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EXPERIMENTAL WORKS

Genovariants of *Vibrio cholerae* Biovar El Tor: Construction, Molecular-Genetic, and Proteomic Analyses

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Abstract—Experimental modeling of the emergence of virulent *Vibrio cholerae* El Tor genovariants is presented. It has been shown that the obtained genovariants differed neither in phenotypic or genotypic traits from natural genetically altered strains that emerged in populations of wild-type strains. It has been established, using the PCR and sequencing methods, that the genovariants formed in the process of conjugation carried in their genome a fragment of the $CTX^{Class}\varphi$ prophage genome with the *ctxB1* gene of classical-type cholera vibrios. It has been shown that changes in the prophage's structure led to higher levels of toxigenicity and virulence in the genovariants compared to a typical recipient strain. A proteomic analysis has also revealed changes in the expression of 26 proteins performing various functions in the cell, such as metabolism, energy exchange, transport of amino acids, etc.). These data are indicative of the effect produced by the new DNA region in the genome of the genovariants on the expression level of some house-keeping genes. The obtained results confirm the idea that horizontal gene transfer is one of the mechanisms leading to the emergence of genovariants in the populations of wild-type strains.

Keywords: *Vibrio cholerae* genovariants, CTXφ prophage, conjugation, recombinants, toxigenicity and virulence, proteome

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INTRODUCTION

A substantial portion of the genome of many human-pathogenic bacteria is represented by mobile elements carrying genes of virulence and adaptation to changing environmental conditions, as well as drug-resistance genes [3, 4, 6]. This circumstance determines the genomic variability of many dangerous infectious agents, which is manifested in the emergence of their genetic variants with a new combination of various genes. Novel genovariants of agents are commonly formed when pathogenic strains acquire mobile DNA regions from other organisms through horizontal gene transfer playing a key role in the evolution of prokaryotes. The most serious epidemiological consequences of such events develop when we observe the formation and a wide range of novel agent genovariants causing infectious diseases of exceptional epidemic hazard, such as cholera, seven pandemics of which (from 1817 to today) have claimed millions of human lives [1]. In the recent period, the interest of many researchers has been focused on studies of the mechanisms of spontaneous formation of genovariants with increased virulence and a high level of adaptation to changing environmental conditions [7, 32, 38]. The etiological agents of the recorded seven cholera pandemics were cholera vibrios serogroup O1 referred either to the classical or to the El Tor biovar [1]. Bacteria of *V. cholerae* classical biovar caused the first six pandemics of Asiatic cholera (1817–1926). Subsequently, in about 40 years (1923–1961), these strains were replaced by bacteria of *V. cholerae* biovar El Tor, the causative agent of the current, seventh, cholera pandemic (from 1961 to today). Vibrios of one biovar were replaced by others due to evolutionary changes in the structure and function of the cholera agent's genome. One of the most significant differences between the classical and El Tor agents is in different structures of the CTXφ prophage carrying the *ctxAB* operon encoding cholera toxin (CT) causing profuse diarrhea, the main clinical symptom of the condition [28]. The structure of phage DNA contains a core region 4.5 kbp long and a DNA fragment 2.4 kbp long designated RS-2. Apart from the *ctxAB* operon, the core region contains the *zot*, *ace*, *orfU*, *cep*, and *psh* genes necessary for the formation of phage particles, as well as for the production of accessory toxins Zot and Ace. The RS-2 sequence contains the *rstA*, *rstB*, and *rstR* genes, as well as two intergenic regions (ig-1 ig-2), controlling regulation, replication, and integration of the indicated phage (Fig. 1a) [41].

It is also known that the CTXφ prophages in the classical and El Tor vibrios have different genetic

