Microbiology (2016), **162**, 1335–1345 DOI 10.1099/mic.0.000310

Genome analysis of a clinical isolate of *Shewanella* sp. uncovered an active hybrid integrative and conjugative element carrying an integron platform inserted in a novel genomic locus

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Shewanella spp. are currently considered to be emerging pathogens that can code for a bla_{OXA} carbapenemase in their chromosome. Complete genome analysis of the clinical isolate *Shewanella* sp. Sh95 revealed that this strain is a novel species, which shares a lineage with marine isolates. Characterization of its resistome showed that it codes for genes *drfA15, qacH* and $bla_{OXA-48.}$ We propose that *Shewanella* sp. Sh95 acts as reservoir of bla_{OXA-48}. Moreover, analysis of mobilome showed that it contains a novel integrative and conjugative element (ICE), named ICE*Sh95*. Comparative analysis between the close relatives ICE*Spu*PO1 from *Shewanella* sp. W3-18-1 and ICE SXT^{MO10} from *Vibrio cholerae* showed that ICE*Sh95* encompassed two new regions, a type III restriction modification system and a multidrug resistance integron. The integron platform contained a novel arrangement formed by gene cassettes *drfA15* and *qacH,* and a class C-attC group II intron. Furthermore, insertion of ICE*Sh95* occurred at a unique target site, which correlated with the presence of a different *xis*/*int* module. Mobility of ICE*Sh95* was assessed and demonstrated its ability to self-transfer with high efficiency to different species of bacteria. Our results show that ICE*Sh95* is a self-transmissible, mobile element, which can contribute to the dissemination of antimicrobial resistance; this is clearly a threat when natural bacteria from water ecosystems, such as *Shewanella*, act as vectors in its propagation.

Received 20 January 2016
Accepted 19 May 2016

INTRODUCTION

Shewanella spp. are Gram-negative rod-shaped bacteria widely spread in sediment and aquatic niches. They are known for their ability to survive under extreme conditions such as high pressure, low temperatures and high salt concentrations (Fredrickson *et al.*, 2008). In recent years, *Shewanella* spp. have attracted interest due to their potential to remediate polluted niches. In addition, some species such as *Shewanella algae* and *Shewanella putrefaciens,* have been implicated in skin and soft tissue infections, bacteraemia,

Abbreviations: ANI, Average nucleotide identity; HS, hot spots; ICE, integrative and conjugative element; MDR, multi-drug resistant; R-M, restriction-modification.

The GenBank/EMBL/DDBJ/PIR accession number for the draft genome of *Shewanella* sp. Sh95 is LGYY00000000.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

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biliary tract infections, empyema, endocarditis, dacryocystitis, intracranial abscess, arthritis, peritonitis, ventilator-associated pneumonia and ear infections (Ananth *et al.*, 2014; Constant *et al.*, 2014; Dey *et al.*, 2015; Goyal *et al.*, 2011; Holt *et al.*, 2005; Sharma & Kalawat, 2010; Yiallouros *et al.*, 2013). *Shewanella* spp. are currently considered to be emerging opportunistic pathogens. Several species of *Shewanella* code within their chromosome for a carbapenemase, a metallo-beta-lactamase that confers resistance to imipenem and meropenem (Poirel *et al.*, 2004; Ramírez *et al.*, 2010; Srinivas *et al.*, 2015; Tsai *et al.*, 2008).

Analysis of the genomes of *Shewanella* spp. has shown that these bacteria are highly versatile and capable of adapting to diverse conditions. The genomes of *Shewanella* spp. may contain several mobile genetic elements that contribute to the rearrangement of bacterial chromosomes and to the acquisition and dissemination of genetic traits, such as insertion sequences, transposons, integron/cassettes systems, group II introns and integrative and conjugative elements (ICE) (Drouin *et al.*, 2002; Quiroga & Centrón, 2009; Ramírez *et al.*, 2010; Romine *et al.*, 2008).

ICE are mobile genetic elements able to self-transfer to a new host through a conjugation mechanism (Carraro & Burrus, 2014). A major ICE family, SXT/R391, is found in Gram-negative bacteria and may carry virulence genes as well as antimicrobial and heavy metal resistance determinants. One of the first ICE from the SXT/R391 family was found in the genome of an epidemic strain of *Vibrio cholerae* serogroup O139 (Waldor *et al.*, 1996). ICE SXT codes for the antimicrobial resistant determinants *dhfr18, sul2, strAB* and *floR*. The presence of these genes turned this strain into a multidrug resistant (MDR) isolate (Hochhut *et al.*, 2001). Furthermore, a class 4 integron harboring the gene cassette *dfrA1* was found within a related ICE from *V. cholerae* O1 El Tor (Hochhut *et al.*, 2001).

ICEs have four conserved modules responsible for their maintenance and mobilization: (i) integration and excision, (ii) replication and DNA processing, (iii) DNA secretion and translocation, and (iv) regulation (Carraro & Burrus, 2014). The ICE structure also has several variable sections identified as hot spots (HSs). Comparative analysis between different SXT/R391 ICE members revealed the presence of at least nine different HS sites (Wozniak *et al.*, 2009). HSs contain additional genes that are beneficial to the host, conferring resistance to antibiotics or heavy metals (such as arsenic, copper and cobalt), as well as for ICE maintenance or regulation of the cellular processes of the host; i.e. toxin-antitoxin systems, restriction and modification systems or diguanylate cyclases (Bordeleau *et al.*, 2010; Wozniak *et al.*, 2009; Wozniak & Waldor, 2009).

