SME-4-producing Serratia marcescens from Argentina belonging to clade 2 of the S. marcescens phylogeny

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SME-4 producing *Serratia marcescens* from Argentina belonging to the clade 2 of the *S. marcescens* phylogeny.

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Synopsis

Background: SME serine carbapenemase are increasingly reported, especially from North and South America. Here, we describe an SME-4-producing *Serratia marcescens (*SME-*Sm)* clinical isolate from Argentina and compared its genome with other SME-*Sm* recovered from different countries.

Methods: *S. marcescens* isolates were characterized by WGS using Illumina technology, susceptibility testing and MIC determination. Carbapenemase activity was revealed by biochemical tests based on imipenem hydrolysis. Additionally, WGS of *S. marcescens* containing or not bla_{SME} genes were retrieved from Genbank database. A whole-genome phylogenetic analysis based on non-recombinant core SNPs was inferred for *S. marcescens* complete genomes and for those encoding any bla_{SME} variant.

Results: *S. marcescens* 163 was resistant to temocillin, aztreonam and carbapenems, remaining susceptible to expanded-spectrum cephalosporins. Analysis of WGS data of S. *marcescens* 163 revealed a genome of 5,139,329 bp, and a chromosomally-encoded class A bla_{SME-4} carbapenemase gene, which was located on a genomic island closely related to SmarG1-1 of *S. marcescens* N11-02820. The comparison of the *S. marcescens* genomes revealed that all SME-*Sm* isolates possess this genomic island inserted at the same loci, form a well-defined subcluster of cluster I of *S. marcescens* clade 1, while *S. marcescens* 163 belonged to clade 2, suggesting that the SME-encoding genomic island may have been transferred between isolates from different clades.

Conclusions: To the best of our knowledge this is the first report of a SME-4-*Sm* from Argentina. Bla_{SME-4} gene is located on a SmarG1-1-like genomic island. The genome of *S. marcescens* 163 belongs to clade 2, unlike all the other SME-*Sm* isolates that belong to a subcluster of clade I.

Introduction

Currently, β-lactamase-mediated resistance does not spare even the newest and most powerful β-lactams (carbapenems), whose activity is challenged by carbapenemases.[1] Class A carbapenemases may be chromosomally encoded (SME, NmcA, SFC-1, BIC-1, PenA, FPH-1, SHV-38), plasmid-encoded (FRI-1) or both (KPC, GES, IMI).[2] Chromosomally-encoded class A carbapenemases have been identified in rare Gram-negative species that appeared sporadically in clinical or environmental samples since their first discovery, more than 20 years ago.[2] SME-1 (for "*Serratia marcescens* enzyme") was first detected in England from two *S. marcescens* isolates collected in 1982. [3,4] SME-1 has a broad hydrolysis spectrum that includes penicillins, cephalosporins, aztreonam, and carbapenems.[1,4] The SME enzymes have been found exclusively in *S. marcescens* and the five variants differing by one or two amino acid substitutions have been found infrequently and sporadically in UK, across North and south America, in Switzerland and in Mexico.[2,3,5-9] Recently, analysis of the genetic environment of bla_{SME} genes in three Canadian *S. marcescens* isolates revealed that it is located on a novel cryptic prophage genomic island, SmarGI1-1. This genomic island can be excised and circularized, which probably contributes to its dissemination amongst *S. marcescens*.[10]

In this work, we have characterized the genome of an SME-4-producing *S. marcescens* (SME-4-*Sm*) from a clinical isolate recovered from an University Hospital in Buenos Aires, Argentina, and compared it to the genomes of SME-*Sm* isolates either sequenced in this study or downloaded from the Genbank database.

