Peruvian *Vibrio cholerae* O1 El Tor strains possess a distinct region in the *Vibrio* seventh pandemic island-II that differentiates them from the prototype seventh pandemic El Tor strains

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A collection of environmental and clinical strains of *Vibrio cholerae* O1 isolated from the beginning of the Latin American epidemic of cholera in 1991 to 2003 from multiple locations in Peru were characterized and compared with *V. cholerae* O1 El Tor strains of the seventh pandemic from the rest of the world (Asia, Africa, Australia and Europe) using a multilocus virulence gene profiling strategy and DNA sequencing. Peruvian strains differed from El Tor strains from the rest of the world by the failure of PCR to amplify genes VC0512, VC0513, VC0514 and VC0515 in the *Vibrio* seventh pandemic island-II (VSP-II) gene cluster. Sequencing of the VSP-II gene cluster and its flanking regions in one Peruvian strain (PERU-130) confirmed the PCR results, indicating that the Peruvian strain had low DNA homology (46.6%) compared to the reference strain N16961 within the VSP-II region encompassing genes VC0511 to VC0515. Based on these differences in VSP-II, and based on the overall similarity between the pulsotypes of the Peruvian strains and the El Tor reference strain N16961, we concluded that the Peruvian, Eurasian and African strains belonged to the same clonal complex, and that the Peruvian strains represented variants that had independently evolved for a relatively short time. Since these ORFs in VSP-II of Peruvian strains are unique and conserved, they could form the basis for tracking the origin of the Peruvian strains and therefore of the Latin American pandemic.

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Abbreviations: ET, electrophoretic type; VSP, *Vibrio* seventh pandemic island.

The GenBank/EMBL/DDBJ accession numbers for the *ctxB* gene sequences of Peruvian *V. cholerae* strains reported in this paper are EU921452–EU921454, and for the sequence of VSP-II is AB300724.

INTRODUCTION

In 1991, after an absence of more than a century, *Vibrio cholerae* O1 biotype El Tor entered into South America and caused explosive epidemics along the coast of Peru. Cholera occurred simultaneously at different sites along the Peruvian coast, over a distance of more than 1200 km (Seas *et al.*, 2000). By the end of 1992, 19 countries in Latin America had reported more than 730 000 cholera cases and 6300 deaths (Kaper *et al.*, 1995; Popovic *et al.*, 1993). The epidemic, thereafter, spread to the rest of South and Central America from Mexico to Argentina (Tauxe *et al.*, 1995).

Since the entry of the El Tor biotype into Latin America, intensive efforts have been made to understand the origin of these strains. Molecular typing methods indicate that isolates from the 1991 Latin American epidemic were clonal and represented an extension of the seventh pandemic strain of El Tor biotype in the Western hemisphere (Wachsmuth *et al.*, 1993). More recent studies have documented a relatively high rate of genetic changes as shown by changing serotypes, electrophoretic types (ETs), ribotypes and pulsotypes among the Latin American strains (Dalsgaard *et al.*, 1997; Popovic *et al.*, 1993; Evins *et al.*, 1995). The current understanding is that there is a mélange of different molecular types among the Latin American strains of *V. cholerae* O1 closely related to the Asian and African seventh pandemic strains.

The past few years have witnessed many advances in our understanding of the genome and genetics of *V. cholerae*. We now know that the cholera toxin is encoded by a filamentous bacteriophage CTXΦ (Waldor & Mekalanos, 1996), and the toxin-coregulated pilus (TCP), an important colonization factor, acts as the receptor for CTXΦ (Kovach *et al.*, 1996; Karaolis *et al.*, 1998). The *rstR* gene of RS1 satellite phage encodes a regulator of CTXΦ replication. The CTXΦ types in *V. cholerae* strains are primarily recognized through the strain-specific *rstR* DNA sequence. The different types of CTXΦ and their corresponding *rstR* types have been described previously (Nusrin *et al.*, 2004). *V. cholerae*, like other members of the genus *Vibrio*, has two circular chromosomes (Trucksis *et al.*, 1998), and the whole genome sequence of an El Tor *V. cholerae* O1 strain N16961 has shown them to be 2.96 and 1.07 Mb in size (Heidelberg *et al.*, 2000). Comparative genomic analysis using DNA microarray showed that although they share a common lineage (O'Shea *et al.*, 2004a) there are differences in gene content between the sixth (classical biotype) (representing the classical strains that caused the first six pandemics) and the current seventh (El Tor biotype) pandemic strains of *V. cholerae* O1, and identified two genomic regions designated the *Vibrio* seventh pandemic island-I (VSP-I) and VSP-II that are unique to seventh pandemic El Tor strains (Dziejman *et al.*, 2002). VSP-I and VSP-II showed several properties of pathogenicity islands, and are believed to be involved with the pandemic tendency of the seventh pandemic El Tor strains.

In light of the new information on the molecular aspects of *V. cholerae*, we decided to re-examine the strains of *V. cholerae* O1 isolated from the beginning of the Peruvian epidemic in 1991 to 2003 from different locations in Peru, using a PCR-based multilocus virulence gene profiling strategy. We report the identification of a distinct and conserved molecular signature in the Peruvian strains in the VSP-II cluster not seen in other seventh pandemic strains of *V. cholerae* O1 El Tor.