SXT/R391 ICE can self-transfer to diverse species of gamma-proteobacteria by a mechanism that involves the excision of the circular form of the ICE from the chromosome followed by its transfer to a new host using its own type IV secretion system (Wozniak *et al.*, 2009). Once transferred, the Int_{SXT} integrase promotes the integration through a site-specific recombination mechanism between the *attP* site of the ICE and the *attB* site of the host chromosome. Most members of the SXT/R391 family integrate specifically at the 5′ end of the *prfC* gene (Hochhut & Waldor, 1999; Taviani *et al.*, 2012; Wozniak *et al.*, 2009).

Ramírez *et al.* (2010) previously reported the presence of antimicrobial resistance genes in 10 clinical isolates of *Shewanella* spp. harbouring class 1 or 2 integrons. *16s rDNA* gene analysis revealed that these isolates were *S. putrefaciens* and *S. algae* (Ramírez *et al.*, 2010). All isolates of *S. putrefaciens* encoded bla_{OXA-48}, a carbapenemase that confers resistance to imipenem (Poirel *et al.*, 2004).

Here, we report the draft genome of bla_{OXA-48}-bearing *Shewanella* sp. Sh95, an isolate previously identified as *S. putrefaciens* Sp95. Comparative genome analysis showed that this strain is indeed closely related to *Shewanella* isolates from marine sources, suggesting an aquatic origin. Furthermore, we describe the mobilome of *Shewanella* sp. Sh95, which contains different families of insertion sequence, prophage-related sequences and an integrative and conjugative element named ICE*Sh95*. ICE*Sh95* is a hybrid element closely related to the SXT/R391 family. It harboured an integron platform with two gene cassettes, *dfrA15* and *qacH,* and a class C-attC group II intron at HS3, and a putative type III restriction-modification system at HS5. Functional characterization of ICE*Sh95* activity showed that it excises forming circular intermediates and self-transfers efficiently to different hosts. Analysis of the integration site revealed that this element inserts at the 5′ end of gene *pabA*, a novel target site. Our results provide evidence supportive of the role of ICE in the acquisition and transfer of antimicrobial resistance determinants among different bacterial hosts, as well as of its participation in genome evolution.

METHODS

Strains and plasmids. Clinical strains of *Shewanella* spp. (Ramírez *et al.*, 2010) and *Escherichia coli* HB101 were grown at 37 °C in Luria-Bertani medium (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl in dH_2O) with shaking at 200 rpm. Total DNA extraction was performed using the Wizard Genomic DNA Purification kit (Promega). Plasmid pCR2.1 (*ori* ColE1, Kan^R) was introduced into *Shewanella* sp. Sh10 (previously Sa10; Ramírez *et al.*, 2010) and into *E. coli* HB101 by electroporation following the protocol described elsewhere (Choi & Schweizer, 2006). Total RNA was isolated from *Shewanella* sp. Sh95 grown at 37 °C during the exponential phase (OD_{600nm} 0.5) using Trizol (Invitrogen) by following the manufacturer's protocol. RNA was treated with 2 U of RQ1 RNase-Free DNase (Promega) for 1 h at 37 °C and heat-inactivated for 10 min at 65 °C.

PCR and RT-PCR assays. For all PCR reactions we used 1 U of *Taq* DNA polymerase (Fermentas, ThermoScientific) in 1 × Taq buffer (Fermentas, ThermoScientific) supplemented with 2 mM $MgCl_2$, 0.14 mM dNTP mix and 0.4 mM of each primer in a final volume of 50 µl. The PCR conditions consisted of an initial denaturation for 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at the appropriate annealing temperature and 1 min at 72 °C, followed by a final extension of 5 min at 72 °C. Amplification of the *16s rDNA* gene of clinical isolates of *Shewanella* spp. was carried out using the specific primers, FD2 (5′-

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AGAGTTTGATCATGGCTCAG-3′) and RP2 (5′-ACGGCTACCTTGTTACGACTT-3′). All positive PCR amplification products were confirmed by sequencing. As ICE*Sh95* was found to be fragmented in two contigs, contig 7 (LGYY01000082.1) and contig 41 (LGYY01000240.1), we confirmed the complete structure by PCR, using the primers, IS4-ICE-F (5′-ACCGACTTTCCGCACCTGAT-3′) and intI9-Sh95-R (5′-TTGAGTAGACGCCGTAACT-3′), and sequence analysis. Detection of the excised form of ICE*Sh95* was performed by using the primers, Sh95-ICE-5′end-R (5′-AACACCACGATAAATAGAG-3′) and ICE-3′end-F (5′-GTGAACAGAAGTACCTAAA-3′). Characterization of the insertion site was carried out by PCR using the primers, ICE-3′end-F (5′-GTGAACAGAAGTACCTAAA-3′) and pabA-R (5′-CTCTTGCCCTAACTGCT-3′) for isolates *Shewanella* sp. Sh95 and *Shewanella* sp. Sh10 :: ICE*Sh95*. Similarly, the insertion site of the ICE in *E. coli* HB101 :: ICE*Sh95* was detected using the primers ICE-3′end-F and pabAEc-R (5′-GCACGCTGGTAATGAGTCAG-3′).