Materials and Methods

Bacterial strains. *S. marcescens* 163 (Sm163) was identified with MALDI-TOF (MALDI Biotyper CA system, Bruker Daltonics, Billerica, MA, USA). *Escherichia coli*

77 TOP10 (Invitrogen, Saint-Aubin, France) was used for cloning experiments. *Bla*$_{SME}$ gene
78 harbouring *Serratia marcescens*, *S. marcescens* S6 (SmS6),[3,4] *S. marcescens* S8 (SmS8), [3,4] *S.*
79 *marcescens* CHE4 (SmCHE4),[8] *S. marcescens* AW (SmAW),[5] *S. marcescens* USA-1
80 (SmUSA-1) and *S. marcescens* Mex-1 (SmMEX-1) were also used in this study. The presence
81 of SME was systematically assessed on all the genome data of *S. marcescens* deposited on the
82 NCBI genome database (December 2017). Genomes of 323 *S. marcescens* isolates were
83 downloaded from Public databases including seven SME-*Sm* and 24 fully-sequenced
84 *S. marcescens* strains (Tables S1 and S2). The sequences of the SmarGI1-1 and flanking
85 regions from *S. marcescens* N10-0408, N11-2820 and N12-0620 were from accession
86 numbers KF445086, KF318367 and KF615855, respectively, in the GenBank database.[10]
87 **Antimicrobial agents, susceptibility testing.** Antimicrobial susceptibilities and MIC
88 values were determined as previously reported[11] and interpreted according to the EUCAST
89 breakpoints (16 May 2018; http://www.eucast.org/clinical_breakpoints/).
90 **Biochemical and molecular detection of carbapenemase-producing**
91 **Enterobacteria.** Carba NP test and β Carba Test were used for carbapenemase detection
92 (BioRad, Marnes-la-Coquette, France) as previously described.[12,13] Genomic DNA of *S.*
93 *marcescens* 163 isolate extracted using the Qiagen DNAamp kit (Qiagen) was used as
94 template for amplification of the most prevalent carbapenemases (*bla*$_{OXA-48}$, *bla*$_{KPC}$, *bla*$_{NDM}$,
95 *bla*$_{VIM}$, *bla*$_{IMP}$).[14]
96 **Whole genome sequencing and bioinformatic analysis.** The whole genome of
97 *Serratia marcescens* 163 was sequenced using Illumina technology, as previously reported.[11]
98 The acquired antimicrobial resistance genes were identified by uploading assembled genomes
99 to the Resfinder server v2.1 (http://cge.cbs.dtu.dk/services/ResFinder-2.1).[15]
100 ProgressiveMauve and the Mauve distributed script stripSubsetLCBs[16] were used to
101 extract all the regions with a minimun length of 500 bp shared by all the strains, generating a

2.877.168 bp core genome alignment. Recombinant variable positions were removed with Gubbins (https://github.com/sanger-pathogens/gubbins), and a Maximum Likelihood phylogeny was inferred for the alignment of the 631207 non-recombinant variable sites with RAxML using a General Time Reversible (GTR) model incorporating a gamma distribution rate among sites.[17] .In parallel a whole genome phylogeny was estimated for all the S. marcescens retrieved from public databases with kSNP3[18] (Supplementary Material and methods). All genomes were annotated with Prokka,[19] and graphic representations were generated with the genoplotR[20] library in R.

Nucleotide sequence accession number. The whole genome sequences generated in the study have been submitted to the Genbank nucleotide sequence database under the accession number detailed in Table S1. The genomic islands constructed from whole genome sequences available at Genbank nucleotide sequence database where submitted to the Genbank Third Party Annotation database under accession numbers BK010577-BK010585 (Table S1). The nucleotide sequence of $bla_{\text{SRT-3}}$ gene has been submitted to the EMBL/Genbank nucleotide sequence database under the accession number MG234451.

Results and discussion

Clinical case. A 62-year-old man with diffuse large B cell lymphoma was admitted at the University hospital in Buenos Aires in July 2016 for his third cycle of chemotherapy. During previous hospital stays in the same year he had received antimicrobial therapy with meropenem, colistin and vancomycin. During his 3rd stay the patient presented fevers and developed pneumonia. He was treated with meropenem and oseltamivir, but cultures remained negative. Twenty days after admission, a carbapenem-resistant *S. marcescens* isolate was recovered from both a surveillance rectal swab and a catheter blood culture, whereas the peripheral blood cultures remained negative. Further carbapenem-resistant *S.*

marcescens isolates were recovered, from the same patient, in subsequent surveillance samples for 3 weeks. The patient was discharged after having completed the 4th cycle of chemotherapy for his underlying disease and he did not suffer from subsequent infections.

