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

We investigated the virulence determinants and phylogeny of a non-O1/non-O139 *Vibrio cholerae* strain that caused an outbreak in Santiago, Chile, 2018. Our results suggest that the high virulence of this strain, as well as other non-O1/non-O139 strains, may be the result of the acquisition of genomic islands carrying antibiotic resistance genes, T3SS and T6SS.

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.

The Study

The *V. cholerae* strain characterized in this study, which we named the Santiago 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 the 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 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 (**Supplementary information**) 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 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 strain lacks the Phage CTXφ and the genomic islands (GIs) (VPI-I, VPI-II, VSP-I and VSP-II) that are generally carried by O1 and O139 strains (**Supplementary Figure 1**). 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 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 (8). 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 2A)**, which is also harbored by the VPI-II (9). The second GI, which we named GI*Vch*-T6SS$_{Santiago}$, was partially identified and contains a CRISPR-Cas region and genes that encode Hcp and VgrG alleles, which are structural T6SS components (**Figure 2B**). The GI*Vch*-T6SS$_{Santiago}$ was also identified in the *V. cholerae* str. HC36A1. The Hcp from GI*Vch*-T6SS$_{Santiago}$ has 96.5% amino acid identity with the Hcp1 and Hcp2 alleles reported in *V. cholerae*. In contrast, the VgrG from GI*Vch*-T6SS$_{Santiago}$ 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 (10). Therefore, we named this uncharacterized allele VgrG-4. Moreover, we found that Hcp and VgrG-4 alleles from GI*Vch*-T6SS$_{Santiago}$ are also harbored by the GI*Vch*S12 (11). 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 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$_{Santiago}$ (**Figure 2C**). This GI harbors the *sul1* and *dfrA15* genes, which confer resistance to sulfonamide and trimethoprim, respectively. The genetic structure of the GI*Vch*-MDR$_{Santiago}$ is also relatively similar to the GI*Vch*Hai6 (12). On the other hand, the Santiago strain has the mutations S83I and A171S in the GyrA protein, which confer resistance to nalidixic acid (13) (**Supplementary information**). Genes or mutations conferring resistance to erythromycin were not identified.

Conclusions

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 strain, as well as the spread of MDR islands (12), are also epidemiological factors that need to be considered by public health authorities. In particular, the Santiago 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 (14). 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* (15). Although less studied, the role of T3SS in the pathogenesis of *V. cholerae* and other pathogenic *Vibrio* species has begun to be understood (8,16). 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, our results highlight the pathogenic potential of the Santiago strain as well as other non-O1/non-O139 *V. cholerae* strains.

About the authors

Mónica Arteaga and Juliana Velasco are medical doctors interested in infectious diseases and the epidemiology of enteropathogens.

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Table 1. Antimicrobial resistance profile of the *V. cholerae* str. Santiago [1]

Antimicrobial agent	Disk diffusion	Zone Diameter (mm)	MIC (µg/ml)	Interpretative criteria
Ampicillin	10 µg	20	-	Susceptible
Azithromycin	-	-	2	Susceptible
Erythromycin [2]	-	-	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 [2]	30 µg	6	-	Resistant

[1] The disk diffusion method or broth dilution was performed according to Clinical and Laboratory Standards Institute guidelines. [2] Interpretive criteria adapted from Enterobacteriaceae

