Comprehensive Functional Analysis of the 18 Vibrio cholerae N16961 Toxin-Antitoxin Systems Substantiates Their Role in Stabilizing the Superintegron.

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ABSTRACT
The role of chromosomal toxin-antitoxin (TA) systems, which are ubiquitous within the genomes of free-living bacteria, is still debated. We have scanned the Vibrio cholerae N16961 genome for class 2 TA genes and identified 18 gene pair candidates. Interestingly, all but one are located in the chromosome 2 superintegron (SI). The single TA found outside the SI is located on chromosome 1 and is related to the well-characterized HipAB family, which is known to play a role in antibiotic persistence. We investigated this clustering within the SI and its possible biological consequences by performing a comprehensive functional analysis on all of the putative TA systems. We demonstrate that the 18 TAs identified encode functional toxins and that their cognate antitoxins are able to neutralize their deleterious effects when expressed in Escherichia coli. In addition, we reveal that the 17 predicted TA systems of the SI are transcribed and expressed in their native context from their own promoters, a situation rarely found in integron cassettes. We tested the possibility of interactions between noncognate pairs of all toxins and antitoxins and found no cross-interaction between any of the different TAs. Although these observations do not exclude other roles, they clearly strengthen the role of TA systems in stabilizing the massive SI cassette array of V. cholerae.

IMPORTANCE
The chromosomal toxin-antitoxin systems have been shown to play various, sometimes contradictory roles, ranging from genomic stabilization to bacterial survival via persistence. Determining the interactions between TA systems hosted within the same bacteria is essential to understand the hierarchy between these different roles. We identify here the full set of class 2 TAs carried in the Vibrio cholerae N16961 genome and found they are all, with a single exception, located in the chromosome 2 superintegron. Their characterization, in terms of functionality, expression, and possible cross-interactions, supports their main role as being the stabilization of the 176-cassette-long array of the superintegron but does not exclude dual roles, such as stress response elements, persistence, and bacteriophage defense through abortive infection mechanisms.

