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The *Vibrio* Pathogenicity Island of Epidemic *Vibrio cholerae* Forms Precise Extrachromosomal Circular Excision Products

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The *Vibrio* pathogenicity island (VPI) in epidemic *Vibrio cholerae* is an essential virulence gene cluster. Like many pathogenicity islands, the VPI has at its termini a phage-like integrase gene (*int*), a transposase-like gene (*vpiT*), and phage-like attachment (*att*) sites, and is inserted at a tRNA-like locus (*ssrA*). We report that the VPI precisely excises from the chromosome and that its left and right ends join to form an extrachromosomal circular excision product (pVPI). Two-stage nested PCR analysis and DNA sequencing confirmed the *int-att-vpiT* junction and that the core *attP* of pVPI is identical to the chromosomal VPI *attR* site. Excision was independent of *toxR* and *toxT*. Excision was independent of *recA*, suggesting that it is mediated by site-specific recombination. Interestingly, while excision was detected in *int* and *vpiT* mutants, excision was abolished in a double (*int vpiT*) mutant and was restored by plasmids containing genes for either recombinase. Excision results in deletion of A361 in the *ssrA* locus, which flanks the right junction of the VPI. Since A361 encodes U70 in the critical G · U base pair in the acceptor stem of the *ssrA* RNA that is the determinant for aminoacylation with alanine, this deletion might have deleterious effects on *ssrA* function. Also, *vpiT* may have undergone interchromosomal translocation or may represent an independent integration event, as it was found downstream of *hutA* in some isolates. Our results provide new insight into the molecular biology of the VPI, and we propose that the process of excision and circularization is important in the emergence, pathogenesis, and persistence of epidemic *V. cholerae*.

Cholera, attributed to the bacterium *Vibrio cholerae*, is a diarrheal disease of humans that results in significant morbidity and mortality (25). Available records show that there have been seven worldwide pandemics of cholera (40). The current seventh pandemic began on the island of Sulawesi in Indonesia and then rapidly spread throughout Asia (1, 24). Because of its high death-to-case ratio, transmissibility, and persistence in the environment and its ability to occur in explosive epidemic form, *V. cholerae* continues to be a public health concern.

Only toxigenic strains of *V. cholerae* that possess two essential virulence gene clusters, i.e., the CTX element, which is a prophage encoding cholera toxin (39, 49), and the *Vibrio* pathogenicity island (VPI) (26, 30), can cause epidemic cholera. The chromosomal VPI is 41.2 kb in size in both sixth-pandemic (classical biotype) and seventh-pandemic (El Tor biotype) strains and encodes 29 potential proteins (26, 27). The VPI encodes proteins with essential roles in virulence, such as toxin-coregulated pili (TCP) (17, 44), and proteins that regulate virulence, such as ToxT, TcpP, and TcpH (4, 6, 7, 14). In addition, the VPI contains several open reading frames with no known or demonstrated function.

The VPI has many features that are typical of pathogenicity islands (PAIs) (2, 12, 13). It has a low percent G+C content (35%) compared to the rest of genome (48%) (15), has phage-like attachment (*att*) sites at its termini, is inserted site specifically in the chromosomes of epidemic *V. cholerae* strains downstream of a tRNA-like locus (*ssrA*), and has at its left and right ends genes with potential roles in DNA mobility, including a transposase-like gene (*vpiT* [formerly *orf1*]) and a phage-like integrase gene (*int*) which belongs to the family of site-specific recombinases (26, 32). Although the genetic properties of PAIs suggest that they were acquired en bloc by horizontal gene transfer, their biology and association with tRNA loci are not well understood.

Understanding the molecular biology of PAIs will provide important insight into the emergence, virulence, and evolution of bacterial pathogens. Previously, we proposed that epidemic *V. cholerae* strains were derived from environmental nontoxigenic strains (29). We and others have also proposed that the emergence of epidemic strains appears to have arisen from the sequential acquisition of the VPI and the CTX element (9, 26). Although the process is not well understood, we recently suggested that the VPI can excise from the chromosome, forming an extrachromosomal circular structure, and that the VPI can be found extracellularly in DNase-protected particles (30). Although it was recently found that the VPI, and presumably other genomic loci, can be mobilized by using the *V. cholerae* generalized transducing phage CP-T1 (38), the excision and circularization of the VPI would appear to be inconsistent with a generalized phage transduction mechanism and suggest that other modes of mobilization can occur. In this paper, we provide genetic evidence showing that the VPI can precisely excise from the chromosome in a site-specific manner to form extrachromosomal circular excision products (pVPI) and that this excision is mediated by the VPI-encoded recombinases *int* and *vpiT*.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Properties	Source (reference)
Strains		
N16961	Wild-type E1 Tor (VPI^+ CT^+)	M. Levine (34)
E7946	Wild-type E1 Tor (VPI^+ CT^+)	J. Kaper (34)
E9120	Wild-type E1 Tor (VPI^- CT^+)	J. Kaper
395	Wild-type classical (VPI^+ CT^+)	J. Kaper (33)
VJ739	E7946 Δ*toxT*	V. DiRita (5)
JJM43	395 Δ*toxR*	J. Mekalanos (44)
DK224	N16961 Sm	51
DK331	DK224 *int*::Km	This study
DK343	DK224 *vpiT*::Km	This study
DK339	DK224 *int*::Km(pDK60)	This study
DK384	DK224 *vpiT*::Km(pDK93)	This study
DK414	DK224(pDK60)	This study
DK591	DK224(pDK62)	This study
DK707	DK224 *int*::Km *vpiT*::Km	This study
DK708	DK224 *int*::Km *vpiT*::Km(pDK60)	This study
DK709	DK224 *int*::Km *vpiT*::Km(pDK93)	This study
CVD50	595B Δ*recA*	J. Kaper
Plasmids		
pDK60	1.4-kb *int* N16961 PCR fragment in pGEM-T	This study
pDK62	pGEM-T::*int*::Km	This study
pDK64	pCVD442::*int*::Km	This study
pDK88	1.35-kb *int-att-vpiT* N16961 PCR fragment in pCR2.1	This study
pDK92	1.35-kb *int-att-vpiT* 395 PCR fragment in pCR2.1	This study
pDK93	2.4-kb *vpiT* N16961 PCR fragment in pGEM-T	This study
pDK94	0.8-kb *hutA-vpiT* 395 fragment in pCR2.1.	This study
pDK95	pGEM-T::*vpiT*::Km	This study
pDK96	pCVD442::*vpiT*::Km	This study
pCR2.1	TA cloning vector, Ap	Invitrogen
pGEM-T	TA cloning vector, Ap	Promega
pCVD442	Suicide vector	8
pUC18K3	pUC18-based vector containing Km	35

