A Study of the Distribution of Regulatory Genes Controlling an Expression of Virulence Genes Among Strains of *Vibrio Cholerae* Biovar Eltor Differing in Their Pandemic Potential

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Abstract—The evolution of the genome of *Vibrio cholerae* biovar eltor, a causative agent of the 7th global cholera pandemic, is conditioned by the acquisition of not only structural virulence genes, but also regulatory systems, which has been caused by a horizontal transfer of a genetic information. Using polymerase chain reaction, we have revealed the presence of the following regulatory genes, controlling the expression of virulence genes, in the chromosomes of pre-pandemic and pandemic strains of *V. cholerae* biovar eltor: *toxR*, *toxT*, *tcpP*, *tcpH*, *luxS*, *luxO*, *hns*, and *pepA*. We have revealed that the avirulent ATCC14033 strain, isolated in 1910 and proposed to be an ancestor of the biovar eltor, lost *toxT*, *tcpP*, and *tcpH* regulatory genes, located in a VPI-1 pathogenicity island and able to realize a positive control over the expression of genes from the ToxR regulon. Virulent strains, isolated during a local cholera episode in Indonesia (1937) from cholera patients, do not differ from the strains that caused the cholera eltor pandemic in 1961, in the composition of the regulatory genes investigated. Only one strain of the four studied isolates lost the *tcpP* gene. Two key regulatory genes, *toxR* and *toxT*, have been sequenced in all studied isolates. The *toxR* nucleotide sequence of three pre-pandemic strains has been shown to be indistinguishable from that of the pandemic isolates. At the same time, the MAK757 strain, isolated prior to the beginning of the epidemic, has demonstrated an altered *toxR* nucleotide sequence. Experiments with the intrainestinal infection of suckling rabbits have demonstrated a similar level of virulence for the pre-pandemic and pandemic clinical strains. These results are evidence of the *in vivo* activity of the *toxT*, *tcpH*, and *tcpP* genes, acquired by *V. cholerae* in the course of evolution and positively regulating the expression of virulence genes in pre-pandemic cholera strains.

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INTRODUCTION

Pathogenic strains of *Vibrio cholerae* biovar eltor were causative agents of the 7th global pandemic of cholera, a serious diarrheal disease, which is registered every year in many developing countries [5, 13, 23]. Virulent cholera vibrios enter a human organism through infected food or water. At first, they contaminate the small intestine; the subsequent production of the cholera toxin (CT) results in the development of diarrhea [5, 23, 29]. To realize the mentioned stages of the cholera infection, there should be a coordinated expression of two key sets of virulence genes: (1) *tcpA-F* genes, which determine the production of the toxin coregulated pili (TCP), a main colonizing factor, and are located in the VPI-1 pathogenicity island (PI) [5, 13, 22]; and (2) CT-encoding *ctxAB* operon, which is a part of the CTX ϕ filamentous prophage [11, 29].

Today, it is known that the virulence of this infectious agent is regulated by several systems, among which is a global regulatory system, consisting of 7 regulatory genes, *aphA* and *aphB*, *tcpP* and *tcpH*, *toxR* and *toxS*, and also *toxT*, occupies a significant place. Via the

regulatory cascade, this system concordantly changes an expression of more than 20 different virulence genes, including *ctxAB* and *tcpA-F* genes [6, 9, 10, 17]. The *toxR* gene acts as the global regulator of this system. Since this gene unites separate structural virulence genes by a common positive regulatory control on the level of transcription, these genes are considered to be included in the ToxR regulon [9, 17]. Transmembrane sensor proteins of this system, ToxR/S and TcpP/H, are able to react to the change in such environmental parameters as temperature, pH, and osmotic pressure; as a result, they “switch on” or “switch off” the regulated genes. One should especially note that the regulatory genes, *tcpP*, *tcpH*, and *toxT*, are located in VPI-1, which was acquired by the cholera vibrio at one of its evolutionary stages [15]. At the same time, other genes, *toxR*, *toxS*, *aphA*, and *aphB*, are located at the core of a chromosome which contains different housekeeping genes. Other important regulatory systems of the cholera vibrio are three quorum sensing (QS) regulatory systems, which act in the tangem and, depending on a bacterial population density, increase or decrease the activity of the virulence genes [1]. The first system

called CA-1 (cholerae autoinducer-1) consists of a cholerae quorum sensing autoinducer (Cqs) protein, involved in the biosynthesis of the autoinducer (AI), and a cholerae quorum sensing sensor (CqsS) protein. The second system consists of the LuxS protein, responsible for the production of the AI-2 autoinducer, and LuxP/Q sensor proteins. Components of the third QS system are still unknown [19]. The transformation of an external AI signal into a translational cell response is carried out via the multistep phosphorylation process. In the case of a low density population of *V. cholerae*, when the AI concentration is low, phosphate is transferred from CqsS and LuxP/Q sensor proteins to the LuxU integral protein, which then transfer it to the LuxO protein. A phosphorylated LuxO molecule, along with G₅₄, activates an unknown receptor, which suppresses the expression of the *hapR* gene, homologous to the *luxR* gene from *V. harveyi*. This, in turn, provides a transcription of the key virulence genes [19]. Recently, it became known that, in addition to the pathogenicity genes, QS also controls the transcription of genes responsible for the formation of the *V. cholerae* biofilm, which essentially increases the probability of survival of the bacteria [2, 14, 28].