Characteristics of *S. marcescens* 163. *S. marcescens* 163 was resistant to amoxicillin (MIC >256 mg/ml), temocillin (MIC of 24 mg/L) aztreonam (MIC >256 mg/ml) and carbapenems (MIC for imipenem, meropenem and ertapenem >32 mg/L), remaining susceptible to expanded-spectrum cephalosporins (MIC for ceftazidime 1 mg/L). *S. marcescens* 163 also showed an MIC of 64 mg/L for amoxicillin+clavulanic acid. (Carba NP and β Carba tests gave positive results indicating the presence of a carbapenemase. PCRs detecting the five most prevalent carbapenemases ($bla_{\text{OXA-48}}$, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}) were negative. High level of resistance to amoxicillin and carbapenems, decrease MIC values in presence of clavulanic acid and susceptibility to expanded-spectrum cephalosporins suggested the presence of a bla_{SME} gene.

WGS of *S. marcescens* 163 revealed a genome of 5,139,329 bp, with a 68 X coverage. Resistome was determined by searching acquired genes and point mutations likely involved in resistance. According to https://cge.cbs.dtu.dk/services/, the isolate presented two β-lactamase genes, the chromosomal and natural $bla_{\text{STR-3}}$ gene coding for cephalosporinase and $bla_{\text{SME-4}}$ gene coding for a chromosomal class A carbapenemase (Table 1S). The isolate also harboured the *S. marcescens* intrinsic *aac*(6')-Ic gene conferring resistance to amikacin and gentamicin,[21] and the tetracycline resistance gene *tet*(41).[22] Isolates SmS6 and SmS8 also harboured the streptomycin/spectinomycin resistance gene *aadA1* and the trimethoprim resistant gene *dfrA1*.

WGS of *S. marcescens* 163, revealed that the $bla_{\text{SME-4}}$ gene was inserted into a SmarGI1-1 prophage-like genomic island initially described in three SME-1 producing *S. marcescens* N11-02820, N10-00408 and N12-00620 isolates (accession numbers KF318367,

KF445086, and KF615855, respectively).[10] This genomic island found in Sm163 is ~28.4 kb in size and bracketed by 25 bp imperfect direct repeats (Figure 1A). While the upstream sequences of the SmarGI1-1 were identical between Sm163 and N11-02820 isolates, downstream of the *attR* site the sequences were similar except for an 8-kb deletion in *S. marcescens* 163 that included genes coding for intCPΔ, GNAT family, RES domain, Helicase, ATP-dependent OLD family protein (Figure 1A).

Phylogeny of SME-producing *S. marcescens*. Phylogeny of the S. marcescens from this study along with the 323 *S. marcescens* assemblies retrieved from the NCBI genome database using KSNP3, revealed the two clades, with clade 1 representing 80% of the isolates (Figure S1). Sixteen phylogenetic clusters were defined using ClusterPicker. 6 clusters were located in Clade 1, with cluster 1 being the main one. In Clade 2, 10 clusters were identified, with Sm163 beeing not related to any cluster. The presence of bla_{SME} as well as other carbapenemase genes was screened in the 323 *S. marcescens* assemblies retrieved from the NCBI genome database (Figure 1S). In total seven bla_{SME} encoding isolates were found (Table S1 and S2) that were added to the seven SME producers sequenced in the present study. A core genome SNP-based Maximum Likelihood phylogenetic tree was obtained for the fourteen SME-producing strains and 24 completed *S. marcescens* genomes retrieved from the NCBI database (Table S1), using the *S. marcescens* fgi94 genome as outgroup. Two main clades, with a mean genetic distance between them of 25.302 non-recombinant SNPs, were revealed in the *S. marcescens* phylogeny (Figure 2, Table S1). The cluster numbers were those from Figure S1 (Figure S1 and 2). Thirteen out of the fourteen SME-producers were found on the clade 1, eleven of them formed a single sub-cluster of SME producers (hereafter named SME sub-cluster within the cluster I), and the two remaining, MGH315 is located outside the SME-sub-cluster but still in Cluster I, while BIDMC44 belongs to cluster III. On

176 the other hand, *S. marcescens* 163, remains as the single strain encoding SME in clade 2
177 (Figure 2).

