

Analysis of *Vibrio cholerae* Genome Sequences Reveals Unique *rtxA* Variants in Environmental Strains and an *rtxA*-Null Mutation in Recent Altered El Tor Isolates

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ABSTRACT *Vibrio cholerae* genome sequences were analyzed for variation in the *rtxA* gene that encodes the multifunctional auto-processing RTX (MARTX) toxin. To accommodate genomic analysis, a discrepancy in the annotated *rtxA* start site was resolved experimentally. The correct start site is an ATG downstream from *rtxC* resulting in a gene of 13,638 bp and deduced protein of 4,545 amino acids. Among the El Tor O1 and closely related O139 and O37 genomes, *rtxA* was highly conserved, with nine alleles differing by only 1 to 6 nucleotides in 100 years. In contrast, 12 alleles from environment-associated isolates are highly variable, at 1 to 3% by nucleotide and 3 to 7% by amino acid. The difference in variation rates did not represent a bias for conservation of the El Tor *rtxA* compared to that of other strains but rather reflected the lack of gene variation in overall genomes. Three alleles were identified that would affect the function of the MARTX toxin. Two environmental isolates carry novel arrangements of effector domains. These include a variant from RC385 that would suggest an adenylate cyclase toxin and from HE-09 that may have actin ADP-ribosylating activity. Within the recently emerged altered El Tor strains that have a classical *ctxB* gene, a mutation arose in *rtxA* that introduces a premature stop codon that disabled toxin function. This null mutant is the genetic background for subsequent emergence of the *ctxB7* allele resulting in the strain that spread into Haiti in 2010. Thus, similar to classical strains, the altered El Tor pandemic strains eliminated *rtxA* after acquiring a classical *ctxB*.

IMPORTANCE Pathogen evolution involves both gain and loss of factors that influence disease. In the environment, bacteria evolve rapidly, with nucleotide diversity arising by genetic modification. Such is occurring with *Vibrio cholerae*, exemplified by extensive diversity and unique variants of the *rtxA*-encoded multifunctional autoprocessing RTX (MARTX) toxin among environment-associated strains that cause localized diarrheal outbreaks and food-borne disease. In contrast, seventh pandemic El Tor *V. cholerae* strains associated with severe diarrhea have changed minimally until the altered El Tor emerged as the most frequent cause of cholera, including in the 2010 Haiti epidemic. These strains have increased virulence attributed to a new variant of the major virulence factor, cholera toxin. It is revealed that these strains also have an inactivated MARTX toxin gene. A similar inactivation occurred during classical cholera pandemics, highlighting that evolution of El Tor cholera is following a similar path of increased dependence on cholera toxin, while eliminating other secreted factors.

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Vibrio cholerae is a diarrheal pathogen that is capable of spreading worldwide (1). The seventh cholera pandemic caused by O1 El Tor strains started in Indonesia, transitioned to India, and has been spreading worldwide since 1960 as a clonal expansion from a common ancestor (1, 2). In 1993, a recombination event occurred in which the typical El Tor O1 strain acquired a novel O antigen, resulting in the O139 epidemic (3). In 1994, “atypical” El Tor strains (also known as CTX-2 [2]) were identified that modified CTX Φ prophage genes and gene arrangement and that carry a classical-like *ctxB* gene, converting the primary virulence factor cholera toxin to the classical toxin (4). Among these atypical El Tor strains are strains, denoted as altered El Tor (5) (also known as CTX-3 [2]), that have a CTX Φ identical to the El Tor strains in sequence and genomic location, except the normal El Tor *ctxB3* allele has two point mutations that convert the gene to the *ctxB1*

allele found in the classical strains (4). Altered El Tor strains were first isolated in Bangladesh in the 1990s (2, 6) and subsequently overtook typical El Tor as the cause of cholera in Bangladesh since 2000 (5). These strains then accumulated an additional mutation in the *ctxB* gene, allele *ctxB7* (4, 7) (also known as CTX-3b [2]), and this strain has subsequently been identified in India, Bangladesh, Cameroon, Nepal, and Haiti (2, 7–11). Compared to typical pandemic El Tor, the altered El Tor variants are linked to increased production of cholera toxin (12) and more severely dehydrating diarrheal disease in humans (6).

V. cholerae also exists in the environment in water and is associated with aquatic animals. Some of these strains can also cause human infections, including localized diarrheal outbreaks or food-borne disease (13–17). In contrast to a calculated rate of mutation of only 3.3 single nucleotide polymorphisms (SNPs) per

year for the El Tor O1 strains (2), the environmental isolates show extensive diversity, with >2% variation between individual isolates (8).

The *rtxA* gene is the largest open reading frame (ORF) of the *V. cholerae* genome (18). All *V. cholerae* strains have an *rtxA* gene except for classical strains, which have a 7,869-bp deletion in the *rtx* locus that removes the 5' end of the *rtxA* gene as well as the *rtx* promoter region and genes for toxin maturation and secretion (19).

The *rtxA* gene encodes a multifunctional autoprocessing RTX (MARTX) family toxin. Toxins in this family range in size from 3,500 to 5,300 amino acids (aa) and are produced by various human, animal, and insect pathogens (20). Similar to all RTX family proteins, the MARTX toxins are exported from the bacteria via C-terminal secretion signals and special dedicated type I secretion systems (21). The MARTX toxins encoded by different bacterial species are comprised of both conserved and variable domains. The conserved domains are characterized by long repeat regions called the A, B, and C repeats and by a cysteine protease domain (CPD) that is required for inositol hexakisphosphate-induced autoprocessing of the toxins (20). The centrally located domains released to eukaryotic cell cytoplasm by autoprocessing are called "effector domains," and these carry cytotoxic and cytopathic activities that affect the cell biology of the target cells, including altered cytoskeleton assembly and signaling. A total of 10 distinct effector domains have been identified by sequence analysis, although a single MARTX toxin carries only from 1 to 5 of these effectors (20). *V. cholerae* has a MARTX toxin that delivers three effectors by autoprocessing: an actin crosslinking domain (ACD) that introduces an isopeptide bond between actin molecules, rendering the actin unable to assemble an actin cytoskeleton (22); a Rho-inactivation domain (RID) that induces actin depolymerization through loss of active RhoA (23); and an alpha-beta hydrolyze enzyme that has not yet been characterized (20).

