure 1a corresponds to the deletion of the pgm locus 1a – coverage of chromosome sequences, the selected segment corresponds to the deletion of the pgm locus. 1b - coverage of plasmid sequences



of 102 thousand b.p. (Fig. 1). The loss of this region, as well as a number of plasmid genes, causes the avirulence of *Y. pestis* I-1 and *Y. pestis* I-3536, which allows us to consider these strains as promising candidates for the subsequent development of a live attenuated vaccine based on them. Of particular interest is *Y. pestis* I-1, in which the loss of the pgm locus occurred independently of the strains of the EV line.

It was found that despite the results of a screening study and assembly of the *Y. pestis* I-1 genome, indicating the absence of the pMT1 plasmid in its composition, a comparison of the sequence of this strain with the *Y. pestis* C092 genome revealed the presence of regions homologous to this plasmid in the chromosome, as well as Plasmids 340 kb long. Similarly, it was shown that the structure of the *Y. pestis* I-3536 genome contains sequences homologous to the pCD1 plasmid lost by this strain.

Subsequently, multiple alignment of the chromosome sequences of the *Y. pestis* I-1 and I-3536 strains relative to the *Y. pestis* EV genome was performed in order to reflect such major evolutionary changes as inversions, translocations, and horizontal gene transfer events (Fig. 2).

According to the alignment data, it was found that the nucleotide sequence of *Y. pestis* I-3536 contains a large region of 146 kb, inverted relative to the *Y. pestis* EV genome. Also, in contrast to the control sequence, the presence of a significant number of non-homologous regions was revealed in the genome of the *Y. pestis* I-1 strain.

Also in this work, an analysis of the *pyrE* orotate phosphoribosyltransferase gene, which was previously characterized

Figure 2. Comparison of the genomic structure of the studied strains:
1 – Y. pestis EV; 2 – Y. pestis I-3536; 3 – Y. pestis I-1

