Genomic characterization of the non-O1/non-O139 *Vibrio cholerae* strain that caused a gastroenteritis outbreak in Santiago, Chile, 2018

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Abstract

Vibrio cholerae is a human pathogen which is transmitted by the consumption of contaminated food or water. *V. cholera* strains belonging to the serogroups O1 and O139 can cause cholera outbreaks and epidemics, a severe life-threatening diarrheal disease. In contrast, serogroups other than O1 and O139, denominated as non-O1/non-O139, have been mainly associated with sporadic cases of moderate or mild diarrhea, bacteremia and wound infections. Here we investigated the virulence determinants and phylogenetic origin of a non-O1/non-O139 *Vibrio cholerae* strain that caused a gastroenteritis outbreak in Santiago, Chile, 2018. We found that this outbreak strain lacks the classical virulence genes harbored by O1 and O139 strains, including the cholera toxin (CT) and the toxin-coregulated pilus (TCP). However, this strain carries genomic islands (GIs) encoding Type III and Type VI secretion systems (T3SS/T6SS) and antibiotic resistance genes. Moreover, we found these GIs are wide distributed among several lineages of non-O1/non-O139 strains. Our results suggest that the acquisition of these GIs may enhance the virulence of non-O1/non-O139 strains that lack the CT and TCP-encoding genes. Our results highlight the pathogenic potential of these *V. cholerae* strains.

Introduction

V. cholerae strains belonging to serogroups O1 and O139 are known to cause cholera outbreaks and epidemics. These serogroups generally produce the cholera toxin (CT) and the toxin coregulated pilus (TCP), which are responsible for secretory diarrhea and intestinal colonization, respectively [1]. Serogroups other than O1 and O139, called non-O1/non-O139, typically lack the CT and TCP-encoding genes [2]. However, several non-O1/non-O139 *V. cholerae* strains harbor additional virulence factors that contribute to pathogenicity [3]. In fact, non-O1/non-O139 *V. cholerae* strains have been isolated from sporadic cases of gastroenteritis, bacteremia and wound infections [1, 2, 4]. To date, the pathogenic mechanisms of non-O1/non-O139 *V. cholerae* strains have not been fully investigated.

There were several cholera outbreaks in South America in the 1990s [5]. In Chile, the last reported cases of cholera corresponded to the 1997-1998 outbreak of San Pedro de Atacama. Until 2017, only sporadic cases of gastroenteritis or bacteremia caused by non-O1/non-O139 *V.* cholerae strains had been reported in the country [6]. On August 2, 2018, an outbreak of acute gastroenteritis started in Santiago, Chile. As of February 16, 2019, 55 gastroenteritis cases were reported, of which 13 required hospitalization, with diarrhea, nausea and vomiting being the main symptoms. The epidemiological study of the outbreak performed at the Chilean National Health Institute (Instituto de Salud Pública de Chile, ISP) identified a clonal strain of non-toxigenic and non-O1/non-O139 *V. cholerae* as the etiology agent in 44/55 cases, although the source of contamination could not be determined [7]. Here, we performed comparative genomic and phylogenetic analyses to decipher the virulence determinants and phylogenetic origin of this outbreak strain.

Materials and methods

Antimicrobial drug susceptibility test

The disk diffusion method or broth dilution was performed according to Clinical and Laboratory Standards Institute guidelines [8].

Genome sequencing

Genomic DNA of the *V. cholerae* str. Santiago-089 was extracted using the Wizard genomic DNA purification kit (Promega Corp., USA) and sequenced at MicrobesNG (University of Birmingham, UK) using the Illumina MiSeq or HiSeq 2500 technology platforms with 2×250-bp paired-end reads. Draft genomes were provided after trimming low-quality ends and assembling reads with SPAdes 3.10 [9]. Assembly statistics were obtained with Quast v4.6.3 [10]. Contigs shorter that 200 nt were removed and sequences were deposited in GenBank under the accession number SRLP00000000.

Publicly available genome sequences

A total of 69 genome sequences of *V. cholerae* strains were downloaded from GenBank on June 1, 2019. Sequence management and BLASTn searches were performed using the Geneious

software (v11.0.5; Biomatters, Ltd). Genome accession numbers are listed in Supplementary Table 1.

Phylogenetic analysis

A maximum likelihood phylogenetic tree based on core single nucleotide polymorphisms (SNPs) of 70 complete or draft genomic sequences of *V. cholerae* strains was built using CSI Phylogeny 1.4 [11]. This analysis was performed using the default input parameters and *V. cholerae* str. N16961 as the reference genome. As a result, 146,534 SNPs were identified in 2,483,145 positions found in all analyzed genomes. The output file in Newick format was downloaded and used to visualize the phylogenetic tree in the Interactive Tree of Life tool [12]. The population structure of the strains was determined with RhierBAPS [13] using the 146,534 SNPs. For this, two depth levels and a maximum clustering size of 14 (default = number of isolates/5; 70/5 = 14) were specified. MLST sequence types were determined using the MLST 2.0 tool [14].

Detection of virulence genes

Virulence genes analyzed in this study and their GenBank accession numbers are listed in Supplementary Table 2. The presence/absence of virulence genes was determined using the BLASTn algorithm implemented in the Geneious software (v11.0.5; Biomatters, Ltd). The absence of a gene was defined as an identity and/or gene coverage of less than 80% and 60%, respectively. The heat map showing the presence, absence and variation of the virulence genes was drawn using the gplots package [15] in R [16].