Recently, we conducted an analysis of 40 biotype 1 *Vibrio vulnificus* strains and identified four variants of the MARTX toxin, each of which carries a different repertoire of effector domains resulting in different cell biological activities and altered pathogenesis in mice (24). Based on that study, we were interested in whether *V. cholerae* MARTX toxins would also show diversity or if *V. cholerae* MARTX toxins would be more conserved, exclusively carrying the same three effector domains.

To address the diversity of MARTX toxins in *V. cholerae*, publicly available complete and whole-genome sequences (WGS) and whole-genome SNP studies were analyzed for variation in the *rtxA* gene. We find that among both clinical and environmental *V. cholerae*, the overall domain structure of the MARTX toxin is highly conserved, but new variants and inactivating mutations can arise that affect function of the secreted toxin.

RESULTS

Bioinformatic analysis cannot resolve discrepancy in *rtxA* gene annotations. At the outset of this project, a problem arose identifying *rtxA* genes in genomes due to conflicting annotation of the large gene. The original annotation of the representative genome *V. cholerae* N16961 *rtxA* gene designated an ATG start site that is 23 bp downstream of the *rtxC* stop codon and preceded by a consensus AGAAGAG Shine-Dalgarno (SD) sequence (Fig. 1). Use of this start site generates a gene of 13,638 bp encoding a deduced protein of 4,545 aa (19). Subsequent annotation of the N16961

complete genome defined a GTG start site within the coding region of *rtxC* preceded by SD sequence GAAAGG (18). Use of this alternative start site generates a gene of 13,677 bp encoding a deduced protein of 4,558 aa.

A BLASTP search using the 4,558-aa translation product as a query against the National Center for Biotechnology Information (NCBI) protein database reveals 14 full-length RtxA proteins annotated as part of sequencing projects since the original publication of the N16961 genome. Seven proteins annotated the GTG start site encoding a protein of 4,558 aa (MO10, M66-2, V52, MZO-3, MZO-2, 623-39, and AM-19226), while the other seven proteins used the ATG encoding a protein of 4,545 aa (IEC224, NCTC 8457, 2740-80, LMA3984-4, HE-48, HE-39, and HE-25). The annotation of the *rtxA* gene of 2010EL-1786 and all closely related strains sequenced to investigate the Haiti cholera epidemic used the ATG start site, but the deduced protein product was only 4,534 aa, indicating a truncated protein that will be discussed below.

It was supposed that the correct start site could be resolved by nucleotide (nt) analysis of additional genomes to determine whether only one start site was conserved. A 104-nt query representing the *rtxC-rtxA* intergenic region was used in a BLASTN search of the NCBI genome sequences database. Two allele variants were identified. Allele 1 was 100% identical to the N16961 query and was present in 88 genomes. Allele 2 was present in 3 genomes, including MZO-2, MZO-3, and *V. cholerae* bv. *albensis* VL426. This second allele has one SNP that introduces a silent change of a GTG codon in *rtxC* to GTA but does not affect either possible *rtxA* start sites or SD sequences (Fig. 1A). Since both start sites and SD elements are conserved in all genomes, bioinformatics could not resolve the proper start site.

Experimental identification of the ATG start site for *rtxA*. To address the issue of the appropriate start site experimentally, point mutations in the DNA sequence on the *V. cholerae* N16961 chromosome I were generated and tested for MARTX toxin-induced cell rounding and actin cross-linking activity after addition of bacteria to HeLa cells (Fig. 1B and C). Mutation 1 (mut1) altered the putative GTG start site to GTT, a mutation that also altered a codon in *rtxC* from Trp to Leu. Mutation 2 (mut2) inserted a C in codon 7 of *rtxA*-4558, resulting in a frameshift creating a premature stop before the SD sequence of the downstream ATG start site. Neither mutation to disrupt the longer *rtxA*-4558 ORF affected cell rounding or actin cross-linking, although actin cross-linking may have been slightly reduced (Fig. 1B and C). Thus, the GTG is not the primary start site of translation, as loss of this site did not significantly affect toxin activity.

Mutation 3 (mut3) altered the ATG start codon to ATC, a mutation that alters the *rtxA*-4558 translation product to have an Ile in place of Met. Disruption of the ATG start codon eliminated cell rounding and reduced actin cross-linking such that only actin dimers were generated (Fig. 1B and C). To determine if the residual cross-linking activity leading to dimer formation represents translation starting at the GTG or at unmapped downstream start sites, mutation 4 (mut4) was created to convert a Glu codon to a stop in both *rtxA*-4545 and *rtxA*-4558. This mutant showed no cell rounding and no actin cross-linking (Fig. 1B and C). Overall, these data show that the ATG is the primary translation start site for *rtxA*, although minor activity may originate from the upstream GTG start site, resulting in actin dimers.

