The Master Activator of IncA/C Conjugative Plasmids Stimulates Genomic Islands and Multidrug Resistance Dissemination

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Abstract

Dissemination of antibiotic resistance genes occurs mostly by conjugation, which mediates DNA transfer between cells in direct contact. Conjugative plasmids of the IncA/C incompatibility group have become a substantial threat due to their broad host-range, the extended spectrum of antimicrobial resistance they confer, their prevalence in enteric bacteria and their very efficient spread by conjugation. However, their biology remains largely unexplored. Using the IncA/C conjugative plasmid pVCR94.AX as a prototype, we have investigated the regulatory circuitry that governs IncA/C plasmids dissemination and found that the transcriptional activator complex AcaCD is essential for the expression of plasmid transfer genes. Using chromatin immunoprecipitation coupled with exonuclease digestion (ChIP-exo) and RNA sequencing (RNA-seq) approaches, we have identified the sequences recognized by AcaCD and characterized the AcaCD regulon. Data mining using the DNA motif recognized by AcaCD revealed potential AcaCD-binding sites upstream of genes involved in the intracellular mobility functions (recombination directionality factor and mobilization genes) in two widespread classes of genomic islands (GIs) phylogenetically unrelated to IncA/C plasmids. The first class, SGI1, confers and propagates multidrug resistance in Salmonella enterica and Proteus mirabilis, whereas MGIVm1 in Vibrio mimicus belongs to a previously uncharacterized class of GIs. We have demonstrated that through expression of AcaCD, IncA/C plasmids specifically trigger the excision and mobilization of the GIs at high frequencies. This study provides new evidence of the considerable impact of IncA/C plasmids on bacterial genome plasticity through their own mobility and the mobilization of genomic islands.

Introduction

Multidrug resistance (MDR) is steadily increasing in Gram-negative bacteria in both community and hospital settings, and represents a growing concern worldwide [1]. MDR usually results from the adaptation of microorganisms through various mutations or from the acquisition of foreign DNA by horizontal gene transfer. In recent years, conjugative plasmids of the IncA/C incompatibility group, which are prevalent in enteric bacteria, have become a substantial threat due to their broad host-range, their extended spectrum of antimicrobial resistance and their efficient spread by conjugation [2]. IncA/C plasmids were first identified more than 40 years ago from diseased fish infected by antibiotic-resistant Aeromonas hydrophila and Vibrio spp [3,4]. For more than three decades IncA/C plasmids received relatively little attention, but the rapid dissemination of these MDR-carrying vectors among enteric pathogens recovered from food-producing animals, food products and humans have sprung intensive research at the epidemiological and genomic level. Several IncA/C plasmids are spreading the New Delhi metallo-lactamase blaNDM-1 gene and its variants, which confer resistance to all β-lactams except for monobactams and are widely distributed throughout all Gammaproteobacteria [5–8]. Resistance to β-lactams, aminoglycosides, chloramphenicol, folate pathway inhibitors (sulfonamides and trimethoprim), quinolones and tetracycline is also commonly conferred by these large plasmids (ca. 140 to 200 kb) [9–13]. IncA/C plasmids have also been shown to mobilize in trans the Salmonella genomic island 1 (SGI1), a 43-kb chromosomal mobile element carrying a class 1 integron that confers resistance to ampicillin, chloramphenicol, streptomycin,
sulfonamides and tetracycline (ACSSuT phenotype) [14–16]. SGI1 and related MDR-conferring genomic islands (GIs) have been found in a large variety of Salmsonella enterica serovars and in Proteus mirabilis [17]. To date, the genetic regulatory network and the nature of the interactions allowing the specific mobilization of SGI1 by IncA/C helper plasmids remain largely unknown.

Comparative genomics studies of IncA/C plasmids isolated from Escherichia coli, S. enterica, Vibrio cholerae, Yersinia pestis, Yersinia ruckeri, Klebsiella pneumoniae and Providencia stuartii have revealed their close relationship [9,10,18,19]. IncA/C plasmids share a common core set of genes exhibiting more than 99% identity that is disrupted by antibiotic-resistance conferring cassettes. While several of these conserved genes are involved in conjugative transfer (tra genes) and replication (repA), most have unknown functions. IncA/C plasmids are distantly related to the integrative and conjugative elements (ICEs) of the SXT/R391 family, which are also broadly distributed among several species of Enterobacteriaceae and Vibrionaceae [10,19,20]. Although many IncA/C plasmids have been fully sequenced to date, little is known about their basic biology, and most importantly about the regulation of their dissemination by conjugation.

Previously, we have identified and characterized the IncA/C conjugative plasmid pVCR94 from the epidemic isolate V. cholerae O1 El Tor F1939 [19]. pVCR94 transfers at very high frequency (10⁻² to 10⁻³) across species and genera, and mediates resistance to co-trimoxazole, chloramphenicol, streptomycin, ampicillin and tetracycline [19]. Here, we have characterized the regulatory mechanisms that control the conjugative transfer function of IncA/C plasmids. Two transcriptional repressors, acr1 and acr2, were shown to repress the expression of two plasmid-encoded conserved genes, acaC and acaD, that are essential for the activation of IncA/C tra gene expression. Chromatin immunoprecipitation coupled to exonuclease digestion (ChIP-exo) and RNA sequencing (RNA-seq) assays were used to characterize the AcaCD regulon. Finally, bioinformatics analyses and experimental evidence revealed that the AcaCD regulon expands beyond IncA/C plasmid-borne genes to include GIs such as SGI1 and another unrelated GI of Vibrio mimicus. Altogether these results reveal a mechanism by which IncA/C conjugative plasmids play a role more important than previously estimated in bacterial genome dynamics.

**Author Summary**

Multidrug resistance is a major health concern that complicates treatments of even the most common infections caused by bacteria. In recent years, IncA/C plasmids have emerged and spread in bacteria infecting humans, food-producing animals and food products, driving at the same time the dissemination of a broad spectrum of antibiotic resistance genes in environmental and in clinical settings. In this study, we have characterized the regulatory pathway that governs IncA/C plasmid dissemination. We have found that AcaCD, the master activator complex encoded by these plasmids, is not only essential for the dissemination of IncA/C plasmids but also activates unrelated mobile genetic elements in bacterial genomes, thereby further promoting the interspecies propagation of multidrug resistance and other adaptive traits at a very high frequency.

**Results and Discussion**

**Identification of repressors of IncA/C plasmids transfer**

Comparative genomics previously revealed a set of six genes coding for putative transcriptional regulators in pVCR94 that are also conserved in nearly all IncA/C plasmids [19] (Table S1). To determine whether these genes are involved in the regulation of pVCR94 transfer, we constructed in-frame deletions and tested the ability of the resulting mutants to transfer by conjugation. For convenience, we carried out all of our assays using the 120,572-bp pVCR94AX mutant (referred to as pVCR94 in the rest of the paper), in which a single cluster containing all antibiotic resistance genes except for sul2 conferring resistance to sulfamethoxazole has been deleted [19]. Deletion of vcrx025, which codes for a putative HUβ-like DNA-binding protein, had no effect on plasmid transfer (3.94 × 10⁻³ exconjugant/donor for pVCR94Δvcrx025 compared to 3.51 × 10⁻³ exconjugant/donor for wild-type, \( P = 0.4400 \), two-tailed Student’s t-test). Despite several attempts, we were unable to delete vcrx027, which codes for a Cro-like transcriptional regulator, suggesting that its absence is lethal. The adjacent gene vcrx028 is predicted to code for a toxin protein (addiction module killer protein, IPR009241), suggesting that the co-transcribed gene vcrx027 encodes its cognate antitoxin (see Dataset S1).

