A genomic island in *Vibrio cholerae* with VPI-1 site-specific recombination characteristics contains CRISPR-Cas and type VI secretion modules

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Cholera is a devastating diarrhoeal disease caused by certain strains of serogroup O1/O139 *Vibrio cholerae*. Mobile genetic elements such as genomic islands (GIs) have been pivotal in the evolution of O1/O139 *V. cholerae*. Perhaps the most important GI involved in cholera disease is the *V. cholerae* pathogenicity island 1 (VPI-1). This GI contains the toxin-coregulated pilus (TCP) gene cluster that is necessary for colonization of the human intestine as well as being the receptor for infection by the cholera-toxin bearing CTX phage. In this study, we report a GI (designated GI_{VchS12}) from a non-O1/O139 strain of *V. cholerae* that is present in the same chromosomal location as VPI-1, contains an integrase gene with 94% nucleotide and 100% protein identity to the VPI-1 integrase, and attachment (att) sites 100% identical to those found in VPI-1. However, instead of TCP and the other accessory genes present in VPI-1, GI_{VchS12} contains a CRISPR-Cas element and a type VI secretion system (T6SS). GIs similar to GI_{VchS12} were identified in other *V. cholerae* genomes, also containing CRISPR-Cas elements and/or T6SS's. This study highlights the diversity of GIs circulating in natural *V. cholerae* populations and identifies GIs with VPI-1 recombination characteristics as a propagator of CRISPR-Cas and T6SS modules.

*Vibrio cholerae* is a species of bacteria commonly found in marine and estuarine waters and the causative agent of the diarrhoeal disease cholera¹. Although more than 200 serogroups of the bacterium are known, only two, O1 and O139, are responsible for pandemics of the cholera disease². Historically, there have been seven pandemics of cholera, with the current seventh pandemic caused by O1 strains of the El Tor biotype and the sixth and presumably the previous five pandemics caused by O1 strains of the classical biotype¹. The evolution of pandemic strains has been greatly influenced by lateral gene transfer (LGT), which led to the acquisition of many novel virulence factors³.

Integrative mobile genetic elements (MGEs) such as transposons and genomic islands (GIs) are particularly important in LGT processes, as they help facilitate integration of non-homologous transferred DNA into replicons such as the chromosome or a resident plasmid(s)⁴. Studies comparing the genomes of *V. cholerae* strains consistently find MGEs as one of the sources, if not the major source, of genome variation⁵,⁶. For example, the seventh pandemic strains contain two GIs called the *Vibrio* seventh pandemic islands I and II not present in the strains isolated from the previous pandemics⁷. Most important to pathogenicity of the pandemic strains are genes found on two MGEs, the cholera toxin bacteriophage (CTXφ) and a GI called the *V. cholerae* pathogenicity island

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1 (VPI-1)\(^9\). Although these MGEs are common in pandemic strains, examples ofCTX\(\phi\) and VPI-1 in non-O1/O139 strains have been documented and are indicative of their mobility across natural populations ofV. cholerae and close relatives\(^10\). TheCTX\(\phi\) contains genes encoding a potent enterotoxin that, when secreted in the human intestinal tract, results in significant loss of fluid that can lead to death within 24 hours if left untreated\(^1\). VPI-1 contains the genes encoding the toxin-coregulated pilus (TCP; thus, VPI-1 is also known as the TCP pathogenicity island) that is required for adhesion to the intestinal wall as well as genes encoding the accessory colonization factor and the regulatory genes toxT and tcpP\(^13\). TCP is also the receptor for CTX\(\phi\) and thus a prerequisite for infection and subsequent lysisogeny in the emergence of toxigenic V. cholerae\(^6\).