Moreover, in the last ten years, new regulatory genes have been revealed in the cholera vibrio, among which we can mention *hns* and *pepA* genes which regulate the expression of virulence genes from the ToxR regulon in certain environmental conditions. The *hns* gene encodes a biosynthesis of the histone-like DNA-binding H-NS protein discovered in many pathogens, including *Escherichia coli* and *Shigella flexneri* [8, 20]. It was shown that if *V. cholerae* is cultivated under anaerobic conditions, then the H-NS protein, binding with the promoter of the *ctxAB* operon, suppresses its expression resulting in a lack of CT production. Strains containing a defective *hns* gene reveal a high level of expression of the *ctxAB* operon under such culture conditions [16]. The *pepA* gene, homologous to the *pepA* from *E. coli*, was discovered in the case of the TnpA-mutagenesis of *V. cholerae*. It seems that at pH 8.0 the PepA protein binds the promoter of the *tcpPH* operon, suppressing its transcription, which ultimately results in a decrease in the *ctxAB* and *tcpA* activity. If pH increases, the mutation in the *pepA* of a cholera vibrio stipulates a heightened expression level not only for the structural *ctxAB* and *tcpA* genes, but also for the *toxT* and *tcpP* genes, being positive regulators of the activity of virulence genes [7].

Therefore, the regulation of the expression of virulence genes in cholera eltor vibrios represents a balanced mechanism providing their “switching on” or “switching off,” depending on the external influence on the cell.

An allocation of many key regulatory genes in mobile genetic elements allows us to propose that epidemically dangerous strains of *V. cholerae* biovar eltor are developed from the avirulent ancestor after it have

acquired both structural pathogenicity genes and regulatory systems which control their expression. Recently, we demonstrated the molecular-genetic differences between the avirulent strain of *V. cholerae* biovar eltor, that was isolated in 1910, prepandemic clinical strains, that were able to provoke only local cholera episodes in 1937, and strains isolated during the 7th global cholera pandemic (1961–1999) [3]. The differences revealed between three groups of strains determine the stages of evolution of the cholera vibrio genome, which are connected with the acquisition of several genes by the avirulent precursor via a horizontal transfer of genetic information. At first, the key structural pathogenicity genes included in PIs (VPI-1 and VPI-2) and prophages CTX ϕ and RSI ϕ were acquired, then the genome acquired pandemicity islands VSP-1 and VSP-2 [3]. These data coincide in general with the results of other studies concerning the evolution of the cholera eltor genome. At the same time, we still do not have enough information about the evolution of regulatory systems of the cholera vibrio. Evolutionary changes in the system of secretion of the pathogenicity factors of this organism are also still unclear.

The purpose of this study was to investigate the distribution of regulatory genes and the genes of the system of secretion of virulence factors among strains of *Vibrio cholerae* biovar eltor isolated before and during the 7th global cholera pandemic.

MATERIALS AND METHODS

Bacterial strains. In our study, we used 11 freeze-dried strains of *V. cholerae* biovar eltor from the national collection of pathogenic germs (Microbe). The characterization of the studied strains, isolated before (prepandemic strains) and during (pandemic strains) the 7th global cholera pandemic, is shown in Table 1. The strains were cultivated on the LB-agar (pH 7.2) for 18 h at 37°C.

Polymerase chain reaction (PCR) was carried out on a Tertsik programmable thermal cycler in 0.6 ml microtubes. Total DNA preparations were obtained by boiling a suspension of bacterial cells (10^7 cells per 1 ml of distilled water) in a water bath for 30 min with subsequent centrifugation (1000 rpm). The preparation obtained was used in an amplification reaction, which was carried out according to [4]. Primers were synthesized by Litech scientific production company (Russia) using an ACM-102U automatic DNA synthesizer. PCR products were separated in 2% agarose (BioRad, USA) and UV-registered. The detection of regulatory genes and genes of the type II secretion system in the chromosomes of the studied *V. cholerae* strains were carried out using the specific oligonucleotide primers shown in Table 2.