METHODS

Bacterial strains. A total of 60 *V. cholerae* O1 strains (48 clinical and 12 environmental) isolated from Lima, Trujillo, Lambayeque, Cajamarca, Arequipa, Ayacucho, Loreto and Ucayali in Peru from 1991 to 2003 were examined. Twenty-three *V. cholerae* O1 El Tor strains from ten other countries (Table 1) were also included in this study for comparison. *V. cholerae* O1 classical O395 and El Tor strain N16961 strains were used as reference strains representing both the biotypes of *V. cholerae* O1. All strains were subcultured on gelatin agar plates and were stored at -80°C in Luria-Bertani (LB) broth (Miller) containing 25 % glycerol until needed for further study.

Serotyping and biotyping. The serogroup of all strains was reconfirmed using polyvalent O1 and monoclonal Inaba and Ogawa antisera prepared at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Biotyping included tests for polymyxin B susceptibility (50 U), chicken red blood cell agglutination and sensitivity to group IV classical and group 5 El Tor phages, and tests were performed as described previously (Nair *et al.*, 2002).

Genomic DNA isolation. In brief, cells were harvested from 3 ml overnight culture in LB broth and they were then subjected to alkaline lysis, and proteinase and RNase treatment. This was followed by phenol/chloroform extraction and precipitation of the nucleic acid in the presence of 2-propanol (Chowdhury *et al.*, 2000). Excess salt was removed by a 70 % alcohol wash and the nucleic acid was air-dried and resuspended in sterile TE buffer. The concentration and purity of the DNA was assayed using a spectrophotometer (GeneQuant) and the DNA was stored at -20°C for subsequent experiments.

PCR analysis

Confirmation of strain identity. All strains were confirmed as *V. cholerae* using species-specific *ompW* PCR (Nandi *et al.*, 2000). Multiplex PCR was used to detect O-antigen biosynthesis genes (*wbe* or *wbf*) and for the cholera toxin A-encoding gene (*ctxA*) (Hoshino *et al.*, 1998). Alleles of the *tcpA* (Keasler & Hall, 1993) and *rtsR* genes (Davis *et al.*, 1999) were identified by PCR using primers and procedures described previously (Nusrin *et al.*, 2004). All PCR reagents and kits were obtained from Invitrogen.

Multilocus virulence gene profiling. Multilocus virulence gene profiling by PCR was used to scan 11 virulence-associated genes and/or gene clusters and a housekeeping gene in the genome of 20 Peruvian *V. cholerae* isolates, including reference O395 classical and N16961 El Tor strains, using 31 sets of PCR primers and conditions described elsewhere (O'Shea *et al.*, 2004a; Chow *et al.*, 2001; Rivera *et al.*, 2001; Nair *et al.*, 2006; Safa *et al.*, 2006). The PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide, visualized under UV light and the results recorded by a gel documentation system (Gel Doc 2000; Bio-Rad). The sizes of the PCR products were determined by comparison with different molecular size markers.

Table 1. Serotypes and genotypes of the *V. cholerae* O1 El Tor biotype strains and the two reference strains examined in this study

Country (no. of strains) or reference strain	Year of isolation	Serotype	Genotype		
			<i>ctxA</i>	<i>tcpA</i>	<i>rstR</i> *
Peru (47)	1991–1999, 2003	Ogawa	+	+	2
Peru (10)	1991–1994	Inaba	+	+	2
Peru (1)	1991	Inaba	+	–	2
Peru (1)	1995	Ogawa	–	+	2
Peru (1)	1993	Ogawa	+	–	2
Australia (1)	1986	Inaba	+	+	1
Australia (1)	1965	Inaba	–	–	–
Australia (1)	1979	Inaba	+	+	1, 2
Bangladesh (4)	2004	Ogawa	+	+	2
India (2)	1975, 1982	Ogawa	+	+	2
India (1)	1981	Ogawa	–	+	2
Maldives (1)	1978	Ogawa	+	+	2
Maldives (1)	1978	Ogawa	–	+	2
Malaysia (1)	1978	Inaba	+	+	2
Malaysia (1)	1978	Ogawa	+	+	2
Myanmar (1)	1970	Ogawa	+	+	1, 2
Macao, China (1)	1970	Inaba	–	+	2
Germany (1)	1975	Inaba	+	+	2
Germany (1)	1975	Ogawa	+	+	2
Zambia (2)	1996, 2003	Ogawa	+	+	2
Mozambique (3)	2004	Ogawa	+	+	1
O395 (classical)	1965	Ogawa	+	+	1
N16961 (El Tor)	1971	Inaba	+	+	2

**rstR1* and *rstR2* represent the *rstR* genes of classical and El Tor type, respectively.

ORF content analysis of VSP-II region. The presence or absence of ORFs in the VSP-II cluster of 83 *V. cholerae* O1 isolates (60 Peruvian strains and 23 strains from different parts of the world) was re-examined by PCR using primers described elsewhere (O'Shea *et al.*, 2004a) with new combinations and PCR conditions, as shown in Table 2. Five sets of primers were designed (Table 2) from the VSP-II DNA sequence of reference N16961 El Tor strain (accession number NC_002505) using GeneFisher software (available at <http://www.genefisher.de/>) to examine the individual ORFs of VSP-II region that encompasses VC0511 to VC0515. PCR conditions are given in Table 2.

