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Article

Novel Mobile Integrons and Strain-Specific Integrase Genes within *Shewanella* spp. Unveil Multiple Lateral Genetic Transfer Events within the Genus

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Abstract *Shewanella* spp. are gram-negative bacteria that thrive in aquatic niches and also cause infectious diseases as opportunistic pathogens. Chromosomal integrons (CI) and mobile integrons (MI) were previously described in some *Shewanella* isolates. Here, we evaluated the occurrence of integrase genes, the integron systems and their genetic surroundings in the genus. We identified 22 integrase gene types, 17 of which were newly described, in different *Shewanella* species, evidencing multiple gain and loss events. Phylogenetic analysis showed that most of them were strain-specific, except for *Shewanella algae*, which seem to have co-evolve within the host as typical CIs. Noteworthy, co-existence of up to 5 different integrase genes, as well as their wide dissemination to *Alteromonadales*, *Vibronales*, *Chromatiales*, *Oceanospirillales* and *Enterobacterales* was observed. Identification of novel MIs suggests that lateral genetic transfer may have occurred resembling the behavior of class 1 integrons. The constant emergence of determinants associated to antimicrobial resistance worldwide, concomitantly with novel MIs in strains capable to harbor several types of integrons may be an alarming threat for the recruitment of novel antimicrobial resistance gene cassettes in the genus *Shewanella,* and its evolution towards the multidrug resistance.

Keywords: *Shewanella*; chromosomal integron; mobile integron; integrase,gene cassette; lateral genetic transfer

1. Introduction

Shewanella spp. are aquatic bacteria commonly found in a wide variety of marine environments including surface freshwater or the profoundest oceanic trenches [1-3]. A few species, such as *S. algae* and *S. xiamenensis*, can occasionally cause skin and soft tissue, peripancreatic, gastrointestinal and biliary tract infections, otitis, bacteremia, endocarditis, arthritis, peritonitis and ventilator-associated pneumonia [4-6]. Genome analyses have revealed that *Shewanella* spp. can act as reservoirs of antimicrobial resistance determinants, such as *qnr* and *bla*$_{\text{OXA-48}}$, which were later on transferred to different pathogenic bacteria [4, 7-10]. In addition, *Shewanella* has a versatile and plastic genome capable of acquiring beneficial genetic traits, such as various antimicrobial resistance genes encoded within integrons [4, 9, 11-14].

Integrons are genetic platforms that participate in the adaptation and evolution of gram-negative bacteria by acquiring and expressing gene cassettes with a wide variety of functions. Several studies showed that integrons can be found in around 17% of bacterial genomes deposited in GenBank [15-17], with as little as 35% aminoacid sequence identity suggesting a long evolutionary history [18]. Integron structure consists of the integrase

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gene *intI*, a regulatory region (P*int* and Pc in class 1 integrons) and an attachment site known as *attI* (Figure 1) [19].

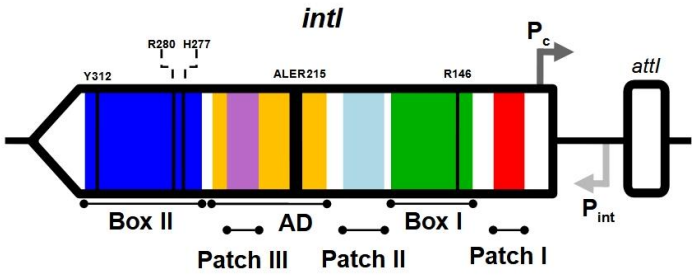

Figure 1. Integron structure. *intI* integrase gene is depicted with a horizontal black arrow; Pc, gene cassette promoter in dark gray, Pint,integrase promoter in light gray; *attI* recombination site with a white rectangle. Colored sections of the integron depict key regions: Box I in green, Box II in blue, Patch I in red, Patch II light blue, Patch III in purple, and the Additional Domain in yellow. The tetrad RHRY is represented with thin black lines and the conserved motif ALER215 with a thick black line.

Integron integrases are tyrosine recombinases responsible for the integration and excision of gene cassettes, preferably at the *attI* site [20]. Gene cassettes are mobile elements usually composed of a single structural gene adjacent to an *attC* site, which together with the *attI* site carry on a site-specific recombination catalyzed by the integron integrase [19]. Expression of gene cassettes occurs upon integration within the integron, [21].