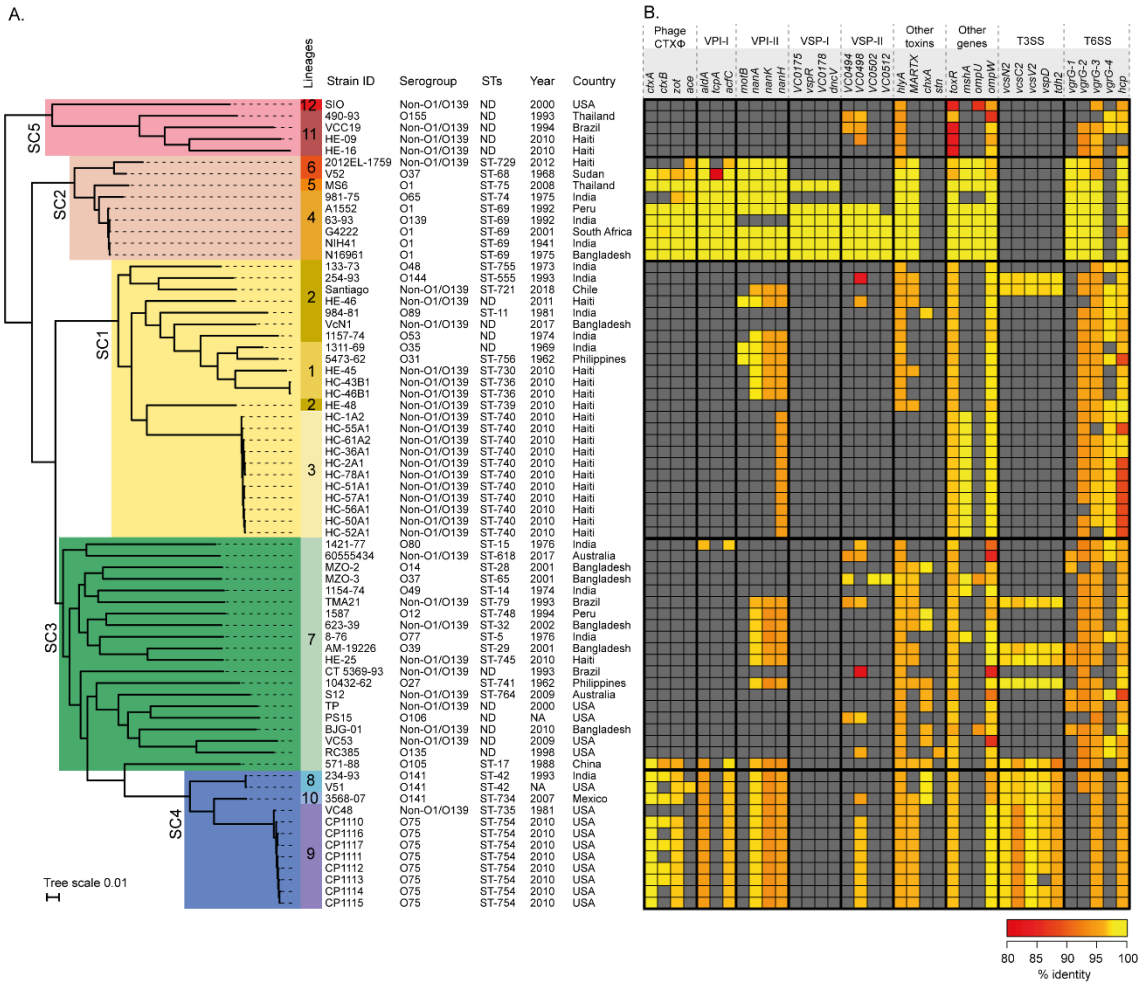

Figure 1. **Phylogenetic relationship between 70 *V. cholerae* strains**. **A)** Maximum likelihood phylogenetic tree (midpoint rooted) based on whole genome SNPs (146,534 SNPs within 2,483,145 positions, which were found in all analyzed genomes). The genome of the *V. cholerae* str. N16961 was used 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.