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1. Before use, the identities of *V. cholerae* strains were confirmed biochemically and serologically.

Isolation of extrachromosomal circular VPI excision product (pVPI). To isolate the VPI circular excision product, we used the Qiagen large-construct kit. Briefly, a 1-liter flask containing 500 ml of Luria-Bertani broth (Difco) was inoculated with a fresh colony and incubated at 37°C for 16 h at 200 rpm. Bacterial cells were harvested by centrifugation at 6,000 × *g* for 20 min at 4°C and then processed according to the manufacturer's instructions. The isolated DNA was gently redissolved in MQ water and analyzed directly or stored at −20°C.

PCR analysis and sequencing. PCR analysis was initially used to detect the formation of the extrachromosomal circular excision product. The primers used in this study are listed in Table 2. Primers used in the initial PCR analysis to generate a 1.35-kb product were KAR393 (located in *vpiT*) and KAR396 (located in *int*). PCR analysis with JumpStart REDAccuTaq DNA polymerase (Sigma) was performed in either 50- or 100-μl reaction mixtures under the following conditions: denaturation at 96°C for 3 min (1 cycle); denaturation at 96°C for 30 s, annealing at 48°C for 45 s, and extension at 72°C for 2 min (30 cycles); 70°C for 10 min; and then holding at 4°C. PCR mixtures were loaded onto 1% agarose gels and stained with SYBR Gold (Molecular Probes). DNA sequencing of all PCR products was carried out with the *Taq* dye-terminator sequencing kit (Perkin-Elmer) and an automated 373A DNA sequencer (Applied Biosystems) at the Biopolymer Core Facility of the University of Maryland. Computer analysis was performed by using the Wisconsin package, version 10.0 (Genetics Computer Group, Madison, Wis.). The 1.35-kb PCR product from strains N16961 and 395 corresponding to the expected size of the *int-att-vpiT* fragment was gel purified by using Geneclean (BIO101) and ligated into the TA plasmid vector pCR2.1 (Invitrogen). The resulting plasmids, pDK88 and pDK92, respectively, carrying the 1.35-kb product were sequenced to confirm the cloned fragment.

Two-stage nested PCR. In order to increase the sensitivity and specificity of PCR, we performed two-stage nested PCR analysis. First-round PCR was performed in order to amplify a fragment containing the pVPI *att* site by using JumpStart RED *Taq* DNA polymerase (Sigma). For stage-two (nested) PCR, primers which targeted an internal fragment of the first-round PCR product were used. Primer locations for two-stage nested PCR analysis are shown in Fig. 1. In two-stage nested PCR, 1 μl of PCR product of the first round was used as the

TABLE 2. Primers used in this study[a]

Primer	Sequence (5′→3′)
KAR218	CGTATTCCACTGACAACC
KAR221	TCCACAAGATTCCATAGC
KAR393	ACTCTATCACCAGGAATTGG
KAR396	CTGTGTTAAACGTTTGACGG
KAR395	GGTAGTGCGTTTGATACTGG
KAR394	ACTTTGCCATTCAAGTGAGG
KAR426	TCTGGTAGCTTCAAAACAAGG
KAR427	AATGGCAAAGTGGAACGC
KAR428	AGAGCAAACAAGATAAACGAGG
KAR429	GTGTTTGACCCAGTATCGTC
KAR433	GCATCACCACATTCCTCATAC
KAR438	ATTCGTTAGCGTGTCGG
KAR439	TTGATGAGACGCTCTGAACC
KAR442	TTTCTCTCTAGGTTTGGAGG
KAR458	CTCTGTCCATAGACACCCAG
KAR463	ATAGGGAGCTGGGCGTTAAT
KAR464	TGTAAGACGGGGAAATCAGG
KAR503	ACTCGTGGGATTTGATCTCG

[a] All primers were made in this study.