The remaining four putative regulator genes cluster near the origin of transfer (oriT) (Figure 1A). Deletion of either vcrx146 (acr1), which codes for a putative Ner-like DNA-binding protein, or vcrx150 (acr2), which codes for a predicted H-NS-like DNA-binding protein, resulted in a 23- and 5.6-fold increase of the frequency of transfer, respectively, thereby suggesting that both genes code for repressors of pVCR94 transfer (Figure 1B). For this reason, vcrx146 and vcrx150 were renamed acr1 and acr2, for IncA/C repressors 1 and 2 respectively. Complementation of the acr1 and acr2 mutations by ectopic expression of the corresponding genes under control of an arabinose-inducible promoter (PRED) did not restore the wild-type transfer frequency (Figure 1B). However, overexpression of either acr1 or acr2 decreased transfer of wild-type pVCR94 by 14 and 3 fold, respectively. Since acr1 is the first gene of an operon structure (see Dataset S1) containing the putative transcriptional activator genes vcrx148 (acaD) and vcrx149 (acaC) (see below), we tested whether acr1 or acr2 are able to repress expression from PACA, the promoter driving expression of the operon containing acr1 (Figure 1A). To do this, we cloned PACA upstream of a promoterless lacZ gene and monitored the β-galactosidase activity upon expression of acr1 or acr2 driven by PREAD. While PACA was transcriptionally active in the absence of arabinose, no β-galactosidase activity was detected upon expression of either acr1 or acr2, confirming that both proteins are capable of directly repressing expression from PACA (Figure 1C).

AcaC and AcaD are key activators of IncA/C plasmids transfer

The cluster of genes acr1-vcrx147-acad-acad2 is extremely well conserved and remain syntenic among all IncA/C plasmids except for XCN1ₚ from Xenorhabdus nematophila in which it is completely absent (Figures 2 and S1). Interestingly, the IncA/C plasmids pAM04528 and pSN254 from S. enterica, which were reported to be non-self-transferable [10,21,22], code for a truncated AcaC protein resulting from a frameshift mutation (acaC263) (Figure 2), suggesting a key role of acaC in the activation of IncA/C-plasmid transfer. Unfortunately, no data is currently available about the intercellular mobility of five other plasmids coding for truncated AcaC or AcaD proteins (Figure 2) [23,24]. However, pRA1 was reported to transfer at a frequency of...
Figure 1. Regulation of IncA/C plasmids. (A) Schematic representation of the regulatory region of IncA/C plasmids. Genes and promoters are represented by arrowed boxes and angled arrows, respectively. Repressors and activators are shown in red and green, respectively. Genes of unknown function are shown in white and the gene coding for a putative lytic transglycosylase is shown in light blue. Locus names vcrxXXX are abbreviated as XXX. The oriT locus is depicted by a blue star. (B) Effect of the deletion and the overexpression of the negative transcriptional regulator-encoding genes acaC and acaR. Conjugation assays were carried out using as donors *E. coli* BW25113 Nx containing pVCR94ΔX2 (WT) or its ΔacaC and ΔacaR mutants. Complementation and overexpression assays were performed with (+) or without (−) arabinose for the expression of *acaR* (pacr1) or acaR (pacc2) from the inducible *P*<sub>lacZ</sub> promoter. *E. coli* MG1655 RF was used as the recipient. Transfer frequencies are expressed as a number of exconjugant per recipient CFUs. The bars represent the mean and standard deviation values obtained from at least three independent experiments. Statistical analyses were performed on the logarithm value of the means using one-way ANOVA with Tukey’s multiple comparison test. P-values are indicated next to the bars when comparison referred to WT or next to the brackets when comparing two bars. (C) The constitutive promoter of *acaR* (*P*<sub>acaR</sub>) is repressed by AcaR and AcaC. Activity of *P*<sub>acaR</sub> was monitored from a single-copy, chromosomally integrated lacZ transcriptional fusion (*P*<sub>acaR-lacZ</sub>). Colorimetric assays were carried out on LB medium supplemented with 40 μg/ml of X-Gal and induction with (+) or without (−) arabinose to express acaR, acaC, or acaCd from *P*<sub>bad</sub> on pacaC, pacc2 or pacaCD, respectively. (D) AcaC and AcaD are essential for conjugative transfer. Transfer assays were carried out using *E. coli* BW25113 Nx containing pVCR94ΔX2 (WT) or the mutants ΔacaC, ΔacaD or ΔacaCD. Complementation assays were performed by expressing acaC, acaD or acaCD from *P*<sub>bad</sub> on pacaC, pacc2 or pacaCD, respectively. Recipient strains and statistical analyses were as described for panel B. All P-values are below 0.0001 when compared to the WT. The asterisk indicates that frequency of exconjugant formation was below the detection limit (<10<sup>−8</sup>). (E) AcaCD is the direct activator of tra gene promoters. Activity of the *P*<sub>vca</sub>, *P*<sub>traF</sub> and *P*<sub>traI</sub> was monitored from single-copy, chromosomally integrated lacZ transcriptional fusions. Colorimetric assays were performed as described in panel C with expression of acaCD or setCD from *P*<sub>bad</sub> on pacaC or pacaCD (pG2B8), respectively. (F) AcaD co-purifies with AcaC. Coomassie blue-stained SDS-PAGE and Western blot analysis of AcaC purified using a Ni-NTA affinity chromatography. AcaD and 6×His-tagged AcaC were co-expressed in *E. coli* BL21(DE3) from pacoC<sup>lacZ</sup>. Western blot analysis was performed using a monoclonal antibody against the 6×His-tag.

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10<sup>−3</sup> [9] despite a GTG insertion at the 3' end of *acaC* (*acaC*<sub>523</sub><sup>GTG</sup>) that slightly alters the primary sequence of AcaC C-terminus (Figure 2). Pbam analyses (database v27.0) revealed that the conserved gene products AcaC and AcaD exhibit weak homology with the FlhC (Pfam PF03262) and FlhD (PF03247) domains, respectively (Table S1) [19]. FlhC and FlhD form a heteromeric complex playing a key role in the transcriptional activation of flagellar genes in Gram-negative bacteria such as *E. coli* [25]. We thus hypothesized that AcaC and AcaD (IncA/C activator subunits C and D) could act as the master activator of *tra* genes in IncA/C plasmids. We constructed three null mutants, ΔacaC, ΔacaD and ΔacaCD, of pVCR94 and tested their ability to transfer by conjugation. All three mutations abolished conjugative transfer, thereby confirming that acaCD plays an essential role for IncA/C plasmid transmission (Figure 1D). Trans-complementation of these mutations under control of *P*<sub>bad</sub> restored and even outperformed transfer of pVCR94 (Figure 1D). Importantly, the ΔacaD mutation could not be complemented by expression of either acaC or acaD. Only the simultaneous expression of both acaC and acaD restored the transfer of pVCR94ΔacaCD, strongly suggesting that these two genes code for an FlhC-like activating complex (Figure 1D). AcaC and AcaD share 34% and 23% identity with the master activator subunits SetC and SetD encoded by SXT/R391 ICEs. As expected from the very poor percentage of identity, the ΔacaCD mutation was not complemented by setCD expressed from the IPTG-inducible promoter *P*<sub>bad</sub> (Figure S2).