178 Eleven isolates encoded other carbapenemases than bla_{SME} genes, 5 of them encoded
179 bla_{KPC} carbapenemase genes, 4 for bla_{NDM-1} genes, one encoded bla_{IMP-1} gene and one bla_{VIM-1}
180 gene (Figure S1, and Table S2). All of them were found in the clade 1, leaving *Sm*163 as the
181 only carbapenemase producing isolate from the clade 2.

182 **Genetic environment of the bla_{SME} genes.** In all cases the bla_{SME} gene was encoded
183 within a conserved SmarGI1-1-like genomic island that varies in size from c.a. 27.2 (in
184 SmS6) to 28.5-Kb (in BIDMC44) and that is integrated after the *ssrA* gene (Figure 1A and
185 1B, and Figure S2). *ssrA* has been identified as a hotspot for integration of mobile genetic
186 elements in enterobacteria.[10] Three different flanking regions have been identified
187 downstream of the *attR* site among the 14 SME producers (Figure 1B). The first one
188 possesses a 2.8-kb long inserted region observed in the S. *marcescens* strains N12-00620 (and
189 N10-00408, data not shown)[10] that is shared by the strains that form the SME subcluster and
190 the strain MGH315 (Figure 1B and Figure S2). The second one possesses an 11.3-kb long
191 region as observed in the strain N11-02820. Finally, the third one has been described in the
192 *Sm*163 (Figure 1B), and in the strain BIDMC44, that is a member of clade 1 (Figure 2),
193 suggesting that the latter might be the donor of this structure.

194 The region downstream the *ssrA* gene was also analysed in the strains umh3 and
195 umh9, both of them are basal to the SME subcluster (Figure 2). In the strain umh3 an
196 incomplete SmarGI1-1 was identified (Figure 1C). It was structurally similar to the first 12.4
197 Kb of the SmarGI1-1, but the hypothetical protein downstream the *intA1* integrase gene was
198 substituted by three completely different genes coding for hypothetical proteins (Figure 1C).
199 No traces of the second half of the SmarGI1-1 could identified in the umh3 genome; however,
200 the *attL* and *attR* sites were present (Figure 1C). Not a single trace of SmarGI1-1 nor of the

truncated version of it was found in the strain umh9 basal to both, umh3 and SME subcluster. No traces for SmarGI1-1 were detected in the closest strains to the other SME-producers analysed in the present study. Despite that the incomplete SmarGI1-1-like in strain umh3 shares a structural similarity with the first 12.4 kb of the SmarGI1-1, the sequence identity is low (about 92%), thus, ruling out the possibility that they share a recent ancestor. In fact, there are a lot of recombination and exchanges between phages and other MGEs, which often makes it impossible to build an evolutionary scenario.

Interestingly, in the phylogeny performed with the 323 sequenced *S. marcescens*, a single isolate GCF_000739215 lacking the SmarGI1-1 genomic island was found within the the SME-subcluster (Figure 1S). In fact, in the isolate GCF_000739215, the insertion site AttB was empty as identified in isolate umh9.

Conclusion

This is the first report of SME-4-*Sm* in Argentina. Until now, SME-4 had only been reported in Brazil,[7] and in one single SME-4 producing *S. marcescens* from US, as reported in GenBank under the accession number KF481967, but not published yet. The increase in the use of carbapenems in Argentina,[23] together with the *S. marcescens* natural resistant to polymyxin, could be factors that helped to select SME-producing *S. marcescens* isolates. Finally, as increasing numbers of the SME carbapenemases are being reported from *S. marcescens*, especially from North and South America, rapid detection systems based on multiplex PCR analyses should also target bla_{SME} genes in addition to the big 5 carbapenemases: KPC and OXA serine carbapenemases, and the IMP, VIM and NDM metallo-β-lactamases, as was previously suggested by Bush *et* al.[24] Thus, underdetection of SME-producers may favor their silent spread.

