IncA/C Conjugative Plasmids Mobilize a New Family of Multidrug Resistance Islands in Clinical Vibrio cholerae Non-O1/Non-O139 Isolates from Haiti

Nicolas Carraro,a Nicolas Rivard,a Daniela Ceccarelli,a,b,c Rita R. Colwell,c,d,e Vincent Burrusa

Laboratory of Bacterial Molecular Genetics, Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada; Central Veterinary Institute of Wageningen UR, Leysstad, The Netherlands; Maryland Pathogen Research Institute, University of Maryland, College Park, Maryland, USA; Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA; Center for Bioinformatics and Computational Biology, University of Maryland Institute for Advanced Computer Studies (UMIACS), University of Maryland, College Park, Maryland, USA

Nicolas Carraro and Nicolas Rivard contributed equally to this work.

ABSTRACT Mobile genetic elements play a pivotal role in the adaptation of bacterial populations, allowing them to rapidly cope with hostile conditions, including the presence of antimicrobial compounds. IncA/C conjugative plasmids (ACP)s are efficient vehicles for dissemination of multidrug resistance genes in a broad range of pathogenic species of Enterobacteriaceae. ACPs have sporadically been reported in Vibrio cholerae, the infectious agent of the diarrheal disease cholera. The regulatory network that controls ACP mobility ultimately depends on the transcriptional activation of multiple ACP-borne operons by the master activator AcaCD. Beyond ACP conjugation, AcaCD has also recently been shown to activate the expression of genes located in the Salmonella genomic island 1 (SGI1). Here, we describe MGVchHai6, a novel and unrelated mobilizable genomic island (MGI) integrated into the 3’ end of trmE in chromosome I of V. cholerae HC-36A1, a non-O1/non-O139 multidrug-resistant clinical isolate recovered from Haiti in 2010. MGVchHai6 contains a mercury resistance transposon and an integron In104-like multidrug resistance element similar to the one of SGI1. We show that MGVchHai6 excises from the chromosome in an AcaCD-dependent manner and is mobilized by ACPs. Acquisition of MGVchHai6 confers resistance to β-lactams, sulfamethoxazole, tetracycline, chloramphenicol, trimethoprim, and streptomycin/spectinomycin. In silico analyses revealed that MGVchHai6-like elements are carried by several environmental and clinical V. cholerae strains recovered from the Indian subcontinent, as well as from North and South America, including all non-O1/non-O139 clinical isolates from Haiti.

IMPORTANCE Vibrio cholerae, the causative agent of cholera, remains a global public health threat. Seventh-pandemic V. cholerae acquired multidrug resistance genes primarily through circulation of SXT/R391 integrative and conjugative elements. IncA/C conjugative plasmids have sporadically been reported to mediate antimicrobial resistance in environmental and clinical V. cholerae isolates. Our results showed that while IncA/C plasmids are rare in V. cholerae populations, they play an important yet insidious role by specifically propagating a new family of genomic islands conferring resistance to multiple antibiotics. These results suggest that non-epidemic V. cholerae non-O1/non-O139 strains bearing these genomic islands constitute a reservoir of transmissible resistance genes that can be propagated by IncA/C plasmids to V. cholerae populations in epidemic geographical areas as well to pathogenic species of Enterobacteriaceae. We recommend future epidemiological surveys take into account the circulation of these genomic islands.

The diarrheal disease cholera remains a serious public health threat worldwide (1). The O1 and O139 toxigenic strains of Vibrio cholerae, the etiologic agent of cholera, produce a toxin that causes profuse diarrhea, vomiting, and subsequent severe dehydration (2). While proper hydration is usually sufficient to treat cholera patients, antibiotic therapy is necessary to treat severe cases and to reduce the release of this infectious agent into the environment (1). Toxigenic V. cholerae is very efficient at rapidly infecting and spreading among and between human populations (1, 3). Stepwise evolution of pathogenic lineages of V. cholerae has involved acquisition of adaptive traits, such as antibiotic resistance genes and virulence factors encoded by diverse mobile genetic elements, including those coding for cholera toxin and Carsonellaceae, including environmental and clinical V. cholerae isolates (8–10). ICEVchInd5, an ICE conferring resistance to sulfamethoxazole-
trimethoprim (co-trimoxazole), streptomycin, and chloramphenicol has been shown to be the most prevalent variant of SXT/R391 ICE in the seventh-pandemic multidrug-resistant V. cholerae lineage (5–7, 11). While the role of SXT/R391 ICEs has been extensively studied over the last two decades, recent studies have also highlighted the sporadic involvement of conjugative plasmids of the IncA/C group (ACPs) in genome plasticity and multidrug resistance (MDR) acquisition in V. cholerae (12–18).