Nucleotide sequence analysis

VSP-II gene cluster. The entire VSP-II region of Peruvian strain PERU-130 was amplified by PCR using Ex *Taq* (Takara Bio) and primers shown in Table 2. The PCR product obtained by using LA *Taq* (Takara Bio) and the primer set VSP-II LaU and VSP-II LaR was digested by *Sau3A*I. The digests were ligated into the *Bam*HI site of pBluescript II SK (–) and the ligation mixture was used to transform *E. coli* JM109. The recombinant *E. coli* strains were cultured in L broth including ampicillin (100 µg ml^{–1}) and an aliquot was boiled in TE buffer. The boiled template was amplified by M13 forward and reverse primers. All the PCR products were purified by QIAquick PCR purification kit (Qiagen) and sequenced using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems), and M13 forward and reverse primers, or sequencing primer designed by the obtained sequence, if necessary. The reactions

were conducted in a GeneAmp 9700 thermal cycler in accordance with the manufacturer's instruction (Applied Biosystems). The nucleotide sequence was determined using an ABI Prism 3100-Avant genetic analyser (Applied Biosystems). VSP-II DNA sequences obtained were analysed using the DNA Lasergene software package (DNASTAR). Homology searches were performed using the BLAST program made available by DNA Data Bank Japan (DDBJ). The DNA sequence of VSP-II was submitted to DDBJ under the accession number AB300724.

***ctxB* gene.** To determine the *ctxB* nucleotide sequence, PCR amplification of *ctxB* gene of three Peruvian *V. cholerae* O1 representative strains (PERU-044, PERU-130 and PERU-296) was performed in an automated Peltier thermal cycler (PTC-200; M. J. Research) using PCR primers and conditions described previously (Mittra *et al.*, 2001). PCR products were purified with a Microcon centrifugal filter device (Millipore) and sequenced using an ABI PRISM BigDye terminator cycle sequencing reaction kit (Perkin-Elmer Applied Biosystems) with an ABI PRISM 310 automated sequencer. The chromatogram sequencing files were inspected using Chromas 2.23 (Technelysium). The *ctxB* nucleotide sequences obtained were compared with that of the reference N16961 El Tor strain (GenBank accession no. NC_002505) and 569B classical strain (GenBank accession no. U25679) retrieved from GenBank using BLAST. Multiple sequence alignments were developed using CLUSTAL_X 1.81.13. The nucleotide sequences of the *ctxB* genes have been deposited in GenBank under accession numbers EU921452–EU921454.

Table 2. PCR primer sequences and conditions for analysing the VSP-II region of *V. cholerae* O1 El Tor strains isolated in Peru and various other countries

Primer designation	Sequences (5'→3')	PCR condition			No. of cycles	Product size (bp)	Reference
		Denaturation	Annealing	Extension			
VC0511F1	CTTGCTGCGTACTTAGCA	95 °C, 1 min	54 °C, 1 min	72 °C, 1 min	30	385	This study
VC0511R1	AGTAGCATCGCTCTCGTA						
VC0512F1	TCCTGATTGGGAGCGAA	95 °C, 1 min	54 °C, 1 min	72 °C, 1 min	30	1 704	This study
VC0512R1	TCACGCGGGTTATTCCA						
VC0513F1	CTGAGGTGTTATATGTTTCG	95 °C, 1 min	54 °C, 1 min	72 °C, 1 min	30	781	This study
VC0513R1	TCAAATTTCTGACAGTTCC						
VC0514F1	GTTTGGGAAGGGTACACA	95 °C, 1 min	52 °C, 1 min	72 °C, 1 min	30	1 651	This study
VC0514R1	GCTCTTCAGCCGCTGA						
VC0515F1	GGTGGTGCTGCATGGA	95 °C, 1 min	50 °C, 1 min	72 °C, 1 min	30	1 135	This study
VC0515R1	TCTAAAGCCTCACACCA						
VC0489F	AGATCAACTACGATCAAGCC	94 °C, 30 s	52 °C, 30 s	72 °C, 4 min	30	5 202	O'Shea <i>et al.</i> (2004a)
VC0490R	TGCAGTTGTTGAATGGAC						
VC0490F	CGTGAAGGGATATAGGAG	94 °C, 30 s	52 °C, 30 s	72 °C, 4 min	30	6 718	O'Shea <i>et al.</i> (2004a)
VC0493R	CGCTCTTCTTTCCACGCTTCA						
VC0493F	AATGCTTCTCAGGGGGGTCTT	94 °C, 30 s	52 °C, 30 s	72 °C, 4 min	30	7 439	O'Shea <i>et al.</i> (2004a)
VC0498R	TGCGGCTCCAATGGAGTCTG						
VC0502F	TCATCAGTTAGCACACGAAC	94 °C, 30 s	52 °C, 30 s	72 °C, 4 min	30	5 808	O'Shea <i>et al.</i> (2004a)
VC0504R	AGCCCGAAATGAATCCCAAAA						
VC0504F	CAGCAAAGGCGGAAGAGGTAG	94 °C, 30 s	52 °C, 30 s	72 °C, 4 min	30	7 616	O'Shea <i>et al.</i> (2004a)
VC0512R	CCCTCCACTGCTATTCCG						
VC0512F	CAGTGGCTTCGCAGAGGA	94 °C, 30 s	52 °C, 30 s	72 °C, 4 min	30	8 384	O'Shea <i>et al.</i> (2004a)
VC0516R	TCCTGATGTCTCTCTTGCCG						
VC0514F	TTATGATCCAAGGAGTAGGG	94 °C, 30 s	52 °C, 30 s	72 °C, 4 min	30	6 736	O'Shea <i>et al.</i> (2004a)
VC0517R	CGCAGTCACAGCTTAAAC						
VSPII LaU	CGCACATACTCTTTGGTGGCATCAG	98 °C, 10 s		68 °C, 10 min	30	10 091	This study
VSPII LaR	ACTCTCTCTGATGGTATGGCCTTC						
VSPII 47U	TGTCACCTGAAGCTACAG	94 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	2 822	This study
VC0517R	CGCAGTCACAGCTTAAAC						