First strand synthesis of gene *pabA* was carried out using SuperScript II Reverse Transcriptase (Life Technologies) and primer pabA-RT-F (5′-TTAGTCATCTCTCCTGGC-3′) by following the manufacturer´s instructions. PCR was performed using the primers, pabA-RT-F (5′-TTAGTCATCTCTCCTGGC-3′) and pabA-RT-R (5′-GTTACCGTTAATGGCTGAT-3′).

Genome sequencing, assembly and annotation. The total DNA of *Shewanella* sp. Sh95 was obtained using the Masterpure DNA purification kit from Epicentre. A draft sequence of this genome was built using Illumina MiSeq at the Argentinian Consortium of Genomic Technology. A total of 1 188 622 high-quality paired-end reads were produced, with an average insertion size of 754. *De novo* assembly was performed with SPAdes assembler version 3.0.0 (Bankevich *et al.*, 2012); 99.7 % of the 1 182 050 paired reads plus 2888 unpaired reads generated were assembled, resulting in a mean nucleotide coverage of 47 (and a *k-mer* coverage of 32). Corrected reads showed an average length of 208. Assembled contigs sum 4823461 bp with an N_{50} contig size of 55 905 (maximum length 209935) and had a G+C content of 46.3 %. Open reading frames were predicted and annotated using the RAST server, which identified 4392 possible coding sequences and 98 tRNA genes (Aziz *et al.*, 2008). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number LGYY00000000. The version described in this paper is version LGYY01000000.

Antimicrobial resistance determinants were identified using the ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) tool and RESfinder v.2.1 software (Gupta *et al.*, 2014; Zankari *et al.*, 2012). Detection of insertion sequences (IS) was carried out using the software ISfinder and other mobile elements, such as phages and genomic islands, were identified using PHAST and IslandViewer3, respectively (Dhillon *et al.*, 2015; Siguier *et al.*, 2006; Zhou *et al.*, 2011). The ICE*Sh95* sequence was manually curated using BlastN, BlastP and BlastX algorithms and genes were confirmed using the best-hit comparison. Comparative analyses between ICE*Sh95,* ICE*Spu*PO1 (*Shewanella* sp. W3-18-1, accession number NC_008750.1) and ICE SXT^{MO10} (*Vibrio cholerae* O139 MO10, accession number AY055428.1) were made using the Artemis Comparison Tool v.13.0.0 (Carver *et al.*, 2008).

Genome analysis. Complete genomes and protein coding sequences of the order *Alteromonadales* were downloaded via ftp from ftp.ncbi.nih.gov/genomes/. The final dataset comprised 54 genomes (53 downloaded from GenBank plus the genome reported in this manuscript, *Shewanella* sp. Sh95), distributed in 10 genera (Table S1, available in the online Supplementary Material). Twenty-four completely sequenced genomes of *Shewanella* spp. were included in the analysis.

Sets of 440 putative orthologous genes were identified among the 54 genomes analysed. The OrthoMCL method (Li *et al.*, 2003) was implemented in the get_homologous software and used for homologous identification (Contreras-Moreira & Vinuesa, 2013). Blast searches were performed with a minimal identity value of 30 % and minimal query coverage of 75 %. Orthologous protein sequences were aligned using ClustalO v1.2.0 (Sievers *et al.*, 2011). A phylogenetic tree was inferred for each alignment using the maximum-likelihood method with an amino acid LG+G model by means of Phyml v3.1 (Guindon & Gascuel, 2003), with three random starting trees. The SH-like test was used to evaluate branch supports (Guindon *et al.*, 2010). Finally, a consensus tree was inferred from the 440 phylograms using the sumtrees.py program (Sukumaran & Holder, 2010). In parallel, phylogenetic tree reconstruction for the *16s rDNA* gene was carried out using neighbour-joining and maximum-likelihood methods with 1000 bootstraps with the software MEGA v6.0 (Tamura *et al.*, 2013).

Assembled contigs longer than 500 bp were used to estimate the average nucleotide identity (ANI) between *Shewanella* sp. Sh95 and the complete genomes of *Shewanella* spp. available in the GenBank database. This index is used to delineate species using genome sequence data (Goris *et al.*, 2007), so, if two genomes display an ANI value of 95 % or higher both genomes belong to strains of the same species. Two-way ANI (reciprocal best hits based comparison) was estimated by means of the ani.rb script developed by Luis M. Rodriguez-R and available at enveomics.blogspot.com.

Bacterial conjugation. Mating assays were carried out on LB agar plates. *Shewanella* sp. Sh95 (Tmp^R) was used as the donor strain, while *E. coli* HB101 :: pCR2.1 (Kan^R) and *Shewanella* sp. Sh10 :: pCR2.1 (Kan^R) were used as recipient strains. Donor and recipient strains were diluted from saturated overnight cultures into 10 ml and grown for 7 h at 37 °C. The cells were harvested by centrifugation, poured onto LB agar plates and incubated at 37 °C for 18 h. The cells were scraped off the mating plates and serial dilutions were plated onto LB agar plates with the specific antibiotic to select for donor, recipient or transconjugant cells. Frequency of transfer was expressed as the number of transconjugant cells per donor cell in the mating mixture at the time of plating. Moreover, transconjugants obtained with both recipient strains were checked for the presence of the ICE*Sh95* by PCR with the original recipient strains used as negative controls.