Another virulence factor associated with frequent LGT is the type VI secretion system (T6SS) ofV. cholerae and other Gram-negative bacteria. Bacteria that harbor T6SS produce a membrane-spanning protein complex capable of puncturing eukaryotic or prokaryotic cells and injecting toxic effector proteins into their targets\(^16\). InV. cholerae, the presence ofT6SS has previously only been reported as a conserved chromosomal element and displays toxic activity against macrophages\(^17\). The T6SS is made up of a series of proteins encoded by genes in three different locations of theV. cholerae genome: a main cluster on chromosome 2 and two smaller auxiliary clusters on chromosomes 1 and 2\(^17\). The characteristic proteins of theT6SS are Hcp and VgrG. Hcp is encoded by two alleles on theV. cholerae chromosome and polymerizes to form the nanotube that protrudes from the bacterial cell surface\(^19\). The tip of Hcp is decorated with VgrG proteins that form a trimer\(^22\). VgrG proteins are conserved at their N-termini but carry specialist C-termini with enzymatic activity\(^21\). For example, V. cholerae contains three VgrG alleles with two encoding proteins with actin cross-linking (VgrG-1) and peptidoglycan degrading (VgrG-3) activity\(^22,23\). Additional effector proteins with diverse enzymatic activities can be added to the VgrG spike for delivery into target cells\(^29\).

Acquisition of novel genes through the uptake of MGEs can have obvious beneficial effects for harmless and pathogenic bacteria alike. However, not all LGT is advantageous, and bacteria have evolved a variety of methods to prevent the spread of harmful DNA sequences in their genomes\(^25\). One recently discovered defense mechanism against unwanted LGT is the CRISPR-Cas system. CRISPR-Cas modules consist of cas genes and an array of short direct repeats separated by highly variable spacer sequences that correspond to genetic elements such as bacteriophages or MGEs\(^26\). Transcription of the CRISPR array with subsequent slicing of the transcript into smaller CRISPR RNAs acts in concert with the Cas proteins to specifically recognize foreign DNA and cleave it\(^26\). The system acts as an adaptive immune system for bacteria as it allows for the synthesis and incorporation of new spacers into the array following invasion of a foreign DNA molecule, thus providing immunity to the host cell.

Non-O1/O139 strains ofV. cholerae have been hypothesized to be an important source of new MGEs that could relegate into pandemic strains. Here, we report a GI with VPI-1 recombination characteristics that harbors both a CRISPR-Cas module and an auxiliary T6SS locus in a non-O1/O139 strain ofV. cholerae from Sydney, Australia. This GI likely provides recipient cells not only with a defense mechanism against maladaptive LGT, but also with a potential competitive advantage over bacteria lacking this GI and perhaps a novel virulence factor. We also show that similar GIs are present in other non-O1/O139 strains from around the globe.

**Methods**

**Bacterial strain, growth conditions, and molecular biology methods.** The non-O1/O139V. choleraeS12 strain was isolated from the Georges River (Sydney, Australia) in 2009\(^25\) and routinely cultured on Luria-Bertani medium at 37 °C. The whole genome ofV. cholerae S12 was paired-end sequenced at the Wellcome Trust Sanger Institute (Hinxton, UK) using Illumina HiSeq 2000 (San Diego, CA, USA) from DNA extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). For extraction of GIvchs12 circles, a plasmid extraction was carried out on S12 using the PureYield Plasmid Miniprep Kit (Promega). PCR was performed using 2X MangoMix (Bioline, London, UK) that consists of DNA polymerase, dNTPs, Mg\(^{2+}\) and an orange dye with 30 cycles of denaturation at 93 °C for 30 sec, the appropriate annealing temperature for 30 sec and an extension of 72 °C (1 min/kb). All primers were acquired from Integrated DNA Technologies (Coralville, IA, USA) (Table 1) and used at a final concentration of 10 \(\mu\)M. PCR amplicons intended for sequencing were excised from1% agarose gels and purified using the Wizard SV Gel and PCR Clean-Up Kit (Promega) and sequenced using the Sanger method at Macrogen (Seoul, South Korea).