PFGE. Intact agarose-embedded chromosomal DNA from the *V. cholerae* strains was prepared and PFGE was performed using a contour-clamped homogeneous electric field apparatus (CHEF-Mapper) (Bio-Rad) following the standardized PFGE protocol (Cooper *et al.*, 2006). Genomic DNA was digested with *NotI* enzyme (Invitrogen). The restriction fragments were separated in 1 % SeaKem Gold agarose in $0.5 \times$ TBE buffer (45 mM Tris/44 mM borate/0.8 mM EDTA). *V. cholerae* N16961 and 569B were used as reference strains. *Salmonella enterica* serotype Braenderup strain H9812 digested with *XbaI* (Invitrogen) was used as the molecular size marker. Following electrophoresis, the gels were stained with ethidium bromide, destained with water and images were captured using a gel documentation system (Gel Doc 2000; Bio-Rad).

RESULTS AND DISCUSSION

Confirmation of strain identity

V. cholerae species-specific *ompW* and serogroup-specific *wbe* PCR were positive for all the 83 strains tested from Peru and 10 other countries (data not shown). Biotyping showed that all strains were resistant to polymyxin B, agglutinated with chicken erythrocytes, were resistant to phage IV and sensitive to phage 5, which are typical traits of the El Tor biotype. Among the 60 Peruvian strains, 2 strains (PERU-067 and PERU-189) were negative for *tcpA* of both the El Tor and classical type, while others were positive for *tcpA* of the El Tor type (Table 1). All Peruvian strains harboured an *rstR* gene of El Tor type. *rstR* types found in other strains are shown in Table 1. The serotype and genotype of the 60 Peruvian strains and the 23 strains of *V. cholerae* O1 from other countries are shown in Table 1. All strains from Peru except one were *ctxA* positive, and only four strains from other countries were negative for this gene. Deduced amino acid sequences from *ctxB* genes of representative Peruvian strains were identical to that of the reference N16961 El Tor strain. Therefore, the *ctxB* of Peruvian strains were categorized as genotype 3, which includes *V. cholerae* strains of Latin American origin and the strains of the current seventh pandemic clone.

Serogrouping and biotyping of Peruvian *V. cholerae* strains revealed that all strains belonged to serogroup O1 and to the El Tor biotype. Examination of biotype-specific genotypic traits also confirmed that the Peruvian strains were of the El Tor biotype. Presence of critical virulence-associated genes *tcpA* and *ctxAB* in the Peruvian strains suggested that almost all strains were virulent and had the potential to cause epidemic outbreaks.

Multilocus virulence gene profiling

Of the 60 Peruvian strains, 20 (Table 3) representative strains and reference strains O395 (classical) and N16961 (El Tor) were examined for 11 virulence-associated genes and/or gene clusters and for a housekeeping gene (*mdh*) by PCR. All 20 strains from Peru showed the presence of all genes comprising VSP-I, MSHA and RTX gene clusters; five individual loci, namely *hlyA*, *pilE*, *tlc*, *intI4* and *rstC*,

were also present in all the Peruvian strains examined (Table 3). Apart from this, two Peruvian strains lacked the genes of the VPI-I region examined and one strain was negative for *ctxAB* genes, and therefore, it was concluded to be non-toxigenic (Table 3). Among the ORFs of VSP-II examined in the preliminary genetic screen, two ORFs, namely VC0512 and VC0514, were negative by PCR in all 20 Peruvian strains tested, including the O395 classical reference strains but they were present in the N16961 El Tor reference strain (Table 3).

Screening of 11 virulence-associated genes and/or gene clusters of Peruvian strains and their comparison with the typical seventh-pandemic El Tor strain indicated that Peruvian strains are similar to the seventh pandemic strains with regard to their pathogenic potential. The only significant disparity observed between the virulence gene content of the Peruvian and typical seventh pandemic strains was confined to the putative virulence gene cluster, VSP-II. The altered gene content of the VSP-II cluster of Peruvian strains distinguished them from the Eurasian and African prototype seventh pandemic El Tor strains.

VSP-II gene cluster analysis

Our next objective was to examine the absence of ORFs 512 and 514 in VSP-II and their flanking regions in further detail. PCR screening of 60 Peruvian strains for 12 ORFs of VSP-II revealed that all strains were positive for ORFs VC0498–VC0502, VC0504–VC0510 and VC0517, whereas ORFs VC0512–VC0515 could not be amplified in any of the Peruvian strains (Table 4). ORF VC0511 was absent in 51 strains and 40 strains were negative for VC0516 (Table 4). ORFs VC0490 and VC0493 were not found in strain PERU-044 and PERU-001, respectively. Except for three strains from Australia, all ORFs of VSP-II examined were found in all strains from other countries (Table 4). Three Australian strains were negative for the complete VSP-II region.