Comparative genomic analysis and identification of genomic islands

Identification and characterization of DNA regions with features of genomic islands were performed using REPuter [17], ISfinder [18] and tRNAscan-SE [19]. Open reading frames (ORFs) were determined using the Geneious software (v11.0.5; Biomatters, Ltd) and RAST server [20]. The ORFs of the GI*Vch*-T6SS$_{\text{Santiago-089}}$ and GI*Vch*-MDR$_{\text{Santiago-089}}$ are listed in Supplementary Tables 3 and 4, respectively. The comparison of the genetic structure of genomic islands was performed using EasyFig [21]. Additionally, the presence/absence of the genomic islands VPI-I (GenBank accession: NC_002505.1, positions 873242 - 915211), VPI-II (GenBank accession: NC_002505.1, positions 1895692 - 1952861), VSP-I (GenBank accession: NC_002505.1, positions 175343-189380) and VSP-II (GenBank accession: NC_002505.1, positions 520634 - 550262) and Phage CTXφ (GenBank accession: NC_002505.1, positions 1550108 - 1574355) were determined using BRIG [22].

Results

The *V. cholerae* strain characterized in this study, which we named the Santiago-089 strain, was isolated from a 12-year-old boy hospitalized with bloody diarrhea and abdominal pain. This was one of the first gastroenteritis cases of the outbreak. The microbiological study of this strain was approved by written consent of the parents and the Ethics Committee of the Clinical Hospital University of Chile. Moreover, a Pulsed-field gel electrophoresis analysis performed at the ISP indicated that this and another 43 strains isolated during the outbreak were clonal (personal communication of Dr. Juan Carlos Hormazabal, Sub-department of Infectious Diseases, ISP).

Initially, an antimicrobial drug susceptibility test showed that the Santiago-089 strain is resistant to trimethoprim-sulfamethoxazole, erythromycin and nalidixic acid (**Table**). Next, the phylogeny of this outbreak strain was investigated. For this, its genomic DNA was sequenced, and the draft genome deposited in GenBank under the accession number SRLP00000000. In addition, a set of genomes of *V. cholerae* strains isolated worldwide that are available in GenBank were included in the phylogenetic analysis (**Supplementary Table 1**). As shown in the maximum likelihood phylogenetic tree (**Figure 1A**), the strains were clustered into twelve lineages. While lineages 4 and 5 clustered the O1, O139 and O65 strains, the rest of the lineages clustered only non-O1/non-O139 strains. In particular, the Santiago-089 strain was clustered in lineage 2 along with strains isolated from India, Bangladesh and Haiti. Moreover, MLST sequence types were consistent with the topology of the tree.

This outbreak strain caused several hospitalizations but lacks the CT and, consequently other virulence genes must be contributing to its pathogenicity. Therefore, we analyzed its genome searching for other virulence genes (**Supplementary Table 2**). As expected, the Santiago-089 strain lacks the Phage CTXφ and the genomic islands VPI-I, VPI-II, VSP-I and VSP-II, which are generally carried by O1 and O139 strains (**Figure 2**). However, this strain carries genes that encode toxins such HlyA and MARTX, and proteins of the Type III and Type VI secretion systems (T3SS/T6SS) (**Figure 1B**). Similarly, most of the non-O1/non-O139 strains lack the Phage CTXφ and the GIs mentioned; rather, they carry genes encoding proteins of T3SS and T6SS.

We analyzed the genetic context of the T3SS and T6SS genes in the Santiago-089 strain and found that they are located in GIs. The GI*Vch*-T3SS identified was previously reported in the *V. cholerae* str. AM-19226 and promotes colonization and infection [23]. We found that this GI of ~64-kb is inserted in the tRNA-*ser* gene and located next to a nan-nag region involved in the sialic acid catabolism (**Figure 3A)**, which is also harbored by the VPI-II [24]. The second GI, which we named GI*Vch*-T6SS$_{\text{Santiago-089}}$, was partially identified and contains a CRISPR-Cas region and genes that encode Hcp and VgrG alleles, which are structural T6SS components (**Figure 3B, Supplementary Table 3**). The GI*Vch*-T6SS$_{\text{Santiago-089}}$ was also identified in the *V. cholerae* str. HC36A1. The Hcp from GI*Vch*-T6SS$_{\text{Santiago-089}}$ has 96.5% amino acid identity with the Hcp1 and Hcp2 alleles reported in *V. cholerae*. In contrast, the VgrG from GI*Vch*-T6SS$_{\text{Santiago-089}}$ has 66.7%, 66.6% and 59.4% amino acid identity with the VgrG-1, VgrG-2 and VgrG-3 alleles reported in *V. cholerae*, respectively [25]. Therefore, we named this uncharacterized allele VgrG-4. Moreover,

we found that Hcp and VgrG-4 alleles from GI*Vch*-T6SS$_{Santiago-089}$ are also harbored by the GI*Vch*S12 [26]. Thus, the identification of T3SS genes and the VgrG-4 allele in non-O1/non-O139 strains of several lineages suggests these GIs are widely distributed (**Figure 1**).

Finally, since the Santiago-089 strain is multidrug-resistant (**Table**), we analyzed its genome searching for genes or mutations that mediate its antimicrobial resistance profile. As a result, we identified a multidrug resistance (MDR) genomic island of ~31-kb that we named GI*Vch*-MDR$_{Santiago-089}$ (**Figure 3C, Supplementary Table 4**). This GI harbors the *sul1* and *dfrA15* genes, which confer resistance to sulfonamide and trimethoprim, respectively. The genetic structure of the GI*Vch*-MDR$_{Santiago-089}$ is also relatively similar to the GI*Vch*Hai6 [27]. On the other hand, the Santiago-089 strain has the mutations S83I and A171S in the GyrA protein, which confer resistance to nalidixic acid [28] (**Supplementary information**). Genes or mutations conferring resistance to erythromycin were not identified.