**Bioinformatic analyses.** The Illumina HiSeq whole genome sequencing reads forV. cholerae S12 were filtered to remove low quality reads with average read quality less than Q20 and low quality trailing ends with base quality less than Q20 using Prinseq-lite v0.20.4\(^24\). Reads were then de novo assembled into contiguous sequences (contigs) using Velvet v1.2.10\(^29\)and the assemblies were improved by scaffolding using SPACER v2.0\(^6\), gap filling usingGapFiller v1.10\(^13\), reordering of contigs against theV. cholerae N16961 reference genome using Mauve v2.4.0\(^12\)and removal of contigs shorter than 300 bp. The final improved draft genome assembled into 83 scaffolds from 4,624,354 read pairs with an average read length of 75 bp to give a total genome size of 4,061,577 bp with average depth of coverage of 171 reads.

The genomic region encoding GIvchS12 was identified on contig 000009 by pairwise comparison of theV. cholerae S12 draft genome to the complete reference genome ofV. cholerae N16961 using the program Mauve v2.4.0\(^12\). Analysis of GIvchS12 on contig 000009 identified three assembly gaps between ORFs 13 and 15 due to a putative repeat of ORF14 (annotated as ORF14a and ORF14b). To confirm this repeat, a PCR with primersGap_F1 and Gap_R1 that anneal outside of this repeat region (see Fig. 1) resulted in an expected 

2.5 kb product (as opposed to a ~1.3–1.5 kb product if only one copy of ORF14 was present). Two of the assembly gaps were closed through sequencing the ends of the amplicon creating the GIvchS12 sequence in GenBank file KU722393. GIvchS12 was annotated using Prokka\(^33\) and the automated annotation was manually curated with the aid of BLAST against the non-redundant NCBI protein database (Bethesda, MD, USA) using BLASTP.
providing putative identification\(^{34}\). The CRISPR-Cas module in GI\(_{Vch}\) S12 was identified using the online tool CRISPRFinder (http://crispr.i2bc.paris-saclay.fr)\(^{35}\).

In order to determine if GI\(_{Vch}\)S12 or similar islands were present in other \(V\). cholerae, additional \(V\). cholerae genomes were obtained from GenBank. The list of genomes used is provided in Supplementary Material. The genomes were annotated with RAST v2.0 (Rapid Annotation Using Subsystem Technology)\(^{36}\). The GI\(_{Vch}\) S12 ORFs were compared against the ORFs of the \(V\). cholerae genomes to determine presence/absence by calculating the BLAST score ratio (BSR)\(^{37}\). Significant hits were considered as putative homologues if the BSR values were at least 0.3 (for 30% amino acid identity)\(^{38}\). Furthermore, the whole genomes were aligned using Mugsy v1.2.3\(^{39}\), and the core alignment (2,539,853 bp in total length) was extracted from the Mugsy output using Galaxy v16.04\(^{40}\) and Geneious v8.1.8\(^{41}\). From this alignment, a maximum likelihood phylogenetic tree was constructed using RAxML v8.1.17\(^{42}\) using the general time reversible (GTR) nucleotide substitution model and gamma distribution pattern. Robustness of branching was estimated with 100 bootstrap replicates. \(Vibrio metoecus\), the closest relative of \(V\). cholerae, was used as an outgroup\(^{43}\).

**GenBank accession numbers.** The full sequence of the GI\(_{Vch}\)S12 including flanking sequences and the sequenced \(attP\) and \(attB\) sites are available in GenBank/ENA/DDBJ and have the accession numbers KU722393, KU722394, and KU722395, respectively. The raw Illumina HiSeq sequencing reads are available under accession number ERR063652 and the improved draft genome assembly of \(V\). cholerae S12 can be accessed at MDST00000000.