The electrophoretical analysis of the protein repertoire of the strains studied was carried out according to Laemmli et al. [18] in the presence of sodium dode-

Table 1. Characterization of *V. cholerae* biovar eltor strains used in the study

Strain	Time and place of isolation	Source	Genotype
ATCC14033	1910, El-Thor quarantine station, the Sinai Peninsula	Human being	<i>ctxA</i> ⁻ , <i>ace</i> ⁻ , <i>zot</i> ⁻ , <i>tcpA</i> ⁻ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁻ , <i>tnp0183</i> ⁻ , <i>pro0490</i> ⁻ , <i>pro0496</i> ⁻
Celebes30	1937, Celebes Island, Indonesia	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁻ , <i>tnp0183</i> ⁻ , <i>pro0490</i> ⁻ , <i>pro0496</i> ⁻
MAK676	1937, Celebes Island, Indonesia	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁻ , <i>tnp0183</i> ⁻ , <i>pro0490</i> ⁻ , <i>pro0496</i> ⁻
MAK757	1937, Celebes Island, Indonesia	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁻ , <i>tnp0183</i> ⁻ , <i>pro0490</i> ⁻ , <i>pro0496</i> ⁻
MAK97	1937, Celebes Island	Cholera patient	<i>ctxA</i> ⁻ , <i>ace</i> ⁻ , <i>zot</i> ⁻ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁻ , <i>tnp0183</i> ⁻ , <i>pro0490</i> ⁻ , <i>pro0496</i> ⁻
B1654	1962, the Taiwan peninsula, Indo-China	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁺ , <i>tnp0183</i> ⁺ , <i>pro0490</i> ⁺ , <i>pro0496</i> ⁺
Malacca1	1963, the Malacca peninsula, Indo-China	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁺ , <i>tnp0183</i> ⁺ , <i>pro0490</i> ⁺ , <i>pro0496</i> ⁺
T4	1963, India	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁺ , <i>tnp0183</i> ⁺ , <i>pro0490</i> ⁺ , <i>pro0496</i> ⁺
M698	1969, Turkmenian Soviet Socialist Republic	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁺ , <i>tnp0183</i> ⁺ , <i>pro0490</i> ⁺ , <i>pro0496</i> ⁺
M818	1970, USSR (Volga region)	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁺ , <i>tnp0183</i> ⁺ , <i>pro0490</i> ⁺ , <i>pro0496</i> ⁺
N16961	1975, Bangladesh	Cholera patient	<i>ctxA</i> ⁺ , <i>ace</i> ⁺ , <i>zot</i> ⁺ , <i>tcpA</i> ⁺ , <i>mshA</i> ⁺ , <i>deo0175</i> ⁺ , <i>tnp0183</i> ⁺ , <i>pro0490</i> ⁺ , <i>pro0496</i> ⁺

Note: The *ctxA*, *ace*, and *zot* genes, encoding the biosynthesis of the A-subunit of the cholera toxin, and the Ace and Zot toxins, respectively, are the parts of the CTX ϕ prophage; the *tcpA* gene, determining the production of the toxin-coregulated adhesion pili, is located in the VPI-1 pathogenicity island; the *mshA* gene, involved in the biosynthesis of the mannose-sensitive adhesion pili, is a part of the EPI persistence island; *deo0175* with *tnp0183*, and *pro0490* with *pro0496* genes are genetic markers of the VSP-1 and VSP-2 pandemicity islands, respectively. All data were obtained by the authors of this study [3].

cyl sulfate. In our study, we used 11% gels in 8 × 7 cm plates.

The sequencing of the *toxR* and *toxT* genes was carried out according to [25], using the forward (SEQtoxRs and SEQtoxTs) and reverse (SEQtoxRr and SEQtoxTr) primers shown in Table 2. DNA was sequenced using an ABI PRISM[®] 3100 Genetic Analyzer (USA). The initial computer analysis of DNA sequences (an assembly of sequenced fragments, the sequence editing and presentation, and a comparison with known nucleotide sequences) was carried out using ABI PRISM[®] 3100 Genetic Analyzer Data Collection software version 1.1 and ABI PRISM[®] DNA Sequencing Analysis software version 3.7.

The virulence of the strains studied was determined by the intrainstestinal infection of suckling rabbits with *V. cholerae* strains according to [12]. The dose of the infection amounted to 10⁷ bacterial cells. Forty-eight hours after infection, the large and small intestines were taken from perished or chlorophorm-euthanized rabbits and triply washed with the physiological solution; the colonization index of the strains studied was evaluated according to the number of bacterial cells per 1 cm² of the walls of both intestines [21].

RESULTS AND DISCUSSION

To reveal different regulatory genes in natural strains of *V. cholerae* biovar eltor, isolated before and during the 7th global cholera pandemic, we used a PCR analysis with 8 pairs of specific primers (see Table 2). Since the cholera eltor vibrio has several separate genes and systems regulating the expression of virulence genes, we felt it was expedient to reveal the presence (or absence) of three groups of regulatory genes in the genome of the strains studied. The first group consists of four key genes involved in the multistage regulation of the activity of genes included in the ToxR regulon: *toxR*, *toxT*, *tcpP*, and *tcpH* [9, 17]. One of these genes, *toxR*, is located in the core chromosome segment, whereas *toxT*, *tcpP*, and *tcpH* are located in VPI-1 [5, 6, 15]. The second group includes *luxS* and *luxO*, the basic genes of the second regulatory QS system of *V. cholerae* [19]. Finally, we thought it necessary to investigate the distribution of *pepA* and *hns* genes acting as negative regulators of the expression of key virulence genes from the ToxR regulon [7, 20].