RESULTS

Genome analysis of the clinical strain *Shewanella* sp. Sh95

In recent years it has been reported that there is an increasing involvement of *Shewanella* spp. in skin and soft tissue infections, thus it is currently considered an opportunistic pathogen (Srinivas *et al.*, 2015; Tsai *et al.*, 2008). *Shewanella* sp. Sh95 was isolated from an ocular secretion and the study of its antimicrobial resistance profile resulted in the identification of bla_{OXA-48}, a gene coding for a carbapenem-hydrolysing class D β-lactamase (Ramírez *et al.*, 2010). Also, it had a class 1 integron, but no gene cassettes were detected in the variable region. So, we have investigated further the mobilome and resistome encoded in its genome in order to understand its role as an emerging pathogen.

De novo assembly of the *Shewanella* sp. Sh95 genome resulted in 4 823 461 bp with a G+C content of 46.3 %. Genome annotation using RAST led to the identification of 4392 coding sequences and 98 tRNA genes (Aziz *et al.*, 2008). Analysis of the 16S subunit of the ribosomal RNA gene, *16S rDNA,* of *Shewanella* sp. Sh95 revealed that this isolate was closely related to the environmental strain

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S. oneidensis MR-1 (Fig. S1a). This feature was also observed in two other clinical isolates of *Shewanella* spp. (strains Sh82 and Sh31; Fig. S1a). In order to corroborate this correlation, we reconstructed a phylogenetic tree using orthologous genes from complete genomes of *Shewanella* spp. isolates available in GenBank. For this analysis we also included the core genome of other members of the *Alteromonadales* family (Fig. S1b). As a result we confirmed that *Shewanella* sp. Sh95 clusters with strains isolated from environmental niches (*S. oneidensis* MR-1, *Shewanella* sp. MR-4, *Shewanella* sp. MR-7 and *Shewanella* sp. ANA-3), rather than clinical ones [e.g. *S. putrefaciens* (Fig. S1b)]. Moreover, we calculated their ANI to estimate the proximity of species, which resulted in 90.25 % (SD, 4.60 %) identity to the closest relative *Shewanella* sp. ANA-3 (cut-off 95 %; Fig. S1). This value confirmed that *Shewanella* sp. Sh95 belongs to a novel species.

Resistome and mobilome of *Shewanella* sp. Sh95

We searched for determinants of antimicrobial resistance and mobile genetic elements encoded within *Shewanella* sp. Sh95. We found a $bla_{\text{OXA-48}}$ β-lactamase gene, which was 99 % identical to $bla_{\text{OXA-48}}$ from *Shewanella xiamenensis* (JX644945.1). A single synonymous substitution was observed at position 783. Analysis of the genetic context showed that $bla_{\text{OXA-48}}$ was located between a gene encoding peptidase, C15, and a putative *lysR* transcriptional regulator. The same context was reported for several $bla_{\text{OXA-48}}$-like genes in *Shewanella* spp. (Zong, 2012). As $bla_{\text{OXA-48}}$-like genes are commonly found in *Enterobacteriaceae*, it has been suggested that this gene has been transferred from the chromosome of *S. xiamenensis* providing evidence for the role of this bacterium as a reservoir (Poirel *et al.*, 2004; Potron *et al.*, 2013; Zong, 2012). Based on our data, we propose that $bla_{\text{OXA-48}}$-like genes are naturally harboured by different species of *Shewanella*, which collaborate with the dissemination of this antimicrobial resistance gene.

However, this search also revealed the presence of a bla-$_{\text{CTX-M}}$ pseudogene and a gene cassette arrangement containing *qacH* and *dfrA15. qacH* confers resistance to quaternary ammonium salts and ethidium bromide, whereas *dfrA15* provides resistance to trimethoprim. The cassette array was located in the variable region of an MDR integron, with an integrase gene (a pseudo *intI9*) that was interrupted by an insertion sequence from the IS*4* family.

We looked for other ISs encoded in the genome of *Shewanella* sp. Sh95 using ISFinder. We found several of them spread throughout the genome, which resembled the occurrence of ISs reported for *Shewanella oneidensis* MR-1 (Romine *et al.*, 2008). Although most hits resulted in short sequences at contig extremities, we were able to identify 10 ISs, three of which belonged to the IS*4* family: IS*10A*, IS*Sba6* and IS*UnCu8*; 3 to the IS*3* family IS*Sba13*, IS*Spu6*, and IS*Shes1*; the remaining ones were ISs from families IS*630* (IS*Sod16*), IS*256* (IS*Sod4*), IS*1634* (IS*Shes12*) and IS*110* (IS*Sba19*). IS*4* and IS*3* are the most studied IS families and are commonly related to composite transposons (De Palmenaer *et al.*, 2008; Sekine *et al.*, 1994). However, the genomic context and the mechanism of transposition of most ISs found in this work are still unclear.

We then used PHAST software to look for phages. We found traces of different prophage elements as we could identify 26 prophage-related coding sequences. Lastly, we used IslandViewer to look for genomic islands. This revealed the presence of a large ICE spanning 110 317 bp. Based on the location of the ICE we were able to establish that the MDR integron harbouring cassettes *qacH* and *dfrA15* was inserted in this genomic island (Fig. 1, orange arrows). Sequence analysis showed that this ICE was closely related to the SXT/R391 family, which are self-transmissible integrative elements. We named this element ICE*Sh95*. As ICE*Sh95* is an MDR element that can increase the resistance profile of a bacterial host, we proceeded with its functional characterization.