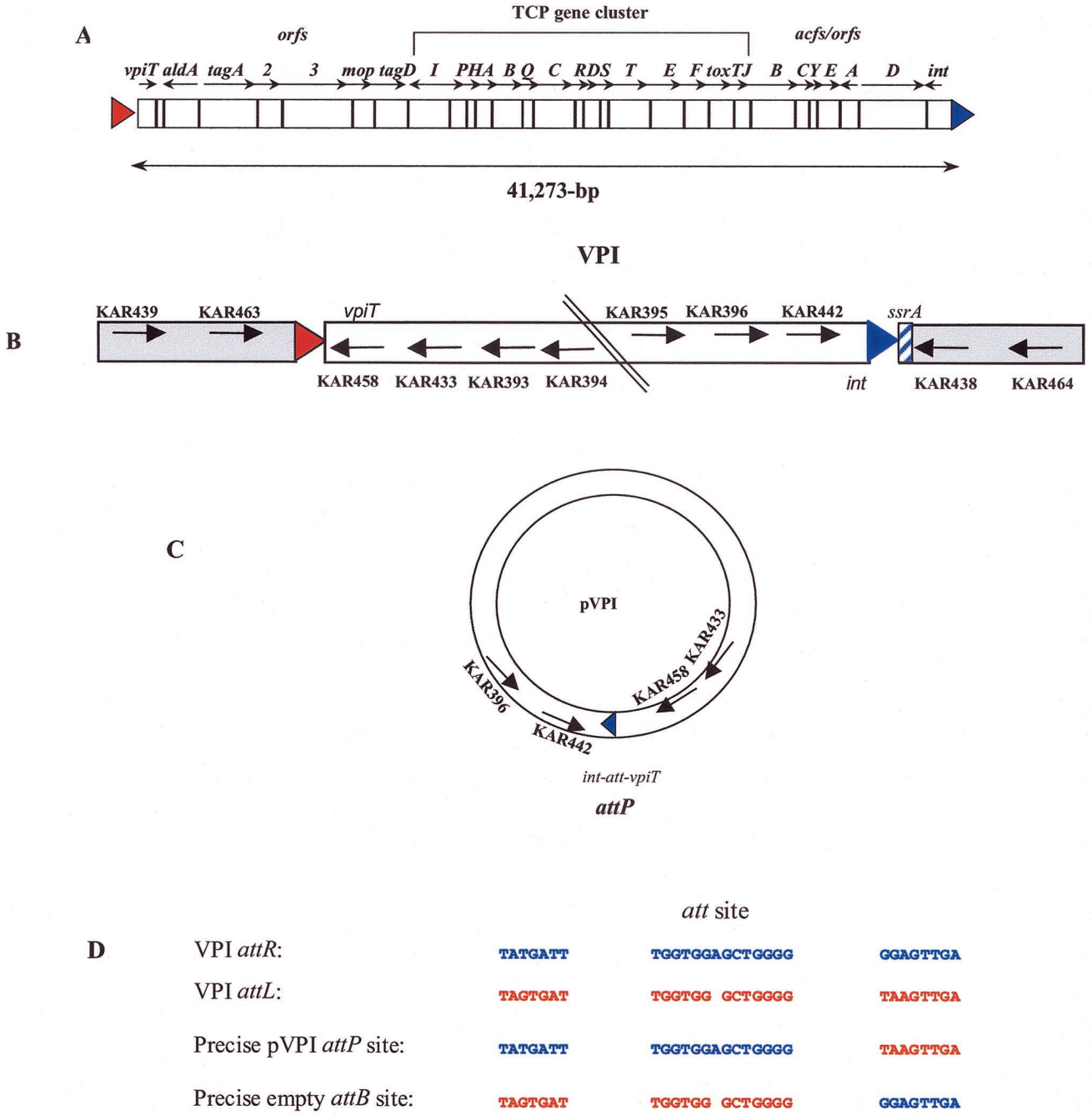

FIG. 1. Chromosomally integrated and extrachromosomal excision products of the VPI in epidemic *V. cholerae* strains. (A) Schematic representation of the VPI in the *V. cholerae* chromosome. (B) Locations of PCR primers in the VPI and flanking chromosome used for analysis of excision events. (C) Junction (*int-att-vpiT*) of pVPI formed by the joining of the left and right ends of the VPI following excision from the chromosome. (D) Sequence of N16961 chromosomal *attL* and *attR*, pVPI *attP* junctions, and empty chromosomal *attB* sites following precise excision. Red, *attL*-derived sequence; blue, *attR*-derived sequence.

template. PCR mixtures were loaded onto 1% agarose gels and stained with ethidium bromide. In all cases, PCR products were confirmed by DNA sequencing.

Frequency of VPI excision. To determine the frequency of VPI excision from the chromosome, an extinction dilution technique along with two-stage nested PCR was used. Briefly, 1 ml of an overnight culture having ~10^9 cells of strains DK224, DK331, DK 339, DK 414, DK 343, DK384, and DK591 per ml were used. Whole-cell lysates were prepared by heating the samples on a dry block at 90°C for 30 min. The lysate (based on 10^9 cells) was then suspended in 100 μl of MQ water and serially diluted 10-fold with MQ, and 1 μl was used as the template in a PCR. First-stage PCR was performed with primers KAR463 and KAR464. The second-stage nested PCR was performed with primers KAR438 and KAR439. The last dilution which served as a template in producing a PCR product was used to estimate the number of cells in that sample and, therefore, the approximate frequency of VPI excision. The experiment was repeated at least three times to determine the average rate of excision.

Cloning, mutagenesis, and complementation of *vpiT* and *int*. The *vpiT* gene was amplified from the chromosome of strain N16961 by using PCR primers

KAR429 and KAR428. The resulting 2.46-kb PCR product containing *vpiT* was cloned into pGEM-T, creating pDK93. Plasmid pDK93 was digested with *Stu*I-*Pml*I, which cuts *vpiT* 65 bp after the start codon and deletes a 357-bp fragment. The *aphA-3* kanamycin cassette obtained after *Sma*I digestion (35) was then blunt-end ligated in frame, creating pDK95. Plasmid pDK95 was digested with *Sph*I and *Sac*I to release the 2.9-kb fragment, which was then cloned into the suicide vector pCVD442 (8), creating pDK96. Plasmid pDK96 was transformed into the mobilizing *Escherichia coli* strain S17-λ*pir* and used in allelic exchange with the spontaneously streptomycin-resistant N16961 strain DK224 to generate *vpiT* mutant DK343. To construct an *int* mutation, the *int* gene from N16961 was amplified by using primers KAR221 and KAR218 on a 1.4-kb fragment which was then cloned into pGEM-T, creating pDK60. A *Sma*I fragment of pUC18K3 containing the *aphA-3* gene, encoding kanamycin resistance, was then ligated into the *Eco*RV site of pDK60, creating plasmid pDK62. The *int*::*aphA-3* fragment was then cloned into pCVD442, confirmed by sequencing, and used in allelic exchange to create an N16961 *int* mutant, called DK331. In order to construct an *int vpiT* double mutant, the *int* strain DK331 was used in allelic exchange with the suicide plasmid pDK96, creating strain DK707.