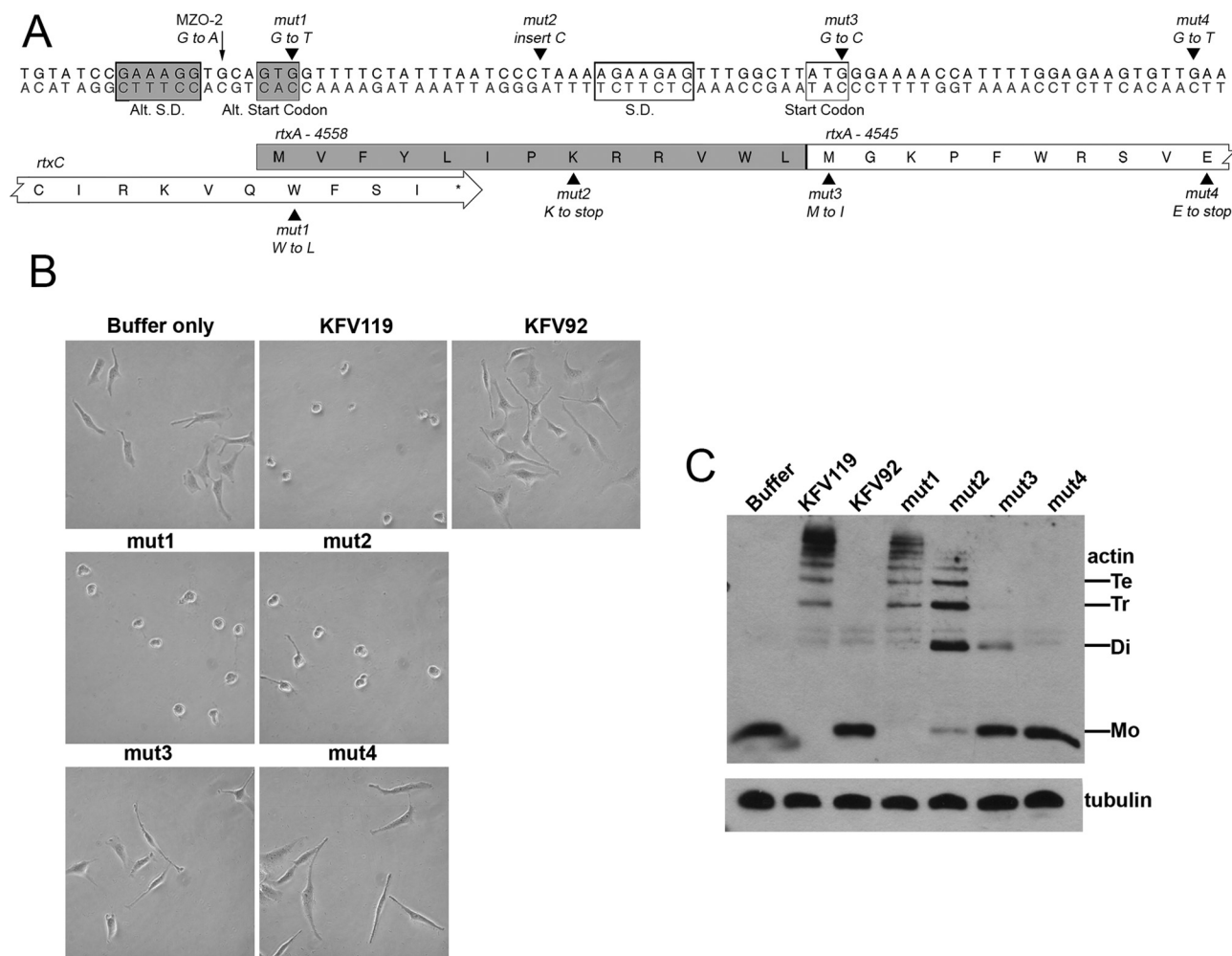


FIG 1 Determining the accurate start site of the *rtxA* gene. (A) The gene sequence for N16961 *rtxC-rtxA* intergenic region is shown. The start site and SD sequence (white boxes) and the alternate start site and SD sequence (gray boxes) are indicated. Partial translation products of *rtxC* and *rtxA* from both potential start sites are shown below. Arrowheads indicate locations of site-directed mutations (above sequence) and the changes introduced in the translated products (below sequences). (B and C) HeLa cells were treated with log-phase *V. cholerae* for 90 min and then were photographed (B) and collected to prepare lysates for immunoblot using anti-actin and anti-tubulin antibodies (C). Strains used were KFV119 (N16961 $\Delta hapA \Delta hlyA$), KFV92 (N16961 $\Delta hapA \Delta hlyA \Delta rtxA$), and mutants as detailed for panel A. Actin laddering in the Western blot is marked as actin monomer (Mo), dimer (Di), trimer (Tr), and tetramer (Te).

Identification of full-length *rtxA* genes in whole-genome sequences. The correct-length 13,658-nt sequence of N16961 *rtxA* (nt 1550147 to 1563784; GenBank AE003852.1) was used in a BLASTN search of 95 complete and WGS genomes available in the NCBI genomes database. A total of 77 genomes were identified that contained a gene sequence that aligned against the full-length *rtxA* gene, while other genomes contained only *rtxA* gene fragments. Eight of these contained out-of-frame mutations and were excluded from analysis. All 69 complete *rtxA* genes were included (see Table S1 in the supplemental material).

Pre-seventh pandemic El Tor and closely related *V. cholerae* strains are linked by an SNP at nt 1558877. Assessment of *rtxA* alignments from *V. cholerae* El Tor strains revealed strong conservation among *rtxA* genes from 57 genomes. These genes could be assorted into seven distinct alleles, varying from representative strain N16961 by only 1 to 4 bp (Table 1). To supplement the analysis, data from three recent genome SNP reports (2, 8, 10) were examined, and synapomorphic SNPs in the *rtxA* gene were

identified. This analysis revealed two additional alleles confirmed by presence in at least three closely related isolates, raising the total El Tor alleles to nine (Table 1).

Allele 6, represented by pre-seventh pandemic strain NCTC 8457 isolated in 1910 in Saudi Arabia, differs from the representative strain N16961 by only a G at *rtxA* nt 8731 (nt 1558877; GenBank AE003852.1). This allele is conserved in a 1986 isolate from Australia (BX 330286). The G8731 residue is also present with a second SNP in allele 7, represented by pre-seventh pandemic strains M66-2 and MAK 757, and in allele 8, represented by 1980 United States Gulf environmental isolate 2740-80. A closely related allele 9 from the O37 strain V52 also shares G8731 along with only 3 other SNPs (Table 1). These data indicate that G8731 is a marker of *rtxA* genes from pre-seventh pandemic strains and El Tor strains that evolved independently of the seventh pandemic.