To test whether AcaCD is necessary and sufficient to drive the expression of *tra* genes of pVCR94, we cloned the promoter regions of *traL* (*P*<sub>traL</sub>), *traF* (*P*<sub>traF</sub>) and *traI* (*P*<sub>traI</sub>) upstream of a promoterless lacZ gene. The genes *traI*, *traF* and *traL* are likely candidate for AcaCD activation as traI codes for the predicted relaxase that would initiate conjugative transfer of pVCR94 at oriT while traL and traF code for two predicted sex pilus assembly proteins. We also tested the promoters *P*<sub>vca</sub>, *P*<sub>traF</sub> and *P*<sub>traI</sub> that drive the expression of acaCD and *vcrX52*, respectively (Figure 1A). All but *P*<sub>vca</sub> were inactive in the absence of acaCD, without arabinose induction or upon expression of setCD (Figure 1C, E). The constitutive expression from *P*<sub>vca</sub> seemed to remain unaffected by AcaCD overexpression. In contrast, expression of AcaCD alone in cells lacking pVCR94 was sufficient to trigger the expression from *P*<sub>traF</sub>, *P*<sub>traI</sub> and *P*<sub>traF</sub> and *P*<sub>traI</sub> (Figure 1E).

Collectively, these results are consistent with a model in which *acaR* represses acaCD expression and its own, thereby preventing expression of the *tra* genes. Accordingly, under the appropriate conditions, repression by AcaR would be alleviated, allowing expression of acaCD that in turn would enable expression of the *tra* genes and other genes such as *vcrX52*. Although *acaR*2 seems to be part of the same operon-like structure as *vcrX52*, its expression is not up-regulated by AcaC and likely not driven by *P*<sub>vca</sub>.
Figure 2. Molecular phylogenetic analysis of the acaC-acaD-acaR locus by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [61]. The tree with the highest log likelihood (−5461.6977) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences (Table S2). Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 2469 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [58]. The background color of each leaf indicates the original host species from which each plasmid was isolated. Insertions (I), deletion (D) and frameshift (F) mutations are indicated where appropriate. *

AcaC and AcaD as a heteromeric activator complex

Because deletion of either acaC or acaD abolished pVCR94 transfer, and both mutations could be individually complemented in trans, we sought to test whether AcaC and AcaD could form a heteromeric transcriptional activator complex. To investigate this possibility, we constructed a C-terminally 6×His-tagged version of acaC, acaC6×His, that was expressed together with acaD from Prac. AcaC6×His was purified by Ni-NTA affinity chromatography and the sample was analyzed on a 12% SDS-polyacrylamide gel. Interestingly, two bands were detected after Coomassie blue staining with molecular weights consistent with AcaC and AcaD. The additional band that co-purified with AcaC6×His has a molecular weight of 22.8 kDa and was confirmed to be AcaD by mass spectrometry. Therefore, our results suggest that, like FlhC and FlhD, AcaC and AcaD assemble as a heteromeric complex.

Identification of AcaCD targets in pVCR94

To get a better understanding of the mechanisms governing transfer regulation of IncA/C conjugative plasmids, and identify genes of pVCR94 expressed under the direct control of AcaCD, we undertook an exhaustive characterization of the AcaCD regulon using transcriptome sequencing and ChIP-exo [31]. For these experiments, we used E. coli MG1655 Rf carrying pVCR94_AcaCD and the same strain bearing a chromosomally integrated single copy of pacaCD3×FLAG that expresses the native AcaD subunit along with a C-terminal 3×FLAG tagged AcaC subunit under control of Prac. Based on the transfer frequency of the ΔcaCD mutant complemented with acaDC3×FLAG, we concluded that the 3×FLAG did not significantly affect the function of the tagged AcaC subunit compared to its wild-type counterpart (Figure S2).

ChIP-exo data analyses revealed 17 major AcaD enrichment peaks, all located within intergenic regions (Figure 3A, B and Table S3). Most of the genes or operons found downstream of these peaks play key roles in conjugative transfer (Figure 3, Figure S4 and Table S3). For instance, AcaCD-binding sites were found upstream of the vraL, vraV, dsbG, vraN and vraF genes that are predicted to be involved in the formation of the mating pore. One peak was also found upstream of the gene coding for the putative relaxase gene vraI. The presence of an AcaCD-binding site is also

The H-NS-like protein Sfi encoded by the IncHI1 conjugative plasmid R27 has been shown to provide a stealth function helping to prevent the transmission of R27 into a naive host by preventing titration of the cellular pool of H-NS by the Avf-rich sequences of the plasmid [27]. The locus occupied by acaR in IncA/C plasmids contains the gene int in the SXT/R391 ICEs (Figure S3) [20], int codes for the integrase which allows SXT/R391 ICEs to remain quiescent in the host chromosome [28].

Although the regulation loci of IncA/C conjugative plasmids and SXT/R391 ICEs encode similar transcriptional activators (AcaCD and SetCD, respectively), IncA/C plasmids carry acaR and an extra acaR gene, while lacking a homolog of setR (Figure S3). In SXT/R391 ICEs, setR codes for a λ CI-related transcriptional repressor that prevents the expression of setC and setD [29,30]. Like λ CI, SetR responds to DNA damage by RecA-dependent autoproteolysis, which alleviates the repression of setCD, thereby allowing excision and transfer of SXT/R391 ICEs [30]. Consistent with the absence of a setR homolog, transfer of IncA/C plasmids has been shown to be recA-independent [19].