ACPs are large plasmids (110 kb) that transfer efficiently by conjugation to and maintenance in a broad range of Gammaproteobacteria (19, 20). ACPs are a threat to human and animal health due to their worldwide prevalence in clinical isolates of bacterial enteric pathogens and their carriage of a large variety of antibiotic resistance genes (18, 19, 21–24). Several reports have associated ACPs with resistance to penicillins, cephalosporins, and carbapenems, conferred by allelic variants of the blaNDM gene (25–28).

Our group has previously described pVCR94, a type 2 IncA/C2 plasmid conferring MDR to the V. cholerae O1 El Tor strain responsible for the 1994 explosive cholera outbreak in Goma refugee camps (Democratic Republic of the Congo) (12). pVCR94 was used to characterize the main regulatory pathway that controls ACP conjugative transfer (12, 20, 21, 29). We have shown that acaCD encodes the master activator of ACP transfer that activates transcription of 18 ACP-borne genes and operons, including those coding for the conjugative machinery (21). In addition, AcaCD was shown to trans-activate excision and dissemination of MDR-conferring Salmonella genomic island 1 (SGI1) and unrelated MGIVHa18 of Vibrio mimicus (Fig. 1) (20, 21, 29).

The presence of an MDR-conferring ACP in a 2012 V. cholerae isolate from Haiti has been reported only once to date (17). We report here the insidious role played by ACPs in the spread of MDR through mobilization of a new family of MDR-conferring genomic islands (GIs) in clinical non-O1/non-O139 V. cholerae isolates from Haiti. The presence of an ACP specifically triggers the excision and conjugative transfer of MGIvHai6, the prototypic member of this new family of mobilizable GIs (MGIs).

**RESULTS**

M brief determinants of V. cholerae HC-36A1 are neither carried on nor mobilized by SXT/R391 ICEs. V. cholerae HC-36A1 is a non-O1/non-O139 clinical isolate recovered in 2010 in the province of Port-au-Prince, Haiti at the beginning of the ongoing cholera outbreak in that country (4). Antibiotic susceptibility tests showed HC-36A1 is resistant to multiple antibiotics, including ampicillin, sulfamethoxazole, trimethoprim, chloramphenicol, kanamycin, streptomycin, and spectinomycin, and exhibits an inducible resistance to tetracycline when the strain is preexposed to trimethoprim (co-trimoxazole), streptomycin, and chloramphenicol has been shown to be the most prevalent variant of SXT/R391 ICE in the seventh-pandemic multidrug-resistant V. cholerae lineage (5–7, 11). While the role of SXT/R391 ICEs has been extensively studied over the last two decades, recent studies have also highlighted the sporadic involvement of conjugative plasmids of the IncA/C group (ACPs) in genome plasticity and multidrug resistance (MDR) acquisition in V. cholerae (12–18).

ACPs are large plasmids (110 kb) that transfer efficiently by conjugation to and maintenance in a broad range of Gammaproteobacteria (19, 20). ACPs are a threat to human and animal health due to their worldwide prevalence in clinical isolates of bacterial enteric pathogens and their carriage of a large variety of antibiotic resistance genes (18, 19, 21–24). Several reports have associated ACPs with resistance to penicillins, cephalosporins, and carbapenems, conferred by allelic variants of the blaNDM gene (25–28).

Our group has previously described pVCR94, a type 2 IncA/C2 plasmid conferring MDR to the V. cholerae O1 El Tor strain responsible for the 1994 explosive cholera outbreak in Goma refugee camps (Democratic Republic of the Congo) (12). pVCR94 was used to characterize the main regulatory pathway that controls ACP conjugative transfer (12, 20, 21, 29). We have shown that acaCD encodes the master activator of ACP transfer that activates transcription of 18 ACP-borne genes and operons, including those coding for the conjugative machinery (21). In addition, AcaCD was shown to trans-activate excision and dissemination of MDR-conferring Salmonella genomic island 1 (SGI1) and unrelated MGIVHa18 of Vibrio mimicus (Fig. 1) (20, 21, 29).