### Table 1. Primers used in this study.

| Primer | Sequence (5’-3’) | Target |
|--------|------------------|--------|
| ML126  | ACTTCTCGAAAGCGGATCAA | attL end of GI\(_{Vch}\)S12 |
| ML127  | AAGGCATCCACCTGCAAAG | attR end of GI\(_{Vch}\)S12 |
| ML130  | GCTACCTGGTCCTCAATCG | attR end of GI\(_{Vch}\)S12 |
| ML131  | TGGCAACAAGATGCTTATCG | attL end of GI\(_{Vch}\)S12 |
| ML134  | TCCCTAGCTCCTGCTTGA | Between VC0848 and attR of GI\(_{Vch}\)S12 |
| ML135  | TCAAGGGATCAGGGTGTC | Within VC0816 |
| ML136  | GGGAAATTGGAGCTGAGG | Between VC0848 and attR of GI\(_{Vch}\)S12 |
| ML137  | ATAGGGACTGGGGGTTAAT | Within VC0816 |
| Gap_F1  | GCCGTTTTTATACATGCCAAC | Within ORF 13 of GI\(_{Vch}\)S12 |
| Gap_R1  | ACACAGGGCTACCTCTAGTGG | Within and just past ORF 15 of GI\(_{Vch}\)S12 |

**Figure 1.** The ~28-kb genomic island, GI\(_{Vch}\)S12, from \(Vibrio cholerae\) S12 containing a CRISPR-Cas module and type VI secretion auxiliary locus. Schematic representation of GI\(_{Vch}\)S12 is shown in (A) with the VPI-1 \(att\) sites given as hatched boxes and the VPI-1 integrase as a black block arrow. Regions of nucleotide identity to VPI-1 and surrounding regions in \(V\). cholerae N16961 are shown. All ORFs and their orientation are shown as block arrows. Numbers shown in, above, or below the block arrows correlate to the putative identification shown in (B). Primer binding sites are shown by black triangles with their direction of extension indicated by the direction of the triangle.
Results and Discussion

A novel variant of the Vibrio pathogenicity island. In order to identify regions of interest in the genome of V. cholerae S12, contigs were compared to the closed genome of V. cholerae N16961. A novel GI in the same respective location as VPI-1 (between VC0816 and VC0848 on chromosome 1) was identified on contig 000009. This GI of ~28-kb has been given the designation GIVchS12 and contains an integrase with 94% nucleotide and 100% amino acid identity to the VPI-1 integrase gene and protein, respectively, and characteristic VPI-1 attL and attR sites abutting the GI. Given GIVchS12's location, with identical integrase protein and att sites, this GI is likely to have recombination functions identical to VPI-1. Previous studies have observed variations in VPI-1 through PCR analysis of V. cholerae strains or BLASTN analysis of V. cholerae genomes for VPI-1 genes. For the most part, the variations identified represent minor gene gain/loss events or sequence changes to known ORFs in VPI-1. GIVchS12 is different in that it shares practically no gene content with VPI-1.

A CRISPR-Cas module for self-preservation. Bioinformatic analysis of GIVchS12 identified a CRISPR-Cas module and a T6SS auxiliary locus at the attR and attL ends, respectively (Fig. 1). The CRISPR-Cas module in GIVchS12 contains genes encoding homologues of Cas1, Cas3, and Cas6. Based on the protein sequences and their organization, this CRISPR-Cas system is most likely similar to those of type 1-F4. Several spacers displayed 100% identity to various bacteriophage genomes consistent with the module having a role in acting against invading foreign DNA. The association of a CRISPR-Cas module within a GI is intriguing for two reasons. From an ecological perspective, the mobilization of a CRISPR-Cas system benefits the host not only with an adaptive immune system but also by the instant addition of the immunity that comes with the various spacer sequences it already carries. Thus, a host would immediately gain protection from various bacteriophages and other invading foreign DNA within that ecosystem. Secondly, an intriguing study identified a CRISPR-Cas system within a bacteriophage genome that was able to counteract an inhibitory GI present in the bacterial host genome, thus improving the bacteriophages’ capacity to successfully infect the bacterial host. As a result, this raises the possibility that the CRISPR-Cas system within GIVchS12 might improve integration efficiency in recipient cells that contain other genetic elements interfering with the GI's integrity and/or integration. Furthermore, once integrated into the host, the CRISPR-Cas system could prevent the replacement of GIVchS12 by VPI-1 or other GIs competing for the same integration sites. The CRISPR-Cas system found on GIVchS12 could therefore promote direct self-preservation or self-preservation by protecting its host.