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well correlated with transcriptional activity since the expression of 86 out of 152 genes, including genes located downstream of AcaCD-binding sites, is clearly increased under the same conditions used for ChIP-exo when compared to a ΔacaCD mutant (Figure 3B and Dataset S1). In contrast, the vast majority of genes that are not significantly affected by the expression of AcaCD appeared to be either inactive or likely constitutively expressed. Their function is unknown or not directly tied to conjugative transfer. Examples of such genes in pVCR94 include repA (vcrx003) involved in plasmid replication, a putative

Figure 3. In-depth analysis of the AcaCD regulon in pVCR94. (A) Genetic organization of pVCR94ΔX adapted from Carraro et al. [19]. The circular map of the plasmid was linearized at the start position of gene vcrx001. The locations and orientations of ORFs are indicated by arrowed boxes. Colors are coded by function as follow: white, unknown; blue, conjugative transfer; light blue, lytic transglycosylase; orange, replication; gray, antibiotic resistance; yellow, putative regulatory function; purple, recombination; green, activator; red, repressor. The origin of replication (oriV) and the origin of transfer (oriT) are also indicated. The position of the scar resulting from the deletion of the antibiotic resistance gene cluster is indicated by FRT. sul2, resistance to sulfamethoxazole. (B) Results of ChIP-exo and RNA-seq experiments on E. coli MG1655 Rf carrying pVCR94ΔacaCD with or without a single chromosomal copy of pacaDC36 FLAG expressing the native AcaD subunit along with a C-terminal 3×FLAG-tagged AcaC subunit induced by IPTG. The first track plots the number of ChIP-exo reads mapped as a function of the position in pVCR94ΔX (black bars). Pink dots at the top of peaks indicate a signal beyond the represented y-axis maximal value. The second track shows the position of ChIP-exo enrichment peaks found by MACS [63] (dark gray). The asterisk at the top of the rightmost peak indicates a MACS false negative result manually incorporated in the figure. The third track is a representation of the pVCR94ΔX genes using the same color code as in panel A. The fourth track indicates the position of the AcaCD-binding motifs found by MAST [64] within ChIP-exo peaks using the AcaCD logo shown in panel C. Green arrows, motifs on positive DNA strand; red arrows, motifs on negative DNA strand. The fifth track represents Rockhopper’s [65] predicted operons. Dark blue, operons transcribed from positive DNA strand; orange, operons transcribed from negative DNA strand. The remaining four tracks show the total RNA-seq read densities (black bars; log scale) and the genome-wide 5′-RACE signals (green and red bars respectively on the positive and negative DNA strands; linear scale) for cells harboring either pVCR94ΔacaCD or pVCR94ΔacaCD complemented with pacaDC36 FLAG and induced by IPTG. Pink dots at the top of 5′-RACE signals indicate a signal beyond the represented y-axis maximal value. (C) Motif sequence recognized by AcaCD in pVCR94ΔX obtained by MEME [64] using the AcaCD-binding sequences generated from ChIP-exo experiments. (D) VAP aggregate profile [66] showing ChIP-exo and 5′-RACE density signals centered on the AcaCD-binding motif (black box). Yellow line, density of reads mapping on the positive DNA strand (Left border); green line, density of reads mapping on the negative DNA strand (Right border). X-axis displays the distance in nucleotides from the aggregated transcription start site shown in blue (TSS). (E) Organization of vcrx059 and traI divergent promoters revealed by ChIP-exo and 5′-RACE for pVCR94ΔacaCD complemented with IPTG-induced pacaDC36 FLAG and induced by IPTG. Pink dots at the top of 5′-RACE signals indicate a signal beyond the represented y-axis maximal value. (C) Motif sequence recognized by AcaCD in pVCR94ΔX obtained by MEME [64] using the AcaCD-binding sequences generated from ChIP-exo experiments. (D) VAP aggregate profile [66] showing ChIP-exo and 5′-RACE density signals centered on the AcaCD-binding motif (black box). Yellow line, density of reads mapping on the positive DNA strand (Left border); green line, density of reads mapping on the negative DNA strand (Right border). 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toxin-antitoxin system (vcrx028 and vcrx027), the sul2 (vcrx029)
genotype conferring resistance to sulfamethoxazole, as well as acc1 and
acc2 that negatively regulate the expression of the acc1-vcrx174-
accDC operon (Figure 1A, C and Dataset S1).

**De novo** motif discovery of DNA sequences bound by AcaCD was
carried out using MEME (Multiple Em for Motif Elicitation) [32] (Figure 3C). We
next used MAST (Motif Alignment and Search Tool) [33] to determine the precise location of potential
AcaCD-binding motifs on the entire sequence of pVCR94 (Figure 3B, Figure S4, and Table S3). Statistically significant
motifs that were localized within ChIP-exo peaks were conserved.

We next analyzed the localization of AcaCD-binding motifs relative to transcription start sites obtained using a genome-wide
5′-RACE (Rapid Amplification of cDNA Ends) methodology (Figure 3B, D, Figure S4 and Table S3). We observed a similar
promoter profile, compatible with a class 2 activator [34], for all
transcription start sites located between an AcaCD-binding motif and a gene in the same orientation (Figure S4). This interpretation is consistent with our high resolution ChIP-exo data that reveals a
protected region containing a distal AcaCD motif (Figure 3D), and
supported by previous observations of the promoter-bound RNA
polymerase holoenzyme complex footprint between position −54
to +22 relative to the transcription start site [28]. An interesting
element of this organization is the regulation of the vcrx059 and
tral divergent promoter region, in which two partially overlapping
transcription initiation complexes are detected (Figure 3E).

In addition to measuring transcriptional expression in
pVCR94-AcaCD and pVCR94-AcaCD complemented with
IPTG-induced pacaDC^-^5^-^FLAG, we also performed RNA-seq on
wild-type pVCR94 (Dataset S1). Our results indicate little differences between the transcriptome expression levels of wild-type pVCR94 and pacaDC^-^5^-^FLAG-complemented pVCR94Δ-
acaCD (Pearson coefficient of 0.87). On the contrary, these two transcriptomes are clearly different from pVCR94-AcaCD
(Pearson coefficients of 0.12 and −0.04, respectively). In total, pVCR94 and pacaDC^-^5^-^FLAG-complemented pVCR94-AcaCD
share 76 differentially expressed genes out of 88 when compared to
pVCR94-AcaCD, which further supports the high similarity between their gene expression profiles (Dataset S1). These findings suggest that AcaCD in wild-type pVCR94 is at least partially active in *E. coli* under laboratory conditions because an appropriate activating signal is already being sensed and/or the expression of accA and accD is not efficiently repressed.

This hypothesis is consistent with the fact that acc1 and acc2 negatively regulate conjugative transfer efficiency while not completely abolishing it (Figure 1B). Our results strikingly contrast with the conclusion drawn by others that most of the backbone of IncA/C plasmid pAR060302, including tra genes, is transcriptionally inactive in *E. coli* [35]. This interpretation is puzzling given the very high nucleotide identity between core regions of IncA/C plasmids, that reads per kilobase per million reads (RPKM) expression values reported for pAR060302 genes expressed at a “low level” such as *repA* reach several thousand units [35], and that pAR060302 is self-transferable at high frequencies by conjugation under laboratory conditions similar to ours [22].