Discussion

V. cholerae remains as a major public health problem, mainly in endemic areas of Asia and Africa, where access to adequate sanitation and safe drinking water is still limited [29]. In Chile, only sporadic or imported cases of gastroenteritis caused by non-O1/non-O139 *Vibrio cholerae* strains have been reported in the last 20 years [6]. Therefore, it was interesting to investigate the virulence determinants of this outbreak strain.

The fact that a *V. cholerae* strain like the one characterized in the present study, which lacks the classical virulence factors (i.e. CT and CTP) yet causes a gastroenteritis outbreak with several hospitalized patients, represents a public health concern that justifies the permanent surveillance of these pathogens. Moreover, the multidrug-resistant phenotype of the Santiago-089 strain, as well as the spread of MDR islands [27], are also epidemiological factors that need to be considered by public health authorities. In particular, the Santiago-089 strain was isolated from a case of bloody diarrhea. Although bloody diarrhea is not a common symptom caused by *V. cholerae*, there are some reports in which non-O1/non-O139 strains have caused it [30]. The above is of major importance for the diagnosis and treatment of these infections.

We show that the population structure of *V. cholerae* and particularly of the non-O1/non-O139 strains is heterogeneous, having a clear phylogenetic diversity and where known virulence factors are widely distributed among lineages. Indeed, we found that in the absence of the classic virulence factors, the non-O1/non-O139 strains have acquired several GIs encoding T3SS and T6SS, which may enhance their virulence. T3SS has a key role in the pathogenesis of *Vibrio parahaemolyticus* [31]. Although less studied, the role of T3SS in the pathogenesis of *V. cholerae* and other pathogenic *Vibrio* species has begun to be understood [23, 32]. These genes could be considered molecular risk markers for these pathogens and may be useful in epidemiological monitoring studies. We also identified new Hcp and VgrG alleles that in future studies will be functionally characterized. In conclusion, this study highlights the pathogenic potential of the Santiago-089 strain as well as other non-O1/non-O139 *V. cholerae* strains.

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Author contributions

The study was conceptualized by DA. Montero and R. Vidal. Laboratory work was done by DA. Montero, M. Arteaga, J. Velasco, S. Rodrigues, C. Arellano, M. Vidal and F. Silva. Genomic analysis was carried out by DA. Montero, M. Arteaga, J. Velasco and LJ. Carreño. *Vibrio cholerae* str. Santiago-089 was provided by F. Silva. The manuscript was drafted by DA. Montero, M. Arteaga and J. Velasco and reviewed and edited by DA. Montero, M. Vidal, LJ. Carreño, and R. Vidal. All authors have read and approved the final version of the manuscript.

Data Availability

For those interested in the original data used for these analyses, please contact the corresponding author.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Table. Antimicrobial resistance profile of the *V. cholerae* str. Santiago-089 *

Antimicrobial agent	Disk diffusion	Zone Diameter (mm)	MIC (µg/ml)	Interpretative criteria
Ampicillin	10 µg	20	-	Susceptible
Azithromycin	-	-	2	Susceptible
Erythromycin **	-	-	8	Resistant
Gentamicin	10 µg	16	-	Susceptible
Tetracycline	30 µg	25	-	Susceptible
Ciprofloxacin	5 µg	22	-	Susceptible
Trimethoprim-Sulfamethoxazole	1.25/23.75 µg	6	-	Resistant
Chloramphenicol	30 µg	31	-	Susceptible
Nalidixic acid **	30 µg	6	-	Resistant

* The disk diffusion method or broth dilution was performed according to Clinical and Laboratory Standards Institute guidelines. ** Interpretive criteria adapted from Enterobacteriaceae