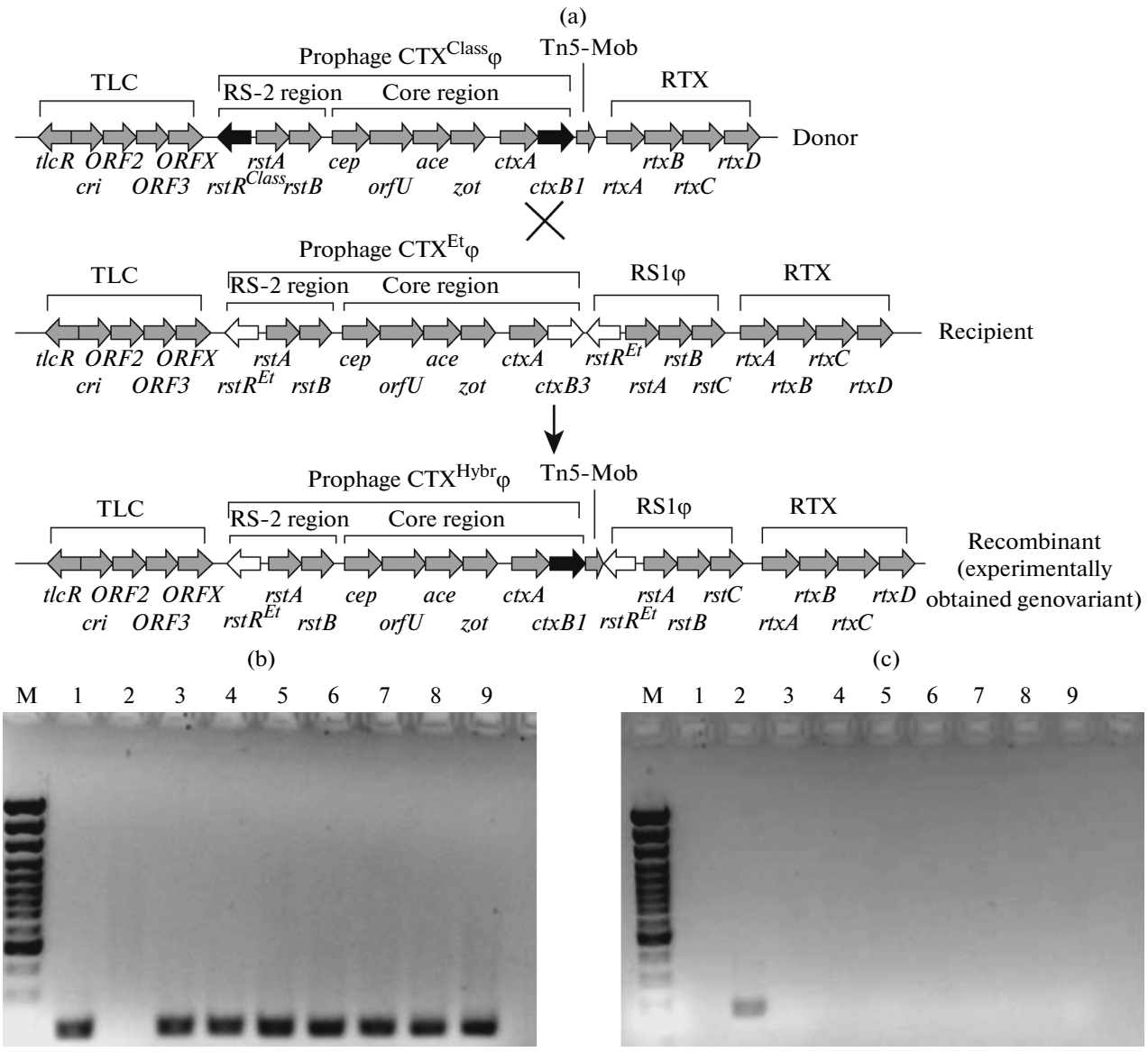

Fig. 1. Scheme for conjugational crossing (a) of the donor and recipient strains of *V. cholerae* biovar El Tor: the KM7P donor strain chromosome has the CTX^{Class}φ prophage with the *ctxB1* gene and the Tn5-Mob transposon, while the recipient has the CTX^{Et}φ prophage with the *ctxB3* gene. The chromosome of the recombinants contains the Tn5-Mob transposon and the genomic region of the donor CTX^{Class}φ prophage, which includes the *ctxAB1* operon with the promoter region; b and c are MAMA-PCR analysis results indicating the presence of the *ctxB1* allele (b) in both the donor and the recombinants and the presence of the *ctxB3* allele in the recipient (c); numbers designate the following tracks: M is the molecular mass marker, 1 is the donor KM7P strain, 2 is the recipient M818 strain, and 3–9 are the recombinant R1–R7 strains.

backgrounds. Whereas in El Tor cholera vibrios three genetic elements (RS1 prophage, TLC-element and RTX gene cluster) are located near their prophage, classical vibrios contain only the last two elements [17, 28, 29]. The RS1φ prophage commonly flanking CTXφ in *V. cholerae* biovar El Tor contains three genes (*rstA*, *rstB* and *rstR*) homologous to those of the CTXφ RS-2 region, as well as one supplementary gene designated *rstC*. The RstC protein is an antirepressor, which secures transcription of genes of CTXφ, including the *ctxAB* genes, by blocking the phage repressor of *rstR* [15, 31]. The second genetic element, TLC (toxin-linked cryptic), which is closely bound to the genes of the CTXφ prophage, is also a filamentous phage (TLCφ) integrated into the chromosome ahead of the CTXφ prophage [17]. The 4.5 kbp long TLCφ prophage genome contains the *cri* and *tlcR* genes encoding, respectively, the Cri replicase homologous to the replication protein of filamentous phages and the TlcR protein, which significantly resembles the RstR protein securing the lysogeny of the CTXφ prophage. The third genetic element, a satellite of the CTXφ prophage, con-

tains a nucleotide sequence RTX about 10 kbp long containing a gene cluster (*rtxA*, *rtxB*, *rtxC*, and *rtxD*) coding for the RTX toxin (repeat in toxin) [21].

Analysis of sequencing results helped establish that structural differences between the genomes of the CTXφ prophages of *V. cholerae* classical and El Tor biovars are determined by differences in the nucleotide sequences of two genes—the repressor gene *rstR* located in the RS-2 region of the prophage and responsible for the lysogenic state of cholera vibrios and the *ctxB* gene in the composition of the *ctxAB* operon, which codes for the CT B-subunit. Due to these differences, the corresponding alleles of the above-mentioned genes are designated $rstR^{Class}$ and $ctxB^{Class}$ or (*ctxB1*) in classical *V. cholerae* and as $rstR^{Et}$ and $ctxB^{Et}$ or (*ctxB3*) in *V. cholerae* biovar El Tor. The prophages were given, respectively, the following designations: CTX^{Class}φ and CTX^{Et}φ [27]. Such differences resulted in a lower level of CT production by the biovar El Tor vibrios compared with the classical *V. cholerae*, which leads to mild and asymptomatic cholera forms, as well as to prolonged cholera-vibrio carrying.

At the same time, a rather rapid evolutionary process in the genome of *V. cholerae* El Tor led to the emergence of its novel variants that have been identified thirty years after the outbreak of the seventh pandemic. Despite their genetic diversity, the *V. cholerae* biovar El Tor genovariants have three common traits. First, their CTXφ prophage contains the *ctxB1* gene belonging to the classical cholera vibrios. Second, these genovariants differ from typical strains in having an increased virulence, which manifests itself in the development of severe clinical cholera forms and in a high mortality rate. Third, they exhibit a high level of adaptation to changing environmental conditions, which has led to the replacement of typical strains by novel genovariants in many countries in Asia and Africa.

Apart from the classical vibrios, *V. cholerae* strains of non-O1/non-O139 serogroups containing the classical CTX^{Class}φ prophage genome could serve as a hypothetical donor for the *ctxB1* gene [7, 32]. Another probable source for the *ctxB1* gene could be the virulent *V. cholerae* O1 biovar El Tor strains isolated before the outbreak of the seventh cholera pandemic and specified as prepandemic. The main differences of these isolates from the typical strains of the seventh pandemic's agent were in the presence of the CTX^{Class}φ prophage against the absence of the RS1φ prophage in their chromosome [30]. The main mechanism securing *ctxB1* gene horizontal transfer is believed to be a transformation taking place in a biofilm formed on a chitin substrate or a conjugation [10, 25]. At the same time, this suggestion has not so far been confirmed experimentally.

To study the onset mechanism for the epidemically hazardous *V. cholerae* El Tor genovariants, we conducted for the first time model experiments on their formation in the process of conjugation, studied the phenotypic and molecular biological traits of the obtained recombinants, and assessed the expression of their virulence and house-keeping genes by proteomic analysis.