The El Tor *rtxA* allele 1 is highly conserved throughout the seventh pandemic isolates. The predominating allele among the

TABLE 1 Nucleotide and amino acid changes in El Tor and El Tor-like *rtxA* alleles

Allele No.	Relevant Description ^a	Representative Genomes ^b	Nucleotide changes (compared to N16961) ^c													Amino acid changes (compared to N16961) ^d											
			2994	2997	3072	3073	3079	3084	3745	5234	5551	6901	8731	11408	12093	12639	13602	1024	1026	1249	1745	1851	2301	2911	3803	4031	4534
1	7 th pandemic ET	N16961	T	G	G	C	T	T	G	G	T	G	A	T	G	A	G	A	F	G	S	S	G	S	V	E	W
	Matlab variant	MJ-1236	T	G	G	C	T	T	G	G	T	G	A	T	G	A	G	A	F	G	S	S	G	S	V	E	W
	O139	MO10	T	G	G	C	T	T	G	G	T	G	A	T	G	A	G	A	F	G	S	S	G	S	V	E	W
	Altered ET, <i>ctxB1</i>	Nepal Group V ^e	T	G	G	C	T	T	G	G	T	G	A	T	G	A	G	A	F	G	S	S	G	S	V	E	W
2	Mozambique variant	B-33	T	G	G	C	T	T	A	G	T	G	A	T	G	A	G	A	F	S	S	S	G	S	V	E	W
3	Altered ET, E. Africa	4784	C	T	C	T	G	C	G	G	T	G	A	T	G	A	G	L	V	G	S	S	G	S	V	E	W
4	Altered ET, <i>ctxB1</i>	CIRS101	T	G	G	C	T	T	G	G	T	G	A	T	G	A	A	A	F	G	S	S	G	S	V	E	X
	Altered ET, <i>ctxB7</i>	2010EL-1798	T	G	G	C	T	T	G	G	T	G	A	T	G	A	A	A	F	G	S	S	G	S	V	E	X
5	Altered ET, S Africa	2011EL-1137	T	G	G	C	T	T	G	G	T	G	A	T	G	G	A	A	F	G	S	S	G	S	V	E	X
6	ET PP	NCTC 8457	T	G	G	C	T	T	G	G	T	G	G	T	G	A	G	A	F	G	S	S	G	G	V	E	W
	ET Environ. Austral.	BX 330288	T	G	G	C	T	T	G	G	T	G	G	T	G	A	G	A	F	G	S	S	G	G	V	E	W
7	ET PP	M66-2	T	G	G	C	T	T	G	G	G	G	T	G	A	G	A	F	G	S	A	G	G	V	E	W	
8	ET, USG, Environ.	2740-80	T	G	G	C	T	T	G	G	T	A	G	T	G	A	G	A	F	G	S	S	R	G	V	E	W
9	O37	V52	T	G	G	C	T	T	G	A	T	G	G	C	T	A	G	A	F	G	K	S	G	G	A	D	W

^a ET, El Tor; PP, pre-7th pandemic; environ., isolated from environment; USG, U.S. Gulf Coast.

^b For a complete list of genomes, see Table S1 in the supplemental material.

^c Green, unchanged; red, nonsynonymous change; yellow, synonymous change.

^d Blue, unchanged; red, amino acid change; X, change to stop codon.

^e As detailed in SNP analysis by Hendriksen et al. (10).

seventh pandemic isolates is allele 1, including the representative strain N16961 and typical El Tor isolates from South America, Kenya, and Malaysia (Table 1; see also Table S2 in the supplemental material). The O139 serotype has allele 1, as do the atypical El Tor Matlab variant MJ-1236, atypical U.S. Gulf Coast isolates from 1991 and 2000, and altered El Tor strains isolated in Thailand and Malaysia. Expansion of the analysis to include studies that reported only SNPs rather than assembled genomes reveals that nearly all typical El Tor O1 and O139 and atypical El Tor strains from Matlab and Vietnam carry *rtxA* allele 1. Of note, this analysis also revealed that four atypical strains from Mozambique (2) have a single SNP at *rtxA* nt 3745, creating a conservative amino acid change from Gly to Ser, and thereby was denoted as allele 2. This mutation was confirmed in the published genome sequence of B-33 (25), which has an incomplete *rtxA* covering only 12,436 nt and was originally excluded from analysis.

Multiple *rtxA* alleles arose within the altered El Tor strains.

Altered El Tor strains are atypical El Tor strains with a classical *ctxB1* allele in an otherwise El Tor CTX Φ strain (4). These strains have spread worldwide as the predominant agent of cholera since 2000 (2, 5). As evidence of emergence from the typical El Tor strains, many isolates of altered El Tor have *rtxA* allele 1, including strains isolated in Thailand and Malaysia (see Table S1 in the supplemental material) and strains isolated in Bangladesh in 1994 and India in 2006 and 2007 (2). The altered El Tor strain with *rtxA* allele 1 was one of two strains circulating in Nepal in 2010 (group V) (10).

From 2005 to 2009, an altered El Tor cholera outbreak occurred in East Africa countries, including Kenya, Djibouti, and Tanzania. Analysis of SNP data revealed that this outbreak strain is

marked by 6 SNPs in the *rtxA* gene (2) (Table 1, allele 3). These SNPs occur in all 21 genomes in this group, verifying this as a unique allele despite the absence of a complete genome. The nucleotide changes are clustered at nt 2994/2997 and nt 3071/3072/3079/3084 (nt 1553141 to 1553231; GenBank AE003852.1), suggesting they may have arisen by a single homologous recombination event rather than spontaneous mutation. Consistent with the extreme conservation of this gene, only two of the mutations are nonsynonymous, and the protein product is highly conserved (Table 1).

Genomic studies revealed that a separate cluster of altered El Tor was isolated in Bangladesh as early as 1999 and subsequently transferred to India in 2004 and Haiti in 2010 (2, 10). This cluster is represented by sequenced strain CIRS101 isolated in Bangladesh in 2002 (25). These strains do not have *rtxA* allele 1, similar to earlier circulating altered El Tor, nor do they have the allele 3 unique to altered El Tor from the East Africa outbreak. Instead, this cluster has an *rtxA* gene that differs from N16961 by only one SNP, an exchange from G to A at nt 13602 (nt 1563760; GenBank AE003852.1). Using gene alignment, this full-length *rtxA* allele 4 was identified in CIRS101 and in 36 additional sequenced genomes, including 22 clinical isolates from the 2010 Haiti cholera epidemic. Two strains with the G13602A mutation isolated in South Africa and Zimbabwe have a second synonymous SNP at 12639 (nt 1562797; GenBank AE003852.1), and this was denoted as allele 5 (Table 1).