The presence of an MDR-conferring ACP in a 2012 V. cholerae isolate from Haiti has been reported only once to date (17). We report here the insidious role played by ACPs in the spread of MDR through mobilization of a new family of MDR-conferring genomic islands (GIs) in clinical non-O1/non-O139 V. cholerae isolates from Haiti. The presence of an ACP specifically triggers the excision and conjugative transfer of MGIvHai6, the prototypic member of this new family of mobilizable GIs (MGIs). Further in silico analyses revealed the presence of MGIVHai6-like elements in several environmental and clinical strains of V. cholerae isolated in North and South America and the Indian subcontinent. Our results demonstrate that ACPs have influenced the evolution of Haitian V. cholerae strains by propagating genomic islands, thereby allowing circulation of a vast reservoir of mobilizable MDR genes.
subinhibitory concentrations of tetracycline (Table 1). The vast majority of Haitian outbreak strains carry ICEVchHai5 (also known as ICEVchHai1), a member of the SXT/R391 family of ICEs that mediates resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (3, 30). Although most SXT/R391 ICEs are easily transferable between V. cholerae and Escherichia coli, our attempts to transfer resistance markers by conjugation, from V. cholerae HC-36A1 to either a rifampin-resistant derivative of E. coli MG1655 (MG1655 Rf) or the tetracycline-resistant E. coli strain CAG18439, did not generate any transconjugants (Fig. 2A). Analysis of the genome of HC-36A1 (GenBank accession no. AXDR01000001) confirmed the presence of an SXT/R391 ICE virtually identical to ICEVchHai2, an 83-kb ICE first identified in non-O1/non-O139 V. cholerae isolate HC-1A2 but lacking the typical antibiotic resistance gene cluster found in SXT/R391 ICEs (30). This result indicates that the resistance determinants carried by HC-36A1 are not carried by an SXT/R391 ICE or a genomic island that they can mobilize in trans or in cis (20, 31–33).

V. cholerae HC-36A1 holds a complex MDR-associated integron reminiscent of In104 from Salmonella genomic island 1. Sequence analysis of the HC-36A1 genome aimed at localizing MDR determinants uncovered a new resistance gene cluster in chromosome I (Fig. 1). This locus contains a complex integron, In36A1, which exhibits structure and gene content relatively similar to those of the In104 resistance complex integron of SGI1 (Fig. 1) (34). Like In104, In36A1 contains the virtually identical dfrA23 (streptomycin/spectinomycin) variant sharing only 89% identity with dfrA23 of SGI1 and a trimethoprim resistance determinant sharing 93% identity with dfrA23, as a larger insertion between two sul1 copies (Fig. 1).

In36A1 is surrounded by additional putative resistance loci: (i) a mercury resistance Tn5053-like transposon, Tn6310, and (ii) a gene cluster coding for an hsd-like type I restriction-modification (RM) system that can confer resistance to bacteriophage infections (35–37). Such accumulation of adaptive determinants within a 38-kb fragment hinted at the presence of a larger genomic island.

---

### TABLE 1 MICs of 12 antibiotics against V. cholerae and E. coli with or without MGIVchHai6

| Antibiotic | V. cholerae HC-36A1 | E. coli CAG18439 | E. coli CAG18439 MGIVchHai6 |
|------------|---------------------|-----------------|-----------------------------|
|            | MIC (µg/ml)         | Phenotype      | MIC (µg/ml) | Phenotype | MIC (µg/ml) | Phenotype |
|            | a                    | b               |            | c                      |            |          |
| Ampicillin | >120                 | R               | <25        | S                    | >800       | R         |
| Chloramphenicol | >32              | R               | 10         | S                    | >160       | R         |
| Ciprofloxacin | 0.5               | S               | <0.13      | S                    | <0.13      | S         |
| Erythromycin | <200               | S               | 100        | S                    | 100        | S         |
| Gentamicin | 2.5                 | S               | <1.25      | S                    | <1.25      | S         |
| Kanamycin | 120                 | R               | 25         | S                    | 25         | S         |
| Nalidixic acid | <10                | S               | 20         | S                    | 20         | S         |
| Rifampin | 100                 | S               | 25         | S                    | 25         | S         |
| Streptomycin | >40                | R               | <50        | S                    | >1,600     | R         |
| Spectinomycin | >80                | R               | 100        | S                    | >400       | R         |
| Sulfamethoxazole | ND                | S               | ND         | ND                   | ND         | R         |
| Tetracycline | 3–40               | I               | –          | R                    | –          | R         |
| Trimethoprim | >40                | R               | <8         | S                    | >256       | R         |

a ND, not determined. Tests were done using solid agar plates.

b R, resistant; S, susceptible; I, inducible resistance.

c Tetracycline resistance of strain HC-36A1 ranges from 3 µg/ml to 40 µg/ml, without and with induction using 3 µg/ml of tetracycline as the inducible treatment, respectively. Tetracycline resistance tests were not carried out for the tetracycline-resistant strain CAG18439 (∼).
In36A1 is part of a new family of genomic islands. Analysis of the nearby sequence revealed proximity of a tRNA modification GTPase-encoding gene trmE, the 3’ end of which is the target site for SG11 integration in Salmonella enterica (38–40). Located next to the trmE integration site is a gene, int, encoding a predicted tyrosine recombinase/integrase (VCHC36A1_0088) distantly related to the integrase of SG1 (67% identity over 386 amino acid residues). Moreover, the gene adjacent to int, xis, codes for a predicted recombination directionality factor (RDF1) (VCHC36A1_0087) sharing 37% identity over 106 amino acid residues with Xis of SG1. Similar to SG11, two imperfect direct repeats (differences underlined) were identified as part of the attL (TTCTGTATGG GAAGTAA) and attR (TTCTGTATTGGCAAGTAA) attachment sites flanking the 47,437-bp genomic island VChvHai6. While VChvHai6 and SG11 share distantly related recombination modules (int/xis) and closely related complex resistance integrons (In36A1 and In104, respectively), the remainder of the gene content is strikingly different (Fig. 1). Therefore, MGI