RESULTS AND DISCUSSION

The molecular basis underlying the horizontal transfer of the VPI in *V. cholerae* appears to be a complex process and is still not fully understood. We previously reported that the VPI appeared to be the genome of a transmissible phage that used TcpA as its coat protein (30). Although several observations at that time led to the conclusions described in that paper, the reexamination of that data by us (data not shown) and studies of others (10) using similarly marked donor strains and methods are currently less able to strongly support conclusions that the VPI encodes a transferable phage with TcpA as its coat. However, in the present paper, we provide several lines of evidence to support our earlier findings that the VPI is not immobilized but can excise from the chromosome in a novel and precise manner involving two VPI-encoded recombinases to form a circular extrachromosomal excision product. We propose that this excision-and-circularization event is important in the emergence, pathogenesis, and persistence of cholera. Whether VPI excision and formation of this circular product are directly involved in a VPI transfer process or serve as an intermediate stage for subsequent phage transfer of the VPI is unclear but is also under investigation.

Detection of an extrachromosomal circular VPI excision product in plasmid preparations. As our previous results suggested that the VPI can form a circular plasmid-like element (30), we decided to further study the molecular biology of the VPI and characterize its excision from the chromosome and formation of circular excision products. Since the left and right ends of the VPI contain phage-like attachment (*att*) sites, a PCR strategy was designed that would amplify a 1.35-kb PCR product only if the VPI excised from the chromosome and its left and right ends joined together at the *att* site to form a circular excision product (Fig. 1). PCR analysis with primer KAR393, located in *vpiT* (VPI left end), and primer KAR396, located in *int* (VPI right end), successfully amplified a 1.35-kb *int-att-vpiT* PCR product from seventh-pandemic strain N16961 and sixth-pandemic strain 395 (Fig. 2, lanes 1 and 2, respectively). All other VPI-positive epidemic strains tested produced a similar fragment (Table 3). PCR analysis detected this *int-att-vpiT* junction in *toxT* and *toxR* mutants, suggesting that the VPI can excise in the absence of *toxR* and *toxT* (Table 3).

In order to increase the sensitivity and specificity of the initial PCR detection system, a two-stage nested PCR strategy was designed to amplify a 483-bp *int-att-vpiT* junction with primer combinations KAR396-KAR433 and KAR442-KAR458 (Fig. 1). This two-stage nested PCR design produced consistent and clear results, confirming the formation of the *int-att-vpiT* junction and demonstrating that the VPI of epidemic *V. cholerae* strains can excise from the chromosome and form an extrachromosomal circular product, which we called pVPI. In order to rule out that this PCR product is a PCR artifact, two-stage nested PCR with KAR395-KAR394 and KAR396-KAR433 was performed on N16961 chromosomal

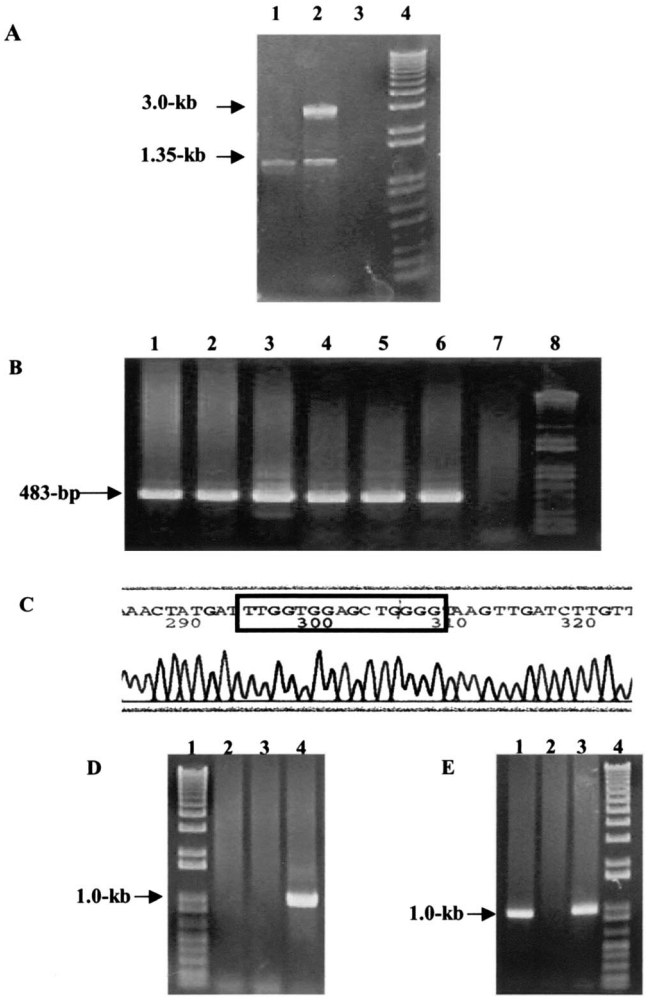

FIG. 2. Detection of the pVPI *int-att-vpiT* product by PCR analysis. (A) Standard PCR analysis of the *int-att-vpiT* fragment of pVPI, showing 1.35- and 3-kb bands. Lane 1, N16961; lane 2, 395; lane 3, negative (buffer) control; lane 4, DNA marker. (B) Two-stage nested PCR analysis of the 483-bp *int-att-vpiT* region of pVPI. Lane 1, N16961; lane 2, DK331; lane 3, DK339; lane 4, DK343; lane 5, DK384; lane 6, DK414; lane 7, buffer control; lane 8, 1-kb DNA marker. (C) Raw sequence printout showing the *attP* site (boxed). (D) Two-stage nested PCR analysis of the 1.0-kb *int-att-vpiT* region of pVPI. Lane 1, 1-kb DNA marker; lane 2, N16961 chromosomal DNA; lane 3, negative (buffer) control; lane 4, N16961 plasmid preparation. Note the absence of the PCR product in the chromosomal sample. (E) Two-stage nested PCR analysis showing production of the *int-att-vpiT* fragment in a *recA* mutant of *V. cholerae*. Lane1, *recA* mutant of 595B; lane2, buffer control; lane 3, N16961; lane 4, 1-kb DNA marker.