Notably, the *rtxA* allele 4 is found in 9 sequenced altered El Tor strains in combination with *ctxB1* of the altered El Tor strain but also in 27 strains with a novel *ctxB* variant, *ctxB7* (see Table S2 in the supplemental material). This analysis revealed that while

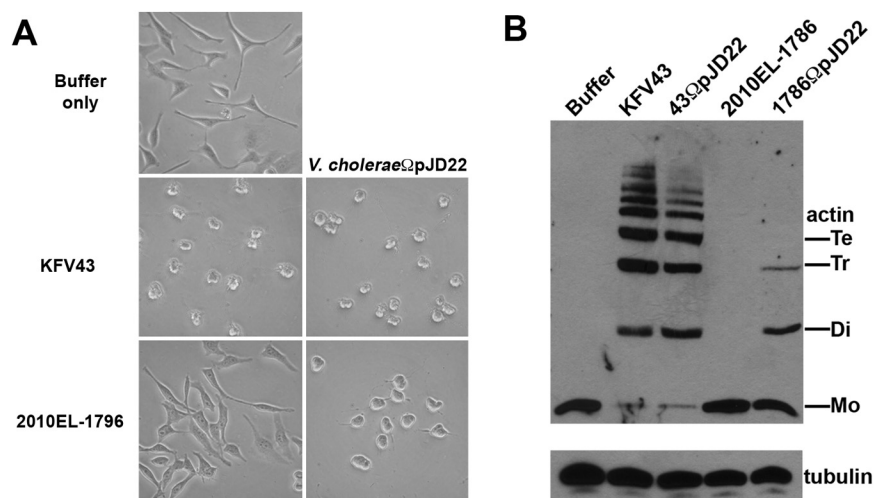


FIG 2 Restoration of MARTX toxin activity to 2010EL-1786. HeLa cells were treated with log-phase *V. cholerae* for 90 min and then were photographed (A) and collected to prepare lysates for immunoblotting using anti-actin and anti-tubulin antibodies (B). Strains used were control strain KfV43 (N16961 Δ *hapA*) and Haiti epidemic strain 2010EL-1786 either alone (left) or after integration of pJD22 to correct stop codon and integrate 6 \times His tag (right). Actin laddering in the Western blot is marked as actin monomer (Mo), dimer (Di), trimer (Tr), and tetramer (Te).

CIRS101 and isolates from Pakistan, Russia, India, and Zimbabwe carry *rtxA* allele 4 in combination with *ctxB1*, all other sequenced *rtxA* allele 4 strains have *ctxB7*. These include strains first identified in India in 2007 and Nepal in 2009 and subsequently in Bangladesh, Cameroon, Haiti, and the Dominican Republic in 2010 (2, 7–11). Assessment of whole-genome data and SNP analysis revealed no strains in which the *ctxB7* mutation occurred independently of *rtxA* allele 4. These data suggest that the *ctxB7* allele arose within the *rtxA* allele 4 background, henceforth called *rtxA4*.

Mutation G13602A generates a premature stop codon.

Translation of the *rtxA4* allele revealed that it would change a TGG codon for Trp to a TGA stop codon, resulting in loss of 12 aa from the C terminus of the protein (Table 1). Since all RTX proteins depend on secretion signals at the C terminus (21), it was considered likely that this stop codon would be an inactivating mutation. We obtained 2010 Haiti epidemic isolate 2010EL-1786 and tested it for cell rounding and actin cross-linking. In contrast to the control strain KfV43, 2010EL-1786 did not cause cell rounding and actin cross-linking (Fig. 2).

To ensure that the loss of MARTX toxin function is due solely to the single *rtxA* point mutation, we modified the strain by integrating plasmid pJD22 at the 3' end of the gene. This integrating plasmid has an insert corresponding to the last 616 nt of the N16961 *rtxA* gene, except with additional codons for a C-terminal 6 \times His tag when the plasmid integrates onto *V. cholerae* chromosome I. We mated plasmid pJD22 into 2010EL-1786 and selected one merodiploid that acquired an uninterrupted ORF, with the 2010EL-1786 TGA stop codon replaced with the N16961 TGG Trp codon. As a control, N16961-derived strain KfV43 was also modified to integrate the plasmid, demonstrating that addition of the 6 \times His tag does not affect MARTX toxin activity. Gain of the N16961 codon sequence restored the ability of 2010EL-1786 to induce cell rounding and actin cross-linking (Fig. 2). The relative activity of the toxin expressed from 2010EL-1786 Ω pJD22 was less than that of the KfV43 Ω pJD22 control, indicating that the strain

naturally produces less toxin. Yet, these data demonstrate that this SNP inactivated the function of the MARTX toxin.

rtxA varies in environmental strains.

Among the remaining 12 genomes with intact *rtxA* genes, the genes were found to be highly diverse (Fig. 3). In contrast to the O37 strain V52 that was closely related to El Tor strains, the O37 strain MZO-3 was highly diverse from N16961, with 176 SNPs, suggesting it evolved in a different lineage from V52, again consistent with the possibility that V52 arose from an El Tor O1 strain rather than linkage to the other O37 strains.

The remaining 11 *rtxA* genes also differ greatly from N16961 by between 207 and 302 nt or 1.5 to 2.2%. The rate of nonsynonymous changes was 4 to 7% (Fig. 3B). Close analysis of the SNP locations suggests the *rtxA* gene has varied extensively by homologous recombination. While many SNPs are independent in genomes, as would be expected if arising by DNA polymerase error, many changes

occur in blocks, and these blocks are shared in multiple genomes.

A map of the SNPs showed a bias away from the 5' end of the gene and the effector regions and a strong bias for changes between the end of the B repeat and the start of the ACD effector domain (Fig. 3A). The function of this highly variable region is unknown, except that it is predicted to be alpha helical in structure. The extent of variation could indicate that this region is not essential for function and thus can tolerate change as long as the total length is retained. Alternatively, it is possible that changes in these regions reflect adaptation to a specific niche, such as colonization of distinct hosts in the environment.

Identification of two novel MARTX variants. An additional search of the NCBI WGS genomes using a TBLASTN algorithm and excluding all genomes previously analyzed revealed two sequences in which there was strong alignment to both the N and the C terminus to the MARTX toxin but not the center of the protein, although the translated sequences originated from the same genome contig. These sequences were analyzed as potential novel *rtxA* variants (Fig. 4).