The PCR analysis of the avirulent pandemic strain ATCC14033 was especially interesting, because this strain, being a representative of the biovar eltor which was discovered more than 100 years ago, can be consid-

Table 2. Sequences of the oligonucleotide primers used in the study

Name	Nucleotide sequence (5'–3')	Amplicon size, bp	Reference
toxR-1	A T G A C T T T G T T T G G C G A G A G	397	[4]
toxR-2	G A T T G G C T T G G G T T A G T G A G		
toxT-1	A T A A C T T T A C G T G G A T G G C	933	[24]
toxT-2	A A A A T C A G T G A T A C A A T C G		
tcpP-1	G G A G T G C T A A A C C G A A T G A A T	687	Designed by the author
tcpP-2	T C A C A G G T A G C A A A G C A A C A T A A A G		
tcpH-1	T G C T T T G C T A C C T G T G A T T T G G	690	"
tcpH-2	G G G T A A A G A T G A A G A G A T G A C		
luxS-1	A G C C G A C A T T G A A G C G A A A G	633	"
luxS-2	T C A T T G A G C A T A G A T T C G G G C		
luxO-1	A T G A G A G A A T G G A T C A A C C A	700	"
luxO-2	T C T A G C G A G A T G T C C A T C A T		
pepA-1	T C G T T G T T G G G G T G T T T G A A	400	"
pepA-2	C G G A T T T C T G G T T T T A C G C T		
hns-1	A G T T T T T T C T T C A C C G T T G G	337	"
hns-2	G G A A A T G G T A A T G T C G G A A A T C A		
epsE-1	G A T T T C T C C C A A G C C A G C A T	652	"
epsE-2	C G G C G G A A A T T A T C A T G G T T		
epsD-1	G A A A A A A A G T T C A T G G C T G T	528	"
epsD-2	A C G A A T A A T T T C C G C T A A A C		
SEQtoxRs	C T A C T C A C A C A C T T T G A T G G	885	"
SEQtoxRr	A T G T T C G G A T T A G G A C A C A A		
SEQtoxTs	A T G A T T G G G A A A A A A T C T T T	831	"
SEQtoxTr	T T A T T T T T C T G C A A C T C C T G		

Table 3. Results of the PCR determination of the presence of regulatory genes in the genome of prepandemic and pandemic strains of *V. cholerae* biovar eltor

Strain	Year of isolation	<i>toxR</i>	<i>toxT</i>	<i>tcpH</i>	<i>tcpP</i>	<i>luxO</i>	<i>luxS</i>	<i>hns</i>	<i>pepA</i>	<i>epsD</i>	<i>epsE</i>
ATCC14033	1910	+	–	–	–	+	+	+	+	+	+
MAK97	1937	+	+	+	+	+	+	+	+	+	+
MAK676	1937	+	+	+	+	+	+	+	+	+	+
MAK757	1937	+	+	+	+	+	+	+	+	+	+
Celebes30	1937	+	+	+	–	+	+	+	+	+	+
B1654	1962	+	+	+	+	+	+	+	+	+	+
Malacca1	1963	+	+	+	+	+	+	+	+	+	+

Note: ATCC14033 is an avirulent strain isolated in 1910; MAK97, MAK676, MAK757, and Celebes30 are the clinical strains having epidemic potential; and B-1654 and Malacca1 are the clinical strains having pandemic potential.

ered as the ancestor of the pathogen that caused the 7th global cholera pandemic. It turned out that its genome contains only one of the studied genes that controls the expression of the ToxR regulon, namely the global regulatory gene *toxR*; other genes, *toxT*, *tcpH*, and *tcpP*, were

lost (Table 3). These data confirmed the earlier information obtained that avirulent strains of the biovar eltor lost VPI-1, which includes not only *toxT*, *tcpP*, and *tcpH* regulatory genes, but also *tcpA-F* structural genes, encoding the TCP biosynthesis [3]. At the same time, the chromo-

some of ATCC14033 contains *luxS* and *luxO* genes, the first of which is involved in the biosynthesis of AI-2 signal molecule, and the second which encodes LuxO sensor protein, being a central “switch” for the functioning of the key virulence genes and also genes involved in the formation of the *V. cholerae* biofilm [19, 28]. Since the ATCC14033 genome does not contain the main pathogenicity genes (*ctxA*, *tcpA*) [3], the presence of *luxS* and *luxO* genes can be evidence of the fact that the QS system participates only in the control of the biofilm formation process. Such explanation agrees with data obtained earlier about the presence of *msh* genes in the ATCC14033 genome; these genes encode the production of the type IV mannose-sensitive hemagglutination pili necessary for the formation of the normal biofilm (see Table 1) [3, 5].