TABLE 3. PCR analysis of the *int-att-vpiT* fragment of pVPI

Strain	Detection of:	
	int-att-vpiT[a] (pVPI)	*vpiT*[b] (chromosome II)
N16961	+	−
E7946	+	+
395	+	+
E9120	+	+
DK224 (N16961 Sm)	+	−
VJ739 (E7946 Δ*toxT*)	+	+
JJM43 (395 Δ*toxR*)	+	+
DK224 *int*::Km	−	−
DK224 *int*::Km(pDK60)	+	−
DK224 *vpiT*::Km	+	−
DK224 *vpiT*::Km(pDK93)	+	−

[a] 1.35-kb fragment (*int-att-vpiT*) of pVPI detected by primers KAR393 and KAR433.

[b] 3-kb fragment (*vpiT-hutA*) on chromosome II detected by primers KAR393 and KAR433.

DNA at the same concentration as the DNA obtained from plasmid preparations. Multiple experimental results revealed that no PCR product (*int-att-vpiT*) was obtained when chromosomal DNA was used (Fig. 2D), demonstrating that the PCR product does not result from a recombination artifact between the two flanking *att* sites, as this would be expected also to be found in the chromosomal sample. Rather, this *int-att-vpiT* product is detected only in plasmid preparations and therefore appears to represent an extrachromosomal circularized VPI excision product. Furthermore, we found that an *int-att-vpiT* product obtained by using primers KAR395-KAR394 and KAR396-KAR433 was consistently produced in a *recA* mutant of classical biotype strain 569B (Fig. 2E). This strongly suggests that pVPI formation is independent of *recA* and that this mechanism is not due to homologous recombination between the *att* sites but involves site-specific recombination.

DNA sequencing of the cloned 1.35-kb *int-att-vpiT* PCR product obtained with primers KAR393 and KAR396 (Fig. 1) showed that the sequences from both N16961 and 395 were identical to that of the predicted *int-att-vpiT* fragment if the VPI excised from the chromosome and its left and right ends joined together. Additionally, the DNA sequence of a cloned 483-bp fragment from two-stage nested PCR analysis with primers KAR396-KAR433 and KAR442-KAR458 was identical to that of the expected *int-att-vpiT* fragment (Fig. 1 and 2B and C). These findings demonstrate that the VPI is active and can excise from the chromosome, forming an extrachromosomal circular excision product.

We have previously reported that there is a deletion of an adenine in the VPI *attL* compared to *attR* in VPI-positive strains (26). PCR sequence analysis of the pVPI *attP* in several epidemic *V. cholerae* strains showed that its sequence is identical to that of *attR* and contains an adenine (Fig. 1D). Additionally, we have previously identified a toxigenic strain (E9120) in which we hypothesized the VPI has been lost from the chromosome (26). This clinical isolate was isolated in 1961 in Indonesia and has a ribotype similar to that of seventh-pandemic strains but is negative for the VPI in its chromosome (26, 28). This hypothesis is further supported by the finding that this strain contains only the *attL* sequence in its genome (26). Since the VPI is generally needed for the acquisition of choler toxin genes, as it encodes the TCP receptor for CTXΦ (49), and the *attB* site in E9120 is identical to *attL* of VPI-positive strains, our findings further support the hypothesis that this toxigenic strain once had the VPI at some stage and subsequently lost it from its chromosome, leaving behind a remnant *attL* site.

Detection of an empty chromosomal VPI site. As our data suggested that the VPI excises from the chromosome and forms a circular product, we hypothesized that there should be an "empty " chromosomal VPI site in these strains. A two-stage nested PCR analysis was designed to detect the empty *attB* site and thus the deletion of VPI from the chromosome. In the first-stage PCR, primers KAR463 and KAR464 were used to target the chromosomal region flanking VPI and amplify a 400-bp fragment if the VPI had excised and left an empty site. In stage-two PCR, primers KAR438 and KAR439 were used to amplify an internal 202-bp fragment. This PCR analysis demonstrated an empty *attB* site on the chromosome of N16961 (Fig. 1 and 3). Interestingly, this excision event leaves an empty *att* site that is identical to the empty *attB* site in strain E9120, further suggesting that the VPI has been precisely lost from the chromosome in E9120. Additionally, growth of N16961 overnight at 37°C in Luria-Bertani broth containing mitomycin C (10 and 20 ng/ml), which is a mutagen commonly used to induce prophage, did not result in an obvious increase in the frequency of excision as detected by empty *att* sites (data not shown), suggesting that VPI excision is independent of *recA* and is not induced by mitomycin C.

The finding that pVPI *attP* and the vacant bacterial *attB* site differ only by an adenine suggests that formation of pVPI can establish a homologous *att* site which may recognize the vacant *attB* site. This might result in site-specific recombination and integration of the VPI into the chromosome of *V. cholerae*. Insertion of the VPI would result in *att* sites (near direct repeats) at the left and right ends that differ by only a single adenine residue. These data also suggest that *V. cholerae* strains have the potential to acquire, excise, and reintegrate the VPI and that strains in which the VPI has excised retain the *attL* site sequence in their genome. It is noteworthy that the mechanisms described above for the VPI are typical of lysogenic and temperate phages (41). The relevance of this specific sequence is described below.