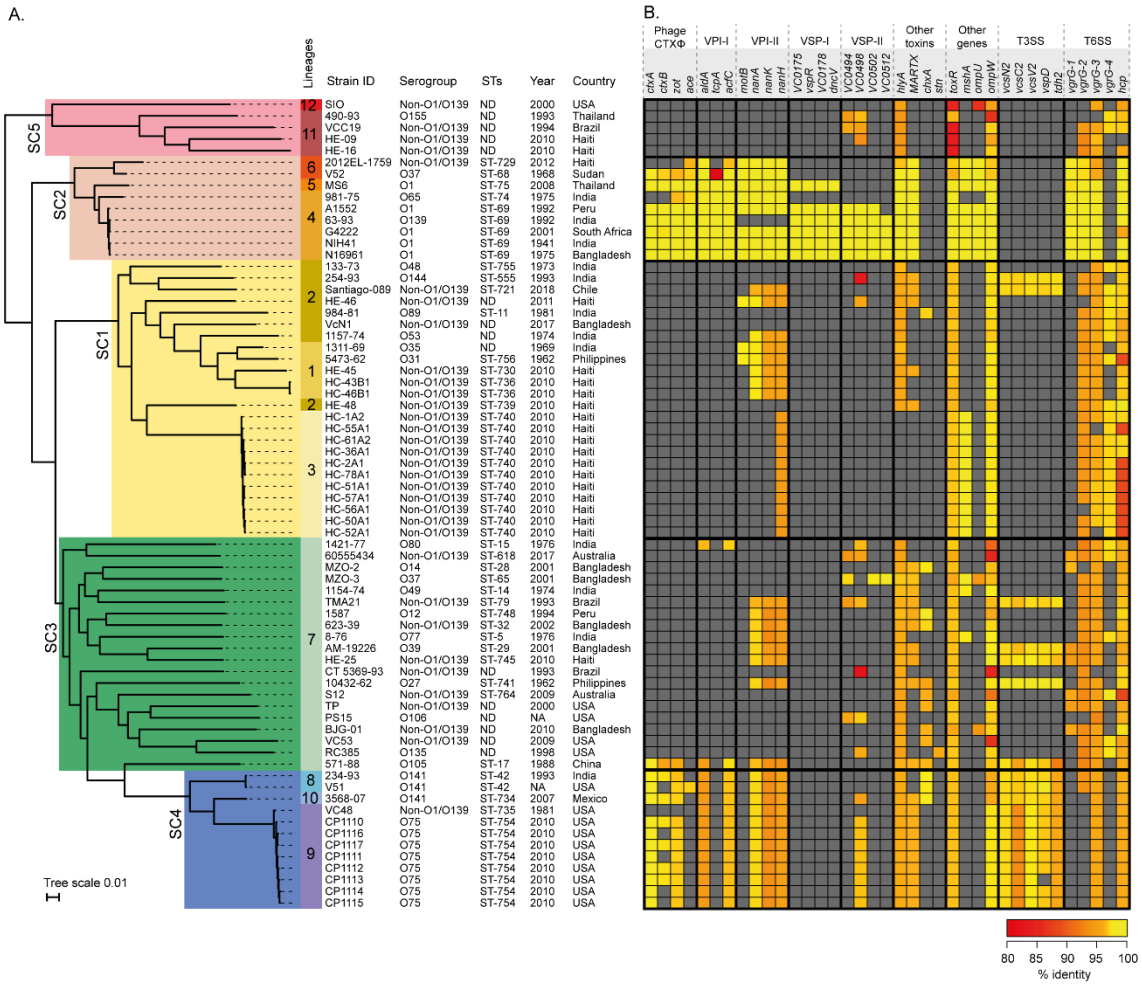

Figure 1. **Phylogenetic relationship between 70 *V. cholerae* strains**. **A)** Maximum likelihood phylogenetic tree (midpoint rooted) based on whole genome SNPs (146,534 SNPs within 2,483,145 positions, which were found in all analyzed genomes). The genome of the *V. cholerae* str. N16961 was used as the reference. Bayesian analysis of population structure grouped the strains into five sequence clusters (SC; SC1 to SC5), which were further divided into 12 lineages. The epidemiological data of each strain is shown, including country and year of isolation. MLST sequence types (STs) are shown. **B)** Heat map showing the presence, absence and variation of major virulence-associated genes distributed among the strains. Presence and variation (nucleotide identity levels, ranging from 80 to 100%) for each gene are indicated by color intensity (red to yellow), as shown in the legend. The analysis was performed using BLASTn. Absence was defined as an identity and/or gene coverage of less than 80% and 60%, respectively and is indicated in gray.