In addition to the *toxR*, *luxS*, and *luxO* regulatory genes, the chromosome of ATCC14033 strain has *pepA* and *hns* genes, realizing the negative regulation of the expression of key virulence genes from the ToxR regulon (*ctxAB* and *tcpA-F*) in the case of a certain external influence on the bacterial cells (anaerobiosis or heightened osmotic pressure). Since the ATCC14033 genome does not contain any of the mentioned structural virulence genes, it is possible that the *pepA* and *hns* genes perform other functions in this strain.

The next stage of our study was to investigate the genomes of four clinical strains of *V. cholerae* biovar eltor (MAK97, MAK676, MAK757, and Celebes30), which caused the local cholera episode in 1937. The appearance of pathogenic properties in these strains was caused by the acquisition of CTX ϕ prophage and VPI-1, which contained, correspondingly, the *ctxAB* and *tcpA-F* genes necessary for the colonization of the human small intestine and CT production. At the same time, however, the MAK97 genome lost the CTX ϕ prophage (see Table 1) [3]. One of the possible reasons for the loss of such a mobile genetic element is a genomial instability of virulent strains that arose at this stage of the *V. cholerae* evolution. The PCR analysis of the mentioned strains showed that, unlike the avirulent ATCC14033 strain, genomes of three clinical strains (MAK97, MAK676, and MAK757) contain *toxP*, *tcpP*, and *tcpH* regulatory genes from the VPI-1 (see Table 3). At the same time, the chromosome of the Celebes30 strain lost the *tcpP* gene; this can be evidence of the instability of VPI-1 in prepandemic virulent strains. B1654, Malacca1, T4, and M818 clinical strains, which were isolated at different times during the 7th global cholera pandemic and acquired some additional foreign gene blocks, VSP-1 and VSP-2, in the course of their evolution (see Table 1), did not differ from the prepandemic virulent strains in the composition of the regulatory genes studied.

In addition, we performed experiments necessary to reveal genes, encoding the type II secretion system, in the chromosomes of pandemic and prepandemic strains; using this system, the cholera vibrio secretes a

series of virulence factors, including CT [24]. An activity of this secretion system, designated as eps (extracellular protein secretion), is determined by *epsC*, *epsD*, *epsE-epsN*, and *vspD* genes [27]. Since the CT secretion is a very important and necessary part of the cholera pathogenesis, we determined the presence of *epsD* and *epsC* genes in the chromosomes of the studied strains. The choice of these genes was conditioned by their great functional significance: EpsD protein forms pores in the outer membrane, which are necessary for the CT translocation out of the cell; one of the functions of EpsE is to regulate the opening and closing of these pores [24].

It turned out that genomes for all the studied strains, regardless of their virulence and pandemicity, contain *epsD* and *epsC* genes (see Table 3). These data allowed us to suppose that the type II secretion system was already functioning in the avirulent ancestor of the cholera vibrio before it acquired any structural and/or regulatory virulence genes in the course of its evolution.

Thus, these data demonstrate that *toxR*, *luxO*, *luxS*, *hns*, and *pepA* are the most ancient regulatory genes, probably existing in the genome of *V. cholerae* biovar eltor long before it acquired pathogenic potential. The appearance of *toxT*, *tcpP*, and *tcpH* regulatory genes in the genome of this pathogen was connected with the acquisition of VPI-1 (containing not only the genes mentioned, but also *tcpA-F* structural genes, encoding the TCP biosynthesis) by its ancestor. A subsequent change in the genetic organization of the cholera vibrio was caused by the acquisition of the CTX ϕ prophage and *ctxAB* genes. The successful combination of different structural and regulatory virulence genes in the genome of *V. cholerae* provided the formation of the epidemically dangerous clone, which, however, still did not possess any pandemic potential. The lack of difference between pandemic and prepandemic virulent strains in the repertoire of regulatory genes testifies that the further *V. cholerae* evolution was not connected with the acquisition of new regulatory systems. The genome transformations, providing the ability of this pathogen to a pandemic spread, represent the acquisition of VSP-1 and VSP-2 pathogenicity islands, which, possibly, provide the heightened survival of the pathogen in the environment and/or more perfect interrelations between this microorganism and the host human cells [3, 5, 22].