MATERIALS AND METHODS

Bacterial strains, plasmid, and transposon. The following strains of *Escherichia coli* and *V. cholerae* were used in the study: *E. coli* C600 (pRP4-4) (Ap^R, Tc^R, Km^S; *V. cholerae* O1 MAK757 biovar El Tor, a clinical strain isolated in 1939 on Celebes Island (Indonesia); *V. cholerae* M818 biovar El Tor isolated in Saratov in 1970; *V. cholerae* O1 classical biovar 569B, a clinical strain isolated in 1949 in India; and *V. cholerae* O1 MAK757 chr:: Tn5-Mob (Km^R Tox^{++}) biovar El Tor, a derivative of the *V. cholerae* O1 strain MAK757. We obtained the donor strain using the previously described methods [36]. *V. cholerae* strains were provided by the State Collection of Pathogenic Bacteria. The *E. coli* C600 (pRP4-4) strain was provided by T.S. Il'ina, Gamaleya Research Institute of Epidemiology and Microbiology, Moscow.

Nutrient media and cultivation conditions for strains. To culture bacteria, we used a Luria Bertani (LB) broth or agar. The minimum medium was a glucose–saline agar with the corresponding liquid medium. We introduced amino acids (Serva, Merk) at concentrations of 20–40 μg/mL, and the antibiotics were kanamycin (50 μg/mL), ampicilline (50 μg/mL), tetracycline (2 μg/mL), and streptomycin (100 μg/mL). Cultivation was carried out at different temperature regimes (30 and 37°C).

Conjugational crossings. Intergeneric (*E. coli* with *V. cholerae*) and intraspecific (*V. cholerae* with *V. cholerae*) crossings were carried out on LB agar by the method described in [11]. The donor counterselection in intergeneric crossings was based on the donor's growth requirements. LB-agar containing kanamycin (50 μg/mL) and streptomycin (100 μg/mL) served as a selective medium in intraspecific crossings. The transfer frequency of a plasmid or chromosomal marker was determined per donor cell.

Determination of CT production. CT production by *V. cholerae* strains was determined using radial passive immune hemolysis (RPIH) on solid media and GM_1 ganglioside enzyme-linked immunosorbent assay (GM_1-ELISA) method. RPIH was performed using a modified method [12]. In accordance with to the replica method, isolated colonies of strains under study were placed into plates with a 1.0% syncase agar containing 1.0% sheep red blood cells washed in a syncase broth. Inoculants were incubated at 30°C for 18 h; then covered by a 1%-syncase agar layer containing sodium azide (NaN_3), a guinea-pig serum complement, and rabbit antitoxic serum; and placed into a thermostat at 37°C for 1–2 h. A zone of specific

hemolysis was formed after the incubation about macrocolonies producing and secreting CT into a culture medium. The CT production was quantitatively determined using GM_1-ELISA [39]. Dilutions of purified cholera toxin (by Sigma Chemicals) at the well-known concentration were used for constructing a standard curve and assessing CT in samples. The ELISA procedure was performed using antitoxic antibodies in rabbits and horseradish peroxidase-conjugated antirabbit IgG (by Gibco-BRL).

Intraintestinal challenge of suckling rabbits with cells of isogenic strains differing in the levels of cholera endotoxin. The virulence of the strains to be studied was determined using the method described in [14] by intraintestinally challenging suckling rabbits. Under anesthesia, 10^7 vibrios in 0.2 mL 0.9% NaCl solution were enterally administered to animals 8–10 days old and weighing 130–160 g. All the animals which died or were killed with chloroform were dissected and macroscopic changes in the large and small intestines were recorded. The procedure for determining the level of virulence was carried out with observance of all the requirements obligatory for dealing with experimental animals.

PCR analysis. Polymerase chain reaction (PCR) was conducted under the conditions described in [13]. Amplicons were distinguished using agarose gel electrophoresis (1.5%) in a 0.5× Tris/borate/EDTA buffer. The products were stained with ethidium bromide, visualized under UV light, and photographed with a gel-documenting system. Mismatch amplification mutation assay (MAMA) PCR was performed in accordance with the methods described in [24]. The primers used in this work are given in Table 1.

Southern blot hybridization and DNA probes. Isolation of chromosomal DNA, a restriction analysis, and Southern blot hybridization with a molecular probe was performed by the method described in [5]. Staining DNA with digoxigenin and its detection were carried out in accordance with the recommendations of the manufacturer (Roche Molecular Biochemicals). The isolated chromosomal DNA was treated with restriction endonuclease PstI (Fermentas). The obtained fragments were separated in a 1% agarose gel by electrophoresis and transferred onto nylon filters for hybridization. The molecular probe used for detecting CT genes was a PCR amplicon of a *ctxA* gene fragment encoding the biosynthesis of the CT A-subunit. The CT probe was 564 bps long.

DNA sequencing. The nucleotide sequence of the CTXφ prophage was determined with 3500xL Genetic Analyzers (Applied Biosystems, USA) according to the method described in [33]. The nucleotide sequences of the studied strains were compared with the corresponding sequences of the reference strains N16961 of biovar El Tor and O395 of the classical biovar taken from GeneBank using the Mega 4 program.

Proteomic analysis. Two-dimensional electrophoresis was performed by the method described in [29] with the following modifications: bacterial cells of the studied strains were lysated in an L buffer containing urea 7 M, thiourea 2 M, DTT 65 mM, 5% Ampholine pH 3–10 (Amersham), 4% CHAPS (Amersham), and 1.5% NP-40. Each specimen (150 μg protein) was subjected to isoelectric focusing with subsequent separation of their molecular masses by electrophoresis under denatured conditions (SDS-PAGE) by the technique described in [19]. Separation in the second measurement (SDS-PAGE) was carried out in a gradient 9–16% polyacrylamide gel (1.5 mm thick) using a PROTEAN II XI 2-D Cell (Bio-Rad) with a current of 35 mA for the gel for 6 h at room temperature. After electrophoresis, the gels were fixed in a solution of 20% ethanol in 10% acetic acid and stained with silver in the presence of thiosulfate. Mass spectra were obtained with a Bruker Reflex III MALDI-TOF mass spectrometer equipped with a UV laser (336 nm) under a regime of positive ions within a mass range of 500 to 8000 D. The obtained mass spectra were calibrated using the well-known masses of internal standards. After trypsinolysis, proteins were identified by sets of peptide mass values using the Peptide Mass Fingerprint option of the Mascot software (Matrix Science) with an accuracy of mass determination MH+ equal to 0.01%, admitting the possibility of modifying cysteines by acrylamide, as well as possible methionine oxidation. The NCBI database was used for searching for proteins.