Toxin-antitoxin (TA) systems were discovered in 1983 on plasmid F of Escherichia coli and were shown to be involved in stable plasmid maintenance by postsegregational killing, a mechanism distinct from replication and partition. These systems typically consist of an operon of two genes, which encode a toxin that targets an essential cellular function and an antitoxin that binds to and inhibits the toxin. Toxin activity is regulated through differential stability of the stable toxin and the labile antitoxin; loss of a TA system by the progeny during cell division results in cell death by the action of the stable toxin (for reviews, see references 2 and 3). The antitoxin, in most cases, also acts as a transcriptional autorepressor of the operon, and the degradation of the antitoxin results in the transcriptional activation of the TA operon. Currently there are three types of TA systems, which are classified according to the nature of their antitoxin. For the type 1 system, such as tisA-tisB (5) and hok-sok (6), toxins are hydrophobic proteins and antitoxins are small RNAs (sRNAs). Type 2 TAs are the most represented among TA systems, and both their toxins and antitoxins are present as proteins (7). The third type of TA system is currently represented only by the toxIN locus, which was discovered in 2009 on a cryptic plasmid of Erwinia carotovora (8). It consists of a protein-RNA toxin-antitoxin pair, where RNA antitoxin ToxI inhibits the toxicity of toxin ToxN by interfering with its biochemical activity (9). Type 2 TA systems are ubiquitous in prokaryotic genomes and have more recently been identified as genuine components of the chromosomes of most free-living bacteria (7, 10, 11), with up to 88 predicted TAs in the Mycobacterium tuberculosis genome (12). Several hypotheses have been proposed for the biological roles of chromosomal TAs, but these are not yet clear and remain under debate. TAs have been proposed to be physiological or developmental regulators of processes such as programmed cell death (PCD), growth and/or development, and persister cell formation. PCD refers to cell death mediated by an...
intracellular death program through a cascade of signalization in response to an external stress (13); however, this hypothesis is controversial, as several groups have failed to reproduce the original observations (4). Gerdes proposed that TA loci function as stress response elements and that they help the cells cope with nutrient starvation by modulating their growth (14). In the context of their role in survival, TA systems have been proposed to induce a dormant cellular condition termed persistence (15–17). Persisters are nongrowing, dormant cells exhibiting low levels of translation, and this state could allow them to overcome stress conditions, such as antibiotic treatment (reviewed in reference 18). TAs have also been involved in bacteriophage defense through abortive infection mechanisms, where bacteria commit suicide to prevent phage spreading (19–21, 56). Two roles for TAs regarding genetic stability have also been proposed. The first is as an antiaddiction module, in which TAs can protect the host genome from colonization by an incoming mobile element or from a plasmid carrying a TA from the same functional family by allowing its harmless loss through neutralization of the invading toxin by the chromosomally encoded antitoxin (22). This hypothesis is supported by the fact that some chromosomal TA systems can cross-interact with their plasmidic homologs; i.e., a chromosomally encoded toxin is neutralized by a plasmid-encoded antitoxin or vice versa (for examples, see references 22 to 24). Recently, we have shown that the chromosomally encoded *phd-doc* system of *Vibrio cholerae* can interact with the homologous *phd-doc* system encoded by phage P1 (25). These observations emphasize that chromosomal TA systems may act as “exclusion” systems to protect the bacteria from an excess of invading mobile genetic elements (plasmids, transposons, and phages) carrying identical TA systems (22, 24). In addition, chromosomal TA systems, by interfering with their homologs encoded by mobile genetic elements, could drive the evolution of the latter ones to select the systems having toxins that are no longer recognized by the antiaddiction modules. Finally, similar to their role in plasmid stabilization, TAs have been proposed to stabilize chromosomal regions by preventing accidental deletions, especially when located in unstable segments such as mobile genetic elements (26–28). The *mosAT* and *tad-ata* systems have been shown to be responsible for the stabilization of two different integrative conjugative elements (ICEs) by postsegregational killing as in plasmids (28, 29). The insertion of two systems (*relBE1* and *parDE1*) from *Vibrio vulnificus* stabilized a 165-kb nonessential chromosomal region in *E. coli* (27). Strikingly, TAs are very common in cassettes of chromosomal integrons, especially those TA systems known to be involved in plasmid stabilization (26, 27, 30), and are present often in high numbers, especially in superintegrons (SIs) (31). Bacterial chromosomes are subject to lateral DNA exchange through genomic parasites such as integrons, conjugative transposons, and bacteriophages. Among these, integrons are very efficient in capturing and rearranging the open reading frames (ORFs) embedded in gene cassettes and converting them to functional genes by ensuring their correct expression. *Vibrio* SIs gather hundreds of highly diverse gene cassettes (32), with a large majority of ORFs of unknown functions. The analysis of available *Vibrio cholerae* genomes in 2007 led to the identification of 1,677 gene cassettes (33). Cassettes are generally promoterless and are thought to constitute a silent reservoir of adaptive functions (26, 33). Expression of these cassettes relies on their relative position from the Pc promoter, and any silent cassette can be called on for expression through recombina-

**MATERIALS AND METHODS**

**Bacterial strains and media.** *E. coli* DH5α (lab collection) and wild-type *V. cholerae* (38) were used to generate recombinant strains carrying various plasmid constructs, including the toxins and antitoxins listed in Table 1. Bacterial strains (see Table S4 in the supplemental material) were grown in Luria-Bertani (LB) medium at 37°C. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml for *V. cholerae* or 50 μg/ml for *E. coli*; and chloramphenicol, 25 μg/ml for *E. coli* or 5 μg/ml for *V. cholerae*. Glucose (1%) or arabinose (0.2%) was used to repress or induce the *PBA* promoter, respectively.

**Construction of expression vectors.** All genes were amplified by PCR using the primers listed in Table S5 in the supplemental material and *V. cholerae* N16961 chromosomal DNA as a template. Each of the toxin genes was cloned into the expression vector pBfad43, carrying the spectinomycin resistance gene under the control of the arabinose-inducible *PBA*.
promoter. Each of the antitoxin genes was cloned into the expression vector pUC18 (Invitrogen), carrying the ampicillin resistance gene, under the control of the promoter P$_{lac}$. Each construction was confirmed by Sanger sequencing.

RNA preparation and RT-PCR. Total RNA was purified from LB cultures harvested in the middle and at the end of the exponential phase as previously described (25). Reverse transcription-PCR (RT-PCR) was performed on 500 ng _V. cholerae_ N16961 total RNA with QF and QR primers (see Table S5 in the supplemental material) using the Access RT-PCR system (Promega) with or without avian myeloblastosis virus (AMV) reverse transcriptase. Real-time quantitative RT-PCR was performed with the same primers and analyzed as previously described (25). The expression level of tested genes was normalized using the 16S rRNA of _V. cholerae_ and previously described primers 16SQF and 16SQR (25). The experiments were repeated twice independently.