Comparative genomic analysis of diverse *V. cholerae* has recently shown that VSP-I and VSP-II gene clusters are unique to the seventh pandemic El Tor biotype strains but absent in strains of the classical biotype. The role of VSP-I and VSP-II in the El Tor biotype is not known (O'Shea *et al.*, 2004a) but their exclusive presence among the seventh pandemic El Tor strains is responsible for some of the distinctive properties of this biotype (Dziejman *et al.*, 2002), and may contribute in some way to its fitness and survival (O'Shea *et al.*, 2004a). VSP-II is a 29.6 kb genomic island encompassing 24 predicted ORFs (VC0490–VC0516) encoding proteins including DNA repair and methyl-accepting chemotaxis proteins, a group of hypothetical proteins and a bacteriophage-like integrase adjacent to a tRNA-encoding gene (O'Shea *et al.*, 2004b). By PCR analysis, we discovered that the Peruvian El Tor strains of *V. cholerae* O1 have an intact VSP-I but found altered gene content for the VSP-II cluster as PCR could not amplify some ORFs, namely VC0512 (methyl-accepting chemotaxis protein), VC0513 (transcriptional regulator),

Table 3. Results of the genetic screen used for identifying 11 virulence regions and one housekeeping gene of the *V. cholerae* O1 strains isolated in Peru between 1991 and 2003

Strain	Biotype	Housekeeping gene	VSP-I					MSHA				<i>hlyA</i>		VSP-II							VPI-I			<i>pilE</i>	RTX		RS1	CTX				TLC	Int14
			<i>mdh</i>	175	178	180	183	185	398	400	403	406	489	490	491–496	498–502	504–510	512–515	516	517	<i>tcpA</i>	<i>toxT</i>	<i>acfB</i>	<i>pilE</i>	<i>rtxA</i>	<i>rtxC</i>	<i>rstC</i>	<i>rstA</i>	<i>orfU</i>	<i>zot</i>	<i>ctxAB</i>	<i>tlc</i>	<i>int14</i>
PERU-130*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-139*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-067*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+
PERU-115*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-044*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-097*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	–	+	+
PERU-120*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-169*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-037*	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-299†	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-189‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+
PERU-200‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-205‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-219‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-306†	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-324‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-327‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-328‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-329‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
PERU-330‡	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
N16961	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
O395	Classical	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	C	+	+	+	+	–	–	+	+	+	+	+	+

C, Classical *tcpA*; E, El Tor *tcpA*.

*Clinical origin.

†Unknown origin.

‡Environmental origin.

Table 4. Comparison of the VSP-II regions of Peruvian *V. cholerae* O1 El Tor isolates with those of the isolates from different countries possessing different ORFs of the VSP-II region

Place of isolation	No. of strains	VSP-II											
		VC0490– VC0492*	VC0493– VC0496*	VC0498– VC0502*	VC0502*	VC0504– VC0510*	VC0511†	VC0512†	VC0513†	VC0514†	VC0515†	VC0516*	VC0517*
Peru	30	+	+	+	+	+	–	–	–	–	–	–	+
Peru	19	+	+	+	+	+	–	–	–	–	–	+	+
Peru	9	+	+	+	+	+	+	–	–	–	–	–	+
Peru	1	–	+	+	+	+	–	–	–	–	–	+	+
Peru	1	+	–	+	+	+	–	–	–	–	–	–	+
Australia	3	–	–	–	–	–	–	–	–	–	–	–	+
Bangladesh	4	+	–	–	+	+	+	+	+	+	+	+	+
Myanmar	1	+	+	+	+	+	+	+	+	+	+	+	+
India	3	+	+	+	+	+	+	+	+	+	+	+	+
Maldives	2	+	+	+	+	+	+	+	+	+	+	+	+
Malaysia	2	+	+	+	+	+	+	+	+	+	+	+	+
Mozambique	3	+	+	+	+	+	+	+	+	+	+	+	+
Macao, China	1	+	+	+	+	+	+	+	+	+	+	+	+
Germany	2	+	+	+	+	+	+	+	+	+	+	+	+
Zambia	1	+	+	+	+	+	+	+	+	+	+	+	+
Zambia	1	+	–	–	+	+	+	+	+	+	+	+	+
Classical (O395)	1	–	–	–	–	–	–	–	–	–	–	–	+
El Tor (N16961)	1	+	+	+	+	+	+	+	+	+	+	+	+

*The ORFs VC0490–VC0504 and the ORFs VC0516–VC0517 were amplified with the primers described by O'Shea *et al.* (2004a).

†The ORFs VC0511–VC0515 were amplified with the primers designed in this study (Table 2).

Fig. 1. Schematic representation of the organization of VSP-II from *V. cholerae* strains N16961 (a) and PERU-130 (b). The position and direction of transcription of the ORFs are indicated by the arrows. Homology between N16961 and PERU-130 at the DNA level is described as a percentage and shown between parts (a) and (b). The numbers refer to the genetic organization of the genes. The regions amplified by PCR using each primer set, described in Table 2, are also depicted (c). The solid bars indicate the regions amplified; the dashed bars indicate the regions that failed to amplify. The numbers below the arrows indicate the names of the PCR primers used.

VC0514 (methyl-accepting chemotaxis protein) and VC0515 (hypothetical protein). At the time of its discovery, VSP-II was described as a 7.5 kb region encompassing eight ORFs (VC0490–VC0497). Later, O'Shea *et al.* (2004b) demonstrated that the 7.5 kb VSP-II region is part of a 29.6 kb island that encompasses ORFs VC0490–VC0516. In the initial study of Dziejman *et al.* (2002), a Peruvian strain C6709 was included but the absence of certain ORFs went unnoticed since at that time these ORFs were not considered as a part of the VSP-II region.