RESULTS AND DISCUSSION

Obtaining *V. cholerae* El Tor genovariants carrying the *ctxB1* gene of classical cholera vibrios. At the first stage of our study we constructed a donor strain able to transfer the *ctxB1* gene during conjugation, as well as selected a recipient belonging to typical virulent strains of *V. cholerae* biovar El Tor carrying the *ctxB3* allele in the composition of the CTX^{Et}φ prophage.

To create the donor strain, we used the binary system described in [34] and consisting of the transposon Tn5-Mob (Km^R) and the helper plasmid pRP4-4 ($Ap^RTc^RKm^S$). The above-mentioned transposon determining resistance to kanamycin was suitable, since it contained the origin of the pRP4-4 $Ap^RTc^RKm^S$ plasmid transfer (oriT) designated as the Mob region. The previously obtained data have shown that the insertion of Tn5-Mob into a bacterial chromosome in the presence in the cell of the pRP4-4 plasmid supplementary tra-operon in transposition can secure directed transfer of chromosomal genes [2, 36]. Furthermore, we used our previously constructed highly toxigenic strain *V. cholerae* biovar El Tor MAK757 chr::Tn5-Mob Km^R, Tox^{++}, a derivative of the prepandemic strain MAK757 carrying the classical CTX^{Class}φ prophage [8]. This choice of this strain was influenced by the introduction of the Tn5-Mob transposon into the

Table 1. Primer sequences used in the study

Primer name	Primer sequence (5'–3')	Target gene	Temperature of annealing, °C	Reference
cep-F cep-R	CAGAACAATTGCCCCCACCAC AAGCACGCTTTCACTCGGGG	*cep*	55	Authors' estimates
orfU1 orfU2	CAAAATGAGCATGGCGGC CCCATTGTGCAATCGGTGT	*orfU*	57	Authors' estimates
ace1 ace2	TAAGGATGTGCTTATGATGGA-CACCC CGTGATGAATAAAGATACTCATAGG	*ace*	60	[40]
zot1 zot2	TCGCTTAACGATGGCGCGTTTT AACCCCGTTTCACTTCTACCCA	*zot*	60	[37]
CTX3 CTX2	CGATGATCTTGGAGCATTCCCAC CGGGCAGATTCTAGACCTCCTG	*ctxA*	62	[16]
Fw-con** Rv-class** Rv-eltor**	ACTATCTTCAGCATATGCACATGG CCTGGTACTTCTACTTGAAACG CCTGGTACTTCTACTTGAAACA	$ctxB^{Class}$ $ctxB^{Et}$	58	[24]
rstA-1 rstA-2	ATCGTCGTGAATTTCCTTAG GCGATTAGTCTCTGAGCC	*rstA*	55	Authors' estimates
rstB-1 rstB-2	CGTGATGGGTCTTCTGGGTC TGGTGCCTCTCATTCTGAAG	*rstB*	58	Authors' estimates
rstC1 rstC2	AACAGCTACGGGCTTATTC TGAGTTGCGGATTTAGGC	*rstC*	55	[39]
Tn5-Mob1 Tn5-Mob2	AGTAGCGTCCTGAACGGAACCTTT AAGAGAACGGAGTGAACCCACCAT	*Tn5*	59	Authors' estimates
TLCR-1 TLCR-2	CTCTTCCTCATCCTCTGTCAG GAGGATCACACTAGACGGGTA	*tlcR*	60	Authors' estimates
cri-TLC-1 cri-TLC-2	AAAAAGGACGTAATCATCGAAATGG CGGACGTTGATTGTCGAAGTTAAAG	*cri*	60	Authors' estimates
rtxA-1 rtxA-2	CACTCATTCCGATAACCAC GCGATTCTCAAAGAGATGC	*rtxA*	55	[21]
rtxCF rtxCR	CGACGAAGATCATTGACGAC CATCGTCGTTAATGTGGTTGC	*rtxC*	59	[13]
rstA-3R* rstR-1F (Class)* rstR-2F (Et)*	TCGAGTTGTAATTCATCAAGAGTG CTTCTCATCAGCAAAGCCTCCATC GCACCATGATTTAAGATGCTC	$rstR^{Class}$ $rstR^{Et}$	58	[9]
ctxB1* ctxB2*	ATGATTAAATTAAAATTTGG TTAATTTGCCATACTAATTG	*ctxB*	42	[35]
prctxAB-1* prctxAB-2*	ATGGGCGACAGGTCATCACATTTAC GAACTCAGACGGGATTTGTTAGGCA	promoter *ctxAB*	57	Authors' estimates

* The primers were used for sequencing.

** The primers for MAMA-PCR: Fw-con is the straight primer common to both alleles. Rv-class is the reverse primer for the *ctxB1* allele; Rv-eltor is the reverse primer for the *ctxB3* allele.

chromosome near one copy of the $CTX^{Class}\varphi$ prophage (Fig. 1a). Such a localization of the transposon allowed us to use Km^R as a selectable marker in selecting recombinants receiving the donor *ctxB1* allele. To obtain a donor strain, the pRP4-4 ($Ap^RTc^RKm^S$) helper plasmid was introduced into the cells of *V. cholerae* MAK757 chr::Tn5-Mob Km^RTox^{++}. The *E. coli* C600 (pRP4-4) strain served as a donor for this plasmid. As a result of conjugational crossings, we have obtained *V. cholerae* MAK757 chr::Tn5-Mob (pRP4-4) transconjugants with the $Ap^RTc^RKm^S$ phenotype. The transfer frequency of the pRP4-4 plasmid was 7×10^4. The obtained transconjugants were tested for the donor traits in the crossings with the recipient strain *V. cholerae* biovar El Tor M818 Str^RTox^+, which carries one copy of the $CTX^{Et}\varphi$ prophage (Fig. 2, track 3)