A novel T6SS auxiliary locus. Also present on GIVchS12 are genes normally associated with three T6SS loci found in all V. cholerae genomes, known as the main locus and auxiliary loci 1 and 2. The GIVchS12 locus structurally resembles the two T6SS auxiliary loci, with the presence of an hcp gene, a copy of vgrG, a gene encoding a protein with a DUF4123 domain, and putative cargo effectors and immunity proteins further downstream. The lack of proteins making up the T6SS machinery indicates that the proteins on this additional auxiliary locus are dependent on the structural proteins encoded on the main chromosomes. T6SS loci for effective translocation into target cells that in S12 is present on contig 00022. The auxiliary loci 1 and 2 are present on contigs 00011 and 00021, respectively, although in contig 00021 the sequence breaks before hcp, presumably due to the difficulty of assembling repeat regions. At the end of contig 00011, the first 392-bp of hcp from auxiliary locus 1 is present before the sequence breaks. In the small contigs of 00079 and 00082 are the first 275-bp and last 108-bp of an hcp homologue, respectively, with hcp from contig 00079 presumably from auxiliary locus 2. The auxiliary 1 and 2 hcp genes in S12 share 99% nucleotide identity with those in V. cholerae N16961 and V52. The hcp from GIVchS12 is clearly different to both the auxiliary 1 and 2 hcp loci sharing 88% nucleotide identity.

The VgrG protein encoded in GIVchS12 is dissimilar to the chromosomal VgrG proteins but, like VgrG-2, lacks a C-terminal effector domain. The DUF4123 domain found in the protein encoded downstream of the vgrG gene indicates a function as an accessory loading proteins like tap-1 (VC1417 in the auxiliary locus of V. cholerae V52), which is responsible for the loading of cargo effectors with antibacterial activity onto VgrG proteins. Due to the structural similarity of this locus with chromosomal auxiliary loci, it is likely that the hypothetical protein encoded by ORF 11 (Fig. 1) represents such a cargo effector. Antibacterial T6SS effectors are always accompanied by immunity proteins that provide protection against self-intoxication, making it likely that the homologous proteins encoded by ORFs 12, 14a and 14b act as such. ORF 12 is 61% and 62% identical at the nucleotide level to ORFs 14a and 14b, respectively. ORFs 14a and 14b share 89% nucleotide identity. Expression of effectors from the GIVchS12 T6SS locus is likely to increase the range of T6SS-mediated microbial toxicity as evidenced by the divergent VgrG protein and one or more putative other effectors encoded on GIVchS12. It is therefore likely that this increase in effector repertoire gives cells harboring GIVchS12 an advantage over other cells in T6SS-mediated competition. A series of other genes encoding hypothetical proteins are in close proximity to the homologous proteins encoded by ORFs 12, 14a and 14b, which may have a role in either forming the T6SS apparatus or act as effector proteins. For example, ORFs 13 and 15 (also homologues of each other) encode Zn-binding Pro-Ala-Ala-Arg (PAAR) proteins. Zn-binding PAAR proteins form a conical extension on the VgrG tip and also function to attach effector proteins to the spike. More research is required to gain insight on how V. cholerae S12 regulates expression of the different Hcp and effector proteins and to determine the enzymatic activity of such effector proteins.