The Int and VpiT recombinases have roles in VPI excision and pVPI formation. In order to better understand the molecular biology of the VPI, its chromosomal excision, and formation of a pVPI molecule, we hypothesized that the P4 phage-like integrase (*int*) gene and transposase-like (*vpiT*) gene, located at the far right and left ends of the VPI, respectively, were involved in the excision event and catalyzed pVPI formation (Fig. 1). To test the role of *int* in VPI excision and pVPI formation, insertional inactivation with the *aphA-3* gene was used to construct a nonpolar *int* mutation in N16961 strain DK224, creating DK331. To our surprise, two-stage nested PCR analysis with primers KAR396-KAR433 and KAR442-KAR458 and DNA sequencing demonstrated that pVPI *int-att-vpiT* junction formation still occurs in the N16961 *int* mutant (DK331) (Fig. 2B). However, complementation of the DK224 *int* mutant with *int* on pDK60 (strain DK339) or intro-

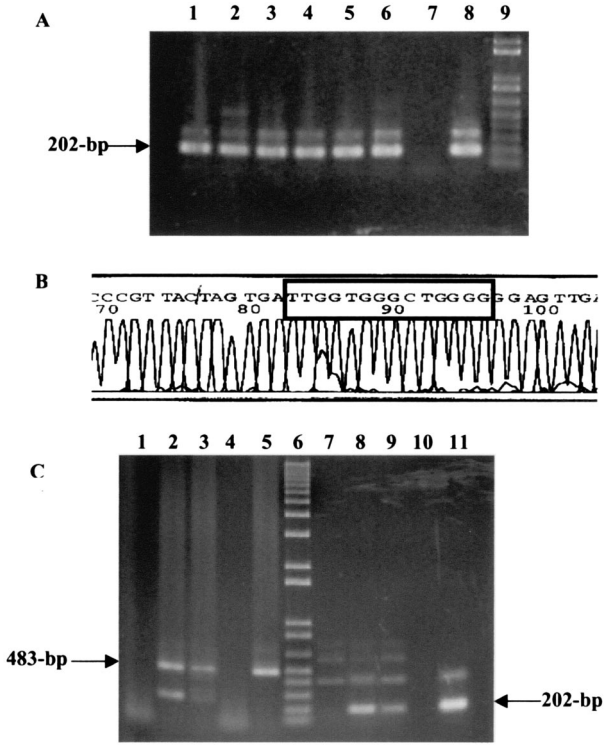

FIG. 3. Analysis of the empty chromosomal *att* site reveals an empty *att* site. (A) PCR analysis of empty *attB* site. Lane 1, N16961; lane 2, DK331; lane 3, DK339; lane 4, DK343; lane 5, DK384; lane 6, DK414; lane 7, buffer control; lane 8, DK70; lane 9, 1-kb DNA marker. (B) Raw sequence printout showing the *attB* site (boxed). (C) Two-stage nested PCR analysis of *int vpiT* double mutant and complemented strains, detecting the *int-att-vpiT* junction (lanes 1 to 5) and empty *attB* site (lanes 7 to 11). Lane 1, absence of the 483-bp circular excision product (*int-att-vpiT* junction) in the double mutant (DK707); lanes 2 and 3, detection of the 483-bp band in the complemented strains (DK708 and DK709, respectively); lane 4, buffer control; lane 5, PCR product detected in the DK224 positive control. Lane 7, absence of the 202-bp band (no empty *attB* site); lanes 8 and 9, detection of 202-bp fragment in complemented strains (DK708 and DK709, respectively); lane 10, buffer control; lane 11, empty *attB* site detected in the DK224 positive control. Lane 6, 1-kb marker.

duction of *int* on pDK60 into DK224 (strain DK414) appeared to increase the frequency of excision as detected by the intensity of the band by PCR analysis (data not shown). To further study the frequency of excision of the VPI, an extinction dilution technique together with two-stage nested PCR analysis was used to target the empty *att* site. The results (Table 4), based on at least three independent experiments, suggest that there is at least one excision event in 10^5 wild-type N16961 cells under the conditions tested. Although the *int* mutation did not appear to affect the frequency of VPI excision compared to that in the wild type, the *int* plasmid (pDK60) in both the *int* mutant and the wild type increased the excision rate by up to 100-fold. We did not observe any increase in frequency when pDK62 (containing *int*::Km) was introduced as a control. These results, although not based on a very quantitative technique, suggested that the *int* gene is active in N16961 and has a role in VPI excision and that increased *int* expression increases excision and pVPI formation.

TABLE 4. Frequency of VPI excision

Strain	Genotype	Excision rate[a]
DK224	N16961 Sm	10^{-5}
DK331	DK224 *int*::Km	10^{-5}
DK339	DK224 *int*::Km(pDK60)	10^{-3}
DK414	DK224(pDK60)	10^{-3}
DK591	DK224(pDK62)	10^{-5}
DK343	DK224 *vpiT*::Km	10^{-5}
DK384	DK224 *vpiT*::Km(pDK93)	10^{-4}

[a] Based on results from at least three independent experiments.