The ICE SXT/R391 element from *Shewanella* sp. Sh95

ICE*Sh95* had the core modules that provide the machinery for excision, integration, replication and transfer (Fig. 1, blue arrows; Table 1). It also contained a regulatory region (*eex, setCD, croS* and *setR*), which can be activated upon DNA damage (Beaber *et al.*, 2002; Garriss *et al.*, 2013; Poulin-Laprade & Burrus, 2015) and a recombination system (*bet/exo*) responsible for generating hybrid forms (Garriss *et al.*, 2009). Moreover, sequence analysis of the entry exclusion gene, *eex*, confirmed that ICE*Sh95* belongs to ICE exclusion group S (99 % nucleotide identity to *eexS* from ICE SXT^{MO10}) (Marrero & Waldor, 2007).

We did a comparative analysis between ICE*Sh95* with its closest relative, ICE*Spu*PO1, from the environmental strain *Shewanella* sp. W3-18-1 (Pembroke & Piterina, 2006), and the reference ICE SXT^{MO10} from *V. cholerae* (Waldor *et al.*, 1996). ICE*Sh95* had a similar genetic content to both ICEs, but shared a higher structural similarity to ICE*Spu*PO1, reflected by the presence of common genes at HS2, HS3 and HS4 (Fig. 1, red arrows; Table 1). However, ICE*Sh95* contained two different regions. The first region, HS5, consisted of genes *sh011* to *sh015* (Fig. 1, orange arrows; Table 1). *sh011* coded for a DEAD-like helicase, *sh012* had an unknown function, and *sh013, sh014* and *sh015* encoded a putative type III restriction-modification (R-M) system (Raghavendra *et al.,* 2012). The R-M system comprised the DNA methylase N-4 gene, the DNA methyltransferase *mod* gene and the restriction endonuclease *res* gene.

The second region, HS3, contained a cation efflux system (*czc*) identical to its counterpart in *Shewanella* sp. W3-18-1, several genes of unknown function (*sh081, sh082, sh083*) and the MDR integron (Fig. 1, red and orange arrows; Table 1). Of note was that the variable region of the MDR integron also contained a class C-attC group II intron, named *Sh.sp.*I4 (Fig. 1, Table 1). Group II introns are ribozymes present in bacterial genomes (Lambowitz &

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Fig. 1. Genetic organization of the ICE*Sh95* compared with ICE SXT^{MO10} and ICE*Spu*PO1. Different hot spots (HS1, HS2, HS3, HS4 and HS5) are highlighted in grey. Blue arrows depict the conserved backbone of the ICEs; green arrowheads represent *oriT*; light blue arrows represent the *xis/int* module of ICE*Sh95*; red arrows indicate the variable genes; orange arrows represent unique genes found in ICE*Sh95* and orange circles indicate the *attC* sites of genes cassettes *dfrA15* and *qacH* and the *attI* of *intI9*.

Zimmerly, 2011). We have previously demonstrated that some group II introns, known as the class C-attC, are associated with class 1 integrons because they specifically attack the *attC* recombination sites of gene cassettes (Centrón & Roy, 2002; Quiroga *et al.*, 2008 ; Quiroga & Centrón, 2009). Analysis of the insertion site, *Sh.sp.*I4, gave evidence that this intron also invaded the *attC* site of the *dfrA15* gene cassette by recognizing the motif TTGTT at the bottom strand (Centrón & Roy, 2002; Quiroga *et al.*, 2008).

The presence of the MDR integron in ICE*Sh95* from *Shewanella* sp. Sh95, which is absent in ICE*Spu*PO1, and the fact that each of these ICEs were isolated from two completely different niches reflects the constant exchange of genetic material among organisms, the adaptive behaviour of bacteria and the potential threat of transferring antimicrobial resistance genes to other species and niches.

ICE*Sh95* is a hybrid element capable of self-transfer

The self-transfer mechanism of ICE SXT/R391 starts with its excision by a site-specific recombination process involving the attachment sites *attL* and *attR* (Carraro & Burrus, 2014). We searched for the excised circular form of ICE*Sh95* by PCR, which resulted in an amplification product of 1021 bp. Sequence analysis of this product revealed that the *attP* attachment site corresponds to sequence 5′-CTACTTATGATCGATAA-3′ (Fig. 2a). We then examined the extremities of ICE*Sh95* and did not find the typical *attL* and *attR* sites (Hochhut & Waldor, 1999); instead we identified two conserved sequences spanning 17 bp, 5′-CTGCTAATGATCGATAA-3′ and 5′-CTACTTATAATAGACAA-3′ (Fig. 2a). Based on the sequences of *attP, attL* and *attR*, we were able to infer that the sequence of *attB* was 5′-CTGCTAATGATCGATAA-3′.