Toxicity assays. All _E. coli_ and _V. cholerae_ strains were grown on LB agar with appropriate antibiotics and 1% glucose to an optical density at 600 nm (OD$_{600}$) of 0.3 to 0.5, washed with LB, resuspended in LB with antibiotics and either 0.2% arabinose or 1% glucose, and then grown at 37°C.

Toxin activity assays in liquid medium. _E. coli_ strains containing either only a toxin or a toxin-antitoxin pair were grown in LB with appropriate antibiotics and 1% glucose to an optical density of 0.3 to 0.5, washed with LB, resuspended in LB with antibiotics and either 1% glucose or 0.2% arabinose, and then grown at 37°C. At successive time points, aliquots were removed, the OD$_{600}$ was measured, and the bacteria were plated onto selective plates containing 1% glucose to enumerate CFU. The functionality of the toxin-expressing plasmids in surviving clones was verified after transformation in a naive _E. coli_ strain.

RESULTS

TA system identification in the _V. cholerae_ N16961 genome. We had previously identified and characterized several TA cassettes carried in different chromosomal integrons of various _Vibrio_ genomes (25–27), and we suspected their presence in chromosomal integrons of other genera such as _Treponema denticola_ (31). A previous analysis of the _V. cholerae_ N16961 genome had identified a total of 13 TA candidates, all located within the SI region (7), a unique property among vibrios. Indeed, analysis of the other available _Vibrio_ genomes showed that TA candidate distribution was not restricted to the integron cassette array, and TAs could be found in other chromosomal contexts (31). This led us to reanalyze the genome of N16961 for the presence of potential TAs. Starting from the data obtained using RASTA (39) on the N16961 genome listed in the toxin-antitoxin database TADB (40), we carefully checked the predicted list of 19 potential TA systems, where 16 were carried in integron cassettes, 1 on chromosome 2 but outside the SI, and 2 on chromosome 1. We found that 2 of the 3 TAs which were not in the SI cassette array were erroneous predictions. The third TA candidate carries gene VC0815, whose 453-amino-acid-long product is related to HipA and which is associated with VC0814, a 160-codon gene with no known homologs but which contains a helix-turn-helix (HTH) domain and whose translation start site overlaps the final codons of VC0815, an organization commonly found in TA operons.

In parallel, we searched all SI cassettes carrying two ORFs, and we recovered 17 cassettes, which included the 16 cassettes listed in TADB (Fig. 1) and the phd-doc cassette that we previously characterized. Ten of the 17 TA systems belong to four well-characterized families (7), and three cassettes were found to be hybrid TA systems (i.e., a TA system whose toxin and antitoxin do not belong to the same family according to the TA search software described in reference 11). These hybrid systems correspond to a phd-parE gene pair (VCA0422-VCA0423), a stb-relE pair (VCA0477-VCA0478), and a relb-parE pair (VCA0488-VCA0489). The last four predicted TA cassettes have at most one gene having poor homology to a known toxin or antitoxin. The identical VCA0318-VCA0319 and VCA0481-VCA0482 cassettes encode proteins having respective homology to _N_. acetyltansferases and ribbon-helix-helix (RHH) transcription factors; the first protein was found to be a toxin and the second its antitoxin (see below). Cassette VCA0486-VCA0487 also encodes an RHH transcription factor-like protein, which was also found to be an antitoxin for the VCA0487 toxic product (also carrying an _N_. acetyltansferase motif). Lastly, the VCA0332-VCA0333 cassette was found to express an RHH transcription factor-like protein acting as an antitoxin for the VCA0332 toxin, which belongs to an uncharacterized family of conserved proteins, COG2929.

Functionality of the sedentary HipAB candidate identified in chromosome 1 of _V. cholerae_. Both candidate HipAB genes, VC0814 encoding the putative antitoxin and VC0815 encoding the HipA-like protein, were independently cloned in compatible plasmids under the control of the P$_{lac}$ promoter for VC0814 in pSU18 and under control of the arabinose-inducible P$_{BAD}$ promoter of the low-copy-number vector pBAD43 (a pSC101 derivative [41]) for VC0815. Both types of plasmids were introduced into _E. coli_ DH5α cells. We found that cells carrying the VC0814 plasmid were able to grow in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside), showing that this gene was not toxic for _E. coli_. Transformants carrying the VC0815 plasmid were selected in the presence of 1% glucose to repress the P$_{BAD}$ promoter. Plating these cells on LB agar plus 0.2% arabinose resulted in cell death, showing that VC0815 indeed encoded a powerful toxin. We then cotransferred the two plasmids into DH5α cells, repeated the arabinose induction test, and found that in the presence of expressed VC0814, VC0815 was no longer toxic. Thus, VC0814-VC0815 forms a genuine TA system.