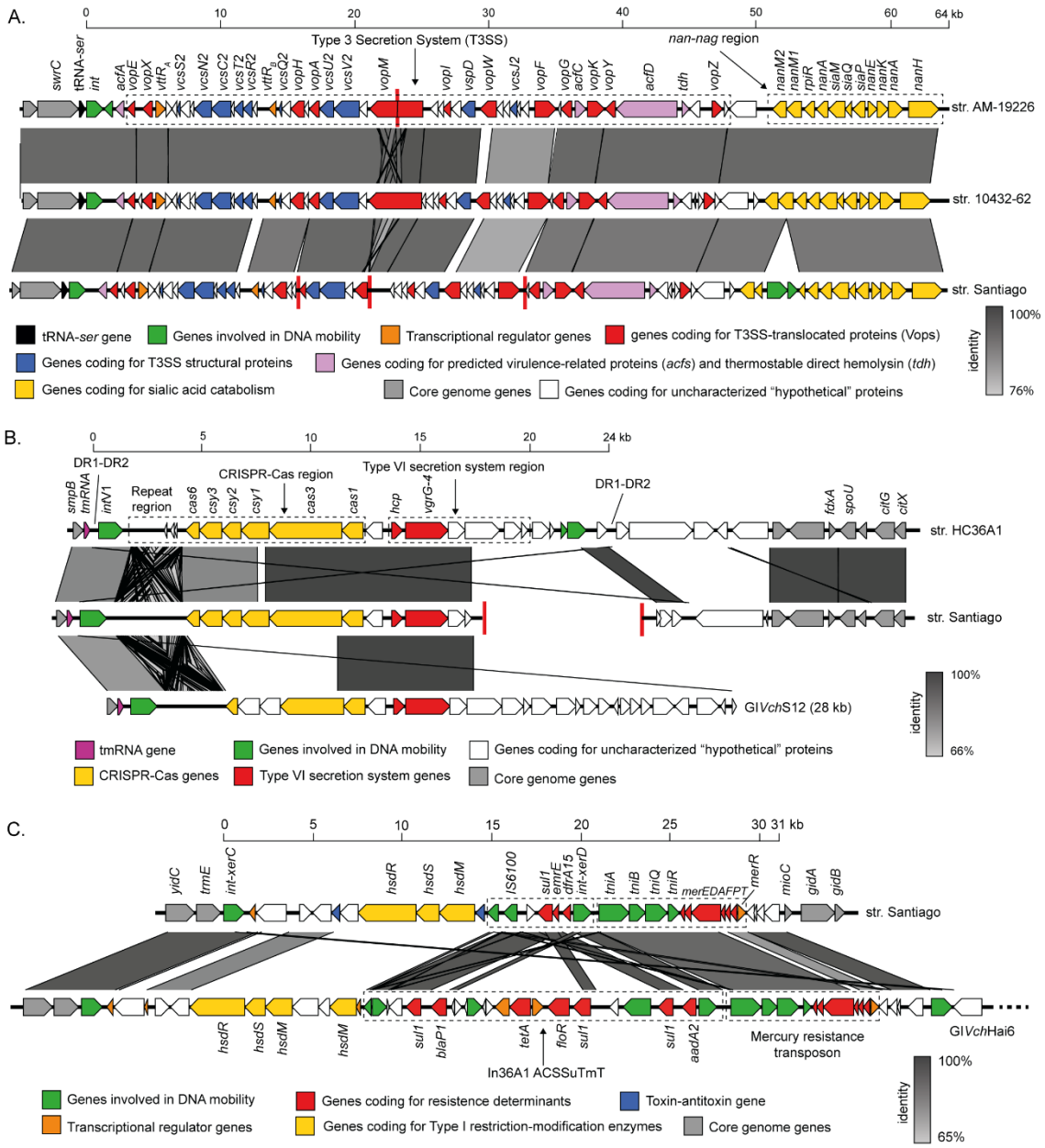

Figure 2. Comparison of the genetic structures of genomic islands carried by the *V. cholerae* str. Santiago. Predicted genes and the direction of transcription are represented as block arrows. Genes are color-coded according to gene function, as indicated in the legends at the bottom. The names of some 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$_{Santiago}$ (SRLP00000000). **B)** Genomic islands encoding CRISPR-Cas and T6SS genes. GenBank accessions: GI*Vch*-T6SS$_{HC-36A1}$ (AXDR01000008.1), GI*Vch*-T6SS$_{Santiago}$ (SRLP00000000), GI*Vch*S12 (KU722393). Open reading frames (ORFs) of the GI*Vch*-T6SS$_{Santiago}$ are listed in Supplementary Table 3. **C)** Multidrug resistance (MDR) islands. The GI*Vch*-MDR$_{Santiago}$ (GenBank accession: SRLP00000000) harbors the *sul1* and *dfrA15* genes, which confer resistance to sulfonamide and trimethoprim, respectively. ORFs of the GI*Vch*-MDR$_{Santiago}$ are listed in Supplementary Table 4. GI*Vch*Hai6 (GenBank accession: AXDR01000001).