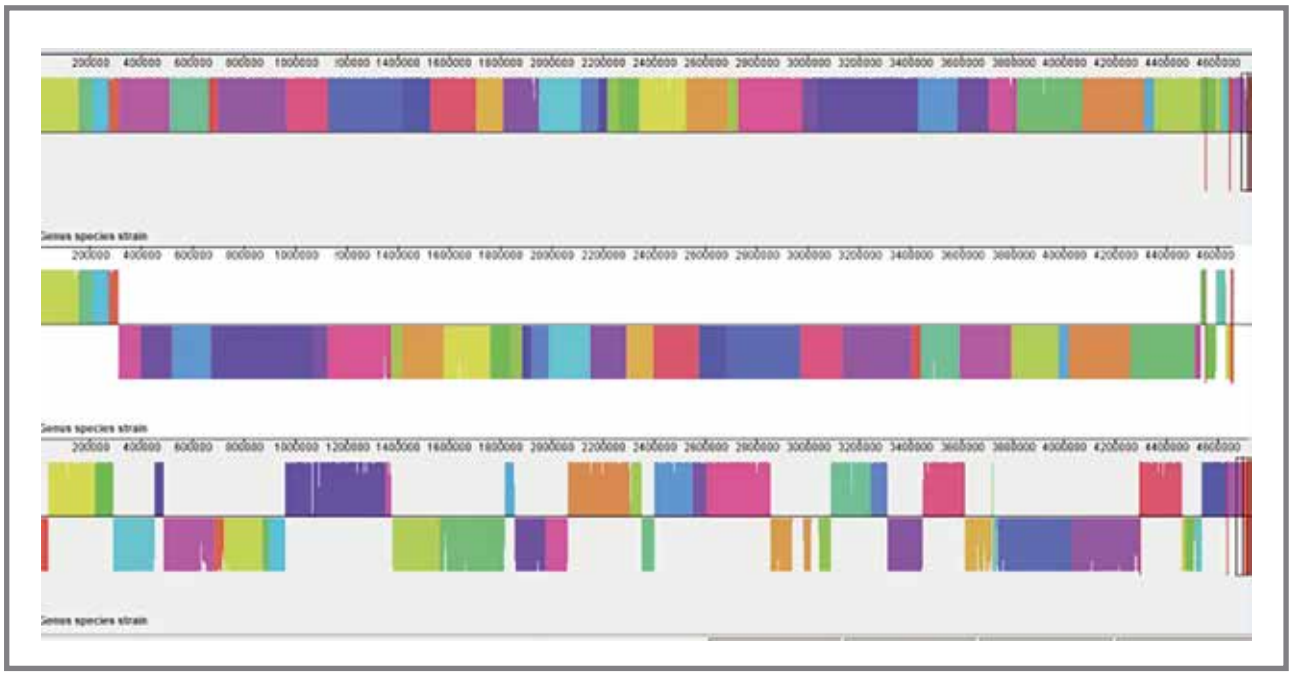


Figure 3. Structure of the target region of the pyrE gene of the Y. pestis strains

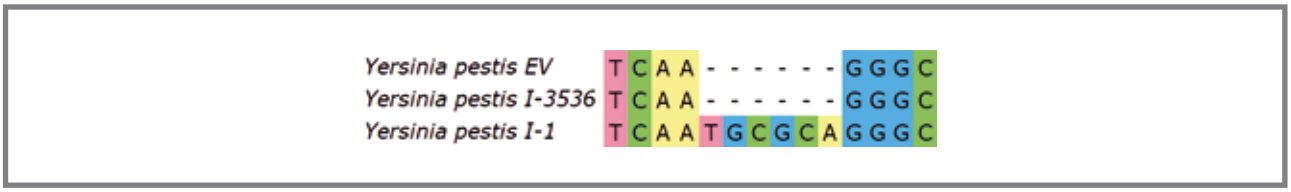
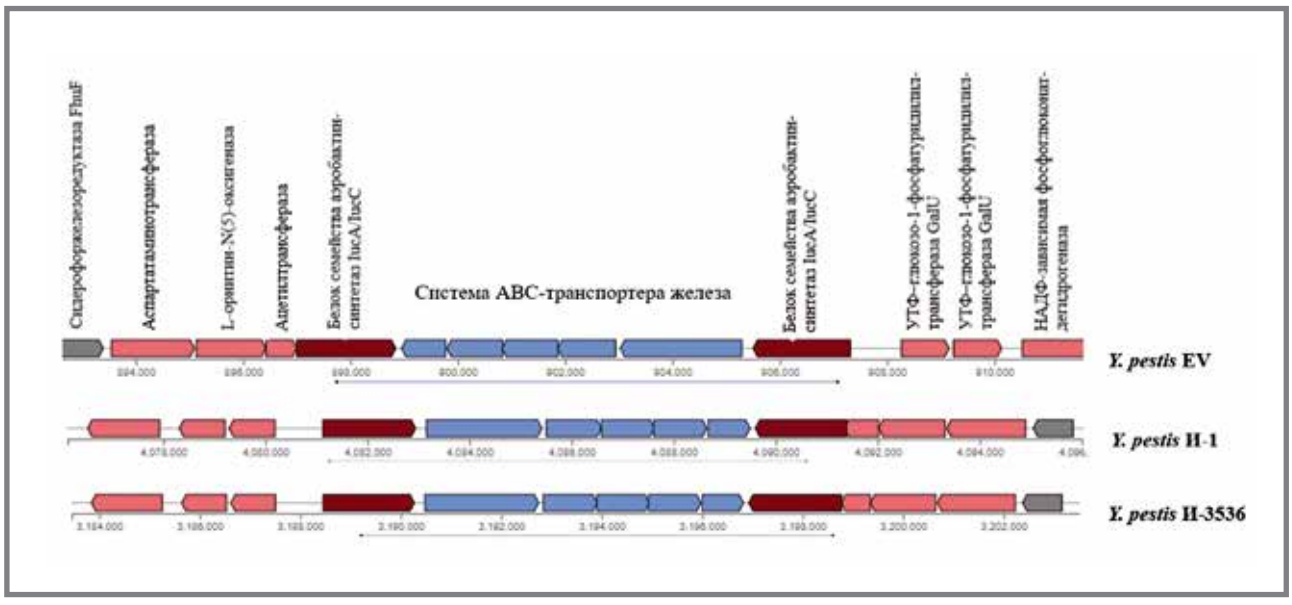