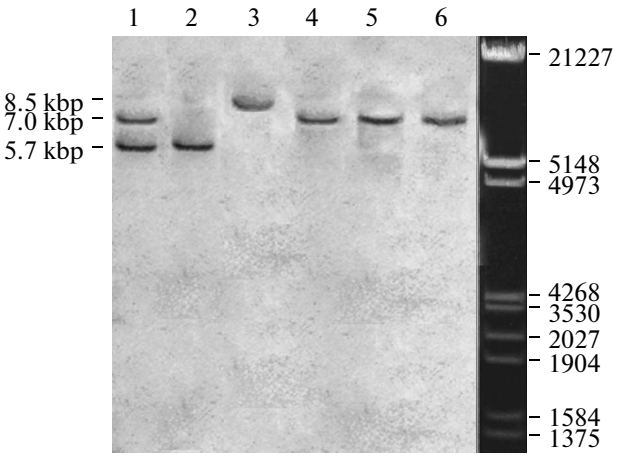

Fig. 2. Results of the DNA–DNA hybridization of donor, recipient, and recombinant *V. cholerae* biovar El Tor strains. Numbers designate tracks: 1 is the initial MAK757 strain, 2 is the KM7P donor strain, 3 is the recipient M818, and 4–6 are the recombinant strains R1–R3.

in its chromosome and produces CT in an insignificant amount. The recombinants were selected on a nutrient medium containing kanamycin. The growth of donor cells was prevented by adding streptomycin into a selective medium. All the tested 15 clones of $Ap^RTc^RKm^R$ proved to be capable of transferring the chromosomal marker Km^R. A clone showing the most effective transfer of this marker (5×10^{-6}) was chosen as the donor and designated *V. cholerae* biovar El Tor KM7P chr::Tn5-Mob (pRP4-4). Thus, the constructed *V. cholerae* KM7P chr::Tn5-Mob (pRP4-4) donor strain contained the *ctxB1* gene in the genome of the $CTX^{Class}\varphi$ prophage and had the phenotype of $Ap^RTc^RKm^RTox^{++}$. The recipient *V. cholerae* biovar El Tor M818 Str^R strain carrying, unlike the donor, $CTX^{Et}\varphi$ with the *ctxB3* genotype had the Tox^+ phenotype.

The second stage of the work was aimed to check how the Km^R recombinants obtained in crossing the donor KM7P with the recipient M818 inherited the nonselectable donor *ctxB1* allele. Since it is known that the donor strain, as well as other natural genovariants carrying the classical *ctxB* (*ctxB1*) gene, usually produces CT in significantly larger amounts than do typical strains [26, 38], we believed that the Km^R recombinants obtaining the donor *ctxB1* gene had to differ from the recipient in having higher production of this protein. Due to this, the presence of the nonselectable donor *ctxB1* allele in the Km^R recombinants was determined by evaluating their CT production using the traditional plate method—RPIH on a solid medium. As a result, seven recombinants differing from the recipient in having a higher level of CT production were detected among the tested 505 Km^R clones (Fig. 3, colonies 3–14). According to the data of the immunoenzymatic GM_1-ELISA assay, the CT production by recombinants was 2.5–3.6 μg/mL, exceeding the values of the recipient strain (0.2 μg/mL) by 12–18 times. According to our presumption, changes in the CT production found in the studied Km^R-recombinants could result from changes in the structure of

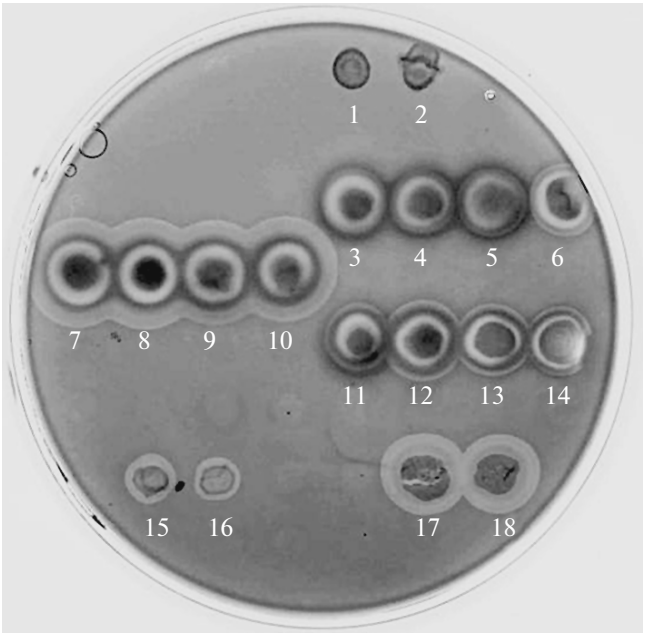

Fig. 3. Production, according to the RPIH data, of cholera toxin by the *V. cholerae* biovar El Tor Km^RTox^{++} recombinants obtained from the crossing of the *V. cholerae* KM7P donor with the *V. cholerae* M818 recipient strain. 1–2 are the recipient M818; 3–6, 7–10, and 11–14 are the recombinants R1–R3, respectively; 15–16 are the KM7P donor strain; and 17–18 are the highly toxigenic *V. cholerae* 569B classical biovar strain taken as a positive control.

Table 2. PCR results on the analysis of genomes of donor and recipient strains and *V. cholerae* El Tor biovar recombinants

Name of strain	CTXφ											Tn5-Mob	RS1φ	TLCφ		RTX	
	core region							RS2 region									
	cep	*orfU*	*ace*	*zol*	*ctxA*	*ctxB1*	*ctxB3*	$rstR^{Class}$	rst^{Et}	*rstA*	*rstB*		*rstC*	*tlcR*	*cri*	*rtxA*	*rtxC*
KM7P	+	+	+	+	+	+	–	+	–	+	+	+	–	+	+	+	+
M818	+	+	+	+	+	–	+	–	+	+	+	–	+	+	+	+	+
R1	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
R2	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
R3	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
R4	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
R5	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
R6	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
R7	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+

ctxB1 is the allele of the $ctxB^{Class}$; *ctxB3* is the allele of the $ctxB^{Et}$.

their CTXφ prophage, due to the acquisition of the classical *ctxB* gene (the *ctxB1* allele) from the donor strain. Therefore, we determined the allele of the *ctxB* gene in the obtained recombinants using the MAMA-PCR method for differentiating single-nucleotide replacements in this gene. All the recombinants were shown to actually carry the donor *ctxB1* allele, whereas the recipient prophage's genome had the *ctxB3* allele (Table 2, see Figs. 1b, 1c). Subsequent sequencing of the *ctxB* gene of the indicated strains confirmed the above data. All the recombinants had cytosine (C) in positions 115 and 203 of the *ctxB* gene sequences, whereas the recipient strain had thymine (T). Thus, changes in the levels of CT production in the recombinants were directly associated with the appearance of a novel gene allele *ctxB1* in their genome. This means that the obtained recombinants are *V. cholerae* biovar El Tor genovariants carrying *ctxB* gene of the classical type (*ctxB1*). The conjugation of the selectable marker Km^R to the nonselectable *ctxB1* was low and accounted only for 1.4%. Nevertheless, we showed for the first time in our model experiments the possibility of forming novel variants of *V. cholerae* biovar El Tor with increased CT production.