V. cholerae HC-36A1 to the donor cell, pVCR94 strains, as well as the possible retrotransfer of MGI

vent the presence of redundant kanamycin resistance in the two

MGI (In36A1 and In104, respectively), the remainder of the gene content

GAAGTAA) and 0087) sharing 37% identity over 106 amino acid residues with Xis

V. cholerae X3 was transferred to E. coli K-12 as a recipient. The presence of pVCR94ΔX3 specifically

The excision of MGIvChHai6 in the presence of an MDR island in the same cell prompted us to test whether pVCR94ΔX3 could mediate MGIvChHai6 conjugal transfer into E. coli. Therefore, V. cholerae HC-36A1 carrying pVCR94ΔX3 was used as a donor in mating assays using E. coli K-12 as a recipient. The presence of pVCR94ΔX3 specifically

Since we were unable to observe MDR transfer from HC-36A1.

ICE 10

traited at a rate of 1.3 × 10^{-3} exconjugant per recipient cell, the rate of cotransfer of both MGIvChHai6 and pVCR94ΔX3 was below the limit of detection, suggesting it might be a rare event (Fig. 2A).

Master activator AcaCD specifically triggers excision of MGIvChHai6. We further investigated whether excision of MGIvChHai6 was specifically dependent upon AcaCD. E. coli bearing MGIvChHai6 with or without a single chromosomally integrated copy of pacaDC_{FLAG}, which expresses AcaCD under control of P_{mcs}, was used in PCR experiments to detect attB and attP sites resulting from MGIvChHai6 excision.

Excision assays revealed that the presence of AcaCD specifically triggers excision of MGIvChHai6, as shown by detection of specific attB and attP sites after IPTG (isopropyl-ß-D-thiogalactopyranoside) induction (Fig. 2B). Excision of MGIvChHai6 was also observed without induction of acaCD expression, likely due to leaky transcription from P_{mcs} allowing sufficient production of AcaCD to induce the AcaCD-dependent promoters of MGIvChHai6 (21, 41). In contrast, attB and attP could not be detected in the strain expressing SetCD, the master activator of XST/R391 ICE conjugation, thereby confirming that MGIvChHai6 excision is specifically triggered by ACP-encoded AcaCD (Fig. 2B). No spontaneous excision of MGIvChHai6 was detected using the control strain devoid of acaCD- or setCD-bearing plasmids.

MGIvChHai6-like elements are widespread among V. cholerae epidemic and environmental strains. To assess the diversity and abundance of MGIvChHai6-like elements among available genome sequences, in silico analysis was conducted using the attL-int DNA portion as a query to retrieve related genomic islands integrated into the 3’ end of trmE. Using this 1,352-nucleotide (nt) sequence in a search of the GenBank nucleotide collection database (nt/nt) targeting Gammaproteobacteria yielded only two significant matches, namely, against Shewanella putrefaciens genomes, one of which was previously detected in S. putrefaciens 200 as GIISp1 (Fig. 3) (29). Further analyses targeting the whole-genome shotgun sequences and narrowing the analysis to Vibrio
revealed the presence of MGI$_{Vch}$Hai6-related elements in the genome of various environmental and clinical _V. cholerae_ strains (Fig. 3). Closer examination of these strains revealed that MGI$_{Vch}$Hai6-related elements are not associated with a specific serotype or geographic location, because different strains were recovered from the Indian subcontinent, as well as from North and South America, including all non-O1/non-O139 clinical isolates from the 2010 outbreak in Haiti (Fig. 3). Interestingly, MGI$_{Vch}$Hai6-related GIs were also detected in strains isolated from various locations in the 1980s, demonstrating that they had existed in _V. cholerae_ populations prior to the 2010 Haitian cholera outbreak.