AcaCD drives the mobility of two unrelated classes of genomic islands

Determination of the AcaCD-bound DNA motif provides the opportunity to investigate the impact of IncA/C plasmids on
genome dynamics. For instance, IncA/C plasmids are known to mobilize SG11 in *S. enterica* by a yet uncharacterized mechanism [14,15,36]. SG11 elements confer and propagate MDR in various pathogenic bacteria [16,17,37]. Data mining using the

AcaCD-binding motif revealed putative sites upstream of the genes *xs*, *s004/rep*, *traN* and *trhH/trhG* likely involved in the mobility (recombination directionality factor and mobilization genes) of SG11 (Figure 4A and Table 1). We carried out a similar analysis on the sequenced genome of *V. mimiws* VM573 and identified three AcaCD-binding motifs upstream of three genes, two of which coding for a predicted recombination directionality factor (VMD_06410, *xs*) and a distant homolog of Vcrx001 (VMD_06490, 29% of identity over 85 amino acid residues), which is a conserved key factor for conjugative transfer of IncA/C plasmids (Table 1) [19]. The third gene, VMD_06480, has no predicted function. The three genes are part of an unannotated 16,511-bp GI integrated into the 3′ end of *yicC*, that we named MGI/m1 (Figure 4A and Table 1). MGI/m1 could confer adaptive traits to its host, notably resistance to bacteriophages conveyed by a putative type III restriction-modification (res-mod) (Figure 4A). SG11 and MGI/m1 are prototypes of two families of GIs that are phylogenetically unrelated to each other and to IncA/C plasmids.

Based on these observations, we hypothesized that expression of *acaCD* either from pVCR94 or from pBAD30 would trigger the excision of both SG11 and MGI/m1 from their respective host chromosome. To verify this hypothesis, we monitored by PCR amplification the formation of a chromosomal *attB* site and of an *attP* site resulting from the circularization of the two GIs (Figure 4B). While no spontaneous excision of either GI was detected in the absence of pVCR94, both GIs excised from the chromosome not only in the presence of pVCR94 but also upon ectopic expression of *acaCD* in cells lacking pVCR94 (Figure 4B).

Finally, to confirm that IncA/C plasmids can mobilize the novel GI MGI/m1, we carried out inter- and intraspecific mating assays using pVCR94 as the helper plasmid and a modified MGI/m1 carrying *res:cat* (*Cm* resistant) as a selectable marker. As a positive control, we used SG11 and took advantage of its natural selectable markers. For both SG11 and MGI/m1, no exconjugant were observed in the absence of pVCR94 (Figure 4C). In contrast, both elements were specifically mobilized in its presence. On one hand, transfer of SG11 occurred at very high frequency (almost all recipient cells received a copy of SG11), but co-acquisition of pVCR94 was infrequent. This observation suggests that negative interactions may exist between these mobile genetic elements. On the other hand, MGI/m1 transferred at a lower rate than pVCR94 (Figure 4C). We confirmed AcaCD-specific expression of the MGI/m1-borne genes *xs* and VMD_06490 by real-time-quantitative PCR (RT-qPCR) (Figure 4D). *int* mRNA level was also specifically increased by AcaCD, despite the lack of a predicted AcaCD-binding motif in its promoter region. This observation suggests that its enhanced expression could be driven by the promoter upstream of *xs* on a large transcript containing *attP* on the circular excised form of MGI/m1. For a reason that remains to be established and despite the presence of a predicted AcaCD-binding motif, VMD_06480 expression was not regulated by AcaCD.

Unlike SG11, which seem to encode a large set of proteins likely dedicated to mobilization (*TraN, TrhG* and *TrhH*), MGI/m1 only encodes a distant homolog of Vcrx001 (VMD_06490), which is strongly induced by AcaCD (Figure 4D). Based on these differences, we speculate that MGI/m1 is not as efficient as SG11 to hijack the conjugative apparatus encoded by IncA/C plasmids. Characterization of the genetic and molecular mechanisms of SG11 and MGI/m1 mobilization by IncA/C helper plasmids are ongoing.

Altogether, these results unraveled a novel example of the multiple and intricate interactions linking phylogenetically unre-
Figure 4. IncA/C-dependent excision and transfer of GIs. (A) Schematic representation of SGI1 from *S. enterica* Typhimurium DT104 and MGIvm1 from *V. mimicus* VM573. The left and right junctions (attL and attR) within the host chromosome are indicated. SGI1 (42 596 bp, Genbank AF261825) and MGIvm1 (16 511 bp, Genbank NZ_A-CV01000005) are integrated into the 3’ end of trmE and yicC (attL sides) in their respective hosts. ORFs with similar function are indicated by colors as follows: black, DNA recombination; orange, DNA replication; blue, conjugative transfer; yellow, regulatory function; pink, putative type III restriction-modification system; white, unknown functions; MDR, multidrug resistance locus. Green chevrons indicate the position and orientation of predicted AcaCD-binding sites (see Table 1). For clarity, ORFs named SXXX were shortened as SXX for SGI1 and VMD_066XX as XXX for MGIvm1. (B) AcaCD induces SGI1 and MGIvm1 excision. Excision was detected by PCR on genomic DNA to specifically amplify the attB chromosomal site and the attP site resulting from the excision of the GIs in *S. enterica* Typhimurium or *E. coli* bearing SGI1 and *V. mimicus* bearing MGIvm1. Integrated GIs were detected by amplification of the attL site. Assays were done in strains devoid of plasmid (−), bearing pVCR94ΔX3 (+) or only expressing acaCD (acaCD) from *pacaDe−3=FLa4* for assays in *E. coli* or pacaCD in *V. mimicus*. (C) Intraspecific mobilization of both GIs was assayed using *E. coli* MG1655 RF bearing pVCR94ΔX3 and SGI1 or MGIvm1 as a donor and the otherwise isogenic strain MG1655 Nx as a recipient. Exconjugants were selected for the acquisition of either GI, pVCR94ΔX3, and for cotransfer of both. Transfer frequencies are expressed as the number of exconjugant per donor CFUs. The bars represent the mean and standard deviation values obtained from three independent experiments. The asterisk indicates that the frequency of exconjugant formation was below the detection limit (<10−6). (D) AcaCD induces the expression of the putative excision and mobilization genes of MGIvm1. Relative mRNA levels of int (VMD_06510), 490 (VMD_06490), 480 (VMD_06480) and xis (VMD_06410) were measured by RT-qPCR assays on cDNA of *V. mimicus* VM573 devoid of plasmid (−) or expressing acaCD from *pacaCD (+). The bars represent the mean and standard deviation values obtained from three independent experiments. Comparison between the strain expressing or not AcaCD were done using two-tailed Student’s t-tests and the P-values are indicated above the bars.

Materials and Methods

Bacterial strains and bacterial conjugation assays

The bacterial strains used in this study are described in Table 2. The strains were routinely grown in Luria-Bertani (LB) broth at 37°C in an orbital shaker/incubator and were maintained at −80°C in LB broth containing 15% (vol/vol) glycerol. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 20 μg/ml for *E. coli*. 30 μg/ml for *S. enterica* and 10 μg/ml for *V. mimicus*; erythromycin (Em), 10 μg/ml; kanamycin (Km), 50 μg/ml or 10 μg/ml for single copy integrants of pQPlacZ; nalidixic acid (Nx), 40 μg/ml; rifampicin (Rf) 50 μg/ml; spectinomycin (Sp), 50 μg/ml; streptomycin (Sm), 200 μg/ml; sulfamethoxazole (Su), 160 μg/ml; tetracycline (Tc), 12 μg/ml; trimethoprim (Tm), 32 μg/ml. Conjugation assays were performed as described elsewhere [19]. To induce expression from pBAD30 and from pH536 in complementation assays, mating experiments were carried on LB agar plates supplemented with 0.02% L-arabinose or 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), respectively.