Strain RC385 is an O137 strain isolated from Chesapeake Bay, MD. It has previously been reported as having a MARTX toxin with a unique effector arrangement but containing a frameshift mutation (26). The updated *rtxA* sequence is now present as a single contig, and the frameshift is corrected to a single uninterrupted ORF. The repeat regions varied from N16961 by 297 nt, consistent with a 3% rate of change similar to other environmental strains (Fig. 4B). Due to the extensive difference among strains, the point of exchange and precursor of RC385 *rtxA* could not be identified among the other *V. cholerae* environmental isolates by SNP analysis.

As previously annotated (26), this MARTX toxin from RC385 has three effectors. Effectors 1 and 3 are duplicated effectors similar to a domain of unknown function (DUF) shared with MARTX toxins from *Photobacterium* spp. The second effector is predicted to be an adenylate cyclase found also in MARTX toxins of *Vibrio*

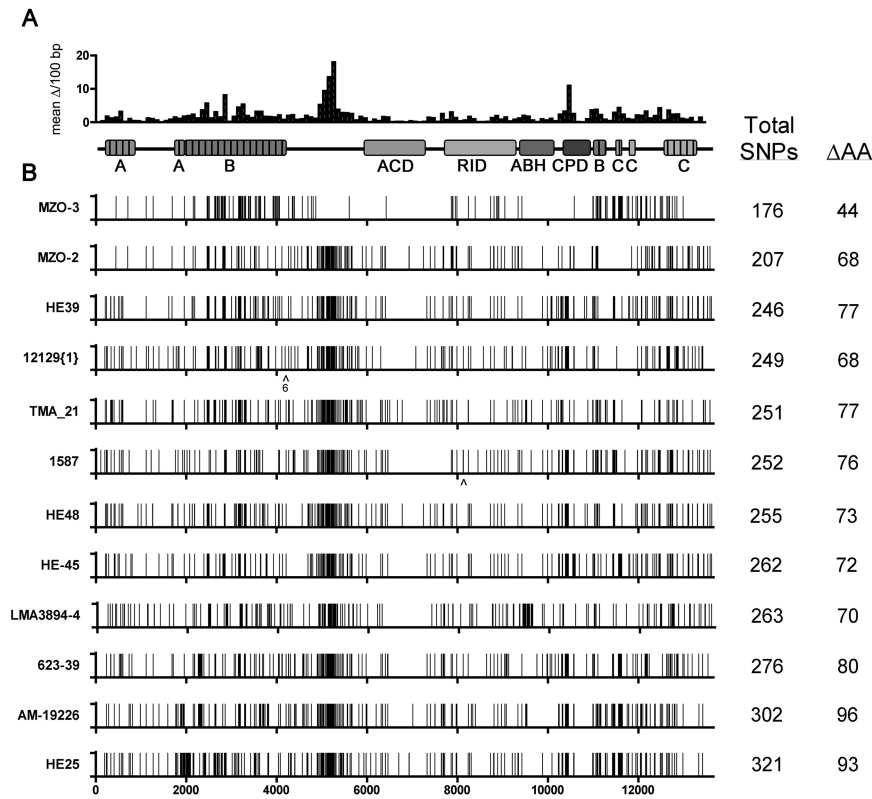


FIG 3 SNP map of non-El Tor *V. cholerae* strains. (A) The domain organization of *V. cholerae* MARTX toxin is shown below a bar diagram representing total SNPs in DNA sequence of *rtxA* from 12 strains compared to reference strain N16961. (B) All SNPs across the 13,638-bp gene are marked by a vertical bar. Strains are in order of increasing nucleotide diversity compared to N16961, as indicated at right.

caribbenthicus (NZ_AEIU01000000), *Vibrio nigripulchritudo* (27), and *Vibrio anguillarum* 775 (28). The putative adenylate cyclase is most closely related to the type III secretion effector ExoY, an adenylate cyclase from *Pseudomonas aeruginosa* (29).

Strain HE-09 is an environmental isolate from Haiti. Annotation of the deduced amino acid sequence revealed extensive variation in the regions aligned with N16961, with 785 SNPs across 8,954 aligned residues (8.7% difference). The encoded MARTX toxin also carried a unique set of effector domains. HE-09 includes a DUF shared with *Vibrio vulnificus* and *Xenorhabdus* species MARTX toxins (20). The next effector is a Rho-inactivation domain (RID). The third effector domain of the HE-09 is a previously unknown MARTX effector. The predicted protein has a 4HBM-type membrane localization domain that predicts that the

effector would be targeted to anionic lipid membranes (30, 31). The tethered effector showed strong homology with the *Bacillus cereus* VIP2 toxin family. VIP2 and related toxins are ADP-ribosylating toxins that modify G-actin (32). Thus, HE-09 is an environmental *Vibrio* strain quite distinct from the four other environmental *V. cholerae* strains also isolated during the Haiti epidemic and from the altered El Tor clinical strains, all of which share an effector arrangement with the reference strain N16961.

DISCUSSION

In this study, we sought to understand how diversity in the gene encoding the *V. cholerae* MARTX toxin might affect the function of the toxin both in clinical isolates and in the environment. Recent studies have shown that the composite MARTX toxin genes can be altered in *V. vulnificus* by homologous recombination to acquire new effector domains (24, 33), and we were interested if similar variation occurred in *V. cholerae*. For genomic analysis, it was first necessary to reconcile discrepancies in the annotation of the *rtxA* gene of the representative strain N16961. We showed experimentally that the correct start site is the ATG downstream of *rtxC*, although some activity may arise from the upstream GTG.

Other *Vibrio* spp. have been identified to have *rtxA* genes for MARTX toxins (20). Analysis of the upstream sequences for *rtxA* genes from *V. vulnificus* (34–36), *V. anguillarum* (28), *V. splendidus* (37), and *V. caribbenthicus* (NZ_AEIU01000000) reveal that these each contain a conserved trinucleotide GTG and ATG start site as well as a conserved TAA stop codon for *rtxC*. However, the intervening sequences vary in comparison to N16961. Most notably, in no cases would use of the GTG as a start site introduce the appropriate frame of translation for the MARTX toxin, indicating that in all *Vibrio* spp., the start site will be conserved with the ATG of *V. cholerae*, as mapped in this study.