The evolutionary history of the _attL-int_ locus of MGI$_{Vch}$Hai6-related GIs was inferred using sequences recovered from BLAST analyses and using SGI1 as an outgroup (Fig. 1 and 3). Three strongly supported clades of elements were delineated. One representative GI of each cluster was chosen for schematic representation: MGI$_{Vch}$Hai6 from strain HC-36A1 (green cluster), the 19-kb element GI$_{Vch}$Hai7 from strain HC-43B1 (orange cluster), and GI$_{Vch}$Bra1 and GI$_{Vch}$Hai8 (17.6 kb) found in their respective strains 116063 and HE-45 (blue cluster) (Fig. 1 and 3). All _V. cholerae_ isolates possessing MGI$_{Vch}$Hai6-like elements were clinical non-O1/non-O139 strains from the 2010 Haitian outbreak (Fig. 3, green cluster). Because analyses had been done on nonassembled draft genome sequences, we were not able to determine the exact structure of each member, but detection ranged from the complete MGI$_{Vch}$Hai6 to partial detection of Tn6310 upstream of _attR_.
of the MGI (21). The presence of an AcaCD-activated activating element such as conjugative plasmids and ICEs (5, 45–47). In particular, both SXT/R391 ICEs and ACPs were shown to be distantly related as they share limited homology in their respective ORFs (7, 11, 21, 41, 48–50). In particular, both SXT/R391 ICEs and ACPs have refined our understanding of the biology of these major determinants circulating among pathogenic and environmental bacteria. Previous studies pointed out the major role of mobile genetic elements in propagation of MDR, notably conjugative elements such as conjugative plasmids and ICEs (5, 45–47).

Recent progress in deciphering the biology of SXT/R391 ICEs and ACPs has refined our understanding of the biology of these major drivers of the distribution of MDR among the Gammaphage-βaetes (7, 11, 21, 41, 48–50). In particular, both SXT/R391 ICEs and ACPs were shown to trans-mobilize many MGIs conferring adaptive traits to their bacterial host, including MDR (21, 29, 31, 32, 51). These MGIs were found to be activated by the master activator of SXT/R391 ICEs (SetCD) or ACPs (AcaCD), thus being part of the extended regulatory network of these autonomous conjugative elements (20). Functional studies of the biology of SXT/R391 ICE-dependent MGIs has revealed key features, but the precise mechanisms allowing dissemination of ACPs-dependent MGIs remain largely unknown (31, 32). Based on recent findings, it is hypothesized that the superfamily of MGIs bearing AcaCD-activated homologs of mobl and xis, including the MGIs described here, as well as other elements previously discovered (MGIVm1, GIVm2, GIVp1, and GISPp1), share a mechanism of mobilization (21, 29). Transcriptional data obtained on MGIVm1 showed that AcaCD specifically triggers transcription of a recombination directionality factor (RDF)-encoding gene (xis), allowing excision of the MGI (21). The presence of an AcaCD-activated mobl homolog strongly suggests that, as shown for SXT/R391 ICEs and ACPs, the upstream intergenic region likely constitutes the origin of transfer (oriT) of the MGI (12, 20, 52). The current working model surmises that, upon AcaCD activation, Mobl of the MGI likely recognizes its cognate oriT and recruits the ACP Mobl-less relaxosome that processes the DNA to transfer one strand of the MGI, regardless of the presence of helper ACP. Functional characterization of each gene and precise determination of the oriT locus are ongoing using different representative of the above-mentioned MGIs.

Current sequence analyses indicate that the size of the core sequence of the MGI family is ca. 8 kb and that it corresponds to DNA regions from attL to mobl and from pinE to attR (Fig. 1). pinE, which codes for a putative recombinase/invertase, is unlikely to be involved in excision, integration, or mobilization of the MGIs as it serves as an insertion site for the In36A1 resistance gene cluster, which moves by transposition (34, 53). While GIVch-Ha16 carries antibiotic resistance genes, as well as a mercuric resistance transposon, the majority of adaptive traits encoded by related MGIs appear to be limited to RM systems (Fig. 1) (20). The presence of RM systems is considered to be ubiquitous in such MGIs, likely conferring strong selective advantage to their host against bacteriophages, which thrive in aquatic environments. Nevertheless, a single event of transposition could lead to acquisition of an In104-like element and its associated MDR phenotype. Moreover, the presence of an integrase likely allows further antibiotic resistance gene acquisition from, and exchange with, other integrons carried by other mobile genetic elements (46).

Although MGIs have been recently discovered, these elements are not specific to recent multidrug-resistant isolates of bacteria. For instance, GIVch-Bra1 was found in the genome of a V. cholerae strain isolated in 1978 in Brazil and appears to have circulated since then, with isolation of its sibling GIVch-Ha16 in 2010 in Haiti (Fig. 1 and 3). Such sequence conservation implies a very recent transfer event or a highly active element that does not accumulate mutations while quiescent in the chromosome of its bacterial host (54). Further large-scale analyses of sequence databases facilitated by very rapid addition of many additional bacterial genomes provided by massive sequencing, together with ongoing functional dissection of their biology, will most likely clarify the dynamics of these MGIs.