Then we analysed the regions adjacent to ICE*Sh95* and noticed that the element did not interrupt the *pfrC* gene, the preferential target site for all members of the SXT/R391 family (Hochhut & Waldor, 1999; Wozniak *et al.*, 2009). On the contrary, ICE*Sh95* was inserted at the 5′ end of *pabA*, which encodes for a para-aminobenzoate synthase involved in folate biosynthesis. When we examined whether ICE*Sh95* alters the reading frame of gene *pabA*, we noticed that the invasion only affected the PabA protein sequence by a single amino acid substitution (MxI; Fig. 2b). Furthermore, RT-PCR assays showed that *pabA* was still

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Table 1. Description of genes associated with ICE*Sh95* and the homologous genes from ICE*Spu*PO1 and SXT^{MO10}

ICE*Sh95*	ICE*Spu*PO1 equivalent	SXT equivalent	Function
sh001	ORF2895a	*xis*	Recombinational directionality factor involved in excision
sh002	ORF2896	*int*	ICE integrase, tyrosine recombinase-type
sh003	ORF2897	*srpR*	DNA-binding protein, type II partitioning system
sh004	ORF2898	*srpM*	Actin-type ATPase, type II partitioning system
oriT	oriT	*oriT*	Origin of transfer
mobI	mobI	*mobI*	DNA transfer and mobilization
rumB	ORF2900	*rumB′*	DNA polymerase UV repair, RumB (truncated gene)
rumA	ORF2901	*rumA*	UV repair RumA
sh008	ORF2902	024	Polymerase III epsilon-like subunit
sh009			Unknown
sh010	ORF2903	025	Unknown
sh011			DEAD-like helicase
sh012			Unknown
sh013			DNA methylase N-4, type III methylase–modification system
sh014			DNA methyltransferase, type III methylase–modification system
sh015			Restriction endonuclease subunit R, type III methylase–modification system
traI	ORF1757	*traI*	TraI relaxase
traD	ORF1756	*traD*	TraD coupling factor
sh018	ORF1755	043	Coupling factor
sh019	ORF1754	044	Unknown
sh020	ORF1753	045	Unknown
traL	ORF1758	*traL*	TraL, sex pilus assembly
traE	ORF1752	*traE*	TraE, sex pilus assembly
traK	ORF1751	048	TraK
traB	ORF1750	*traB*	TraB, sex pilus assembly
traV	ORF1749	*traV*	TraV, sex pilus assembly
traA	ORF1748	*traA*	TraA pilin subunit
sh027	ORF1747		Heavy metal translocating P-type ATPase
sh028	ORF1746		*merR* family transcriptional regulator
sh029	ORF1745		Transcriptional regulator
sh030			Unknown
sh031	ORF1744		Unknown
sh032	ORF1742		*merR* family transcriptional regulator
sh033	ORF1741		Unknown
sh034			Transcriptional regulator, ArsR family
sh035	ORF1735		Dihydrolipoamide dehydrogenase (E3) subunit
sh036	ORF1734		Cation efflux protein
sh037	ORF3953		Heavy metal-(Cd/Co/Hg/Pb/Zn)-translocating P-type ATPase
sh038			Unknown
sh039	ORF3950		Secretion protein HlyD-like
sh040	ORF3949		RND multidrug efflux transporter
sh041	ORF3948		Outer membrane lipoprotein, NodT-like, RND efflux system
sh042	ORF3947		Cation efflux protein
sh043	ORF3946		Cation efflux protein
sh044	ORF3945		Cation efflux protein
sh045	ORF3944		Cation efflux protein
sh046	ORF3958		Unknown
sh047	ORF3957		transposase IS*66* (truncated gene)
sh048	ORF3943	054	DsbC isomerase thiol-disulfide interchange
traC	ORF3942	*traC*	TraC, sex pilus assembly
sh049	ORF3941		Unknown

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Table 1. cont.

ICE*Sh95*	ICE*Spu*PO1 equivalent	SXT equivalent	Function
sh050	ORF3940	*trsF*	TrsF conjugational signal peptidase
traW	ORF3939	*traW*	TraW, sex pilus assembly
traU	ORF3938	*traU*	TraU, sex pilus assembly
traN	ORF3937	*traN*	TraN, mating pair stabilization
sh054			Unknown
sh055	ORF3936		Unknown
sh056	ORF3956		Unknown
sh057	ORF3934	062	DNA-specific endonuclease I
sh058	ORF3933	063	Unknown
sh059			Plasmid-related protein
ssb	ORF3932	*ssb*	Single-stranded DNA-binding protein
bet	ORF3931	*bet*	RecT/Bet homologue involved in recombination
exo	ORF3930	*exo*	Phage-like endonuclease
cobS	ORF3929	*cobS*	ATPase
sh064	ORF3928	068	Unknown
sh065	ORF3927	069	Unknown
sh066	ORF3926	070	Unknown
radC	ORF3925	*radC*	RadC-like protein involved in recombination
sh068	ORF3924		Unknown
sh069			Unknown
sh070	ORF3923		Plasmid-related protein
sh071	ORF3922	072	DNA primase
sh072	ORF3921	073	Unknown
sh073	ORF3920		Heavy metal efflux pump, CzcA family
sh074	ORF3919		Co/Zn/Cd efflux system membrane fusion protein
sh075	ORF3918		Heavy metal RND efflux transporter
sh076	ORF3917		Unknown
sh077	ORF3916		Cation transporter
sh078	ORF3915		Putative transcriptional regulator, MerR family
sh079	ORF3914		Cobalt transporter
sh080	ORF3913		Unknown
sh081			Unknown
sh082			Unknown
sh083			Unknown
*Sh.sp.*I4			Class C-attC group II intron
dfrA15			Resistance to trimethoprim
qacH			Resistance to quaternary ammonium salts and ethidium bromide
Δ*intI9-like*			Integrase *intI9* (truncated gene)
tnp			Insertion sequence IS*4*
Δ*intI9-like*			Integrase *intI9* (truncated gene)
sh090			Unknown
sh091			DEAD-like helicase
sh092			*merC* family transcriptional regulator
sh093			*merR* family transcriptional regulator
traF	ORF3912	*traF*	Thiol isomerase –TraF, sex pilus assembly
traH	ORF3911	*traH*	TraH, sex pilus assembly
traG	ORF3910	*traG*	TraG, entry exclusion system
eexS		*eexS*	EexS, entry exclusion system
setC	ORF3909	*setC*	FlhC-like transcriptional activator
setD	ORF3954	*setD*	FlhD-like transcriptional enhancer
sh100	ORF3908	082	Similar to lytic transglycosylase

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Table 1. cont.