Finally, the phylogeny of all the available *S. marcescens* genomes assemblies revealed that all SME-*Sm* isolates possess a SmarGI1-1 inserted at the same loci, that they form a well-defined subcluster of cluster I of *S. marcescens* clade 1, while *S. marcescens* 163 belonged to clade 2, suggesting that the SmarGI1-1 may have been transferred between isolates from different clades.

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Transparency declarations

No competing interests to declare.

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309
310

Figure legends

Figure 1. (A). Schematic representation of the genetic structure of SmarGI1-1 of *S. marcescens* N11-02820 (KF318367) and *S. marcescens* 163 and flanking regions. Common structures are highlighted in gray. Genes and putative functions of genes are shown; H means hypothetical protein. **(B)**. Comparison of the SmarGI1-1 and its genetic environments among the different bla_{SME}-encoding strains. (**C)**. Comparison of the insertion site for SmarGI1-1 in the SME subcluster and its basal strains. Numbers below the representative SME cluster represent: 1. Phage regulatory protein, 2. Phage capside protein, 3. AlpA phage transcriptional regulator, CI repressor-like protein, 5. primase. H above the CDS B) AttL and AttR sequences comparison between umh3 and SmarGI1-1 (UCI-88).

Arrows colored in blue, green, purple, red and yellow correspond to genomic CDS, variable genomic CDS, TmRNA[25], Bla_{SME} gene, and genomic island CDS, respectively. Horizontal bars (I) in black and in red represents, Att (L and R) sites and contig boundaries, respectively. Small light green boses (▯) represent RNA genes. Insertion sequences are indicated by an arrow in a rectangle light green for IS*Ec31* and IS*Sm3*, and grey for the interrupted IS*Ec14*.

Figure 2. *S. marcescens* Maximum Likelihood tree obtained from core non-recombinant SNPs. All bla_{SME}-encoding strains were bolded and capital letters. Those sequences in this study are highlighted in grey, and bla_{SME-2}-expressing isolates are underlined. *S. marcescens* isolates not carrying bla_{SME} gene are represented in lower case letters.