The most notable event in the evolution of the regulatory systems of *V. cholerae* is the appearance of new functions of the *toxR* gene. In nonpathogenic strains, the ToxR protein controls only a biosynthesis of the outer membrane proteins OmpU and OmpT, but in pathogenic strains it is involved in the regulation of the foreign *ctxAB* and *tcpA* virulence genes. The N-terminal cytoplasmic domain of the ToxR protein is able to interact with a certain site on the *toxT* promoter and induce its transcription. As a result, the ToxT protein is

	30	40	100	110	120
N16961 Reference	CGTTAGGGTTAGCAACGATGCGTAA.....	TAACTGGTTATTTTGTCCACCTGTGGC			
Celebes30					
MAK676					
MAK97					
MAK757	A.....G.....				
T4					
M818					

	130	140	150	160	170	180
N16961 Reference	AATGACTTCTATCGGCTTGAGCCCAACAGTATGCTTTTCATTGTATTTTAAACGCACAGTT					
Celebes30						
MAK676						
MAK97						
MAK757	C.....					
T4						
M818						

	190	290	300	310	320	330
N16961 Reference	CGATTGA.....AGTGAGCAGTAATACTGCGAGGGGAAGTAAGACCGCTATCAGAATAAG...					
Celebes30						
MAK676						
MAK97						
MAK757						
T4						
M818						

Fig. 1. A comparison of the nucleotide sequences of the global *toxR* regulatory gene taken from the clinical strains of *V. cholerae* biovar eltor isolated before and during the 7th global cholera pandemic. Identical nucleotide sequences are shown by dots; deletions and substitutions are shown by bold type. Celebes30, MAK676, MAK97, and MAK757 are pre-pandemic strains; N16961, M818, and T4 are pandemic isolates. Nucleotide sequences in the positions 49–96, 193–288, 337–816 were identical for all compared strains.

synthesized, which carries out a direct positive control of the *ctxAB* and *tcpA* expression. A new function, connected with (1) the regulation of the activity of virulence genes and (2) their secretion, also was acquired by the QS system and the type II secretion system, correspondingly.

The presence of the regulatory *toxR* gene in all studied strains served as the basis for a comparative analysis of its structure in the virulent *V. cholerae* strains developed during the different evolutionary stages. To carry out such an analysis, we performed a sequencing of the *toxR* gene from four pre-pandemic strains (MAK97, MAK676, MAK757, and Celebes30) and strains isolated from cholera patients during the 7th global cholera pandemic (M818, T4). An analysis of the *toxR* nucleotide sequence showed its identity in five compared strains: MAK97, MAK676, Celebes30, M818, and T4 (Fig. 1). In the case of MAK757, we revealed three nucleotide substitutions (G/A, T/G, and A/C in positions 30, 104, and 145, respectively) and one deletion (lack of 18 base pairs in positions 306–325) in the *toxR* gene (Fig. 1). Thus, we established, that the nucleotide sequence of this global regulatory gene is the same for the five studied strains isolated in different years and exhibiting different degrees of epidemic risk. The reason for such an appreciative change in the *toxR* nucleotide sequence of only one pre-pandemic strain is still unknown.

As for the nucleotide sequence of the *toxT* gene, which also plays a significant role in the regulation of the virulence genes, it was revealed to be the same for all virulent strains.

Since *toxR* acquired new functions connected with the control of the expression of virulence genes in pathogenic strains which acquired the foreign DNA (VPI-1, CTX ϕ prophage, and other gene blocks) during the course of its evolution [3], a question emerges as to whether the activity of this gene regarding the structural porine-encoding genes, *ompU* and *ompT*, has changed in the new genetic environment. To find the answer, we compared the profile of the outer membrane proteins of the avirulent ATCC14033 strain, where *toxR* controls only the *ompU* and *ompT* activity, with the virulent strains. It turned out that the *toxR* activity was the same in the avirulent ATCC14033 strain and B1654 and Malacca1 pandemic isolates (Fig. 2, lanes 1, 6, 7). The marked production of the OmpU protein and only trace amounts of the OmpT protein in the virulent strains indicate that the regulatory protein ToxR activates the *ompU* transcription, but suppresses the *ompT* activity. At the same time, in vitro activity of the *toxR* gene varied between different pre-pandemic virulent strains. For example, MAK97 and MAK676 strains demonstrated about the same level of biosynthesis of both proteins (OmpT and OmpU), whereas in the case of the Celebes30 isolate, we detected only OmpT protein

Fig. 2. Electrophoresis of cell lysates of *V. cholerae* biovar eltor strains with different pandemic potential in 11% SDS-polyacrylamide gel. 1 ATCC14033, an avirulent strain isolated in 1910; 2–5 MAK97, MAK676, MAK757, and Celebes30 strains, respectively (clinical strains isolated during a local cholera episode in 1937); 6–7 B1654 and Malaccal strains, respectively (strains with pandemic potential isolated from cholera patients during the 7th global cholera pandemic).

(Fig. 2, lanes 2, 3, 5). The picture observed in both cases was probably caused by a decrease in the *toxR* activity. Only one strain (MAK757) demonstrated the same high level of OmpU production, as in the case of pandemic strains, in spite of some changes in the nucleotide sequence of the *toxR* gene (Fig. 2, lanes 4, 6, 7). Based on these data, one can suppose that the acquisition of foreign gene blocks by the cholera vibrio during the early stages of its evolution could cause a functional instability of the *toxR* gene as a result of its involvement in the execution of an additional function, namely, the regulation of the activity of acquired virulence genes.