ICE*Sh95*	ICE*Spu*PO1 equivalent	SXT equivalent	Function
sh101	ORF3907	083	Unknown
croS	ORF3906	*croS*	Cro-like repressor
setR	ORF3905	*setR*	Regulator C1 phage-like repressor

transcribed, which suggests that there is an outward-oriented promoter at the 3′ end of ICE*Sh95* (Fig. 2c).

In addition to the differences observed in the insertion site and the attachment sites, detailed analysis of the *xis/int* module of ICE*Sh95* showed almost no identity when compared to its counterparts in ICE SXT^{MO10} and ICE-*Spu*PO1 (Fig. 1, light blue arrows). This module contains two genes that code for a putative recombination directionality factor (Xis), which belongs to the HTH-mer superfamily, and a phage integrase (Int), a tyrosine recombinase from the Int-P4 integrase superfamily. Since the Xis and Int proteins from the SXT/R391 group are also members of the same superfamilies, it is possible to assume that they are responsible for the integration and excision of ICE*Sh95*.

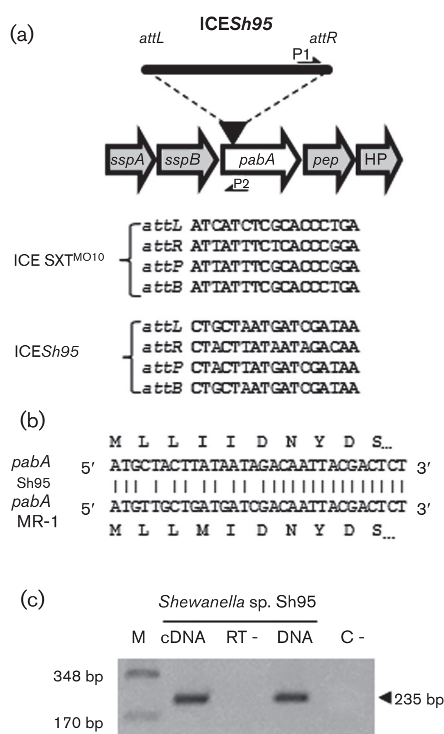

Fig. 2. Insertion site of ICE*Sh95*. (a) Schematic representation of the genomic surroundings of ICE*Sh95* in *Shewanella* sp. Sh95. Genes *sspA* and *sspB* code for the stringent starvation proteins A and B, gene *pabA* codes for para-aminobenzoate synthase, gene *pep* codes for a peptidase, HP is a hypothetical protein. The black arrowhead indicates the ICE*Sh95* insertion site. *attL, attR, attP* and *attB* are the attachment sites of each ICE. Bold residues represent the conserved nucleotides. P1 and P2 correspond to primers pabA-RT-F and pabA-RT-R respectively. (b) Sequence alignment of the 5′ end of *pabA* gene from *S. oneidensis* MR-1 and *Shewanella* sp. Sh95 with their translated amino acid sequences. MxI substitution is indicated in bold letters. (c) 1.2 % agarose gel shows transcription of *pabA*. cDNA is the reverse transcribed mRNA from *Shewanella* sp. Sh95, RT- is the RT control, DNA is genomic DNA of *Shewanella* sp. Sh95, C- is the RT-PCR control and M corresponds to the marker.

While the *xis/int* module was different from other ICEs from the SXT/R391 family, the remaining core modules showed high identity values (>95 %) against ICE*Spu*PO1. It has been reported that the *bet/exo* recombination system promotes the generation of hybrid versions of ICEs (Garriss *et al.*, 2013, 2009). Thus, it is likely that the *xis/int* module was incorporated into the ICE*Sh95* structure by a recombination event, which was probably mediated by its own Bet/Exo system resulting in the generation of a novel ICE.

Lastly, we tested whether this element was actively capable of self-transfer. In order to assess this, we used a mating assay between *Shewanella* sp. Sh95 (Tmp^R) and *Shewanella* sp. Sh10 :: pCR2.1 (Kan^R) (which did not have an SXT/R391 ICE; data not shown) and calculated the efficiency of the conjugation. The conjugation efficiency of ICE*Sh95* to other species of *Shewanella* was 2.85×10^{-3} ($\pm2.15\times10^{-3}$). We repeated this assay using *E. coli* HB101 :: pCR2.1 (Kan^R) as a recipient cell to evaluate its transfer range, which showed values of 1.04×10^{-5} ($\pm0.60\times10^{-5}$). Conjugation efficiencies revealed that ICE*Sh95* is a highly efficient mobile element that can self-transfer at great rates to other species of *Shewanella* and at standard rates to different bacteria, despite having a different recombination system and target site. PCR assays confirmed that upon transfer ICE*Sh95* integrated at the *pabA* gene in *Shewanella* sp. Sh10 and *E coli* HB101 genomes.