Functionality of the toxins carried in the superintegron cassettes. Among these 17 potential TA systems, 3 cassettes were found in two identical copies. These correspond to 2 parDE loci (parDE1 and parDE3), 2 relBE loci (relBE2 and relBE4), and the new TA encoded by VCA0318-VCA0319 and VCA0481-VCA0482, thus downsizing the number of different systems to 14.

### TABLE 1 Relative expression of the TA cassettes according to the growth phase

<table>
<thead>
<tr>
<th>TA cassette</th>
<th>Expression ratio, exponential/stationary phase$^a$</th>
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</thead>
<tbody>
<tr>
<td>highB1</td>
<td>2.4</td>
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<tr>
<td>highB2</td>
<td>0.9</td>
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<tr>
<td>parD1-3</td>
<td>7.1</td>
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<tr>
<td>parD2</td>
<td>1.6</td>
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<tr>
<td>phd</td>
<td>3</td>
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<tr>
<td>relB1</td>
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<tr>
<td>relB2-4</td>
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</tr>
<tr>
<td>relB3</td>
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<tr>
<td>332</td>
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<td>486</td>
<td>0.9</td>
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<tr>
<td>488</td>
<td>2.8</td>
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</table>

$^a$ Quantitative RT-PCR was performed on total RNA from exponential- and stationary-growth-phase cultures.

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To test the functionality of the toxins of *V. cholerae* N16961 chromosomal TA modules, each of its 14 putative toxin genes was cloned into pBAD43 (see Table S1 in the supplement material), as described above for VC0815, and introduced into *E. coli* DH5α cells in the presence of glucose. The toxin activity of each candidate was first tested in an *E. coli* background to avoid potential interference resulting from the presence of resident chromosomal toxin and/or antitoxin genes. Under glucose repression, *E. coli* recombinants carrying each of the 14 toxin genes were able to grow on LB agar plates. When replicated on plates containing 0.2% arabinose, the cells did not grow for any of the tested toxins, except those expressing VCA0423. The gene coding for this putative toxin ends in the middle of the VCR (the *V. cholerae* cassette-specific attC site). This rare configuration generally reflects a frameshift mutation or a fusion between unrelated cassettes. Comparison with other *V. cholerae* genomes revealed the origin of this odd feature. Indeed, in strains O395/569B, we found that the VCA0423 equivalent (VC0395-0843, here called VCA0423c) has a different and shorter C-terminal end, with a stop codon properly located upstream of the VCR. The N16961 VCA0423 odd C-terminal end corresponds to the sequence of a cassette (VC0395-0861) located 18 cassettes downstream of VC0395-0843. We then tested the functionality of VCA0423c and found that it was toxic. Thus, it is very likely that an accidental fusion between these two cassettes has occurred in an ancestor of N16961, inactivating the toxin gene while keeping the promoter and antitoxin gene operational. The rest of the study was made with the O395 VCA0423c toxin gene.

Hence, all the 14 putative toxin genes of *V. cholerae* TA modules are active, and thus toxic, in *E. coli* DH5α, confirming previous characterizations of the HigBA, ParDE, and Phd-Doc subset (25, 30, 36, 37, 42).

**Functionality of the antitoxins of the *V. cholerae* TA cassettes.** Each of the 14 putative antitoxin genes was cloned into a high-copy-number expression vector pUC18 under the control of the Plac promoter and introduced into *E. coli* DH5α cells (see Materials and Methods; also see Table S1 in the supplement material). The functionality of the 14 antitoxins was tested through the fact that expression of a functional antitoxin should prevent cell death due to toxin expression triggered in the presence of arabinose. *E. coli* cells containing each antitoxin cloned into pUC18 were transformed with the cognate toxin-compatible plasmid and grown in the presence of glucose. Cotransformants were then replicated on medium containing either arabinose or glucose. Growth under these two conditions was found to be similar for the 14 tested TA modules. This showed that all of the TA cassettes found in the *V. cholerae* SI encode functional toxins and antitoxins.