SXT/R391 ICEs were defined as major drivers of MDR among V. cholerae strains, generating a strong bias toward their detection in epidemiological reports (9, 10, 55). More recent studies highlight the presence of ACPs as MDR determinants in clinical and environmental V. cholerae strains (12–18). While ACPs are widely distributed in many species of pathogenic bacteria, their occurrence in V. cholerae is less well characterized. It is plausible that ACPs are not stably maintained in V. cholerae or that the role of ACPs in MDR acquisition by V. cholerae was overlooked. In agreement with the first hypothesis, sequence analyses of V. cholerae strains analyzed in this study using the IncA/C2_repA gene revealed that only the Indian environmental strains 116-14, 116-17a, and 116-17b contain an ACP (18). Nevertheless, ACPs likely have a more insidious role in the dissemination of MDR by not remaining in Vibrion strains but efficiently promoting circulation of MDR-associated MGIs and the adaptive traits that they may confer. In the future, circulation of GIVch-Ha16 and related elements driven by ACPs should be monitored as they could enhance and accelerate dissemination of MDR in V. cholerae and related pathogens.

**MATERIALS AND METHODS**

**Bacterial strains and media.** The bacterial strains and plasmids used in this study are described in Table 2. The strains were routinely grown in lysogeny broth (LB-Miller; EMD) at 37°C in an orbital shaker/incubator.
Table 2: Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or phenotype | Reference(s) |
|-------------------|-------------------------------|--------------|
| V. cholerae HC-36A1 | Clinical, non-O1/non-O139, Haiti (Tabarre) 2010 (Ap Cm Km Sn Sm Tc Tm) | 4 |
| E. coli MG1655 Rf | Rf derivative of MG1655 (Rf) | 52 |
| CAG18439 | MG1655 lacZU18 lacI42::Tn10 (Tc) | 63 |
| β2163 | (F−) RP4-2-Tc::Mu dapA::erm-par (Kn Em) | 43 |
| Plasmids | | |
| pVCR94ΔX3 | Kn’ derivative of IncA/C plasmid pVCR94 (Kn Su) | 21 |
| psaCD*×FLAG | pAH56::psaCD*×FLAG (Kn) | 21 |
| psx DC*×FLAG | pAH56::psx DC*×FLAG (Kn) | 21, 41 |

* Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Kn, kanamycin; Rf, rifampin; Sm, streptomycin; Sp, spectinomycin; Su, sulfamethoxazole; Tc, tetracycline; Tm, trimethoprim.

Table 3: Primers used in this study

| Name | Nucleotide sequence (5’ to 3’) | Reference |
|------|-------------------------------|-----------|
| thdF_attBF | TATAGCCCCAGCAACACCTTA | This study |
| int1_attPR | GGAATTCTGTTTTATGATGCGTA | This study |
| 43_attPF | GCAATATTATGATAAAGACCGGTA | This study |
| int2_attBR | GGATATACCGGTGCTAATATGAT | This study |
| EcU7-L12_for | AACTCTACACAGGCACCAG | 38 |
| Ec164D_rev | ACCATTGCATTGACCATACCA | 38 |
| promvcrx60ralpsL.for | NNNNNNCTGACGATCAAAATTGTCATGTA | 21 |
| promvcrx60ralpsL.rev | NNNNNNCTGACGATCAAAATTGTCATGTA | 21 |
| GIVchIverif.for | TGCAGATGGTCGAAGAAAGTTC | This study |
| GIVchIverif.rev | AATCCGGTGTTTATAGGTCCTC | This study |

Detection of MGIvchHai6 excision. Excision of MGIvchHai6 was detected by PCR on genomic DNA of the appropriate strains, using the primers listed in Table 3. The attR site was amplified using primer pair 43_attPF/int2_attBR in V. cholerae and pair 43_attPF/Ec164D.rev in E. coli. The attB chromosomal site was detected using thdF_attBF/int2_attBR in V. cholerae and EcU7-L12.for/Ec164D.rev in E. coli (38). The attP site carried by the extrachromosomal circular form of MGIvch-Hai6 was amplified using the primer pair 43_attPF/int1_attPR.

Sequence annotations. Detection of the attL and attR attachment sites flanking MGIvchHai6 and related genomic islands was carried out using the software YASS to identify the direct repeats (57). Genes were predicted using the RAST pipeline (58), and spurious annotations were manually curated. Antibiotic resistance determinants were detected using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and The Comprehensive Antibiotic Resistance Database (http://арарd.mcmaster.ca/?q=CARD/tools/RLGI). Precise locations of AcaCD binding motifs were searched using FIMO (59) against the sequence of MGIvchHai6, GIVchHai7, and GIVchBra1 with the AcaCD and SetCD MEME logos, as described elsewhere (21, 41).