Comparative analysis of virulent traits of the recipient *V. cholerae* M818 biovar El Tor strain and the genovariants with a high level of CT production. Since increased virulence distinguishes natural *V. cholerae* biovar El Tor genovariants from typical strains, the problem was to determine a degree of virulence in the recombinants containing the *ctxB1* allele in their genomes. To solve this problem, suckling rabbits were intraintestinally challenged with cells of the recipient strain M818 and two randomly selected genovariants at three different doses (10^7, 10^5, and 10^2 CFU) and the pathologoanatomical pictures were compared with reference to the character of the animals' intestinal lesions.

As a result, we have established that when young rabbits were infected not only with high (10^5 and 10^7 CFU), but even at low (10^2 CFU) doses, the recombinants caused, unlike the recipient, the development of a specific cholerogenic effect (serious enlargements of the small and large intestines in the animals due to the presence of a transparent opalescent fluid). This fact shows a higher level of virulence in the obtained recombinants compared to the recipient, which is a typical *V. cholerae* biovar El Tor strain, and confirms their geno- and phenotypical similarity with natural *V. cholerae* genovariants.

Molecular-genetic analysis of experimentally obtained *V. cholerae* biovar El Tor genovariants with a high level of CT production. The presence of a sizeable portion of homology in both the core and the RS-2 region of the donor and recipient prophage could secure the obtaining of recombinants containing the entire genome of CTX^{Class}φ prophage. However, PCR analysis of the selected Km^RTox^{++} recombinants has shown that the core region alone of the prophage seemed to be inherited in all cases. This is confirmed by the fact that the recombinants preserved all the checked genes belonging to the core region (*ctxA*, *ctxB1*, *zot*, *ace*, *orfU*, and *cep*), but did not inherit the donor allele of the *rstR* ($rstR^{Class}$) gene present in the composition of the RS-2 sequence (Table 2). The preservation of $rstR^{Et}$ in the recipient allele was confirmed by sequencing this gene. We also compared the nucleotide sequence of the *ctxAB1* operon promoter in the recombinants with those of the recipient and donor strains. It is known that the transcription of the *ctxAB* operon starts with its own promoter. The *ctxA* gene is read first, and then *ctxB* is read. The genes are read in different reading frames and have their own ribosomal binding sites, each consisting of the Shine-Dalgarno (SD) sequence and the start ATG triplet [22]. The particular trait of the promoter region of the

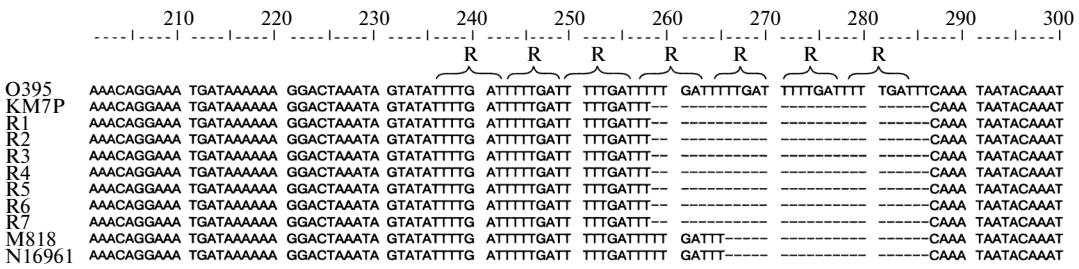

Fig. 4. Nucleotide sequences of the promoter region in the obtained *V. cholerae* biovar El Tor recombinants R1–R7. The letter R designates hepta-nucleotide repeats TTTTGAT, O395 is the reference strain of the *V. cholerae* classical biovar, KM7P is the donor strain of *V. cholerae* MAK757 biovar El Tor, R1–R7—$Km^{R}Tox^{++}$ recombinant strains, M818 is the recipient strain M818 of *V. cholerae* biovar El Tor, and N16961 is the reference strain of *V. cholerae* biovar El Tor.

ctxAB operon is that the tandem-repeat sequence 5'-TTTTGAT-3' being located at a distance of 77 bp from the start codon enhances the operon transcription by interacting with the regulatory ToxR and ToxT proteins. The expression level of the *ctxAB* operon depends on the number of 5'-TTTTGAT-3' repeats [42]. According to the sequencing results, the promoter region of the donor strain had three 5'-TTTTGAT-3' repeats, with four copies of this sequence being detected in the recipient. As for the recombinants, their *ctxAB1* operon had three 5'-TTTTGAT-3' repeats in its promoter region (Fig. 4). These data indicate that the recombinants obtained at least the entire *ctxAB1* operon with the promoter region from the donor strain. It was difficult to draw a more precise conclusion, since other genes (*zot*, *ace*, *orfU*, and *cep*) in the core region of the $CTX^{Class}\varphi$ and $CTX^{Et}\varphi$ prophage genomes are identical, which makes their transfer identification difficult under PCR analysis. It has also been found that, according to Southern blot-hybridization of the PstI-fragments of chromosomal DNA with a CT probe, all the checked recombinants contained one copy of the prophage (Fig. 2, tracks 4–6).

Thus, it has been shown that the experimentally obtained genovariants were formed as a result of including the donor DNA containing the Tn5-Mob transposon and the adjacent fragment of the $CTX^{Class}\varphi$ prophage genome with the *ctxAB* operon and its promoter region into the chromosome of the recipient strain. We should also note that the genes of *V. cholerae* of both the El Tor (*rstR*^{Et}) and the classical (*ctxB1*) biovars are present in the prophage genome of these genovariants. This fact confirms that they had a hybrid prophage, $CTX^{Hybr}\varphi$.