The different in vitro activity of the *toxR* gene in the cells of prepandemic strains could correlate with a varying level of virulence of these isolates. OmpU⁺ and OmpT⁺ isolates usually demonstrated an effective in vitro TCP and CT expression, whereas the biosynthesis of basic pathogenicity factors in OmpU[−] and OmpT[−] isolates is significantly reduced [26]. To check this

hypothesis, we carried out an intrainstestinal infection of suckling rabbits with six *V. cholerae* strains (Table 4). As a result, we determined that only the MAK97 strain was avirulent among the examined prepandemic strains. These data agree with information obtained earlier about the structure of its genome, including (1) the lack of *ctxA*, *zot*, and *ace* genes, encoding a biosynthesis of three different toxins (including CT) and being a part of the CTXφ prophage; and (2) the presence of the VPI-1 pathogenicity island containing the *tcpA* gene, which determines the production of the main TCP subunit [3]. Having the key structural and regulatory genes of virulence, the MAK757 strain effectively colonized the small intestine and after 24–48 h caused the development of a typical cholerogetic syndrome, which appeared through a significant stretching of loops of the small and large intestines because of their filling with a transparent fluid (Table 4). Furthermore, MAK97, MAK676, and Celebes30 strains also proved to be virulent (see Table 4) despite the fact that in vitro activity of their *toxR* gene was markedly decreased (see Fig. 2, lanes 2, 3, 5). Since the virulence of strains is connected with their TCP and CT production, regulated by the *toxR* gene, then it is obvious that the activation of this gene in these strains occurs *in vivo*. Such an explanation agrees with the data obtained by Simonet et al. [26], who showed that if OmpT⁺ strains infect the intestines, then a switching of biosynthesis of both outer membrane proteins and virulence factors occurs. The bile contained in the intestines acts as a signal for these events; it enters the periplasm through the pores of the outer membrane, which are formed mainly by the OmpT protein, and activates the ToxR protein via the interaction with its C-terminal domain located in the periplasm. As a result, an effective expression of the OmpU protein begins; this protein protects cells from the damaging action of the bile and also TCP and CT [22, 26]. The observed virulence of the Celebes30 strain requires a separate explanation, since its genome does not contain the functionally active *tcpP* gene (see Table 3). Together with ToxR, TcpP activates the transcription of the *toxT* gene, which directly realizes a pos-

Table 4. The virulence of prepandemic strains of *V. cholerae* biovar eltor determined through the intrainstestinal infection of suckling rabbits

Strain	Infection dose, cells/ml	Cholerogetic effect	Strain virulence	Colonizing ability of the studied strains	
				small intestine	large intestine
ATCC14033	10 ⁷	Absent	Avirulent	3.5 × 10 ⁶	2.1 × 10 ⁶
MAK676	10 ⁷	Appreciable	Virulent	6.2 × 10 ⁶	4.7 × 10 ⁶
Celebes30	10 ⁷	Appreciable	Virulent	4.3 × 10 ⁶	3.8 × 10 ⁶
MAK757	10 ⁷	Appreciable	Virulent	5.1 × 10 ⁶	3.9 × 10 ⁶
MAK97	10 ⁷	Absent	Avirulent	1.8 × 10 ⁶	1.0 × 10 ⁶
M698	10 ⁷	Appreciable	Virulent	5.8 × 10 ⁶	3.2 × 10 ⁶

Note: The number of cells adhering to 1 cm² of the small or large intestine of the infected rabbit was taken as the index of the colonizing ability of strains; the virulent M698 strain isolated during the 7th global cholera pandemic was taken as the positive control.

itive control of the expression of the virulence genes [10]. The lack of TcpP usually results in the marked decrease in the *in vitro* activity of *ctxAB* and *tcpA* genes [9, 17]. However, this lack does not change significantly the *in vivo* expression of the pathogenicity genes, because there are many other or additional signal molecules in a macroorganism, which evidently induce an activity of not only *toxR*, but also any genes from other regulatory systems.

Thus, we did not reveal any significant differences between the level of virulence of prepandemic MAK676, Celebes30, and MAK757 strains isolated during the 7th global cholera pandemic (Table 4).

As for the avirulent ATCC14033 strain, which contains only one regulatory *toxR* gene among all the studied genes, it proved to be able to effectively colonize the small and large intestines of our biomodels, but it did not induce any cholero-genic reaction (see Table 4). These data are worthy of mention, because the marked colonizing activity of this strain was not conditioned by TCP due to the lack of the *tcpA* gene in its genome [3]. The high colonization index (3.5×10^6 and 2.1×10^6 for the small and large intestines, respectively) is probably connected with the production of still unknown adhesion pili in this strain.