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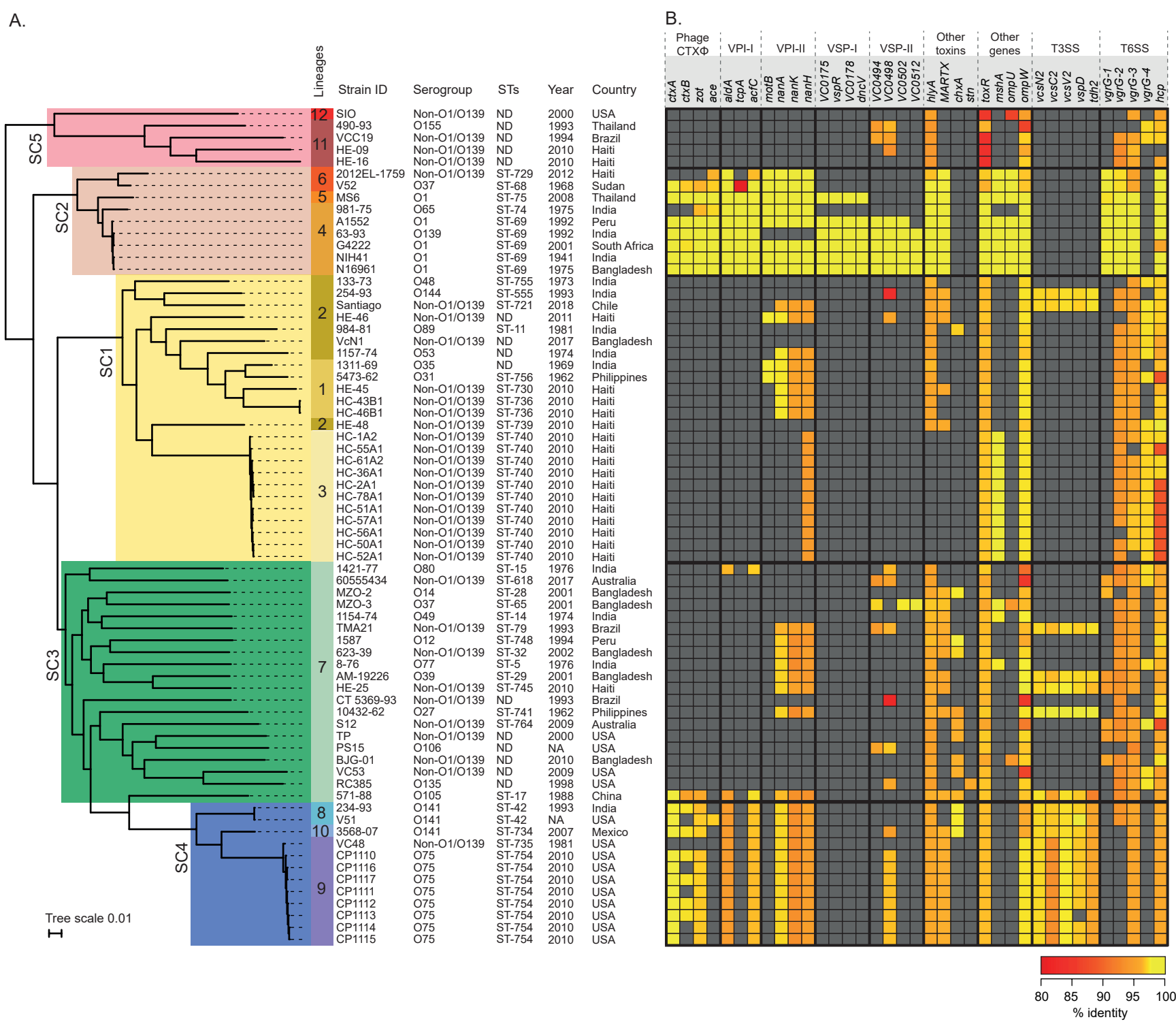
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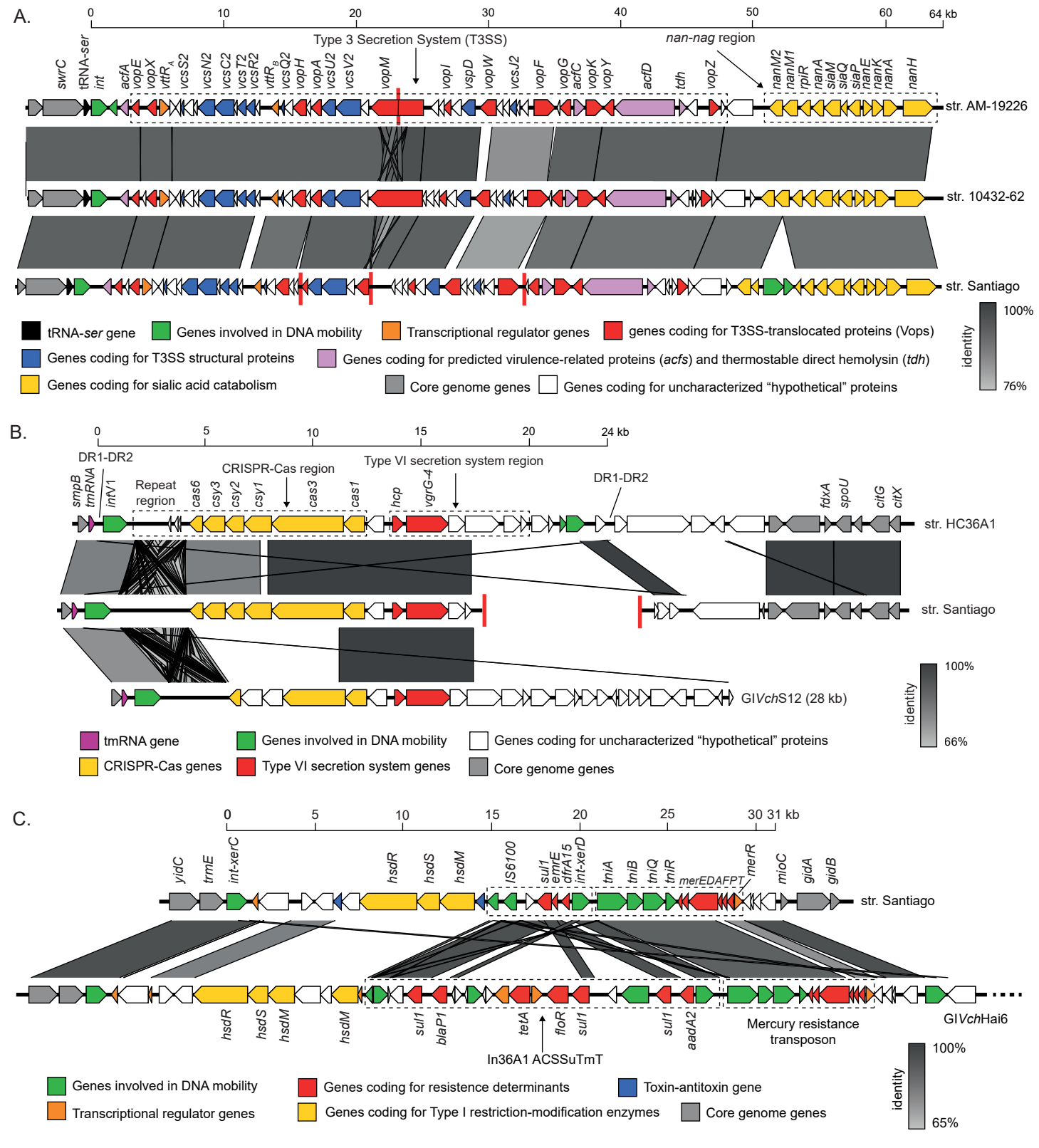
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tRNA-ser gene
Genes involved in DNA mobility
Transcriptional regulator genes
genes coding for T3SS-translocated proteins (Vops)
Genes coding for T3SS structural proteins
Genes coding for predicted virulence-related proteins (acfs) and thermostable direct hemolysin (tdh)
Genes coding for sialic acid catabolism
Core genome genes
Genes coding for uncharacterized "hypothetical" proteins
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