Phylogenetic analyses. Molecular phylogenetic analysis of the attL−int locus was performed in MEGA6 (60). The 1,351-bp nucleotide sequence of MGIvchHai6 encompassing 18 nt at the end of tsrM that correspond to the attL site direct repeat and ending at the top codon of int was used to search for homologous sequences in the GenBank nucleotide collection database (nt/nt targeting Gammmaproteobacteria and WGS targeting Vibrionaceae) using nucleotide BLAST (61). The corresponding sequence in Salmonella genomic island 1 (SGI1) was manually added to the data set. Phylogenetic analyses were computed using a nucleotide alignment generated by MUSCLE (62). The evolutionary history was inferred by using the maximum likelihood method. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and selecting the topology

and preserved at −80°C in LB broth containing 15% (vol/vol) glycerol. For E. coli, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; gentamicin, 10 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 40 μg/ml; rifampin, 50 μg/ml; spectinomycin, 50 μg/ml; streptomycin, 200 μg/ml; sulfamethoxazole, 160 g/ml; and tetracycline, 10 g/ml. For V. cholerae, antibiotics were used at the following concentrations: chloramphenicol, 2 μg/ml; kanamycin, 30 μg/ml; streptomycin, 10 μg/ml; and tetracycline, 10 μg/ml. When required, bacterial cultures were supplemented with 0.3 mM N-2,6-diaminopimelic acid (DAP) or 0.02 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Antibiotic susceptibility profiling and MIC determination were performed using broth dilution tests (56).

Bacterial conjugation assays. Conjugation assays were performed as described by Carraro et al. (12). Donors, recipients, and exconjugants were selected on LB agar plates containing the appropriate antibiotics. If needed, additional controls were done on isolated clones using selective media, including thiosulfate-citrate-bile salts-sucrose (TCBS) agar for identification of clones using selective media, including thiosulfate-citrate-bile salts-sucrose (TCBS) agar for identification of V. cholerae and PCR amplification of the promoting region of pVCR94ΔX3 and/or an internal fragment of ORF86 of MGIvchHai6 using primer pairs promvcrx60ralpsL.for/promvcrx60ralpsL.rev and GIVchIverif.for/GIVchIverif.rev, respectively (Table 3).

Molecular biology methods. All enzymes used in this study were purchased from New England Biolabs. PCR assays were performed employing the plateforme de Séquençage et de Génotypage du Centre de Recherche du CHUL (Québec, QC, Canada).
REFERENCES

1. Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB. 2012. Cholera. Lancet 379:2466–2476. http://dx.doi.org/10.1016/S0140-6736(12)60436-X.

2. Waldor MK, Mekalanos JJ. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272:1910–1914. http://dx.doi.org/10.1126/science.272.5270.1910.

3. Weil AA, Ivers LC, Harris JB. 2014. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 345:10014. http://dx.doi.org/10.1126/science.aaa5092.

4. Garriss G, Déry C, Burrus V, Waldor MK. 2009. Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. PLoS Genet 5:e1000939. http://dx.doi.org/10.1371/journal.pgen.1000939.

5. LeClerc JE, Hinshaw JM, Lindler LE, Cebula TA, Carniel E, Ravel J. 2012. Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. PLoS Genet 5:e1000939. http://dx.doi.org/10.1371/journal.pgen.1000939.

6. Carattoli A. 2015. IncA/C conjugative plasmids. Plasmid 80:63–82. http://dx.doi.org/10.1016/j.plasmid.2015.04.003.

7. Poulin-Laprade D, Carraro N, Burrus V. 2015. The extended regulatory networks of SXT/R391 integrative and conjugative elements and IncA/C conjugative plasmids. Front Microbiol 6:837. http://dx.doi.org/10.3389/fmicb.2015.00837.

8. Carraro N, Matteau D, Luo P, Rodrigue S, Burrus V. 2014. The master activator of IncA/C conjugative plasmids stimulates genomic islands and multidrug resistance dissemination. PLoS Genet 10:e1004714. http://dx.doi.org/10.1371/journal.pgen.1004714.

9. Carattoli A. 2013. Plasmids and the spread of resistance. Int J Med Microbiol 303:298–304. http://dx.doi.org/10.1016/j.ijmm.2013.02.001.

10. Welch TJ, Fricke WF, McDermott PF, White DG, Rosso M-L, Rasko DA, Mammel MK, Eppinger M, Rosovitz MJ, Wagner D, Rahalison L, LeClerc JE, Hinshaw JM, Lindler LE, Cebula TA, Carniel E, Ravel J. 2007. Multiple antimicrobial resistance in plague: an emerging public health risk. PLoS One 2:e309. http://dx.doi.org/10.1371/journal.pone.0000309.

11. Fricke WF, Welch TJ, McDermott PF, Mammel MK, LeClerc JE, White DG, Cebula TA, Ravel J. 2009. Comparative genomics of the IncA/C multidrug resistance plasmid pIP1202-like plasmids. J Bacteriol 191:4750–4757. http://dx.doi.org/10.1128/JB.00923-09.