To test the role of *vpiT* in VPI excision and pVPI formation, we constructed a nonpolar *vpiT* mutant of N16961, called DK343. Again, to our surprise, two-stage nested PCR analysis revealed that VPI excision and pVPI formation still occurred in the N16961 *vpiT* mutant (DK343) (Fig. 3A and B). However, complementation of *vpiT* by using pDK93 appeared to increase the frequency of excision, as detected by two-stage nested PCR analysis and sequencing (data not shown). This result was supported by combining two-stage nested PCR and an extinction dilution technique (Table 4), which shows a 10-fold increase in excision frequency in DK384 containing *vpiT* on plasmid pDK93 and suggests that *vpiT* is required for efficient excision and pVPI formation. We have previously shown that the VPI-encoded VpiT (previously Orf1) has significant homology to BfpM of enteropathogenic *E. coli*, which appears to be truncated and may be defective (26, 43, 45). In enteropathogenic *E. coli*, this gene is located on a plasmid and, like the *V. cholerae* VPI-encoded TCP gene cluster (44), is associated with genes encoding a type IV bundle-forming pilus intestinal colonization factor (11, 43).

Our data suggested that precise excision and the formation of pVPI are regulated by the VPI-encoded phage CP4-57-like (P4-like) site-specific recombinase (*int*) gene and the recombinase-transposase-like (*vpiT*) gene. To further study and confirm the role of these VPI-encoded recombinases in VPI excision, we performed allelic exchange with pDK96 and the *int* mutant DK343 to construct an *int vpiT* double mutant, DK707. Two-stage nested PCR with primers KAR396-KAR433 and KAR442-KAR458 as described above failed to detect an expected 483-bp PCR product resulting from circularization of the VPI in the *int vpiT* double mutant DK707 (Fig. 3C). This suggests that the VPI is defective and is unable to excise in the double mutant. This defect could be restored when the double mutant was complemented either with *int* alone in DK708 (containing pDK60) or with *vpiT* alone in DK709 (containing pDK93). In addition, we found that the double mutant did not contain an empty VPI *attB* site, further suggesting that the VPI is unable to excise in the double mutant (Fig. 3C). The detection of an empty *attB* site, presumably due to VPI excision, was restored in both complemented strains. It is important to note that since neither the *int* plasmid nor the *vpiT* plasmid contains the *att* site sequences, the increase in excision frequency in strains containing these plasmids cannot be due to an increase in the availability of *att* sites provided by fragments on these plasmids. The results from these studies confirm that *int* and *vpiT* have a role in VPI excision and, importantly, provide compelling evidence suggesting that *int* and *vpiT* are analogous and that one of them can compensate for the role in excision

of the other recombinase. Recently, it has been reported that the PAI (SaPIbov2) from bovine *Staphylococcus aureus* is able to excise to form a circular element and can integrate site specifically and RecA independently at a chromosomal *att* site (48). Since the VPI-carried *int* and *vpiT* genes have homologs in many different PAIs, our data support the hypothesis that similar homologous recombinase genes may be active and involved in the excision, circularization, and potential mobilization of PAIs in other pathogens.

Attempts to detect transfer of pVPI from wild-type N16961 into VPI-negative recipients by using electroporation have so far been unsuccessful with a Km^r-marked VPI strain. This can be explained if the pVPI is present at a very low copy number or if it is unable to self-replicate under the conditions tested. We were unable to visualize pVPI as a band in plasmid and chromosomal preparations from N16961Sm (strain DK224) after staining with SYBR Gold or by using dot blots on Zeta-probe blotting membrane with enhanced chemiluminescence direct nucleic acid labeling of an *int-att-vpiT* PCR product as a probe. These results further suggest that excision and formation of pVPI occur at a very low frequency (~1 in 10,000 cells) under the laboratory conditions tested. Interestingly, the self-transmissible SXT element of *V. cholerae* can be detected by PCR analysis but cannot be visualized in plasmid preparations (50). We propose that the excision of the VPI (albeit detected at a low frequency under the conditions tested) is biologically significant and may represent a particular stage in the life of the VPI in *V. cholerae*. We are currently developing more sensitive detection systems for these events. Despite the low frequency, we propose that a similar two-stage nested PCR strategy (targeting the joining of PAI termini) can specifically detect similar excision events, which can then be further studied.

VPI excision affects *ssrA*. The chromosomal integration site of the VPI, like those of most other PAIs, is at the 3′end of a tRNA-like gene (*ssrA*) (26, 32). The *ssrA* gene encodes a small stable RNA (~370 nucleotides in length) that has a tRNA-like tertiary structure and an acceptor stem with a terminal CCA end that can be aminoacylated with alanine (18, 31). The *ssrA* RNA is also known as the tmRNA because it functions as an alanine-specific tRNA and also contains a short reading frame coding for 10 amino acids (47). The 11-amino-acid tag (alanine plus the 10 encoded amino acids) is added to polypeptides translated from mRNAs lacking a termination codon, and the added 11 amino acids serve as a tag to mark the protein a target for specific proteolysis. Besides the VPI, a PAI of *Salmonella enterica* serovar Typhimurium is also inserted into the *ssrA* gene (23). Insertion of PAIs into tRNA or tRNA-like genes is typical of many pathogenic bacteria (3), and this feature is also shared among lysogenic and temperate phages (19, 41, 42).

Comparison of the *attP* site in pVPI and the vacant bacterial *attB* site reveals the absence of an adenine in the *attB* following excision. This deletion was confirmed by sequencing the chromosome from four independent cultures in strain N16961(data not shown). The deleted adenine corresponds to A361 in the *ssrA* gene, which encodes the U of the G · U base pair that is critical for aminoacylation of the *ssrA* RNA with alanine (31). The G · U base pair is conserved in the evolution of all cytoplasmic alanine-specific tRNAs and *ssrA* RNA (20, 21). Biochemical analysis has shown that substitution of the U in the G · U base pair completely eliminates aminoacylation with alanine in vitro and inactivates the acceptor activity in vivo (21). In the case of excision of VPI, deletion of U may also alter the structure of the acceptor stem to prevent aminoacylation. Thus, excision might have a major deleterious impact on the cellular function of the *ssrA* RNA and impede the homeostasis of the host cells to target protein degradation. Conceivably, because the retention of the VPI maintains the integrity of the *ssrA* RNA, this may provide a selective advantage for pathogenicity. The potential relevance of this specific sequence in *V. cholerae* is being further studied.