Table 3. Screening data for biosynthetic clusters

Наименование Name	Известный кластер с наибольшим сходством Known Cluster with Most Similarity	Процент сходства, % Similarity percentage, %
Кластер синтеза тиопептидных антибиотиков Cluster for the synthesis of thiopeptide antibiotics	–	–
Гены, подобные поликетид-синтазам Genes similar to polyketide synthase	Taxlllaids A	15
Кластер синтеза сидерофора Cluster of siderophore synthesis	Путребактин/ аваробактин Putrebactin/Avaroferrin	100
Гены, кодирующие синтез серлактона Genes encoding serlactone synthesis	–	–
Гены, подобные нерибосомным пептидсинтетазам Genes similar to nonribosomal peptide synthetases	Колицин V Colicin V	1
Кластеры, кодирующий синтез сидерофора Clusters encoding siderophore synthesis	Аэробактин Aerobactin	66
Гены нерибосомных пептидсинтетаз Genes of nonribosomal peptide synthetases	Колицин V Colicin V	1
Кластер, кодирующий β-лактонсодержащий ингибитор протеазы Cluster encoding a β-lactone-containing protease inhibitor	Колицин V Colicin V	1

Figure 4. Structure of the detected siderophore synthesis gene cluster



as a mutation hotspot for strains of the *Y. pestis* EV line, was carried out [31]. It was found that *Y. pestis* strain I-3536, like *Y. pestis* EV of the NIIEG line, is characterized by the presence of a 6 b.p. deletion located at the 3' end of *pyrE* (Fig. 3). Unlike them, *Y. pestis* I-1, like wild-type strains, contains the intact sequence of this locus.

In the course of the study, a search was made for clusters responsible for the synthesis of bacterial secondary metabolites. Eight similar regions were identified (Table 3).

Of particular interest was a cluster presumably associated with the synthesis of the siderophore putrebactin/avaroferrin. Currently, data have been accumulated on the important role of transition

metal transport systems in the survival of *Y. pestis* in the macroorganism and their influence on the pathogenesis of plague [5]. The identified cluster (Fig. 4) contained two copies of the gene encoding a protein belonging to the IucA/IucC family of aerobactin synthetases, the genes for sideroforge iron reductase FhuF, aspartate aminotransferase, L-ornithine-N(5)-oxygenase, and acetyltransferase, and the gndA locus encoding NADP- dependent phosphogluconate dehydrogenase, two copies of the UTP-glucose-1-phosphate uridylyltransferase GalU gene, genes for the components of the ABC iron transport system - ATP-binding protein, two permeases of the iron transporter ABC, substrate-binding protein, TonB-dependent siderophore receptor.

It was noted that for *Y. pestis* I-1 and *Y. pestis* I-3536 the organization of the gene cluster is identical. A different picture is typical for *Y. pestis* EV: inversion and translocation of this structure is observed in the genome of this strain. The question of the possible role of this cluster in the virulence and reactogenicity of the plague microbe remains open, which requires its further study.

Conclusion

Thus, within the framework of the work, the study of the genomic organization of two avirulent strains *Y. pestis* I-1 and *Y. pestis* I-3536 was carried out, and the genetic structure of the *Y. pestis* EV vaccine strain was refined. According to the data obtained, all three strains are characterized by a deletion of the pgm chromosomal region, which, by analogy with *Y. pestis* EV, makes it possible to use *Y. pestis* I-1 and *Y. pestis* I-3536 as the basis for creating a vaccine culture. Of particular interest was the *Y. pestis* I-3536 strain, which is characterized by the loss of the pCD1 plasmid (if there are regions homologous to the plasmid in the genome structure), which in theory may indicate a lower reactogenicity of this strain compared to *Y. pestis* EV and the preservation of its immunogenicity due to the presence of the pMT1 plasmid. In turn, *Y. pestis* I-1 lost the pMT1 plasmid, whose homologous sequences were found in the chromosome and 340 kb plasmid.

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