Molecular biology methods

Genomic and plasmid DNA were prepared using the Wizard Genomic DNA Purification Kit (Promega) and EZ-10 Spin Column Plasmid DNA Miniprep Kit (Biobasic), respectively, according to manufacturer’s instructions. All the enzymes used in

Concluding remarks

IncA/C conjugative plasmids and SXT/R391 ICEs both rely on transcriptional activator complexes that are reminiscent of the FlhDC master activator of flagellar genes to enable expression of their tra genes. Nevertheless, AcaCD recognizes DNA motifs that are unrelated to the recognition motif of FlhDC [25]. Moreover, AcaCD and SetCD, the master activator of SXT/R391 ICEs, are not exchangeable and are thus expected to recognize completely unrelated DNA motifs. Our study revealed that the AcaCD region extends beyond the sole genes involved in the dissemination of IncA/C plasmids and that AcaCD acts as a beacon signaling the presence of a helper plasmid, allowing the propagation of two unrelated classes of GIs. Similarly, SetCD has been shown to trigger the excision of MGI plasmids originating from various pathogenic *Vibrio* species [38,39,41], thereby suggesting that sequences bound by AcaCD- and SetCD-like activators can be easily mimicked by unrelated GIs to regulate their own gene expression. Clearly, our results and observations from others indicate that GIs are not necessarily defective or decaying mobile genetic elements, unable to propagate. Instead, most are likely quiescent parasites awaiting opportunities to hijack helper self-transmissible elements using diverse strategies [14,38,42]. Such intricate connections between various genetic elements support their major impact on the evolution of genomes and on the adaptation of bacteria to their environment, particularly in the current context of massive emergence of multidrug resistant pathogens worldwide. Ultimately, future research investigating the regulation of other metabolic genetic elements relying on similar transcriptional activator complexes to regulate their own dissemination will unravel unforeseen regulatory networks linking self-transmissible and mobilizable elements.
and other genes of unknown function in MGI S004 secretion system (T4SS), as well as genes of unknown function such as unknown function (green arrows). AcaCD activated the excision of SGI1. AcaCD triggered the excision of SGI1. AcaCD activates the expression of the transfer genes of pVCR94, as well as the expression of the master interaction with genomic islands. Figure 5. Model of regulation of IncA/C plasmids and Fig. 5. Model of regulation of IncA/C plasmids and interaction with genomic islands.

Plasmids and strains construction

Plasmids and oligonucleotides used in this study are listed in Table 2 and S4. Complementation vectors derived from the pBAD30 vector [45]. The ORFs acr1, acr2, acaC, acaD and aca CD were amplified using primers pairs vcrx146.for/vcrx146.rev, vcrx150.for/vcrx150.rev, vcrx149(acaC).for/vcrx149(acaC).rev, vcrx148(acaD).for/vcrx148(acaD).rev, vcrx148(acaD).for/vcrx149(acaC).rev and cloned into the EcoRI restriction site of pBAD30 to generate pacr1, pacr2, pacac, pacad and pacoCD, respectively. The transcriptional fusion vector pOPlacZ contains the promotorless lacZ gene from E. coli K12 MG1655 with its native Shine-Dalgarno sequence. This plasmid was constructed by amplifying the lacZ gene using primer pair Op-lacZ-F/Op-lacZ-R and subsequently cloning into the 2462-bp fragment of PsI-digested pAH56 using the Gibson assembly method [46]. PCR products containing the promoter region upstream of acr1, traF, traL, trha and vcrx152 were cloned into the PsI restriction site of pOPlacZ to produce pPromacr1, pPromtraL, pPromtraF, pPromtraH and pProm152, respectively. The vectors pacaDC37FLAG and psetDC37FLAG used for complementation assays and ChIP-exo experiments derived from pAH56. Briefly, the acaDC and setDC loci were amplified using primer pairs acaDF-NdeI/acaCR-HindIII and setDF-NdeI/setCR-HindIII, respectively, and cloned into pCR2.1. (Invitrogen) to generate pCR2.1:acaDC and pCR2.1:setDC. 3xFLAG was amplified from pJL148 [47] using the primer pair FlagV/FlagR and subsequently cloned into the HindIII site of pCR2.1:acaDC and pCR2.1:setDC to generate pCR2.1:acaDC37FLAG and pCR2.1:setDC37FLAG. The acaDC37FLAG and setDC37FLAG inserts were recovered by NdeI/SalI
| Strains or plasmids | Relevant genotype or phenotype | References |
|---------------------|-------------------------------|------------|
| **Escherichia coli** |                               |            |
| BW25113            | F<sup>−</sup>, Δ[araD-araB]567, ΔlacZ4787::rrnB-3, λ<sup>−</sup>, rph-1, ΔrhaD-rhaB568, hsdR514 | [50]       |
| BW25113 Nx         | Nx<sup>−</sup>-derivative of BW25113 | [19]       |
| MG1655 Nx          | Nx<sup>−</sup>-derivative of MG1655 | [51]       |
| MG1655 Rf          | Rf<sup>−</sup>-derivative of MG1655 | [51]       |
| BL21(DE3)          | F<sup>−</sup>, ompt<sup>−</sup> ΔhsdS gal dcm (DE3) | Novagen    |
| j2163              | (F<sup>−</sup>) RP4-2-Tc::Mu ,adapA<sup>−</sup> (erm-pir) (Kn Em) | [49]       |
| **Salmonella enterica** |                               |            |
| St038              | serovar Typhimurium; SGI1<sup>+</sup> (Ap Cm Tc Su Sm) | F. Malouin |
| **Vibrio mimicus** |                               |            |
| VM573              | Patient with diarrhea; USA, 1990s; CT<sup>+</sup> MGIvmt1<sup>+</sup> (Ap Sp Su) | [68]       |
| **Plasmids**       |                               |            |
| pVCR94             | IncA/C conjugative plasmid; V. cholerae O1 F1939; Rwanda, 1994 | [19]       |
| pVCR94 LX          | Su<sup>−</sup>-derivative of pVCR94 (Su) | [19]       |
| pVCR94 RX2         | Sp<sup>−</sup>-derivative of pVCR94 LX (Sp Su) | This study |
| pVCR94 DX3         | Kn<sup>−</sup>-derivative of pVCR94 DX (Kn Su) | This study |
| pVCR94 DX25        | pVCR94 ΔX2 Δvcrx025 (Sp Su) | This study |
| pVCR94 ΔacAC       | pVCR94 ΔX2 Δacac (Sp Su) | This study |
| pVCR94 ΔacAD       | pVCR94 ΔX2 Δacad (Sp Su) | This study |
| pVCR94 ΔacCD       | pVCR94 ΔX2 Δacac (Sp Su) | This study |
| pVCR94 Δacr1       | pVCR94 ΔX2 Δacr1 (Sp Su) | This study |
| pVCR94 Δacr2       | pVCR94 ΔX2 Δacr2 (Sp Su) | This study |
| pBAD30             | ori<sub>p15A</sub> araC P<sub>BAD</sub> (Ap) | [45]       |
| pacac              | pBAD30::pacac (Ap) | This study |
| pacD               | pBAD30::pacD (Ap) | This study |
| pacCD              | pBAD30::pacCD (Ap) | This study |
| pacr1              | pBAD30::pacr1 (Ap) | This study |
| pacr2              | pBAD30::pacr2 (Ap) | This study |
| pGG2B              | pBAD30::setDC (Ap) | [69]       |
| p5W23T             | ori<sub>Tn5</sub> ori<sub>V<sub>lac</sub></sub> (Cm) | [49]       |
| pAH56              | ori<sub>V<sub>lac</sub></sub>; attP<sub>lac</sub>; lacI; lacZ<sup>+</sup> (Kn) | [70]       |
| pJL148             | SPA-tag (CBP-TEV site-3×FLAG) (Kn) | [47]       |
| pacDC<sup>3×FLAG</sup> | pAH56::pacDC<sup>3×FLAG</sup> (Kn) | This study |
| psetDC<sup>3×FLAG</sup> | pAH56::psetDC<sup>3×FLAG</sup> (Kn) | This study |
| pOPlacZ            | pAH56 lacZ (Kn) | This study |
| pPromtraL          | pOPlacZ P<sup>prom</sup>~lacZ (Kn) | This study |
| pPromtraL          | pOPlacZ P<sup>prom</sup>~lacZ (Kn) | This study |
| pPromtraF          | pOPlacZ P<sup>prom</sup>~lacZ (Kn) | This study |
| pPromacr1          | pOPlacZ P<sup>prom</sup>~lacZ (Kn) | This study |
| pProm1S2           | pOPlacZ P<sup>prom</sup>~lacZ (Kn) | This study |
| pINT-ts            | ori<sub>R101</sub>; cI857; λ<sub>R101</sub>-Int<sub>a</sub> (Ap Ts) | [70]       |
| PET-2A(+)<sup>+</sup> | ori<sub>V<sub>lac</sub></sub>; lacI; P<sub>T<sub>lac</sub></sub>; 6×His expression vector (Kn) | Novagen    |
| pacDC<sup>6×His</sup> | pET-2A(+):pacDC<sup>6×His</sup> (Kn) | This study |
| pSIM6              | λ Red recombination thermo-inducible encoding plasmid (Ts Ap) | [52]       |
| pKD3               | PCR template for one-step chromosomal gene inactivation (Cm) | [50]       |
| pKD13              | PCR template for one-step chromosomal gene inactivation (Kn) | [50]       |
| pV36               | PCR template for one-step chromosomal gene inactivation (Sp) | [51]       |
digestion and subsequently cloned into the NdeI/SalI-digested pA156 to generate pacaDC<sub>6</sub>-His<sub>6</sub> and psetDC<sub>6</sub>-His<sub>6</sub> [46]. pacaDC<sub>6</sub>-His<sub>6</sub> was obtained by cloning of acaDC amplified with acaDF-NdeI/acaCR-HindIII into pET-24b(+) (Novagen), pRes for insertion of a Gm marker into MGI/m1 was obtained cloning into EcoRI/BamHI-digested pSW23T [49] the 732-bp PCR fragment amplified using the primer pair GIVmi-res2F/GIVmi-res2R on genomic DNA of V. mimiuc VM573.