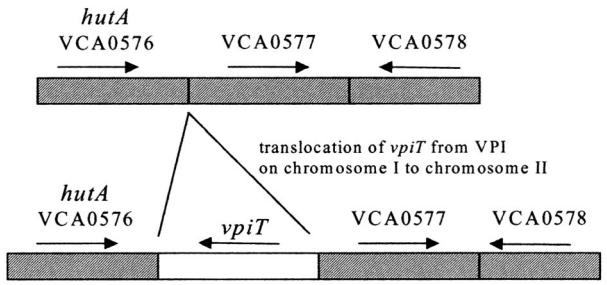

FIG. 4. Schematic representation showing translocation of *vpiT* from the VPI on chromosome I to the *hutA* region on chromosome II in some *V. cholerae* strains.

Interchromosomal translocation of *vpiT* into a hot spot on chromosome II. As our initial PCR analysis and sequencing (with primers KAR393 and KAR396) identified an additional strong 3-kb product in all other sixth- and seventh-pandemic strains tested except N16961 (Fig. 1 and Table 3), the intensity of this band prompted us to hypothesize that it was derived from chromosomal DNA. DNA sequencing of the 3-kb PCR product from sixth-pandemic strain 395 and a BLAST search showed that part of this fragment had homology with the reverse strand of *vpiT* (including 54 bp upstream of *vpiT*) encoded on the VPI on chromosome I and that part had homology to a region on chromosome II adjacent to *hutA* (VCA0576) which is involved in heme transport (16). A computer search indicated that the chromosome II insertion site is 5 bp downstream of the *hutA* stop codon. Interestingly, *hutA* and the downstream gene VCA0577 have low percent G+C contents (38 and 39%, respectively).

In order to further show that *vpiT* had translocated to chromosome II, we designed primers KAR426 (located in *hutA*) and KAR427 (located in *vpiT*) and performed PCR analysis with the chromosome of strain 395 as a template. A single expected 0.8-kb product was obtained, cloned into pCR2.1 to create pDK94, and sequenced. Sequencing confirmed that *hutA* and a second copy of *vpiT* were linked in chromosome II of strain 395 and presumably in the other strains (Fig. 4). Examination of the DNA sequence of the *vpiT* insertion near *hutA* shows the absence of *att* sites. We then determined whether *vpiT* could excise from chromosome II in strain 395 by using PCR analysis and primers KAR426, located in *hutA*, and KAR503, located in VCA0578 (gene designation from the published TIGR N16961 genome). We did not detect any excision of *vpiT* from chromosome II under the conditions tested, suggesting that it is stable (data not shown). These

results suggest that *vpiT* has undergone or can undergo interchromosomal translocation. Alternatively, it is possible that the insertion of *vpiT* into chromosome II might represent the insertion of some form of independent transposable element.

Interestingly, a recent study found that the two genes downstream of *hutA* in chromosome II (VCA0577 and VCA0578) had translocated into the left end of the VPI in several *V. cholerae* non-O1 strains (37). These findings are consistent with our previous report that the VPI is mosaic in structure and contains both conserved and divergent regions (27), a feature that is also common in temperate phages (22). We suggest that these regions (*vpiT* and *hutA*) may represent hot spots for gene translocation. *V. cholerae* is known to contain two circular chromosomes (46), and while the reason for the association between regions of the VPI (on chromosome I) and *hutA* (on chromosome II) in some strains is not clear, these genetic rearrangements might be adaptive and confer some evolutionary advantage to the cell.

Conclusion. We have previously put forward a hypothesis that the VPI can be found as extrachromosomal circular products and can be detected extracellularly in DNase-protected particles (30). As noted recently by Miller (36) in a commentary on the topic "the case for horizontal transfer of the TCP pathogenicity island remains as strong as ever," it was recently found that the VPI, and presumably other chromosomal fragments, can be transferred among *V. cholerae* O1 strains by using the generalized transducing phage CP-T1 (38). Whether the process of VPI excision and formation of the circular product reported in the present paper is itself involved in transfer needs further study. While the VPI might be mobilized by a generalized transduction mechanism, our genetic analysis presented here clearly shows a specialized excision and circularization of the VPI (involving VPI-encoded recombinases), suggesting that there might be a specialized mechanism for VPI mobilization. In our present work, we provide compelling evidence that the VPI of epidemic *V. cholerae* (in both El Tor and classical biotype strains) can excise from the chromosome at its terminal *attL* and *attR* sites to potentially form an extrachromosomal circular excision product (pVPI) joined at a specific *attP* site. The finding that the VPI is capable of excision and circularization is relatively novel for PAIs. We suggest that this particular mechanism is important in the biology of the VPI and in epidemic *V. cholerae*. Although excision occurs independently in individual *int* and *vpiT* mutants, excision does not occur in an *int vpiT* double mutant, suggesting the *int* and *vpiT* recombinases have a role in the excision process. The mechanism and function of VPI excision and circularization are being further studied by us, including the specific roles of *int* and *vpiT* (and other genes) and the conditions required for excision and integration. We suggest that these events are important in the horizontal transfer of the VPI and in the emergence and virulence of epidemic *V. cholerae* strains. We are also studying the association of the VPI with *ssrA*, since the excision of the VPI could have a major deleterious effect on the cellular function of the *ssrA* RNA and is likely to perturb the balance between protein stability and degradation. This raises the possibility that maintaining the VPI imparts a selective advantage. A better understanding of the molecular biology of PAIs not only will greatly improve our understanding of bacterial virulence but may lead to new and improved vaccines, novel therapeutics, and better environmental monitoring of pathogenic strains.

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