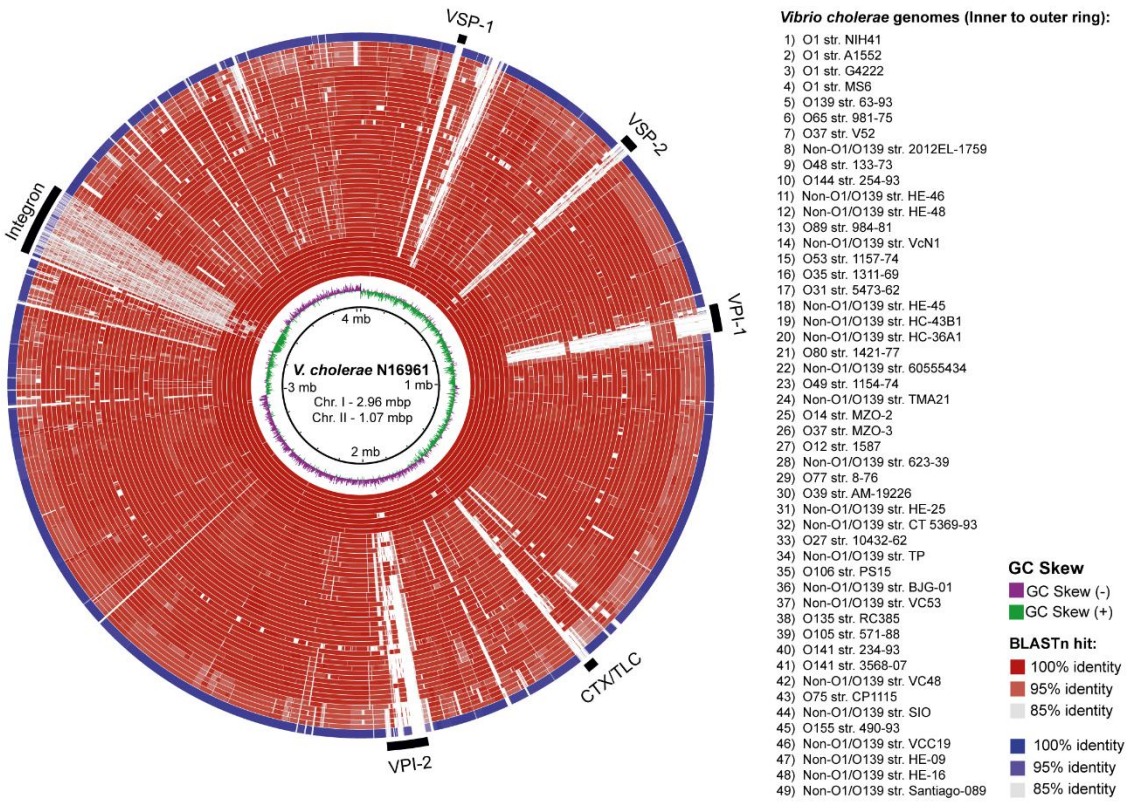

Figure 2. **Comparison of the genomes of the *V. cholerae* str. Santiago-089 and 48 representative *V. cholerae* strains**. The genomes are compared against the reference genome of *V. cholerae* str. N16961. The outermost ring (blue) shows the genome of *V. cholerae* str. Santiago-089. All other genomes are shown in red rings. BLASTn comparisons between reference genome and query genomes are shown as % identity according to the legend at the right. White regions indicate absence of genes or identity levels below 85%. The location of the major genomic islands is indicated as black bars. VSP, *Vibrio* seventh pandemic island; VPI, *Vibrio* pathogenicity island; CTX/TLC, cholera toxin/toxin-linked cryptic. The figure was prepared using BRIG [22].