Deletion mutants of pVCR9AX were constructed using the one-step chromosomal gene inactivation technique and are listed in the Table 1 [19,50]. Primers used are listed in Table S4. The pVCR9AX derivatives pVCR9AX2 (Sp) and pVCR9AX3 (Kn) were constructed using primer pair 94DelXnoFRT.for/94DelXnoFRT.rev and pV136 and pKD13 as templates, respectively [50,51]. Subsequent deletions of vcrX25, arc1, arc2, acaC, acaD and acaCD were done on pVCR9AX2 using primer pairs 94Delvcrx25.for/94Delvcrx25.rev, 94Delvcrx146.for/94Delvcrx146.rev, 94Delvcrx150.for/94Delvcrx150.rev, 94DelacaC1.for/94DelacaC1.rev, 94DelacaD.for/94DelacaD.rev and 94DelacaC.rev respectively, and pKD3 as the template. The λRed recombination system was expressed using pSIM6 as described by Datta [52]. When possible, the antibiotic resistance cassette was removed from the resulting construction by Flp-catalyzed excision using the pCP20 vector [53]. All deletions were designed to be non-polar and verified by PCR and antibiotic resistance profiling.

MGI/m1 was labelled with a Cm resistance marker in V. mimiuc VM573 by inserting the pSW23T-derivative suicide plasmid pRes into the putative type III restriction gene Vmi. Briefly, pRes was mobilized from E. coli BL21(DE3) carrying pacaDC<sub>6</sub>-His<sub>6</sub> into MGI/m1. pRes and pV136 were selected on LB agar plates on a CFX-96 thermocycler (Bio-Rad) with 5 μl of 2 x Taq Universal SYBR Green Supermix (Bio-Rad), 10 ng (3 μl) cDNA, and 200 nM final (2 μl) primer pair solutions. The following cycling conditions were used: 3 min at 95°C; 50 cycles: 15 s at 95°C, 30 s at 60°C, 30 s at 72°C. Relative expression levels of int (VMD_06510), VMD_06490, VMD_06480 and xis (VMD_06410) were calculated using a model taking into account multiple stably expressed reference genes [55] and housekeeping genes rpoZ and gyrA evaluated by geNorm [56]. Primer design (see Table S4) and validation were evaluated as described elsewhere [57]. In every qPCR run, a no-template control was performed for each primer pair and a no-reverse transcriptase control was performed for each cDNA preparation. Experiments were carried out three times on three biological replicates and combined.

RNA isolation and qRT-PCR

Cells from V. mimicus VM573 that contain no vector or pacaCD were recovered after 2.5 hours of induction with 0.02% of arabinose. Total RNA extraction was done using an RNasy minikit (Qiagen) following the manufacturer’s instructions. Purified RNA samples were subsequently subjected to qDNA digestion using Turbo DNase (Ambion) following the manufacturer’s instructions. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). qRT-PCR assays were performed on the RNomecs platform of the Laboratoire de Génomique Fonctionnelle de l’Université de Sherbrooke (http://lgus.ca). Reverse transcription was performed on 2.2 μg total RNA with Transcriptor reverse transcriptase, random hexamers, dNTPs (Roche Diagnostics), and 10 units of RNAaseOUT (Invitrogen Life Technologies) following the manufacturer’s protocol in a total volume of 20 μl. All forward and reverse primers were individually resuspended to 20–100 μM stock solutions in Tri-EDTA buffer (IDT) and diluted as a primer pair to 1 μM in RNase DNase-free water (IDT). Quantitative PCR (qPCR) reactions were performed in 10 μl volumes in 96 well plates on a CFX-96 thermocycler (Bio-Rad) with 5 μl of 2 x Taq Universal SYBR Green Supermix (Bio-Rad), 10 ng (3 μl) cDNA, and 200 nM final (2 μl) primer pair solutions. The following cycling conditions were used: 3 min at 95°C; 50 cycles: 15 s at 95°C, 30 s at 60°C, 30 s at 72°C. Relative expression levels of int (VMD_06510), VMD_06490, VMD_06480 and xis (VMD_06410) were calculated using a model taking into account multiple stably expressed reference genes [55] and housekeeping genes rpoZ and gyrA evaluated by geNorm [56]. Primer design (see Table S4) and validation were evaluated as described elsewhere [57]. In every qPCR run, a no-template control was performed for each primer pair and a no-reverse transcriptase control was performed for each cDNA preparation. Experiments were carried out three times on three biological replicates and combined.