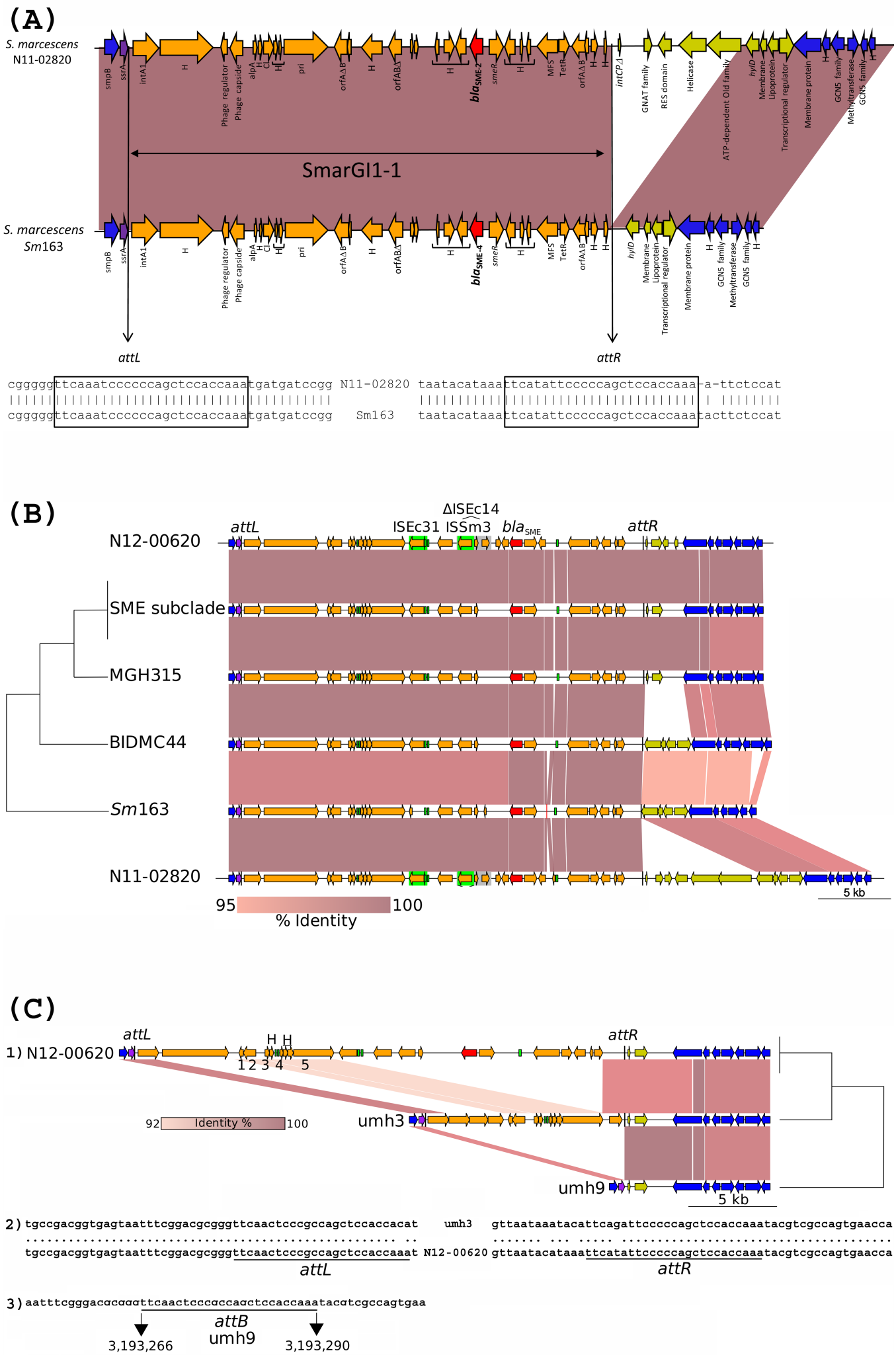
(A)
S. marcescens N11-02820
S. marcescens Sm163
SmarGI1-1
attL
attR
cgggggttcaaatccccccagctccaccaaatgatgatccgg N11-02820 taatacataaactcatattccccccagctccaccaaaa-a-ttctccat
cgggggttcaaatccccccagctccaccaaatgatgatccgg Sm163 taatacataaattcatattccccccagctccaccaaaatacttctccat
(B)
ΔISEc14
attL
ISEc31 ISSm3 bla SME
attR
N12-00620
SME subclade
MGH315
BIDMC44
Sm163
N11-02820
5 kb
95
100
% Identity
(C)
1) N12-00620
attL
attR
1 2 3 4 5
92
Identity %
100
umh3
umh9
5 kb
2) tgccgacggtgagtaatttcggacgcgggttcaactcccgccagctccaccacat umh3 gttaataaatacattcagattcccccagctccaccaaatacgtcgccagtgaacca
tgccgacggtgagtaatttcggacgcgggttcaactcccgccagctccaccaaat N12-00620 gttaatacataaattcatattccccccagctccaccaaatacgtcgccagtgaacca
attL
attR
3) aatttcgggacgccgattcaactcccgccagctccaccaaatacgtcgccagtgaa
attB
umh9
3,193,266
3,193,290