**Functionality of the toxins of TA modules in *V. cholerae.*** Each of the 14 toxins found to be toxic in *E. coli,* was introduced by transformation into its natural host *V. cholerae* N16961 in order to establish whether the TA systems were expressed from their original SI location and whether expression of the antitoxin could prevent the toxin’s deleterious activity. The functionality of the Pbas promoter in *V. cholerae* has already been established (see, for example, references 30, 36, 42, and 43), and pBAD43 derivatives carrying the toxin genes were introduced by transformation and selection on glucose-containing medium. The expression of the toxins was then induced by replica plating on a 0.2% arabinose-containing medium, and cell viability was determined. We found that the chromosomally encoded antitoxins counteracted the toxicity of the overexpressed toxins and that the cells showed a similar growth on arabinose or glucose for all the toxins tested. We used the ccdAB TA system previously characterized in the *Vibrio fischeri* SI (26) as a control of our setup. Indeed, N16961 does not carry a
TABLE 2 Cross-interactions between toxin and antitoxins carried in the N16961 TA cassettes

<table>
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<tr>
<th>Antitoxin</th>
<th>HigB1</th>
<th>HigB2</th>
<th>ParE1-3</th>
<th>ParE2</th>
<th>RelE1</th>
<th>RelE2-4</th>
<th>RelE3</th>
<th>Doc</th>
<th>318-481</th>
<th>332</th>
<th>423c</th>
<th>478</th>
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ccdAB locus, and we found that once transformed with a pBAD43::ccdB plasmid, transfer from glucose- to arabinose-containing medium triggered cell death.

Expression of the TA cassettes in the native SI context. In order to formally demonstrate the expression of these TA cassettes and confirm the results obtained in the N16961 transformation experiments described above, we performed a semiquantitative RT-PCR, with or without AMV reverse transcriptase, for each of those TA cassettes, with the exception of phd-doc, as its expression under the same conditions has previously been published (25). Amplification of the expected products was obtained only after reverse transcription (see Fig. S1 in the supplemental material), demonstrating their efficient transcription. Furthermore, their expression in cells harvested at mid-log or early stationary phase was analyzed by real-time quantitative RT-PCR on total RNA. We found that apart from higBA2, which is compatible with the vector used to express all antitoxins, all toxins were expressed from the same vector, which is compatible with the vector used to express all antitoxins. We thus transformed all strains carrying the different antitoxins with each of the plasmids carrying the toxins, selected transformants on glucose, and subsequently grew the cells in arabinose-containing medium to determine if growth could occur under toxin expression conditions. The cells were unable to grow when the expression of a toxin was induced in the presence of a noncognate antitoxin (Table 2).

Growth with toxin:

Toxin activity assays in E. coli. In order to better understand the activity of these toxins, as we could not test their toxicity in Vibrio cholerae, we investigated the activity of 11 of these toxins in E. coli under conditions where toxins were induced in a liquid medium for specific periods of time (60 to 180 min), with the cells then being plated on a glucose-containing medium and incubated to grow with the toxins repressed. We found that all toxins induced at least a 3-log drop in CFU in the first hour of induction, but in all cases a small fraction of cells was able to survive at least as long as 3 h following induction of the toxin (see Fig. S2 in the supplemental material). We verified the presence of active toxin in these cells, as when these surviving cells were replicated on LB agar plates containing either glucose or arabinose, in all cases more than 90% of cells were unable to grow on arabinose (data not shown). This demonstrates that the toxins were still toxic for these cells and they were not spontaneously inactivated mutants. The consistent blockage of the toxicity by the expression of each protein’s cognate antitoxin suggests that this toxicity is due to a discrete activity that can be neutralized and is not a nonspecific consequence of protein overexpression.

Cross-interactions between all toxins and antitoxins carried in the 17 superintegron cassettes. Among these 17 TA systems, 10 belong to four different, well-characterized families, i.e., Hig, Rel, Par, and Phd-Doc. The HigB and RelE toxins belong to the RelE superfamily, but their antitoxins are unrelated (11). The Hig family contains two remotely related higBA loci. The Rel family has four relBE loci, where two relBE loci (relBE2 and relBE4) have identical ORFs and regulatory sequences. The Par family consists of 3 parDE systems, and this family also has two identical parDE loci (parDE1 and parDE3), while there is only 1 phd-doc locus. To gain insight into the degree of specificity of these TA modules, we explored whether or not an antitoxin can counteract the activity of a noncognate toxin. All toxins were expressed from the same vector, which is compatible with the vector used to express all antitoxins. We thus transformed all strains carrying the different antitoxins with each of the plasmids carrying the toxins, selected transformants on glucose, and subsequently grew the cells in arabinose-containing medium to determine if growth could occur under toxin expression conditions. The cells were unable to grow when the expression of a toxin was induced in the presence of a noncognate antitoxin (Table 2). No cross-interaction was observed among all 87 crosses between antitoxins and noncognate toxins, showing that interactions are highly specific, even between TA systems of the same family (Table 2).