So, we experimentally proved that the reorganization of the cholera eltor genome during the course of evolution was connected with the acquisition of not only structural virulence genes, but also foreign regulatory genes, located in the mobile genetic element. New data about the *in vitro* and *in vivo* activity of regulatory genes were obtained. These data are of undoubted interest and will serve as a basis for further investigations directed at a comparative analysis of actively expressing genes in prepandemic and pandemic strains.

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REFERENCES

- Ginzburg, A.L., Il'ina, T.S., and Romanova, Yu.M., *Zh. mikrobiol.*, 2003, no. 5, pp. 86–93.
- Il'ina, T.S., Romanova, Yu.M., and Ginzburg, A.L., *Genetika*, 2004, vol. 40, no. 11, pp. 1445–1456.
- Osin, A.V., Nefedov, K.S., Eroshenko, G.A., and Smirnova, N.I., *Genetika*, 2005, vol. 41, no. 1, pp. 1–10.
- Smirnova, N.I., Kirillina, O.A., Cheldyshova, N.B., and Kutyrev, V.V., *Mol. Gen.*, 2001, no. 3, pp. 23–28.
- Smirnova, N.I. and Kutyrev, V.V., *Mol. Gen.*, 2004, no. 4, pp. 3–13.
- Beck, N.A., Krukonis, E.S., and DiRita, V.J., *J. Bacteriol.*, 2004, vol. 186, no. 24, pp. 8309–8316.
- Behari, J., Stagon, L., and Calderwood, S.B., *J. Bacteriol.*, 2001, vol. 183, no. 1, pp. 178–188.
- Bertin, P., Benhabiles, N., Krin, E., et al., *Mol. Microbiol.*, 1999, vol. 31, no. 1, pp. 319–329.
- Bina, J., Zhu, J., Dziejman, M., et al., *Proc. Natl. Acad. Sci. USA*, 2003, vol. 100, no. 5, pp. 2801–2806.
- Cotter, P.A. and DiRita, V.J., *Annu. Rev. Microbiol.*, 2000, vol. 54, pp. 519–565.
- Dawis, B.M. and Waldor, M.K., *Curr. Opin. Microbiol.*, 2003, vol. 6, pp. 35–42.
- Dutta, N.K. and Habbu, M.K., *Br. J. Pharmacol.*, 1955, vol. 10, no. 2, pp. 153–159.
- Farique, S.M., Naser, I.B., Islam, M.J., et al., *Proc. Natl. Acad. Sci. USA*, 2005, vol. 102, no. 5, pp. 1702–1707.
- Hammer, B.K. and Bassler, B.L., *Mol. Microbiol.*, 2003, vol. 50, no. 1, pp. 101–114.
- Karaolis, D.K., Johnson, J.A., Bailey, C.C., et al., *Proc. Natl. Acad. Sci. USA*, 1998, vol. 95, pp. 3134–3139.
- Krishnan, H.H., Ghosh, A., Paul, K., and Chowdhury, R., *Infect. and Immun.*, 2004, vol. 72, no. 7, pp. 3961–3967.
- Krukonis, E.S., Rosa, R.Yu., and DiRita, V.J., *Mol. Microbiol.*, 2000, vol. 38, no. 1, pp. 67–84.
- Laemmli, U.K., *Nature*, 1970, vol. 227, pp. 680–685.
- Miller, M.B., Skorupski, K., Lenz, D.H., et al., *Cell*, 2002, vol. 110, pp. 303–314.
- Nye, M.B. and Taylor, R.K., *Mol. Microbiol.*, 2003, vol. 50, no. 2, pp. 427–444.
- Pierce, N.F., Cray, W.C., and Kaper, J.B., *Infect. and Immun.*, 1988, vol. 56, no. 1, pp. 142–148.
- Provenzano, D. and Klose, K.E., *Proc. Natl. Acad. Sci. USA*, 2000, vol. 74, no. 18, pp. 10220–10224.
- Reidl, J. and Klose, K.E., *FEMS Microbiol. Rev.*, 2002, vol. 26, pp. 125–139.
- Sandkvist, M., Hough, L.P., Bagdasarian, M.M., et al., *J. Bacteriol.*, 1999, vol. 181, no. 10, pp. 3129–3135.
- Sanger, F., Nicklen, S., and Coulson, A.R., *Proc. Natl. Acad. Sci. USA*, 1977, vol. 74, pp. 5463–5467.
- Simonet, V.C., Basle, A., Klose, K.E., et al., *J. Biol. Chem.*, 2003, vol. 278, no. 19, pp. 17539–17545.
- Tauschek, M., Gorrell, R.J., Strugnelli, R.A., et al., *Proc. Natl. Acad. Sci. USA*, 2002, vol. 99, no. 10, pp. 7066–7071.
- Vance, R.E., Zhu, J., and Mekalanos, J.J., *Infect. and Immun.*, 2003, vol. 71, no. 5, pp. 2571–2576.
- Waldor, M.K. and Mekalanos, J.J., *Science*, 1996, vol. 272, pp. 1910–1994.