A successful and globally distributed genomic island. In order to determine whether GIs similar to GIVchS12 were present in the VPI-1 site of other V. cholerae genomes, we compared GIVchS12 to 28 other V. cholerae strains for which genome sequences are available in public databases. The non-O1/O139 strain from Haiti (2012Env-2) contained a complete GIVchS12-like island with similar CRISPR-Cas and T6SS modules and a complete set of ORFs 16–25 (Fig. 2). RC385 has an almost complete GIVchS12 but lacks ORF14b. A further three GIVchS12-like islands had minor variations. The non-O1/O139 strain 2012Env-32 from Haiti lacks ORF24 and...
three other ORFs in the CRISPR-Cas module and the non-O1/O139 strain 8–76 and O1 strain A325 from India and Argentina vary in their CRISPR-Cas modules. A325 also lacks ORF14b. Fifteen other GIs were identified containing a CRISPR-Cas module and/or a T6SS, although for many (in the upper most clade; see Fig. 2), we were unable to locate a contig containing VC0816 so it is unclear if the GI continues beyond ORF14a. One GI (in strain VCC19) harboured a T6SS but contained other genes instead of the CRISPR-Cas module and ORFs 16–25. Two other GIs in strains 490–93 and HE-48 contained a divergent CRISPR-Cas modules and other genes instead of ORFs16-25. Given that all these strains have been isolated from different geographic locations (Supplementary Material) including Europe, South America, North America, and Asia, this data indicates that GIs with VPI-1 recombination characteristics are active in disseminating CRISPR-Cas and T6SS modules across the globe.

A genomic island that readily excises as a circle. To confirm that the GI could excise as a circle, a nested PCR strategy was employed using primers annealing within the GI and facing outward toward the attL and attR ends. First, primers ML130 and ML131 were used in a PCR reaction with template derived from a plasmid preparation of V. cholerae S12. Next, 1 μl from the ML130/ML131 PCR was used as template for a fresh PCR reaction employing primers ML126 and ML127 (relative primer binding sites are shown in Fig. 1A) giving an expected fragment of ~580-bp (Fig. 3). As expected, sequence of the PCR product showed that excision occurs at the att sites abutting the GI producing an attP site identical to what is observed when VPI-1 excises from the genome44.
previously seen with excision of VPI-1\textsuperscript{44}. VPI-1 uses both a phage-like integrase and a transposase protein called VpiT to facilitate excision of the GI\textsuperscript{44}. Genes encoding these proteins are present within VPI-1.

However, in some \textit{V. cholerae} pandemic strains, vpiT is in a different location\textsuperscript{44}. VpiT or a homologue of VpiT was not found in \textit{GiVchS12} or in the S12 genome.

In conclusion, we report an interesting GI with VPI-1 recombination characteristics housing a CRISPR-Cas element and a T6SS auxiliary locus. This GI is likely to provide its host with a competitive advantage by protecting from bacteriophages as well as adding T6SS-associated bactericidal effectors proteins. Furthermore, this study shows that GIs with VPI-1 recombination characteristics carrying CRISPR-Cas and T6SS modules are circulating in natural \textit{V. cholerae} populations globally.

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M.L. conceived the experiments and wrote the manuscript. A.M. and N.R.T. sequenced the
V. cholerae genome. N.D.J., W.L.K., and C.A. conducted the experiments. M.L., F.D.O., N.K.P., P.C.K., and Y.B. performed recombination characteristics contains CRISPR-Cas and type VI secretion modules.

Author Contributions

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Author Contributions

M.I. conceived the experiments and wrote the manuscript. A.M. and N.R.T. sequenced the V. cholerae S12 genome. N.D.J., W.L.K., and C.A. conducted the experiments. M.L., F.D.O., N.K.P., P.C.K., and Y.B. performed bioinformatic analyses. G.M. and I.G.C. helped supervise the project. All authors reviewed the manuscript.

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