We believe that the point of the genetic background in the recombinant strains was also of importance, since the donor and recipient strains differed in this trait. The donor strain contained only two (TLCφ and RTX) of the identified three genetic elements (RS1φ, TLCφ and RTX) located on the chromosome of *V. cholerae* biovar El Tor near the CTXφ prophage, whereas all the indicated nucleotide sequences were present in the recipient strain (Fig. 1a). Two chromosomal DNA fragments adjacent to their $CTX^{Hybr}\varphi$ prophage on both sides of the recombinants were PCR-analyzed. All the recombinants have been found to contain RS1φ and TLCφ prophages, as well as RTX. This fact indicates that the genetic environment of the $CTX^{Hybr}\varphi$ prophage of the recombinants did not differ from the corresponding recipient $CTX^{Et}\varphi$ prophage (Table 2, Fig. 1a). All that has been mentioned above indicates that, during conjugation, the recipient strain obtained from the donor only a chromosome fragment containing the Tn5-Mob transposon, as well as a $CTX^{Class}\varphi$ prophage segment containing the *ctxAB1* operon with the promoter region. The integration of the indicated DNA fragment into the recipient strain's chromosome has led to the emergence of *V. cholerae* biovar El Tor recombinants with the same specific genetic traits as in natural genovariants, i.e., the presence in the chromosome of the *ctxB1* gene of classical cholera vibrios.

Comparative proteomic analysis of the recipient *V. cholerae* M818 strain and the genovariant with CT hyperproduction. The significant increase in toxigenicity and virulence in the obtained recombinants (genovariants) may be associated with changes in the activity of their different genes responsible for pathogenicity and house-keeping. If this is true, the recombinants must differ from the recipient strain in the expression of not only CT, but also other proteins responsible for various cell functions. To confirm this idea, proteomic maps of the recipient M818 strain and the R1 recombinant were produced using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry in cooperation with the Center of Proteomic Studies, Orekhovich Institute of Biomedical Chemistry (IBMC), Russian Academy of Medical Sciences, Moscow.

Computer analysis of proteomic maps of the studied strains has revealed 2338 proteins. Comparative analysis of them has really shown changes in the levels of expression of 26 proteins in the R1 recombinant's cells, with 17 proteins exhibiting increased levels and nine proteins showing decreased levels of their biosynthesis (Fig. 5). Subsequent mass-spectrometry identification helped select 13 proteins with levels of expres-

sion being reliably more than twice as high as that of other strains being compared (Table 3). Of 13, eight proteins demonstrated levels of expression in the recombinant by 2.3–4.3 times higher than in the recipient strain. These proteins participate in vital cell processes, such as energy and metabolic exchanges (dihydrolipoamide dehydrogenase, KF1.8.1.4; 4-hydroxyphenylpyruvate dioxygenase, KF1.13.11.27; alanine dehydrogenase, KF1.4.11) and transport of amino acids, peptides, and amines (ABC oligopeptide carrier, periplasmic oligopeptide-binding protein), as well as protein folding and cell protection from the damaging effect of higher temperatures (chaperon GroL). In addition, in the recombinants, we observed a more than three times increased expression of the OmpT porin protein contained in the cell wall, which supports the resistance of bacteria to the action of organic acids and anion detergents by regulating the transport of water, nutrients, and various compounds into the cell [23]. At the same time, we have revealed five proteins the content of which halved in the R1 recombinant. The latter group includes uridine phosphorylase (KF2.4.2.3) and iron-contaning alcohol dehydrogenase (KF1.1.1.1), as well as modified forms of alanine dehydrogenase and 4-hydroxyphenylpyruvate dioxygenase. All these enzymes participate in metabolism and energy exchange. A significant decrease in the universal stress protein UspE, which is responsible for the resistance of bacteria to oxidase stress, in the recombinant's cell deserves special attention (Table 3).

Thus, analysis of the obtained results allows us to conclude that the inclusion of a new DNA fragment containing the Tn5-Mob transposon and the *ctxAB1* operon into the chromosome of the recombinants was accompanied by changes in the activity of some of its genes. In total, we have revealed 26 genes the expression of which in the recombinant differed from their expression in the recipient strain. A significant portion of the genes encoding the identified proteins was located on the first chromosome known to contain the genes necessary for manifestation of virulence and vitality in the cell. Only one gene encoding iron-containing alcohol dehydrogenase is located on the second chromosome. The identified proteins with the level of expression differing in the recipient strain and the recombinant participate in a variety of processes in the cell, namely, in metabolism, energy exchange, and transport of various substances, as well as in cell protection from stress exposures to environmental conditions. However, we have failed to succeed in detecting any differences in the expression of proteins directly associated with pathogenicity. This may mean that these proteins exhibiting high levels of virulence could belong to secreted proteins. The mechanism behind changes in the expression of the genes encoding the identified proteins, as well as CT, remains unknown so far. We can assume that the induction or repression of the mentioned proteins in a recombinant may result from changes in the activity of some global regulatory