VSP-II gene cluster sequence analysis

The VSP-II gene cluster of one representative Peruvian strain Peru-130 was sequenced to gain better understanding of its gene content. When primer sets, 498F and 490R, 490F and 493R, 493F and 498R, 502F and 504R, and 47U and 517R, were used, PCR products of the expected sizes were obtained as shown in Fig. 1. When primer sets, 504F and 512R, 512F and 516R, and 514F and 517R, were used, however, no amplicons were obtained. PCR primer set, VSP-II LaU and VSP-II LaR (designed using the DNA sequence obtained from PCR products amplified by 502F and 504R, and by 47U and 517R, which encompass the negative region), produced an amplicon, which was close to the size of the prototype VSP-II of N16961 El Tor strain. Comparison of the obtained nucleotide sequences of the entire VSP-II including flanking regions (31,299 bp) of PERU-130, and other parameters, with those of the N16961 El Tor strain are shown in Fig. 1 and Table 5. Analysis showed that the 26.5 kb (26,519 bp) VSP-II of PERU-130 contained 26 ORFs, which correspond to the 26.9 kb (26,866 bp) containing 24 ORFs of N16961 El Tor

strain (O'Shea *et al.*, 2004b). In the VSP-II sequences from PERU-130 and N16961, four regions could be identified based on the extent of similarity. These were ORFs 2–14 of PERU-130 (100 % similarity), ORFs 15–19 (94.4 % similarity), ORFs 20–24 (46.8 % similarity) and ORF 25 (89 % similarity) (Fig. 1). The sequencing data confirmed our PCR results, indicating that ORFs 20–24 of the Peruvian strain were, indeed, different from the corresponding VC0511–VC0515 region of N16961.

The hypothetical proteins encoded by ORFs 20 and 21 showed 46.7 and 33.2 % homology to proteins of *Azoarcus* sp. BH72, a mutualistic N_2 -fixing endophyte of rice and other grasses (Krause *et al.*, 2006). ORF 22 representing another hypothetical protein that showed 46.7 % homology to a protein encoded by a gene designated Mmcs_4842 of *Mycobacterium* sp. MCS (Table 5). The transposase OrfAB, subunit A, encoded by ORF 23 of PERU-130 showed 87.2 % homology to a similar protein encoded by VCA0372 of N16961, while subunit B of transposase OrfAB encoded by ORF 24 showed 83.6 and 86.1 % homology to similar proteins of *Nitrococcus mobilis* Nb-231 and VCA0371 of *V. cholerae* N16961, respectively (Table 5). However, ORF 23 and ORF 24 (1214 bp) have high homology to *V. cholerae* serogroup O103 insertion sequences IS_{alg} with 98.8 % identity (Sozhamannan *et al.*, 1999). ORF 25 of PERU-130 shared close homology to the phage integrase of *Vibrio vulnificus* YJ016 (98.5 %) and *V. cholerae* strain N16961 (93.7 %). The 5' flanking ORF 1 and the 3' flanking ORF 26 of PERU-130 were identical to VC0489 and VC0517 of N16961 strain, respectively.

The complete nucleotide sequence analysis of VSP-II showed little difference in the overall size of the VSP-II of PERU-130

Table 5. The 26 ORFs encompassing the 26.5 kb VSP-II island in *V. cholerae* O1 El Tor Peru-130 strain