Taken together our results suggest that ICE*Sh95* is a highly active hybrid mobile element capable of invading a novel target site. These data provide new information on the ability of ICEs to evolve and adapt in order to extend their host range, and to ensure survival in bacterial genomes while providing the host cell with beneficial traits, such as heavy metal and antimicrobial resistance determinants.

DISCUSSION

In recent years *Shewanella* spp. have been frequently found to be associated with various infectious diseases (Holt *et al.*,

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2005; Srinivas *et al*., 2015; Tsai *et al*., 2008). To date, few genomes of *Shewanella* originating from hospital settings have been studied. The present work involved the analysis of the genom of the clinical isolate, *Shewanella* sp. Sh95, a novel species responsible for an ocular infection, and the characterization of its mobilome and resistome. Comparative analysis showed that this strain is closely related to isolates recovered from marine niches. This finding suggests that our strain, or a recent ancestor, has been transferred from aquatic niches to nosocomial environments, a process during which antimicrobial pressure played a key role in the selection and survival of this bacterium. Furthermore, we observed that strain Sh95 coded for several determinants of antimicrobial resistance. Among them we found a bla_{OXA-48}-like gene located at the same loci previously described for other OXA-48-like encoding genes (Zong, 2012). The similarities between these species suggest that they may share a role as a reservoir of this carbapenemase, which is commonly found in *Enterobacteriaceae*.

Characterization of the mobilome of *Shewanella* sp. Sh95 revealed the presence of an ICE closely related to the SXR/R391 family, which are major contributors in the spread of antimicrobial resistance in gamma-proteobacteria (Beaber *et al*., 2002; Burrus *et al*., 2006; Hochhut *et al*., 2001; Wozniak *et al*., 2009). Sequence analysis and experimental assays demonstrated that ICE*Sh95* from *Shewanella* sp. Sh95 is a hybrid element that efficiently self-transfers to a new host and integrates into the chromosome, despite having a different *xis/int* module. The limited nucleotide identity of *xis* and *int* genes to their respective homologues found in SXT/R391 ICEs, as well as the opposite orientation observed for *xis* in the ICE*Sh95* structure, suggest that this element may have gone through a recombination event by way of its own *bet/exo* system, a process previously proposed for the atypical element ICE*VchBan8* (Garriss *et al*., 2009; Taviani *et al*., 2012).

In addition to a different *xis/int* module, ICE*Sh95* had different recombination sites that resulted in efficient integration at a novel locus. Consequently, the *Shewanella* sp. Sh95 genome still has a free slot at the *pfrC* gene available for acquiring an ICE from exclusion group R. The unique properties shown in this study for ICE*Sh95* provide evidence that its dissemination can contribute to the generation of extreme MDR organisms.

A major difference in ICE*Sh95* was the presence of an MDR integron at HS3, which harboured a novel cassette arrangement in its variable region along with a C-attC group II intron. This integron has probably gone through several recombination processes that led to the accumulation of different genetic elements before reaching strain Sh95. Despite the *intI9*-like type integrase being interrupted by an IS4, it is well known that integron integrases can act *in trans* and reorganize the variable region of other integrons (Hall, 2012). A functional IntI1 integrase acting *in trans* may introduce new cassettes at the integron located within ICE*Sh95*, which in turn will be able to self-transfer to a new organism and collaborate in the spread of antimicrobial resistance.

ICE*Sh95* also had a type III R-M system and a DEAD-like helicase at HS5. R-Ms are widely spread in bacterial genomes and are often linked to mobile genetic elements (Kobayashi, 2001). These systems are useful traits for the host as they act as cellular defence mechanisms against foreign DNA (Kobayashi, 2001; Raghavendra *et al*., 2012). The presence of an R-M system at this position has been previously reported for ICE*Spu*PO1, which contained a type I R-M system (Pembroke & Piterina, 2006). Taken together, the differences observed at regions HS3 and HS5 and the similarities at HS1, 2 and 4, it is possible to assume that the ICEs found in both strains of *Shewanella* (Sh95 and W3-18-1) may have evolved from a close ancestor.

Our work reports a novel hybrid ICE, ICE*Sh95,* in a clinical isolate of *Shewanella* spp., which shares a strong genomic core with species of marine origin. ICE*Sh95* has the potential to provide additional benefits to bacteria, such as protection against foreign DNA and an integron platform for the acquisition and accumulation of antimicrobial resistance genes, which ensures its success in the selection process. In addition, the unique and efficient activity of ICE*Sh95* to self-transfer and integrate at a novel site makes this element a clear threat to the control of the dissemination of antimicrobial resistance; a threat that becomes more troublesome when natural bacteria from water ecosystems, such as *Shewanella* spp., act as vectors for its propagation.

ACKNOWLEDGEMENTS

We are very grateful for the contributions done by the members from the Argentinian Consortium of Genomic Technology at INTA. G. P. D. is a recipient of a doctoral scholarship from Consejo Nacional de Investigaciones en Ciencia y Tecnología (C.O.N.I.C.E.T.). C. Q. and D. C. are career investigators from C.O.N.I.C.E.T. This work was supported by grants BID/OC ANPCyT (2013–1978) to C. Q., and by BID/OC ANPCyT (2012–0014) and UBACYT Programación 2014–2017 to D. C.

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