Integron integrases possess key residues that define their activity, i.e., R146, H277, R280 and Y312 [22-24]. In addition, they have several conserved residues (boxes I and II and patches I, II and III) and an singular additional domain (AD) of about 36 amino acids (near Patch III), with the conserved motif ALER215 and the residue K219 [23-25]. Some residues are involved in the catalytic reaction (K171, H277, and G302), while others (E121, W229, and F233) are non-catalytic residues (coordinates assigned based on the IntI1 sequence) [25].

Although there are different schools of thought concerning the classification of integrons [26-34], two main groups are identified, mobile integrons (MI) and chromosomal integrons (CI). MI are embedded in mobile genetic elements (MGE), such as transposons, genomic islands and/or plasmids that facilitate their dissemination to a variety of gram-negative bacilli by lateral genetic transfer (LGT) [29-31, 35]. This group comprises integrons from classes 1 to 5, which are defined by their respective integrase gene. These integrase genes share 40-58% sequence identity and some of them, such as *intI1* and *intI2*, are commonly found in clinical isolates [26, 30, 36-40]. MI can harbor up to 9 gene cassettes, most of which encode antimicrobial resistance mechanisms to almost all antibiotic families, except for tetracycline and colistin [30, 41]. Class 1 integrons have thrived in nosocomial settings, where they can capture and collect antimicrobial resistance (AMR) gene cassettes. This ability is directly linked to the emergence of multi-, extensively or pan-drug resistant bacteria [30, 35, 42-43]. On the other hand, CI are located in the bacterial chromosome and they are proposed to co-evolve with their host over long evolutionary periods of time [26, 34, 38, 44-45]. CIs may contain a few and up to 150 gene cassettes depending on the bacterial host and can be found in different environmental species, such as *Nitrosomonas europaea*, most *Vibrio* spp., some *Treponema* spp., *Geobacter sulfurreducens*, and several isolates from the genera *Shewanella*, *Xanthomonas* and *Pseudomonas* [44, 46-51]. Most integrase genes found in CI have most key residues and motives; however, integrase genes from *Xanthomonas* spp. have lost their activity due to frameshifts in their sequences, interruptions by insertion sequences or deletions [51].

Regarding integron integrases in *Shewanella* genus, previous studies described the presence of these genes in *Shewanella oneidensis* MR-1 and in *Shewanella amazonensis* SB2BT,

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identified as SonIntIA and SamIntIA respectively [48, 52]. SamIntIA shared 64.8% sequence identity with SonIntIA and 44.6% with IntI1. Furthermore, SamIntIA amino acid sequence analysis showed that it had different residues in the ALER motif [52]. Although both integrases were able to excise antimicrobial resistance gene cassettes at low level frequencies [52], their implication in the threat of antimicrobial resistance requires further studies.

Here, we analyzed the occurrence of integron integrases and their genetic surroundings in the genus *Shewanella*, in order to evaluate their association with specific lineages relevant to clinical infections and their contribution with the widespread of antimicrobial resistance determinants. Furthermore, the analysis of our results led us to delve into the evolution and dissemination of chromosomal and mobile integrons.

2. Materials and Methods

2.1. Shewanella genomes dataset

Our analysis included 304 complete and draft genomes of *Shewanella* spp. retrieved from the Genbank database [53] published until April 1st, 2021 (Table S1).

2.2. Identification of integron-integrases and attC sites

Integron-integrase genes were searched in the 304 complete and draft genomes of *Shewanella* spp. with TBLASTN comparative analysis using IntI1 protein (ADW78905.1) as query. Only sequences containing the additional domain were included and any partial IntI protein sequences were excluded from the analysis [33]. Identification of empty integrons (In0), complete integron (integron integrase with their respective variable regions), *attC* sites and CALIN elements was done using the software IntegronFinder v2.0 with default setting and the --eagle-eyes option, as recommended by the developers [16]. When necessary, the *attC* sites were analyzed and confirmed using the UNAFold web server [54]. Classification of integron integrases was based on previous nomenclature [30, 51-52, 55], and the amino acid coordinates were based on the IntI1 sequence from plasmid pVS1 [25].

2.3. Pairwise similarity and identity analyses

Similarity and identity values of integron-integrase protein sequences were calculated using the software MatGAT v2.03 (Matrix Global Alignment Tool) as recommended by the developers [56] (Table S2). Values were plotted as a heat map using GraphPad Prism v8.2.0 software [57] (Figure 2).