ORF	Length in bp (no. of aa)	Homologue	Length in bp (no. of aa)	Amino acid identity (%)	DNA identity (%)	Gene designation	Strain	Accession no.	Reference
1	1302 (433)	Haemolysin	1761 (586)	432/433 (99.8)	1301/1302 (99.9)	VC0489	<i>V. cholerae</i> N16961	NP_230143	Heidelberg <i>et al.</i> (2000)
2	1962 (653)	Hypothetical protein	1962 (653)	653/653 (100)	1962/1962 (100)	VC0490	<i>V. cholerae</i> N16961	NP_230144	Heidelberg <i>et al.</i> (2000)
3	537 (178)	Hypothetical protein	537 (178)	177/178 (99.4)	537/537 (100)	VC0491	<i>V. cholerae</i> N16961	NP_230145	Heidelberg <i>et al.</i> (2000)
4	1164 (388)	Hypothetical protein	1164 (388)	388/388 (100)	1164/1164 (100)	VC0492	<i>V. cholerae</i> N16961	NP_230146	Heidelberg <i>et al.</i> (2000)
5	876 (291)	Hypothetical protein	876 (291)	291/291 (100)	876/876 (100)	VC0493	<i>V. cholerae</i> N16961	NP_230147	Heidelberg <i>et al.</i> (2000)
6	645 (214)	Hypothetical protein	645 (214)	214/214 (100)	645/645 (100)	VC0494	<i>V. cholerae</i> N16961	NP_230148	Heidelberg <i>et al.</i> (2000)
7	675 (224)	Hypothetical protein	675 (224)	224/224 (100)	675/675 (100)	VC0495	<i>V. cholerae</i> N16961	NP_230149	Heidelberg <i>et al.</i> (2000)
8	600 (199)	Hypothetical protein	600 (199)	199/199 (100)	600/600 (100)	VC0496	<i>V. cholerae</i> N16961	NP_230150	Heidelberg <i>et al.</i> (2000)
9	201 (66)	Transcriptional regulator	201 (66)	66/66 (100)	201/201 (100)	VC0497	<i>V. cholerae</i> N16961	NP_230151	Heidelberg <i>et al.</i> (2000)
10	441 (146)	RNase H	441 (146)	146/146 (100)	441/441 (100)	VC0498	<i>V. cholerae</i> N16961	NP_230152	Heidelberg <i>et al.</i> (2000)
11	525 (174)	Type IV pilin	525 (174)	174/174 (100)	525/525 (100)	VC0502	<i>V. cholerae</i> N16961	NP_230153	Heidelberg <i>et al.</i> (2000)
12	1281 (426)	Hypothetical protein	1281 (426)	426/426 (100)	1281/1281 (100)	VC0503	<i>V. cholerae</i> N16961	NP_230154	Heidelberg <i>et al.</i> (2000)
13	228 (75)	Hypothetical protein	228 (75)	75/75 (100)	228/228 (100)	VC0504	<i>V. cholerae</i> N16961	NP_230155	Heidelberg <i>et al.</i> (2000)
14	369 (122)	Hypothetical protein	369 (122)	122/122 (100)	369/369 (100)	VC0505	<i>V. cholerae</i> N16961	NP_230156	Heidelberg <i>et al.</i> (2000)
15	735 (244)	Hypothetical protein	735 (244)	236/244 (96.7)	708/735 (96.3)	VC0506	<i>V. cholerae</i> N16961	NP_230157	Heidelberg <i>et al.</i> (2000)
16	177 (58)	Hypothetical protein	177 (58)	53/58 (91.4)	165/177 (93.2)	VC0507	<i>V. cholerae</i> N16961	NP_230158	Heidelberg <i>et al.</i> (2000)
17	444 (147)	Hypothetical protein	444 (147)	143/147 (97.3)	424/444 (95.5)	VchoR_ 02001367	<i>V. cholerae</i> RC385	ZP_01482712	Direct submission
18	444 (147)	Hypothetical protein	444 (147)	137/147 (93.2)	410/444 (92.3)	VV0523	<i>V. vulnificus</i> YJ016	NP_933316	Chen <i>et al.</i> (2003)
				137/147 (93.2)	419/444 (94.4)	VC0508	<i>V. cholerae</i> N16961	NP_230159	Heidelberg <i>et al.</i> (2000)
				140/144 (97.2)	397/427 (93.0)	VV0524	<i>V. vulnificus</i> YJ016	ZP_01482712	Chen <i>et al.</i> (2003)
				133/144 (92.4)	398/434 (91.7)	VC0509	<i>V. cholerae</i> N16961	NP_230160	Heidelberg <i>et al.</i> (2000)
19	474 (157)	DNA repair protein RadC family protein	474 (157)	154/157 (98.1)	443/474 (93.5)	VV0525	<i>V. vulnificus</i> YJ016	NP_933318	Chen <i>et al.</i> (2003)
20	1812 (603)	Hypothetical protein	1776 (591)	151/157 (96)	440/474 (92.8)	VC0510	<i>V. cholerae</i> N16961	NP_230161	Heidelberg <i>et al.</i> (2000)
21	2466 (821)	Hypothetical protein	2187 (728)	278/595 (46.7)	982/1836 (53.5)	azo2045	<i>Azoarcus</i> sp. BH72	YP_933549	Krause <i>et al.</i> (2006)
22	2043 (673)	Hypothetical protein	2043 (680)	246/741 (33.2)	432/914 (47.3)	azo2046	<i>Azoarcus</i> sp. BH72	YP_933550	Krause <i>et al.</i> (2006)
				319/683 (46.7)	1072/2054 (52.2)	Mmcs_4842	<i>Mycobacterium</i> sp. MCS	YP_642002	Direct submission
23	345 (114)	Transposase OrfAB, subunit A	345 (114)	107/114 (93.9)	300/345 (87.2)	VCA0372	<i>V. cholerae</i> N16961	NP_232767	Heidelberg <i>et al.</i> (2000)
24	870 (289)	Transposase OrfAB, subunit B	921 (306)	265/289 (91.7)	727/870 (83.6)	NB231_17565	<i>N. mobilis</i> Nb-231	ZP_01128962	Direct submission
			873 (290)	264/289 (91.3)	749/870 (86.1)	VCA0371	<i>V. cholerae</i> N16961	NP_232766	Heidelberg <i>et al.</i> (2000)

Table 5. cont.

ORF	Length in bp (no. of aa)	Homologue	Length in bp (no. of aa)	Amino acid identity (%)	DNA identity (%)	Gene designation	Strain	Accession no.	Reference
23 + 24*	1214	ISalg (IS element found in <i>V.</i> <i>alginolyticus</i>)	1258	–	1200/1214 (98.8)	ISalg	<i>V. cholerae</i> O103	AF133213	Sozhamannan <i>et al.</i> (1999)
25	1242 (413)	Phage integrase	1230 (409) 1242 (413)	402/408 (98.5) 387/413 (93.7)	1193/1224 (97.5) 1110/1242 (89.4)	VV0560 VC0516	<i>V. vulnificus</i> Y1016 <i>V. cholerae</i> N16961	NP_933353 NP_230167	Chen <i>et al.</i> (2003) Heidelberg <i>et al.</i> (2000)
26	1878 (625)	RNA polymerase sigma factor (RpoD)	1878 (625)	625/625 (100)	1878/1878 (100)	VC0517	<i>V. cholerae</i> N16961	NP_230168	Heidelberg <i>et al.</i> (2000)

*The DNA sequences of ORF 23 and ORF 24 have high homology to *V. cholerae* serogroup O103 insertion sequence ISalg.

and N16961 but the flanking regions were conserved. Of the 26 ORFs in the VSP-II of PERU-130, 20 showed extensive homology with the corresponding ORFs of N16961. Only one region comprising ORFs 20–24 of PERU-130 was different from the corresponding VSP-II region constituting VC0511–VC0515 of the prototype El Tor strain N16961. The presence of hypothetical proteins homologous to those present in the nitrogen-fixing endophyte symbiotic *Azoarcus* sp. in the VSP-II region (ORFs 20 and 21) indicates that the progenitor of the Peruvian *V. cholerae* strains may have acquired these genes from micro-organisms in the environment. The extensive DNA homology of ORFs 23 and 24 of the VSP-II of PERU-130 to *V. cholerae* serogroup O103 insertion sequence ISalg is interesting since ISalg is distributed in 69 % of the *V. cholerae* non-O1 non-O139 strains, and has extensive homology to the IS element of *Vibrio alginolyticus* and IS911 of *Shigella dysenteriae* (Hayashi *et al.*, 1997; Sozhamannan *et al.*, 1999). We are not sure if these five divergent ORFs of PERU-130 assembled together or were independent acquisitions, but the presence of ISalg suggests genetic rearrangements or horizontal transfer.