Detection of SGI1 and MGI/v/1 excision

Excision of the GIs was detected by PCR on genomic DNA of the strains containing either SGI1 (S. enterica or E. coli) or MGI/v/1 (V. mimicus) using the primers listed in the Table S4. For SGI1, the attL site was amplified using primer pair SGI-lattLfor/SGI-lattL.rev in S. enterica and EcU7-L12.for/SGI-lattL.rev in E. coli [15]. The chromosomal site attB was detected using SGI-lattLfor/SGI-lattR.rev in S. enterica and EcU7-L12.for/EcU7-L12.rev in E. coli. The attP site carried by the extrachromosomal circular form of the element was amplified using the primer pair SGI-lattRrev/SGI-lattLfor. Based on the same methodology for MGI/v/1 in V. mimicus, primer pairs GIVm/1-YGIVm/1 and GIVm/1-YGIVm/1 were used to detect attL, attB and attP, respectively.

Phylogenetic analyses

The molecular phylogenetic analysis of the acr1-vcr147-acDC-acr2 locus was conducted in MEGA6 [58]. The nucleotide

| Strains or plasmids | Relevant genotype or phenotype | References |
|---------------------|--------------------------------|------------|
| pCP20              | Flp recombinase thermo-inducible encoding plasmid (Ts Ap Cm) | [53] |

Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Kn, kanamycin; Nl, nalidixic acid; Sp, spectinomycin; Rf, rifampicin; Sm, streptomycin; Su, sulfonamides; Tc, tetracycline; Tm, trimethoprim; Ts, thermostable; CT, cholera toxin; TCP, toxin co-regulated pilus.

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Phylogenetic analyses

The molecular phylogenetic analysis of the acr1-vcr147-acDC-acr2 locus was conducted in MEGA6 [58]. The nucleotide
sequence of the 2452-bp sequence of pVCR94 starting at the initiation codon of act1 and ending at the initiation codon of act2 was used to search for homologous sequences in the Genbank Nucleotide collection (nt/nu) database using Megablast [59]. Phylogenetic analyses were computed using a nucleotide alignment generated by MUSCLE [60]. The evolutionary history was inferred by using the Maximum Likelihood method. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Identical procedures were used for the molecular phylogenetic analysis of the 1101-bp repA gene of pVCR94 (Figure S1).

Data availability
Fastq files for each experiment were deposited at the NCBI Sequence Read Archive (SRA) under accession numbers SRR1544479 and SRR1544064 for ChiP-exo, SRRX675502 and SRR1544143 for total RNAseq as well as SRRX675014 and SRR1544479 for 5′-RACE. Complete data from aligned reads for ChiP-exo and RNA-seq can also be visualized using the UCSC genome browser at http://bioinfo.ccs.usherbrooke.ca/pVCR94.html.

Supporting Information
Figure S1 Molecular phylogenetic analysis of the repA replication initiation gene by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model [67]. The tree with the highest log likelihood (−2386.8588) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories +G, parameter = 0.2014). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1101 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [58]. The background color of each leaf indicates the original host species from which each plasmid was isolated. *, XNC1_p lacks the act1-vcrx147-acaDC-acr2 regulation cluster.

Figure S2 The 3×FLAG epitope does not alter the function of AcaCD. The donor strain E. coli MG1655 Rf was used to transfer pVCR94AX or its AcaCD mutant (Su Sp) to the recipient strain MG1655 Nx. AcaCD and SetCD complementation assays were carried out with the native D subunit and the C subunit C-terminally fused to the 3×FLAG epitope expressed from an IPTG-inducible promoter (prom) provided by pAH56, i.e. pacoCD3×FLAG (acaCD3×FLAG), psetDC3×FLAG (setDC3×FLAG) or empty pAH56 (0) integrated into the attB locus. Exconjugants were selected as Nx Su Sp colonies. The bars represent the mean and standard deviation values obtained from at least 3 biological replicates. The asterisks indicate that the frequency of transfer was below the limit of detection (<10−8).

Figure S3 Comparison of the genetic context of genes coding for AcaCD and SetCD orthologs in IncA/C plasmids and SXT/R391 ICEs. Schematic representation of regulatory regions of pVCR94 from V. cholerae O1 El Tor (NC_023291.1) and SXT from V. cholerae O139 (AY055428.1). Arrows of similar color represent genes predicted to have similar functions: green, transcriptional activator; yellow, putative transcriptional regulator; red, transcriptional repressor; blue, conjugative transfer; light blue, putative lytic transglycosylase; orange, replication; black, site-specific recombination; white, unknown function. Blue stars indicate the position of origins of transfer (oriT). The orange star indicates the position of the origin of replication (oriV) of pVCR94 based on identity with pRA1 from Aeromonas hydrophila (NC_012885). The black star indicates the position of the attP site for chromosomal integration of SXT by site-specific recombination. The percent of identity of orthologous proteins are indicated on dashed lines.

Figure S4 Alignment of AcaCD-dependent promoters in pVCR94. The AcaCD motif is as represented in Figure 3C. AcaCD boxes obtained by MAST analysis are shown in bold green capital letters with their respective p-value and downstream regulated gene. The positions of the transcription start sites obtained from 5′-RACE data are indicated in bold blue capital letters and underlined (TSS). Shine-Dalgarno sequences (SD) are underlined while start codons are in capital letters. The approximate positions of the −35 and −10 regions are highlighted in gray. The length of spacers between the represented transcription start sites and the Shine-Dalgarno regions is indicated in base pairs. Since no clear 5′-RACE signal was observed for vcrx035 and vcrx098, the approximate positions of expected transcription start sites are underlined and shown in bold black capital letters.

Table S1 Open reading frames (ORFs) of pVCR94 coding for putative transcriptional regulators.

Table S2 akr1-vcex147-acaDC-acr2 orthologous clusters in IncA/C plasmids.

Table S3 AcaCD-regulated promoters identified by ChiP-exo and 5′-RACE.

Table S4 Primers used in this study.

Table S5 Illumina libraries sequenced in this study.

Dataset S1 RNAseq transcriptome profiling of wild-type pVCR94, pVCR94AacaCD and complemented pVCR94AacaCD.

Text S1 Additional experimental procedures.

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Author Contributions
Conceived and designed the experiments: NC DM SR VB. Performed the experiments: NC DM PL SR VB. Analyzed the data: NC DM PL SR VB.

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