Using the correct 13,638-bp gene, we examined diversity of this gene in 71 *V. cholerae* strains for which the entire gene was repre-

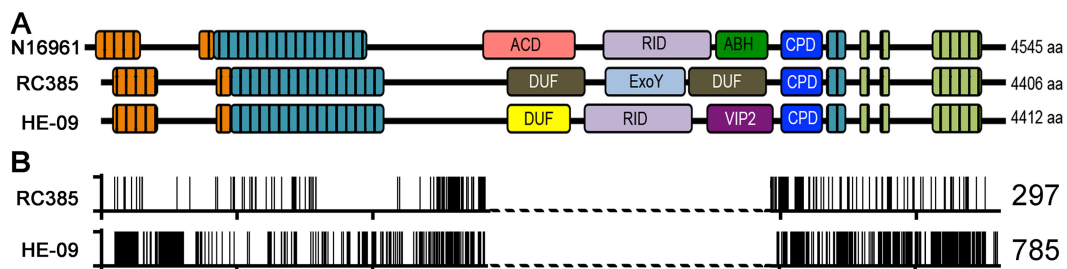


FIG 4 Schematic diagram of N16961 MARTX toxin and new variant toxins from RC385 and HE-09. Abbreviations are as detailed in the text.

sented in a sequenced genome supplemented by available SNP data from recent whole-genome phylogeny (2, 8, 10). Whole-genome studies have revealed that the El Tor O1 lineage differs from N16961, with a calculated rate of only 3.3 SNPs/year (2). Not surprisingly, given the global conservation of these strains, few SNPs were identified in the *rtxA* gene despite the 100-year history of available data. In total, only 9 *rtxA* alleles were identified across all El Tor O1, O139, atypical, and altered El Tor strains.

All of the El Tor seventh pandemic strains shared an adenosine nucleoside at *rtxA* nt 8731, distinct from a guanosine present in pre-seventh pandemic strains, other El Tor-like lineage strains from the United States Gulf Coast and Australia, and the O37 strain V52. The nucleoside change results in a codon change from glycine in pre-pandemic strains to serine in pandemic strains within the RID effector domain. Notably, among other MARTX toxins with an RID, a Gly at this position is most prevalent (38). This finding of a conservative mutation unique to the El Tor seventh pandemic MARTX RID is consistent with the El Tor strains, representing a clonal expansion from a single predecessor at some point in the 1950s diverging away from other El Tor-like lineages (2). All together, the strong conservation of this gene, with only 1 to 6 SNPs across 100 years and worldwide geography, reinforces that these strains are closely related evolutionarily and distinct from other *V. cholerae* isolates.

The conservation of the *rtxA* sequence in the El Tor O1 and closely related strains is in stark contrast to the extensive variation in the non-El Tor group, whether the strain was of environmental or clinical origin. The database for these strains is more limited, although each of the 12 isolates had a unique *rtxA* gene that differed from the reference strain by between 176 and 321 nt, or 1 to 2%. This variation matches the calculated 2% rate of change for whole-genome variation of 3 environmental isolates recently reported (8). This dramatic variation was not expected, because in a recent multi-virulence locus sequence typing analysis, including both O1 and non-O1 strains, non-O139 isolates showed limited variation in the *rtxA* gene (39). Interestingly, this analysis was focused to 332 bp from nt 1339 to 1671, and assessment of the SNP map for our analysis showed limited SNPs in this region (Fig. 4A). Conservation in this region is in contrast to the spacer region between the B repeats and the ACD effector domain where extensive variation occurred, indicating that conservation of *rtxA*, as previously reported (39), was biased by the region analyzed and does not reflect total diversity across the large gene. Many of the SNPs in the genomes seemed to arise in clusters across several genomes, suggesting that variation in this gene arises extensively from homologous recombination and the regions amenable to homologous recombination may be defined in some way by DNA structure or nucleotide sequence. In all, in contrast to the lack of variation within the El Tor group, there is a large capacity for the *rtxA* gene to vary in the environmental strains.

Two genes were identified that are new toxin variants due to a change in the central portion of *rtxA* that encodes the effectors. These changes converted one of the toxins to a potential adenylate cyclase toxin and another to a putative actin ADP-ribosylating toxin. Due to extensive nucleotide diversity in the conserved regions, the site of recombination events could not be identified. The consequences of these genetic changes will need to be further explored in the future, as will whether these changes affected the biological niche these strains occupy.

The most clinically relevant change in the *rtxA* toxin gene was

one SNP that emerged within the currently circulating strains known as the altered El Tor strain. This SNP inactivated the function of the MARTX toxin by introducing a premature stop codon that would result in a protein truncated by 12 amino acids, probably disrupting the C-terminal secretion signal. Strains 2011EL-1098, 2009EV-1131, and 2011EL-1132 were deleted from analysis for out-of-frame mutations, but if these mutations could be validated, they would indicate that this group of strains is accumulating additional null mutations in *rtxA*, generating a pseudogene that could not be reversed by a single nucleotide reversion.

Subsequent to the emergence of *rtxA* allele 4, another significant mutation apparently arose within the *rtxA*-null background in which a point mutation in *ctxB* created a CtxB with a never-before-observed Asn at aa 20. As no strains were identified in which this *ctxB7* allele occurred with an intact *rtxA* gene, it is suggested that *ctxB7* arose within the *rtxA*-null background. This genetic cluster is represented by 27 different sequenced strains and extensive additional WGS analysis, demonstrating that strains with both mutations have been transmitted globally, reaching India, Nepal, Cameroon, Haiti, and the Dominican Republic (2, 8–11). The data also indicate that in addition to comprising a clonal cluster that may have originated in India (7), these strains have a distinct virulence factor profile from other El Tor strains (6, 12). Therefore, it is necessary when categorizing the altered El Tor to distinguish not only the *ctxB* allele but also the *rtxA* allele to determine if the strain produces an active MARTX toxin. It is yet to be determined which of these mutations, if either, can account for the increased virulence of these strains in humans (6), although 2010EL-1786 is not statistically different in virulence from typical El Tor strains in infant mice (12) or in adult mice (our unpublished findings).