Occurrence of these TA cassettes in Vibrio species. We first explored the conservation of the 14 unique TA cassettes of N16961 among the other Vibrio cholerae genomes that have been entirely or partially sequenced. We found that these cassettes are highly conserved among the V. cholerae strains and isolates with fully assembled genomes (see Table S2 in the supplemental material), while their distribution in strains with partially sequenced genomes is extremely variable. However, this does not reflect the actual TA cassette content of the SI in these strains.

We also carried out a study of the distribution of these TAs in the genomes of other Vibrio species. We observed that these TAs are variably present in other Vibrio genomes and sometimes are not embedded in cassettes (Table 3). Three TAs, HigB2A, VCA0477–478, and Phd-Doc, were uniquely found in V. cholerae (Table 3), but it is possible that this apparent lack of cassette exchange reflects a bias in the sampling, as we previously showed that the Phd-Doc cassette was also present in the Vibrio metchnikovii SI (26).
<table>
<thead>
<tr>
<th>Bacterial species and strain</th>
<th>Location of TA family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio cholerae N16961</td>
<td>C, CNC</td>
</tr>
<tr>
<td>Vibrio fischeri ES114</td>
<td>CNC, CC</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>C, CNC, P, C</td>
</tr>
<tr>
<td>Vibrio splendidus</td>
<td>CNC, CC, GP, CNC</td>
</tr>
<tr>
<td>Aliivibrio salmonicida</td>
<td>CNC, CC, P, C, C, C, C</td>
</tr>
<tr>
<td>Vibrio vulnificus CMCP6</td>
<td>C, CNC</td>
</tr>
<tr>
<td>Vibrio vulnificus YJ016</td>
<td>CNC</td>
</tr>
</tbody>
</table>

- C, cassette; CNC, chromosomal not in cassette; P, plasmid.
- HigBA1 (N16961 genome), VCA0391-392; HigBA2, VCA0468-469.
- ParDE1 (N16961 genome), VCA0311-312; ParDE2, VCA0359-VCA0360; ParDE3, VCA0385-386.
- RelBE1 (N16961 genome), VCA0324-325; RelBE2, VCA0348-349; RelBE3, VCA0444-445; RelBE4, VCA0503-504.
- Phd-Doc (N16961 genome), VCA0475.
- Seven hybrid and new systems of the N16961 genome.
- Embedded in cassette but mobilized away from SI.
- Cassette repeated twice.
DISCUSSION
In a previous analysis in 2005, Pandey and Gerdes identified 13 TA operons in the *V. cholerae* N16961 genome. Strikingly, their analysis revealed that all of these were embedded within cassettes of the superintegron (7). Since then, TA systems have gained even more attention, as they have been the subject of multiple analyses and many new systems have been described (11). This, together with the fact that the role of these genetic modules is highly debated, led us to undertake a reanalysis of the toxin-antitoxin system content of *V. cholerae* genomes. We uncovered 5 novel TA modules in N16961, 4 of which were carried within integron cassettes, giving a total of 17 TA cassettes (Fig. 1). These cassettes are highly conserved among *V. cholerae* strains and isolates (see Table S2 in the supplemental material). The single TA system that we identified and characterized which is not carried in the SI encodes a toxin of the HipA family and its antitoxine. This toxin has been shown to inactivate the glutamyl-tRNA synthetase through phosphorylation and was identified through its major role in persiste formation in *E. coli* (15, 16).