Fig. 1

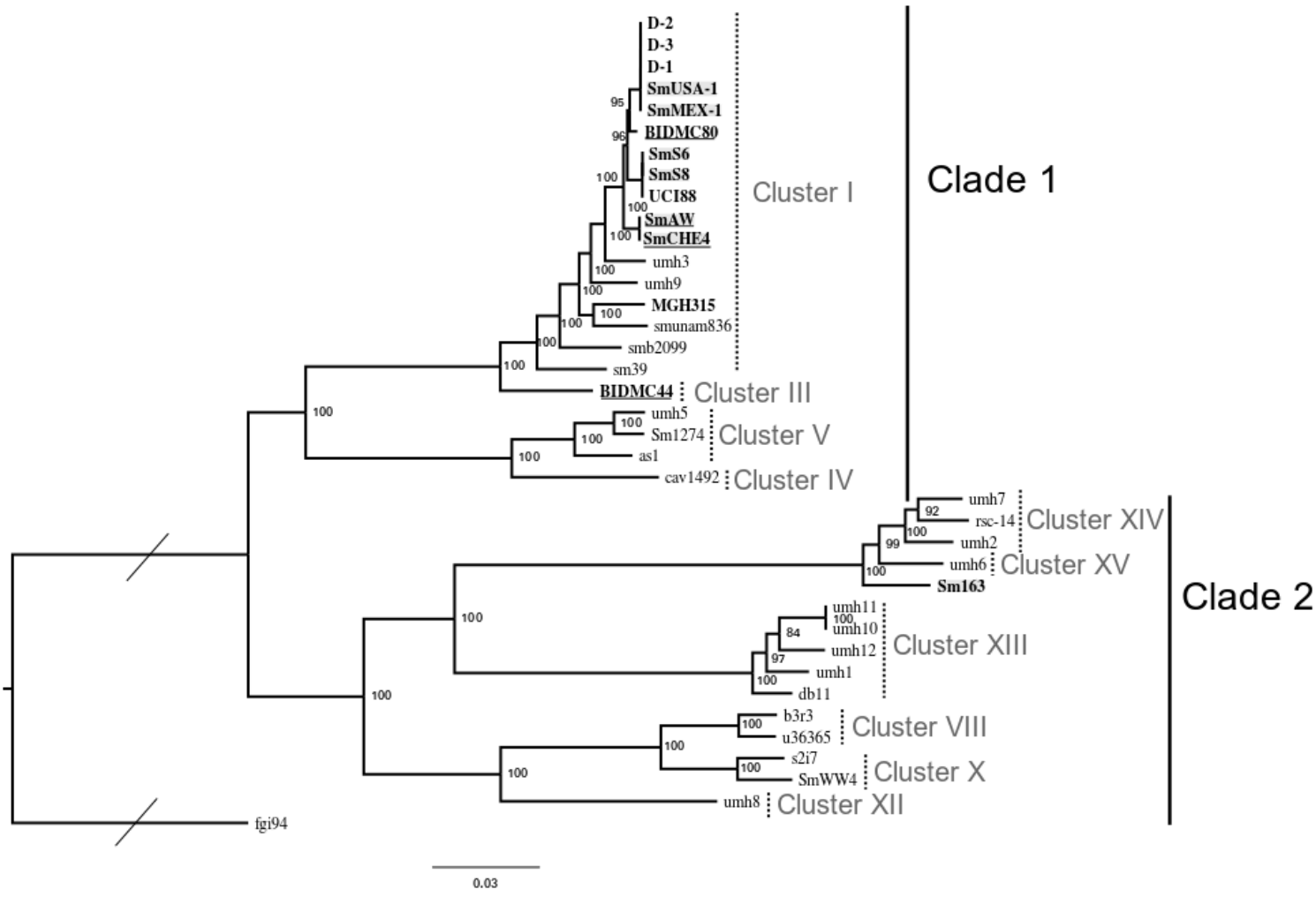
D-2
D-3
D-1
SmUSA-1
SmMEX-1
BIDMC80
SmS6
SmS8
UCI88
SmAW
SmCHE4
umh3
umh9
MGH315
smunam836
smb2099
sm39
BIDMC44
umh5
Sm1274
as1
cav1492
Cluster I
Cluster III
Cluster V
Cluster IV
Clade 1
umh7
rsc-14
umh2
umh6
Sm163
Cluster XIV
Cluster XV
umh11
umh10
umh12
umh1
db11
Cluster XIII
b3r3
u36365
Cluster VIII
s2i7
SmWW4
Cluster X
umh8
Cluster XII
Clade 2
fgi94
0.03

Fig. 2