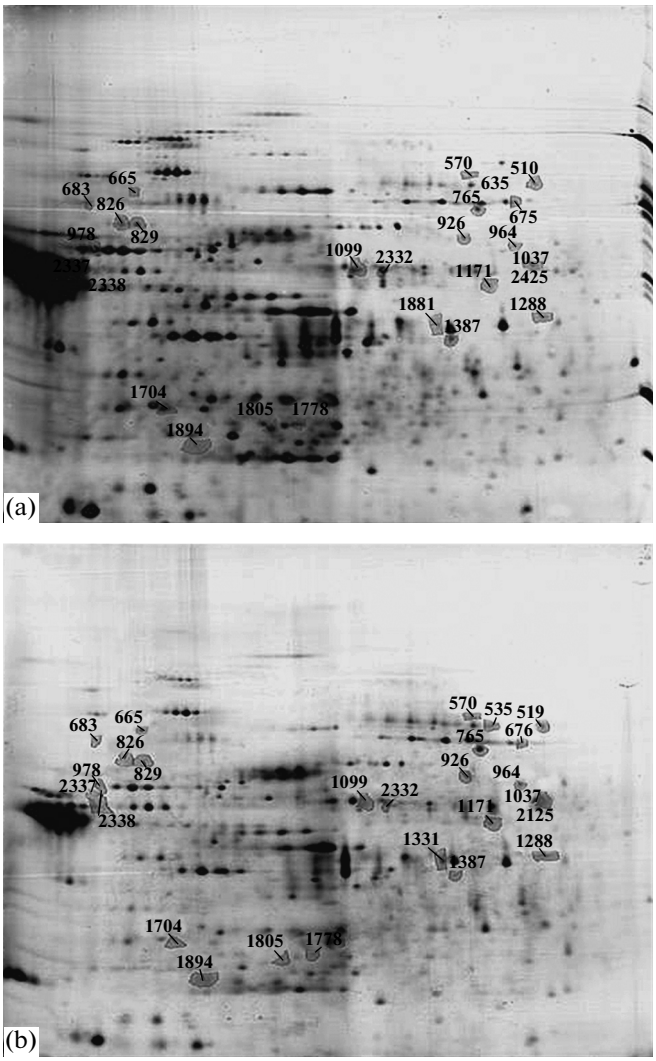

Fig. 5. Proteinogram of the recipient strain of *V. cholerae* M818 biovar El Tor (a) and the recombinant R1 with higher CT production. The pH range in isofocusing is from 4 to 7. Numbers designate proteins the expression of which changed two or more times in the compared strains.

systems controlling the expression of genes associated not only with virulence, but also with maintenance of life in the cell [17, 20, 34]. In general, the changed expression of the indicated genes in the recombinants may evidently increase the resistance of these strains to the impact of unfavorable environmental factors.

Finally, the emergence of virulent *V. cholerae* biovar El Tor genovariants was experimentally simulated for the first time and their molecular-genetic and phenotypic properties were presented in the results of our studies. The *V. cholerae* biovar El Tor recombinants carrying the *ctxAB1* operon of classical cholera vibrios were obtained in the process of conjugational crossings of the constructed donor strain with the recipient containing different CTXφ types ($CTX^{Class}\varphi$ and $CTX^{Et}\varphi$, while their studied genetic and phenotypic traits did

Table 3. Identification of proteins in the recipient *V. cholerae* M818 biovar El Tor strain and the $Km^{R}Tox^{++}$ R1 recombinant using mass spectrometry

Number of blot	Name of protein	Chromosome locus	Gene	NCBI database designation	Molecular mass, D	pI*	Intensity of blots		Function
							R1	M818	
				Metabolism and energy exchange					
765	Uridine phosphorylase	VC1034	*udp-1*	gi|229505371	27063	5.7	1.4 ± 0.06	5.9 ± 0.05	Metabolism of purines, perimidines, nucleosides, and nucleotides
826	Iron-containing alcohol dehydrogenase	VCA0702	*adh*	gi|15601458	41961	5.79	4.1 ± 0.05	8.4 ± 0.05	Alcohol metabolism
676	Dihydrolipoamide dehydrogenase	VC2412	*lpdA*	gi|15642409	50963	5.79	**5.5 ± 0.05**	**2.3 ± 0.05**	Energy exchange: dihydrolipoamide
1099	4-Hydroxyphenylpyruvate dioxygenase;	VC1344	*hppD*	gi|153802276	40321	5.46	1.4 ± 0.06	3.3 ± 0.05	Energy exchange: amino acids and amines
2332	4-Hydroxyphenylpyruvate dioxygenase	VC1344	*hppD*	gi|153802276	40321	5.46	**7.1 ± 0.05**	**1.6 ± 0.06**	Energy exchange: amino acids and amines
1037	Alanine dehydrogenase	VC1905	*ald*	gi|15641907	39814	5.88	**9.6 ± 0.05**	**4.0 ± 0.05**	Energy exchange: amino acids and amines
2425	Alanine dehydrogenase	VC1905	*ald*	gi|15641907	39814	5.88	1.4 ± 0.06	5.0 ± 0.05	Energy exchange: amino acids and amines
				Transport and binding					
619	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein	VC1091	*oppA*	gi|15641104	61102	6.02	**8.7 ± 0.05**	**2.5 ± 0.05**	Transport and binding of proteins: amino acids, peptides, and amines
635	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein	VC1091	*oppA*	gi|15641104	61102	6.02	**7.2 ± 0.05**	**2.2 ± 0.05**	Transport and binding of proteins: amino acids, peptides, and amines
978	Long-chain fatty-acid transporter	VC1043		gi|15641056	46768	4.85	**6.0 ± 0.05**	**2.3 ± 0.06**	Transport of fatty acids
				Outer-membrane proteins					
2337	Protein OmpT	VC1854	*ompT*	gi|121591724	36208	4.48	**9.9 ± 0.05**	**3.0 ± 0.06**	Porin protein of the outer membrane
				Protective function					
1331	Universal stress protein UspE	VC1433	*ydaA*	gi|15641444	35281	5.6	1.9 ± 0.06	8.0 ± 0.05	Protection from oxidative stress
				Protein folding and stabilization					
665	Chaperon Grol	VC2664	*groL-1*	gi|356443417	55459	4.71	**6.9 ± 0.05**	**2.3 ± 0.05**	Protein folding and stabilization

* Isoelectric point; protein-blotting intensity values differing in the levels of expression of the corresponding proteins by two or more times are given in bold; average intensity indicators are given for protein blots after three experiments with statistically insignificant differences.

not differ from those of natural genovariants of the seventh pandemic's cholera agent. Introducing a fragment of the $CTX^{Class}\varphi$ prophage genome into the chromosome of a typical *V. cholerae* biovar El Tor strain has led to a significant increase in the toxigenicity and virulence of the obtained recombinants (genovariants). Moreover, the genovariants have been found to acquire significantly changes in the level of expression of more than 1% of the house-keeping genes. This circumstance may possibly explain the high level of adaptation in natural genes to stress exposures caused by environmental conditions. The obtained data on the mechanism of formation of the genovariants carrying the classical *V. cholerae* genes, as well as data on the structure and function of their genomes, are important for more than only understanding of the key events occurring in the agent's wild populations at its contemporary stage of evolution. The obtained data may also be of interest for studies aimed at constructing producer strains of immunogenic proteins to be used in the manufacturing of preparations for the diagnostics and prophylaxis of cholera.

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