We have demonstrated the functionality of the 17 *V. cholerae* TA cassettes and explored their cross-interactions. Among these, 10 correspond to well-defined TA families, and six of these cassettes have been previously characterized to various extents. These are the three ParDE, the two HigBAs, and the single Phd-Doc cassettes (25, 30, 36, 42). Two of the three ParDE family cassettes correspond to a duplication of the same cassette (parDE1 and parDE3) separated by 43 kb in N16961. The genes of both ParDE cassettes, parDE1-3 and parDE2, are in opposite orientation compared to the majority of cassettes whose expression relies on the Pc promoter (Fig. 1) (44). The ParE toxin is a gyrase inhibitor that induces DNA degradation and activates the SOS response to DNA damage, resulting in inhibition of cell division and reducing cell viability. In agreement with the recent findings of Yuan and coworkers (36, 42), our results confirm that parDE1-3 and parDE2 encode functional toxins whose toxicity was completely blocked by their cognate antitoxins. We further confirmed no cross-interaction between the three members of Par family (36, 42). As in *Caulobacter crescentus*, which carries three ParDE systems (45), transcription of the two functional paralogous parDE loci is differentially regulated, as parDE1-3 is highly induced during exponential growth phase, while parDE2 is induced only 1.6-fold (Table 1), supporting the idea that these paralogous systems act as distinct functional units (45). The two HigBA cassettes, higBA1 and higBA2, carry operons oriented as classical cassette genes, i.e., in the same direction as transcription from the Pc promoter (Fig. 1). Both HigB toxins have been shown to inhibit the global rate of translation and preferentially cleave translated mRNA (30). Our results are similar to those published previously (30, 37) and confirm that both of the *V. cholerae* higBA loci encode functional toxins and that their toxicity is neutralized only by expression of their cognate antitoxins. As for parDE, we found that the two paralogues are differentially regulated during growth (Table 1).

We characterized in this study, in terms of both functionality and expression, the remaining 11 TA cassettes. The *V. cholerae* N16961 SI contains 4 relBE-homologous cassettes, two of which (relBE2 and relBE4) are identical. As with ParDE cassettes, the relBE genes are in opposite orientation compared to canonical integron cassettes. RelE toxins are translation inhibitor cytotoxins that cleave mRNAs from their 5’ ends, leading to a rapid translational shutdown that is lethal or inhibitory to the host cells (46). We show here that all the relBE loci of *V. cholerae* N16961 encode functional toxins and that the activity of these toxins is completely counteracted by their cognate antitoxins. We also observed that no cross-interactions exist between the noncognate RelE and RelB proteins. In contrast to the ParDE and HigBA TA systems, the four paralogues RelBE are similarly regulated during growth (Table 1).

The seven other TA systems in the superintegron are either hybrid or novel systems. There is one phd-parE-like (VCA0422/0423), one stbD-relE-like (VCA0477/0478), and one relB-parE-like (VCA0488/0489) system according to the TA search software described previously (11) and four systems with ribbon-helix-helix (RHH) transcription factor-like antitoxins (VCA0319 [its duplication in VCA0482], VCA0333, and VCA0486 [see Table S3 in the supplemental material]) associated with toxins that are either N-acetyltransferases (VCA0318/0481, VCA0332, and VCA0487) or conserved proteins of unknown function (VCA0332). We showed that all are expressed and that their transcription, except for VCA0486-0487, is induced during exponential growth (Table 1). We demonstrate that all but one of these TA loci encode functional toxins-antitoxins. The nonfunctional cassette of N16961, VCA0422-0423, carries a toxin (VCA0423) that has been inactivated through an illegitimate recombination with another cassette, which exchanged the 3’ part of the carried gene, leading to a new C-terminal part for the original VCA0423. By chance, we found that this was a recent event, as several *V. cholerae* strains, including 569B and O395, carry the original VCA0422-0423c cassette, for which we demonstrated toxin functionality.

We tested all possible cross-interactions between the different antitoxins and toxins that could lead to toxin inhibition and found that all the antitoxins block only their cognate toxin activity, even when TAs belonged to the same family (Table 2). The 17 identified TA cassettes are scattered throughout the 176-cassette-long array of the N16961 superintegron (Fig. 1). The first TA cassette corresponds to cassette 18 in the array, and the last is located in position 174, 2 cassettes before the end cassette (see the annotated *V. cholerae* N16961 genome of the MicroScope platform at the French Genoscope for a precise mapping [https://www.genoscope.cns.fr/technologies/microscope/mage/]). As mentioned above, these cassettes are highly conserved among *V. cholerae* strains and isolates (see Table S2 in the supplemental material).