12. Sarkar A, Pazhani GP, Chowdhury G, Ghosh A, Ramamurthy T. 2015. Attributes of carbapenemase encoding conjugative plasmid pNDM-SAL from an extensively drug-resistant Salmonella enterica serovar Senftenberg. Front Microbiol 6:969. http://dx.doi.org/10.3389/fmicb.2015.00969.

13. Wailan AM, Sartor AL, Zowawi HM, Perry JD, Paterson DL, Sidjabat HE. 2015. Genetic contexts of blaNDM-1 in patients carrying multiple NDM-producing strains. Antimicrob Agents Chemother 59:7405–7410. http://dx.doi.org/10.1128/AAC.00319-15.

14. Liu C, Qin S, Xu H, Xu L, Zhao D, Liu X, Lang S, Feng X, Liu H-M. 2015. New Delhi metallo-β-lactamase 1 (NDM-1), the dominant carbapenemase detected in carbapenem-resistant Enterobacter cloacae from Henan Province, China. PLoS One 10:e0135044. http://dx.doi.org/10.1371/journal.pone.0135044.

15. Rahman M, Shukla SK, Prasad KN, Ovjerco CM, Pati BK, Tripathi A, Singh A, Srivastava AK, Gonzalez-Zorn B. 2014. Prevalence and molecular characterisation of New Delhi metallo-β-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant Enterobacteriaceae from six countries. Int J Antimicrob Agents 44:30–37. http://dx.doi.org/10.1016/j.ijantimicag.2013.03.003.

16. Carraro N, Matteau D, Burrus V, Rodrigue S. 2015. Unraveling the regulatory network of IncA/C plasmid mobilization: when genomic islands hijack conjugative elements. Mob Genet Elem 5:34–38. http://dx.doi.org/10.1801/2159256X.2015.1045116.

17. Ceccarelli D, Spagnolotti M, Hasan NA, Lansiing S, Huq A, Colwell RR.
Kholodii GY, Yurieva OV, Lomovskaya OL, Gorlenko Z, Mindlin SZ, Mulvey MR, Boyd DA, Olson AB, Doublet B, Cloeckaert A, Daccord A, Mursell M, Poulin-Laprade D, Burrus V. 2010. Replication and active partition of integrative and conjugative elements (ICEs) of the SXT/R391 family: the line between ICEs and conjugative plasmids is getting thinner. PLoS Genet 11:e1005298. http://dx.doi.org/10.1371/journal.pgen.10005298.

Garriss G, Waldor MK, Burrus V. 2009. Mobile antibiotic resistance encoding elements promote their own diversity. PLoS Genet 5:e1000775. http://dx.doi.org/10.1371/journal.pgen.1000775.

Carraro N, Poulin D, Burrus V. 2015. Identification of a new gene required for mobilization of the SXT/R391 family of conjugative elements. J Bacteriol 190:5328–5338. http://dx.doi.org/10.1128/JB.01500-08.

Plasterk RH, van de Putte P. 1985. The invertible P-DNA segment in the chromosome of Escherichia coli. EMBO J 4:237–242.

Touchon M, Bobay L-M, Rocha EP. 2014. The chromosomal accommodation and domestication of mobile genetic elements. Curr Opin Microbiol 22:22–29. http://dx.doi.org/10.1016/j.mib.2014.09.010.

Bellanger X, Payot S, Leblond-Bourget N, Guédon G. 2011. Integration enzymes and their relatives. Nucleic Acids Res 40:844–872. http://dx.doi.org/10.1093/nar/gkr847.

Levings RS, Lightfoot D, Partridge SR, Hall RM, Djordjevic SP. 2005. Type I restriction enzymes and their relatives. Nucleic Acids Res 33:W540–W543. http://dx.doi.org/10.1093/nar/gki478.

Noé L, Kucherov G. 2005. YASS: enhancing the sensitivity of DNA similarity search. Nucleic Acids Res 33:W320–W324. http://dx.doi.org/10.1093/nar/gki478.

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Gerdes K, Glass EM, Kubal S, Losey GE, Overbeek R, Parrello B, Pusch GD, Ratsch P, Visweshwar E, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics 9:75. http://dx.doi.org/10.1186/1471-2164-9-75.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37:W202–W208. http://dx.doi.org/10.1093/nar/gkp335.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. http://dx.doi.org/10.1093/molbev/ms317.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410. http://dx.doi.org/10.1016/0022-2836(90)90306-2.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. http://dx.doi.org/10.1093/nar/gkh340.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37:W202–W208. http://dx.doi.org/10.1093/nar/gkp335.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37:W202–W208. http://dx.doi.org/10.1093/nar/gkp335.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37:W202–W208. http://dx.doi.org/10.1093/nar/gkp335.