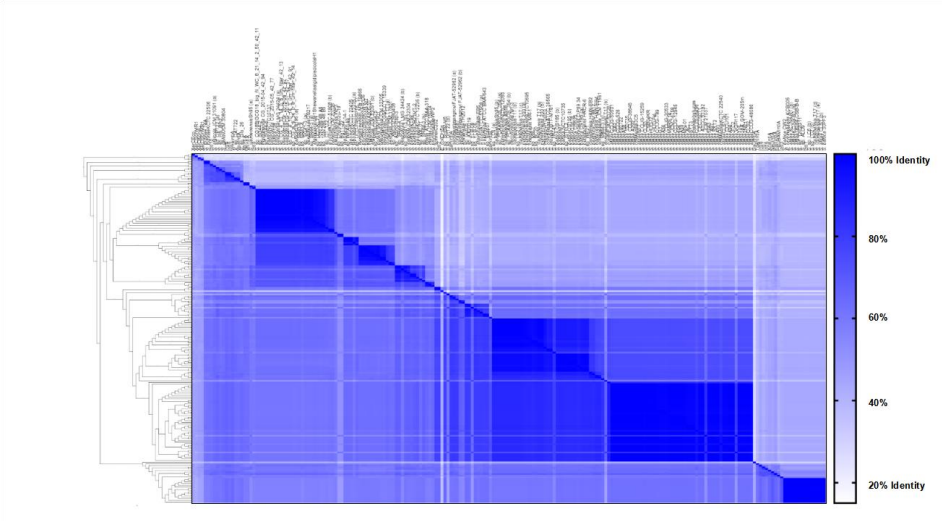

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Figure 2. Heat map with identities and similarities of integron integrases from *Shewanella* spp. based on their amino acid sequences. The orders of rows and columns correspond to the phylogenetic tree. The colored scale depicts the percentages of similarity and identity.

2.4. Phylogeny and sequence analyses

Phylogenetic trees were constructed with IQ-TREE v1.5.5 software [58] using the maximum-likelihood method with model LG+I+G4 and theultrafast bootstrap parameters (1000 replicates) to evaluate the node support [59] (**Figure 3 and Figure S1**). The Integrase protein sequences included in the analysis were listed in **Table S3**, and corresponds to 182 integron integrases from *Shewanella* spp., 24 reference IntI integrases (IntI1: ADW78905.1, ADC80454.1; IntI2: ADH82153.1, CAA05031.1, AAT72891.1; IntI3: AAO32355.1; IntI4: AAD53319.1; IntI5: AAD55407.2; IntI6: AAK00307.1; IntI7: AAK00305.7; IntI8: AAK00304.1; IntI9: AAK95987.1; IntI10: CAC35342.1; XcaIntIA: AAAK07444.1; *V.* sp. DAT722, IntIA: ABA55859.1; VmeIntIA: AAK02074.1; VpaIntIA: AAK02076.1; AfiIntIA: AAW87733.1; VfiIntIA: AAK02079.1; GsulIntIA: AAR35840.1; TdenIntIA: AAS12359.1; NeuIntIA: CAD86100.1; PalcIntIA: AAK73287.1; PstuBAMIntI: AAN16071.1) and 4 XerC and XerD proteins sequences used as outgroups. Sequence alignment was done using MUSCLE in MEGA X version 10.0.4 software (Molecular Evolutionary Genetics Analysis across computing platforms) [60].

2.5. Genetic context of integrons analyses

Genetic surroundings of integron integrase genes (n=121) were analyzed in complete genomes (47/121) and in contigs with more than 1000 bp adjacent to an In0 or to a complete integron (74/121) using Geneious Prime v.2021.0.3 software [61] to visualize the integron integrases genomic context. SoptIntIA-like (*S.* sp. HS_Bin2) was also included as it was the only representative of the group C (**Figure S2**). Adjacent proteins were analyzed by doing sequence comparison with the tools blastp and tblastn using Genbank and Pfam databases. Insertion sequences were detected using the webserver from ISFinder [62] (Figure S2). Genomes that did not have an available annotation (n=20) were excluded except for *Shewanella* sp. Glo_26, since it was the only representative of the group E (**Figure S2**).