As mentioned above, we have shown that all of these cassettes are expressed and carry their own promoter, an extremely rare situation for integron cassettes. This rare property guarantees that they are transcribed independently of their location inside the cassette array. Indeed, integron cassettes seldom carry a promoter, and their expression occurs when they are recombined at, or close to, the attl site, which carries a strong promoter, Pc (44, 47). This dynamic expression model, which allows the expression of adaptive genes only when needed, is considered to be central for integron evolutionary success (31). In the *V. cholerae* N16961 SI, as for other integrons, this constraint impacts the orientation of the cassette ORFs; among the 188 consecutive ORFs of its SI, only 27 are in opposite orientation relative to the Pc promoter. Strikingly, among these, 18 correspond to genes carried in 9 of the 17 TA cassettes. It is interesting to note that in this configuration, even if transcription extends beyond the cassette boundary, it will not express the surrounding genes, as they are carried on the opposite, complementary, strand. The SI TA expression was found to be higher during exponential growth than during entry into station-
ary phase, except for two systems (Table 1). It was previously suggested that chromosomal TA loci function as stress response elements, as they help the cells cope with nutrient starvation (14). Indeed, transcription of TA loci was found to be strongly increased during nutrient starvation in Escherichia coli (48–50) and during heat shock in Sulfolobus solfataricus (51). This has also been observed for the two higBA cassettes, whose transcription is induced during amino acid starvation (30). All but two antidotes are predicted to carry a structural domain known to mediate interactions with DNA (see Table S3 in the supplemental material), suggesting that they can regulate the expression of the TA operons under certain conditions. Our results advocate for a major role of these 17 TA cassettes in the stabilization of the SI cassette arrays, as previously proposed when we characterized the first two TA cassettes (26, 27). Chromosomal TA loci may act to stabilize their neighboring DNA, mobile genetic elements, and large genomic islands because the loss of any given TA locus (e.g., by slippage during replication or illegitimate recombination) will result in a cessation of de novo antitoxin production, toxin activation, and arrest of cell growth and/or viability. This hypothesis is reinforced by the absence of cross talk between the SI TAs we observed (Table 2).

This possible role better explains the presence of 17 functional TA loci grouped in the SI of V. cholerae (Fig. 1), as the V. cholerae SI is tightly packed with genes (one gene per 600 bp and at least 176 attC sites) (26). The V. cholerae attC sites, called VCR, are 123-bp-long specific sequences which show more than 90% identity in 149 out of the 176 VCRs (26). Thus, any recombination between repeated copies of cassettes that can result in a large-scale deletion will be counterselected by one of the 14 unique TA loci, as far as the TA is located in that fragment. The V. cholerae TA loci are likely to constrain the emergence of daughter cells carrying deletions within the SI, which may otherwise survive if there were no functional TA loci and thus contribute to the stable perpetuation of the V. cholerae genome. The facts that all these TA cassettes are expressed from their own promoter and that 15 out 17 are more expressed during exponential growth, which is when the replication is active, support this stabilization role. Indeed, recombination and slippage, which would lead to accidental cassette loss, are more frequent during active replication than when bacteria reach stationary phase and stop replication. However, it is likely that the TA cassettes could also have additional roles. These cassettes can protect their host genome from colonization by incoming mobile elements or plasmids carrying TAs from the same functional families by allowing their harmless loss through neutralization of the invading toxin by the chromosomal antitoxin (22, 25). These TAs can also play a role in bacteriophage defense through abortive infection mechanisms, as already demonstrated in other bacteria (19–21, 56). Vibrio cholerae ecology is intrinsically linked to phage infection. Indeed, phage predation has been reported to be a factor that influences seasonal epidemics of cholera in Bangladesh (52). Furthermore, phage infections have been shown to occur in the human host, leading both to a decreased load of bacteria (53) and to an alteration of the virulence potential of V. cholerae shed from cholera patients (54).

We observed that when ectopically expressed in E. coli, the efficiencies of the different tested toxins on cell viability vary to a large extent. However, all lead to the formation of a fraction of cells that, if not dividing, survive toxin activity and are in a state very similar to persistence (see Fig. S2 in the supplemental material). Persisters are nongrowing, dormant cells having low levels of translation, and their state may be related to viable-but-nonculturable (VBNC) conditions, which have been commonly observed in Vibrio species, including V. cholerae (for a review, see reference 55). The molecular mechanisms underlying this phenomenon have not yet been identified, but those TAs may have similar properties in V. cholerae and play a key role in persister formation, even when carried in SI cassettes, as found by the groups of Lewis and Hill for E. coli TA systems (16, 17). Indeed, in E. coli, the overexpression of different TA loci enriched for persister cells and ectopic expression of TA loci dramatically increased the number of cells exhibiting multidrug resistance (16–18).

The quasiabsence of TA loci from the V. cholerae core genome is puzzling, especially as we identified TAs in other vibrios both inside and outside their integrons in their genome (31) (Table 3). The presence of expressed TAs within the integron cassettes may have allowed the deletion of TAs located outside the SI due to TA cross-interactions, given the possibility that these cassettes could fulfill the physiological role(s) played by chromosomal TAs. This hypothesis now needs to be tested.

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References

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