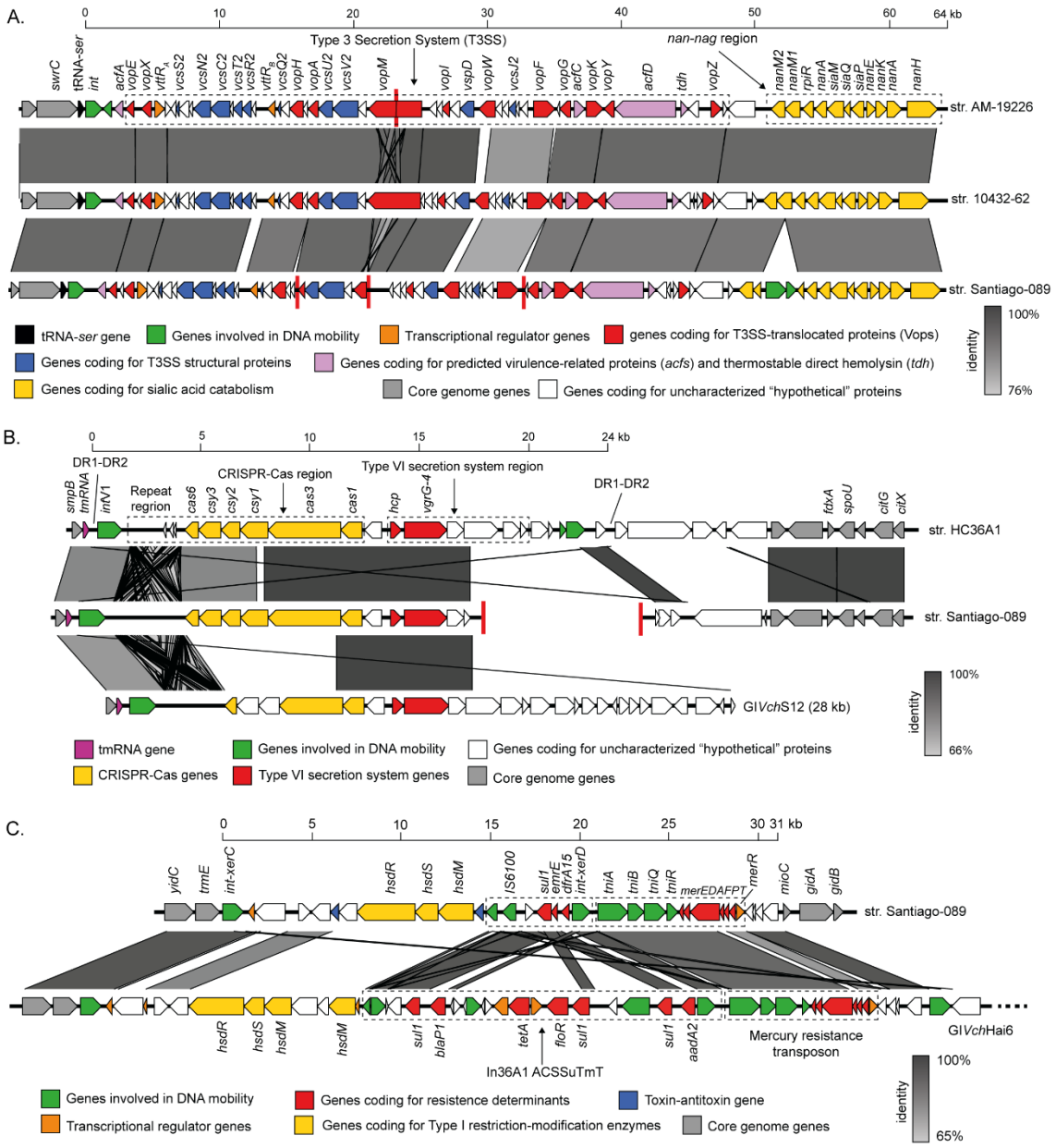

Figure 3. Comparison of the genetic structures of genomic islands carried by the *V. cholerae* str. Santiago-089. Predicted genes and the direction of transcription are represented as block arrows. Genes are color-coded according to gene function, as indicated in the legends at the bottom. The names of some genes are indicated. Conserved regions are shaded in gray and the intensity of the color indicates nucleotide identity levels, as indicated in the legends at the right. Contig boundaries are shown as red lines. **A)** Genomic islands encoding a T3SS. GenBank accessions: GI*Vch*-T3SS$_{AM-19226}$ (AATY02000004 and AATY02000003), GI*Vch*-T3SS$_{10432-62}$ (CP010812), GI*Vch*-T3SS$_{Santiago-089}$ (SRLP00000000). **B)** Genomic islands encoding CRISPR-Cas and T6SS genes. GenBank accessions: GI*Vch*-T6SS$_{HC-36A1}$ (AXDR01000008.1), GI*Vch*-T6SS$_{Santiago-089}$ (SRLP00000000), GI*Vch*S12 (KU722393). Open reading frames (ORFs) of the GI*Vch*-T6SS$_{Santiago-089}$ are listed in Supplementary Table 3. **C)** Multidrug resistance (MDR) islands. The GI*Vch*-MDR$_{Santiago-089}$ (GenBank accession: SRLP00000000) harbors the *sul1* and *dfrA15* genes, which confer resistance to sulfonamide and trimethoprim, respectively. ORFs of the GI*Vch*-MDR$_{Santiago-089}$ are listed in Supplementary Table 4. GI*Vch*Hai6 (GenBank accession: AXDR01000001).

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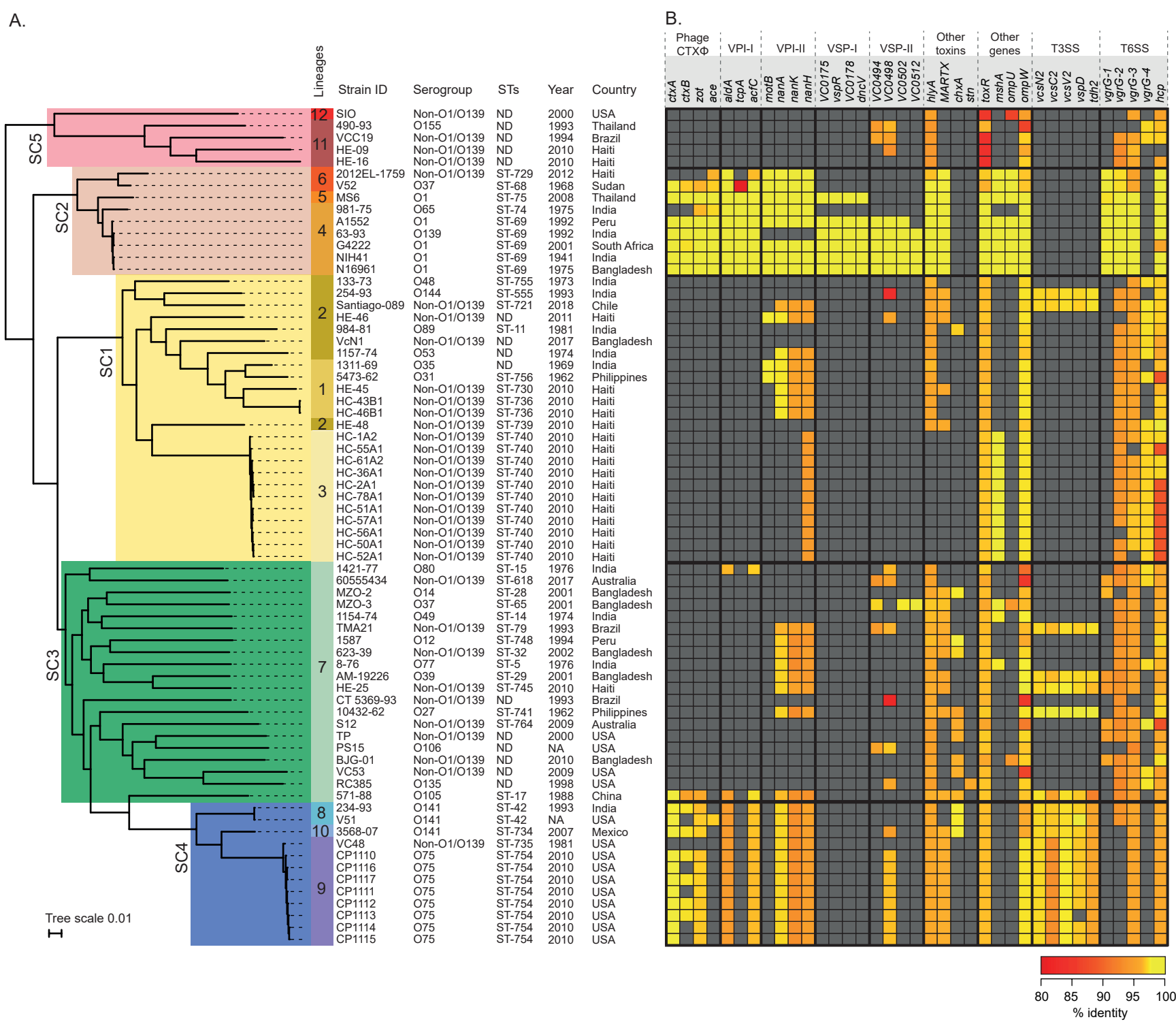
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motB nanA nanK nanH
VC0175 vspR VC0178 dncV
VC0494 VC0498 VC0502 VC0512
hlyA MARTX chxA stn
toxR mshA ompU ompW
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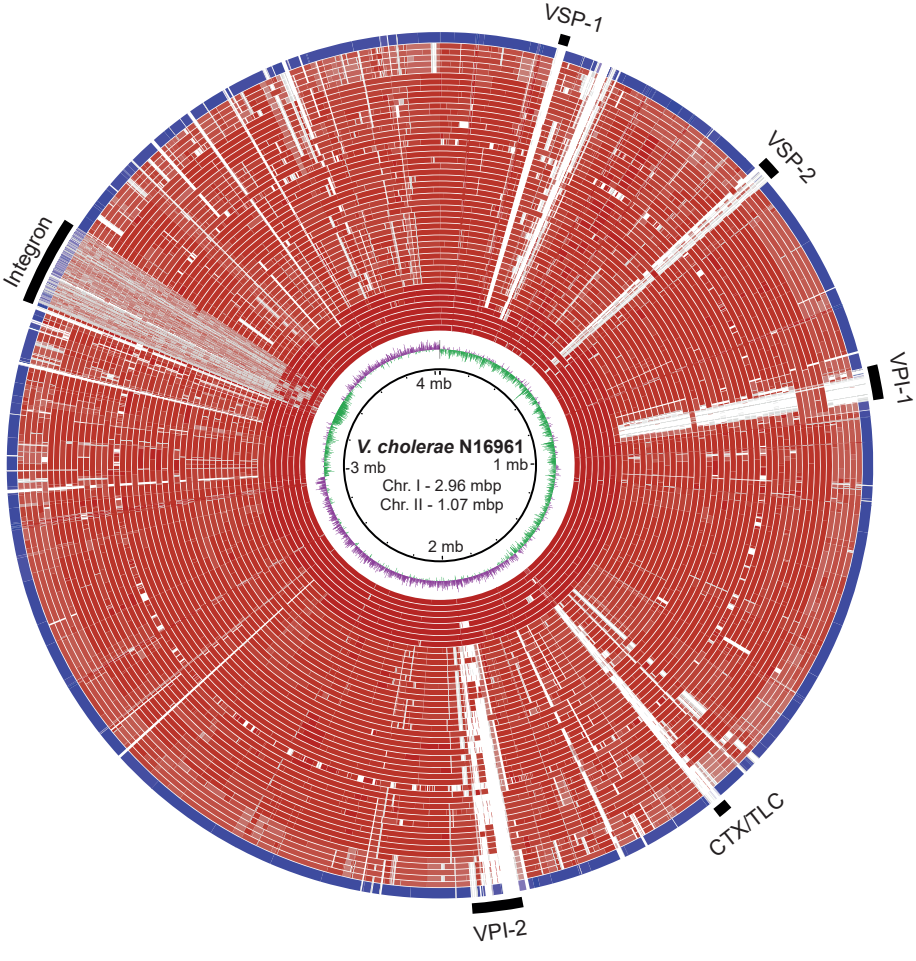
% identity