Interestingly, the classical strains responsible for historical cholera pandemics are also noted for a naturally occurring deletion that removes >7 kb of the *rtx* locus, deactivating the MARTX toxin (19). The question then is, as strains become more virulent and adapted for human-to-human passage, why do they deactivate MARTX? The toxin is very large and may be detrimental to growth due to energy expenditure reducing rapid growth necessary for increased dissemination. If the toxin has a role in the environment, it may no longer be necessary if the strain acquires the ability to transfer host to host, making circulation through the environment less essential. The toxin has been shown in mice to have a function in virulence by promoting colonization of the intestine through compromising the innate immune system during early colonization (40, 41). However, it has been shown that the toxin is fully redundant in function with hemolysin, a pore-forming toxin (42). Strain 2010EL-1786, the representative strain used in this study, has been reported to be hemolytic, similar to other El Tor strains (12), and we independently confirmed this result (data not shown). Thus, the loss of function of the *rtxA* gene may be circumvented by the action of secreted hemolysin.

Yet, classical strains are nonhemolytic in addition to not producing a MARTX toxin. It has been postulated that innate immune evasion is a key function of CT, in addition to its ability to induce enterocyte secretion (43). It is possible that in the context of excess production of classical-type CT, as occurs in classical strains and the altered El Tor strains now circulating, neither MARTX nor hemolysin is required for innate immune evasion, their function being replaced by the immunomodulatory function of the classical form of CtxB.

Overall, we have found that the MARTX toxin in general has been highly conserved in *V. cholerae*, but when variants do arise, they can dramatically alter protein function and possibly overall process of pathogenesis. Thus, while it is clear that analysis of whole genomes can provide phylogenetic evidence of how strains are evolving over time, detailed analysis of individual genes is still necessary to reveal how small changes in nucleotide sequence can dramatically affect protein function, and these mutations may contribute to increased fitness and global spread.

MATERIALS AND METHODS

Genome sequences and analysis. All genome sequences used for this analysis were publically available at NCBI (<http://www.ncbi.nlm.nih.gov/>). The database included 95 complete and incomplete (i.e., WGS) genomes as of 3 October 2012. A slightly expanded database was reexamined on 10 March 2013. Genomes selected for analysis are listed in Table S1 in the supplemental material with NCBI accession numbers. SNP data from published supplemental data were used to augment analysis of the El Tor pandemic strains. All genome database searches were conducted using BLAST interfaces within the NCBI website. Multiple alignments of identified sequences were performed by the ClustalW algorithm using the MacVector 12.6.0 software package. SNPs were diagrammed by importing sequences into Excel to compare individual SNPs. These were then graphed using Prism for MacIntosh 4.0.

Strains and reagents. *V. cholerae* strain KfV119 (N16961 Δ hlyA Δ hapA) and KfV43 (N16961 Δ hapA) are from our collection (44, 45), and 2010EL-1786 (9) was obtained from the American Type Culture Collection (ATCC BAA2163). All strains were cultured on Luria-Bertani agar or broth supplemented with 100 μ g/ml streptomycin, 50 μ g/ml ampicillin, or 5% sucrose as necessary. Cells were cultured in Dulbecco's minimal Eagle medium (DMEM; Life Technologies) containing 10% fetal bovine serum (FBS; Gemini Bioproducts or Atlanta Biologicals) and in the presence of penicillin and streptomycin antibiotics. All custom primers for DNA amplification and site-directed mutagenesis were obtained from Genosys (The Woodlands, TX) or Integrated DNA Technologies (Coralville, IA) and are listed in Table S2 in the supplemental material. Enzymes were obtained from New England Biolabs and chemicals and media from Sigma or Fisher. Sequencing was conducted at the Northwestern University Genomics Core Facility.

Generation of modified *rtxA* promoter sequences in N16961. To generate site-directed mutants in the *rtxA* promoter, DNA encompassing gene sequences from VC1449, *rtxC*, and the 5' end of *rtxA* was amplified. During amplification, the sequence was modified using crossover PCR to insert 6 nt for an EcoRV site immediately adjacent to the ATG putative start site for *rtxA* using techniques as previously described (44). The product was cleaved within *rtxC* by BspM1 to reduce the fragment size, converted to blunt ends using Klenow, and cloned into pCR2.1 (Invitrogen). This clone was digested with restriction enzymes SpeI and XhoI and moved into the similarly digested *sacB* counterselectable plasmid pWM91 (46) to generate plasmid pJD12. The modified *rtxA* sequence was recombined onto the *V. cholerae* KfV119 chromosome I by double homologous recombination with sucrose counterselection as previously described (44), generating *V. cholerae* strain JD8. Gain of the EcoRV site was confirmed by PCR amplification across the region and subsequent processing of the PCR product with EcoRV. A second pWM91-based plasmid, pJD11, encompassing the same region without the EcoRV site was also generated and then modified as detailed in Results using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). These single-nucleotide changes (mut1 to mut4) were exchanged into strain JD8 as described above, except that strains that gained the point mutations were identified by loss of the EcoRV cleavage and by sequencing of the PCR products.

Restoration of the stop codon in 2010EL-1786 using plasmid integration. Plasmid pKJF344 is a derivative of integrating plasmid pGP704 (47) that has a segment of N16961 DNA corresponding to the 3' end of the

rtxA gene, except the sequence was modified during amplification to include 18 nt for a 6 \times His C-terminal tag immediately before the TAA stop codon. It has been previously shown that addition of six histidines to the MARTX toxin does not affect function (48). The plasmid was transferred to *V. cholerae* 2010EL-1786 or KfV119 by mating from *Escherichia coli* SM10 λ pir, selecting for single homologous integration of the plasmid by gain of ampicillin resistance. Isolated colonies were screened by amplifying the integrated insert and sequencing to ensure that the recombination event resulted in gain of the N16961 Trp codon replacing the stop codon in 2010EL-1796.

Assessment of cell rounding and actin cross-linking. A total of 10⁵ HeLa cells were seeded into 6-well culture dishes. Before assay, medium was exchanged for DMEM without antibiotics or FBS. Bacteria grown to logarithmic phase were washed in phosphate-buffered saline and added to wells at a multiplicity of infection of 20. Plates were incubated under 5% CO₂ at 37°C for 90 min. Cells were viewed using a Nikon T200 phase microscope and photographed using a Nikon E995 digital camera. Cells were collected by scraping, pelleted at 500 \times g, and lysed by boiling in SDS-PAGE buffer. Cross-linking of actin was assessed by Western blotting using anti-actin and anti-tubulin monoclonal antibodies (Sigma) as previously described (38).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00624-12/-/DCSupplemental>.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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