PFGE analysis

In this study, PFGE analysis of the representative Peruvian strains isolated between 1991 and 1996 showed diverse PFGE banding patterns or pulsotypes (Fig. 2). Most of the pulsotypes were different from that of the reference O395 classical strain but similar to the N16961 El Tor strain, except pulsotype A1, which is almost identical to the pulsotype of N16961.

The Latin American isolates, at the time of introduction, were clonal and were described as ET4 by multilocus enzyme electrophoresis (MLEE); the seventh pandemic strains in Asia and Africa belonged to ET3 (Wachsmuth *et al.*, 1993). Later studies showed at least two distinct clones easily distinguishable by MLEE, ribotype, PFGE pattern and antimicrobial resistance pattern (Evins *et al.*, 1995). By ribotyping, the Latin American epidemic was caused by strains of ribotype 5, which were isolated from several other geographical locations but could be differentiated from the Latin American strains by other molecular typing techniques (Popovic *et al.*, 1993). The evolution of *V. cholerae* O1 biotype El Tor strains isolated in Lima, Peru, from 1991 to 1995 showed the continuous and more frequent occurrence than previously, of genetic changes in the Latin American cholera epidemic strains (Dalsgaard *et al.*, 1997). The Peruvian strains that we examined were isolated from the beginning of the epidemic in 1991 through 2003. Overall PFGE analysis revealed that the Peruvian strains are clonal and similar to the prototype seventh pandemic clone but different from the classical reference strain.

The origin and source of the Latin American strains of *V. cholerae* O1 in Peru remains a mystery. The 'single introduction hypothesis' states that the Latin American strains may have originated as a result of the emptying of

Fig. 2. *NotI*-restricted patterns of chromosomal DNA of Peruvian *V. cholerae* O1 El Tor strains isolated between 1991 and 1996. Lanes 1, 8 and 15, *Salmonella braenderup* strain H9812 as a molecular mass marker; lanes 2, 3 and 5, represent pulsotype A1 – PERU-180 (1991), PERU-139 (1992) and PERU-067 (1993), respectively; lane 4, represents pulsotype B1 – PERU-011 (1993); lane 6, represents pulsotype B2 – PERU-086 (1994); lane 7, represents pulsotype A2 – PERU-115 (1994); lane 9, represents pulsotype C – PERU-097 (1995); lane 10, represents pulsotype D – PERU-132 (1995); lane 11, represents pulsotype A3 – PERU-120 (1996); lane 12, represents pulsotype A4 – PERU-137 (1996); and lanes 13 and 14, represent 569B classical and N16961 El Tor strains, respectively.

contaminated bilge waters of ships arriving from cholera endemic areas of Asia or Africa (McCarthy & Khambaty, 1994). The difference in the VSP-II between the Peruvian strains and those from Asia and Africa does not support this hypothesis. Another, less-accepted hypothesis, consistent with simultaneous appearance of the disease in a number of coastal Peruvian sites within a few days, relates to the sudden and massive expansion of a pre-existing small population of pathogenic cholera vibrios on zooplankton or phytoplankton in Peruvian coastal waters (Craig, 1996). Once introduced into the coastal communities in concentrations large enough for human infection to occur, cholera spreads by the well-known means of contaminated water and food (Seas *et al.*, 2000). The Peruvian strains may be genetic derivatives of *V. cholerae* O1 from the coastal environs of Peru because the VSP-II of environmental Peruvian strains from 1991 examined in this study were similar to all the clinical Peruvian strains and different from strains isolated from other countries and continents. Seas *et al.* (2000) also postulate that cholera vibrios, autochthonous to the aquatic environment, were present in multiple coastal locations, and this resulted from the environmental conditions that existed during an El Niño phenomenon.

The Peruvian El Tor O1 strains are genotypically not very distinct from the seventh pandemic Eurasian and African strains. The pulsotypes, while very different to the classical biotype, were very similar, almost indistinguishable, to *V. cholerae* O1 El Tor (see Peruvian pulsotypes A1 and N16961); all Peruvian strains contained VSP-I and part of VSP-II with identical flanking regions. It is difficult to accept that strains with this high similarity arose independently. VSP-II seems to be highly variable, in fact Peruvian strains differ within the VSP-II gene cluster; among the 60 strains examined 9 were positive for VC0511 and 20 were positive for VC0516. The presence of insertion

elements suggests that the missing region in the Peruvian strains could be due to genetic rearrangements via horizontal transfer.

From a functional viewpoint, we do not know the significance of the difference between VSP-II of the Peruvian strains and those isolated from other continents. It is noteworthy that despite the genetic diversity, changing serotypes, and different year and place of isolation, the alteration in the VSP-II region of the Peruvian strains isolated for more than a decade was stably conserved. Therefore, from an identity point of view, this difference in the VSP-II lends a unique molecular signature to the Peruvian strains that could form the basis of tracking the origin of the Peruvian strains and therefore the Latin American pandemic. Based on the overall result obtained we suggest that it is more likely that the Peruvian and Eurasian and African strains belong to the same clonal complex, and the Peruvian strains represent variants that have independently evolved for a relatively short time. Optimally, we should have examined strains from other Latin American countries but did not do so since we had none in our collection. Further studies are required to understand what the difference means functionally.

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