***Vibrio cholerae* genomes (Inner to outer ring):**

1) O1 str. NIH41
2) O1 str. A1552
3) O1 str. G4222
4) O1 str. MS6
5) O139 str. 63-93
6) O65 str. 981-75
7) O37 str. V52
8) Non-O1/O139 str. 2012EL-1759
9) O48 str. 133-73
10) O144 str. 254-93
11) Non-O1/O139 str. HE-46
12) Non-O1/O139 str. HE-48
13) O89 str. 984-81
14) Non-O1/O139 str. VcN1
15) O53 str. 1157-74
16) O35 str. 1311-69
17) O31 str. 5473-62
18) Non-O1/O139 str. HE-45
19) Non-O1/O139 str. HC-43B1
20) Non-O1/O139 str. HC-36A1
21) O80 str. 1421-77
22) Non-O1/O139 str. 60555434
23) O49 str. 1154-74
24) Non-O1/O139 str. TMA21
25) O14 str. MZO-2
26) O37 str. MZO-3
27) O12 str. 1587
28) Non-O1/O139 str. 623-39
29) O77 str. 8-76
30) O39 str. AM-19226
31) Non-O1/O139 str. HE-25
32) Non-O1/O139 str. CT 5369-93
33) O27 str. 10432-62
34) Non-O1/O139 str. TP
35) O106 str. PS15
36) Non-O1/O139 str. BJG-01
37) Non-O1/O139 str. VC53
38) O135 str. RC385
39) O105 str. 571-88
40) O141 str. 234-93
41) O141 str. 3568-07
42) Non-O1/O139 str. VC48
43) O75 str. CP1115
44) Non-O1/O139 str. SIO
45) O155 str. 490-93
46) Non-O1/O139 str. VCC19
47) Non-O1/O139 str. HE-09
48) Non-O1/O139 str. HE-16
49) Non-O1/O139 str. Santiago-089

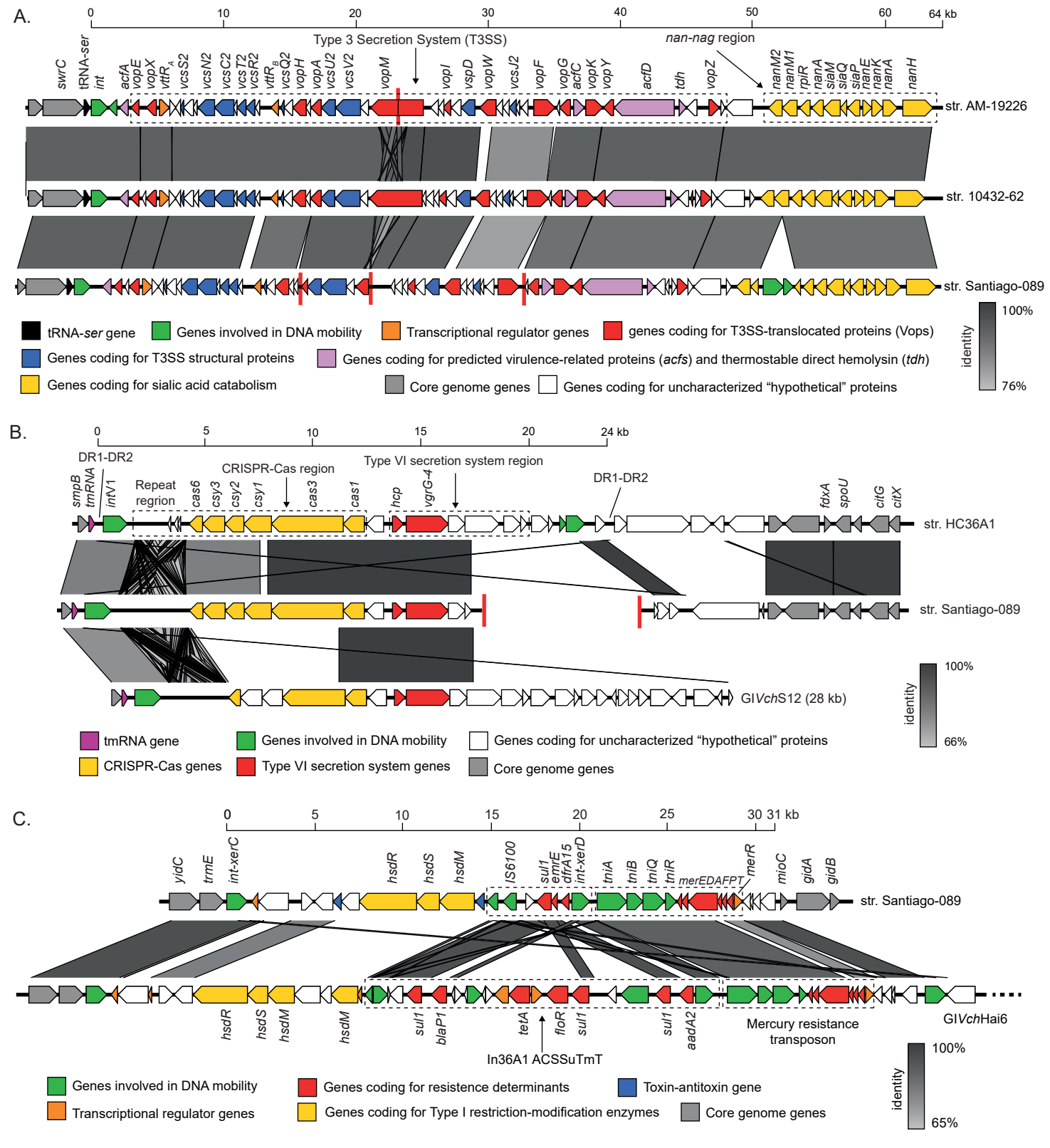
GC Skew

- GC Skew (-)
- GC Skew (+)

BLASTn hit:

- 100% identity
- 95% identity
- 85% identity

- 100% identity
- 95% identity
- 85% identity


A.
0
10
20
30
40
50
60
64 kb
Type 3 Secretion System (T3SS)
nan-nag region
swrC
tRNA-ser
int
acfA
vopE
vopX
vttR_A
vcsS2
vcsN2
vcsC2
vcsT2
vcsR2
vttR_B
vcsQ2
vopH
vopA
vcsU2
vcsV2
vopM
vopI
vspD
vopW
vcsJ2
vopF
vopG
acfC
vopK
vopY
acfD
tdh
vopZ
nanM2
nanM1
rpiR
nanA
slaM
slaQ
slaP
nanE
nanK
nanA
nanH
str. AM-19226
str. 10432-62
str. Santiago-089
tRNA-ser gene
Genes involved in DNA mobility
Transcriptional regulator genes
genes coding for T3SS-translocated proteins (Vops)
Genes coding for T3SS structural proteins
Genes coding for predicted virulence-related proteins (acfs) and thermostable direct hemolysin (tdh)
Genes coding for sialic acid catabolism
Core genome genes
Genes coding for uncharacterized "hypothetical" proteins
identity
100%
76%
B.
0
5
10
15
20
24 kb
DR1-DR2
CRISPR-Cas region
Type VI secretion system region
smpB
tmRNA
intV1
Repeat region
cas6
csy3
csy2
csy1
cas3
cas1
hcp
vgrG-4
DR1-DR2
fdxA
spoU
citG
citX
str. HC36A1
str. Santiago-089
GIVchS12 (28 kb)
identity
100%
66%
tmRNA gene
Genes involved in DNA mobility
Genes coding for uncharacterized "hypothetical" proteins
CRISPR-Cas genes
Type VI secretion system genes
Core genome genes
C.
0
5
10
15
20
25
30
31 kb
yidC
trmE
int-xerC
hsdR
hsdS
hsdM
IS6100
sul1
emrE
dfrA15
int-xerD
tniA
tniB
tniQ
tniR
merEDAFPT
merR
mioC
gidA
gidB
str. Santiago-089
hsdR
hsdS
hsdM
hsdM
sul1
blaP1
tetA
floR
sul1
sul1
aadA2
Mercury resistance transposon
GIVchHai6
In36A1 ACSSuTmT
Genes involved in DNA mobility
Genes coding for resistence determinants
Toxin-antitoxin gene
Transcriptional regulator genes
Genes coding for Type I restriction-modification enzymes
Core genome genes
identity
100%
65%