3. Results

3.1. Identification of integrase genes encoded in Shewanella spp..

In order to evaluate the incidence of integron integrases in *Shewanella* spp., we searched for the presence of the integrase gene (*intI*) in genomes available in GenBank [53] until April 1st, 2021 (81 complete and 223 draft genomes). We found 182 integrase genes in 158 genomes showing a 52% occurrence. Fourteen sequences were incomplete genes and thus were excluded from the analysis (**Table S3**). Alignment of the 182 IntI sequences resulted in the identification of all main residues (E121, K171, K219, W229, F233, G302, ALER215, and RHRY). Residues K171, K219, W229, F233, H277, R280 were conserved in all integrases. E121 showed a high conservation in this dataset (96.1%; 175/182), however, some sequences had an E121Q substitution (3.8%; 7/182), which has been previously reported in marine integrons [63]; residue G302 was conserved in 99.4% of integrases (181/182) (**Table S3**). Regarding the motif ALER, we found that 95 out of 182 IntI (52.2%) had the canonical sequence, whereas 21 integrases had different motives, with SLIR (14.3%; 26/182) as the most frequent variant (**Table S3 and Figure S3**). L216 from the ALER motif was the single conserved residue in all but one sequence corresponding to the animal gut isolate *S. waksmanii* (ATCC BAA-643). Last, the residue Y312 was conserved in all integrases, except for the one found in *S. corallii* A687 (Y312C). Overall, integrase sequences detected in this genus showed considerable differences that may reflect variations in the recombination processes.

We then evaluated the relationship among integrase proteins by phylogenetic analysis and primary sequence identity. The phylogenetic tree revealed a wide distribution of these enzymes along different *Shewanella* species showing a very heterogeneous

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distribution (**Figure 3 and Figure S1**). Taking into account the cut-off values for the respective % of identity (% ID) at the protein level, we defined the same integrase type to those that have >95% ID; an IntI-like when values ranged from < 95% - > 70% ID; or a different IntI type when values were < 70% ID (**Figure 2 and Table S2**). Based on this criteria, we organized the data in 9 groups (A to I) (depicted in Figure S1). Groups A to F comprised a few integrases, showing a limited occurrence of these variants in this genus. Since we found an unexpected diversity of integrases among *Shewanella* species, we assigned each cluster based on previous nomenclature [30, 51-52, 55]. Group A contained 2 integrases found in *S. fodinae* (SfoIntIA; TCN90131.1, GGY88300.1). Group B consisted of an integrase in *S. algicola* JCM31091 (SalIntIA; GGP58595.1). Group C contained an integrase identified as SoptIntIA (*S. sp.* OPT22; RYV01218.1) and one SoptIntIA-like integrase (*S. sp.* HS_Bin2; MBE8168179.1). Group D and E consisted of a single integrase from *S. gelidii* JCM30804 (SgeIntIA; GGI87225.1) and *S. sp.* Glo_26 (SgIoIntIA; PIWH01000266.1), respectively. Group F contained an IntI9-like integrase previously reported in *S. xiamenensis* Sh95, located in an ICE from the SXT/R391 family (KPN75525.1) [9]. As seen in Figure S1, most integrases clustered into Groups G and H. Group G contained a large number of integrases divided in 7 lineages (Figure S1). We identified these lineages as i) SveIntIA (23 members) and SveIntIA-like (3 members); ii) SpieIntIA (ACJ28675.1); iii) SchoIntIA (2 members; PJBE01000013.1, PJAZ01000066.1) ; iv) SjaIntIA (5 members); v) SfriIntIA (7 members) and SfriIntIA-like (5 members); vi) SactIntIA (4 members) and SactIntIA-like (6 members); and vii) SmarIntIA (WP_025822232.1) and SmarIntIA-like (2 members; AQS38529.1, WP_037410996.1). Group H was the most abundant, with 102 members divided in 7 lineages (Figure S1). Within this group we found the already described integrases SonIntIA (QKG96659.1) [48] and SamIntIA (ABL99562.1) [52]; however, our analysis showed a wider diversity. These lineages were identified as: i) SshIntIA(NMH67033.1); ii) SamIntIA (ABL99562.1) and SamIntIA-like (2 members; QSX38667.1, WP_115137975.1); iii) SfjIntIA (QSX39015.1); iv) SkhiIntIA (AZQ10419.1); v) SoptIntIB (RYV02274.1); vi) SguIntIA (2 members) and SguIntIA-like (6 members); and vii) SonIntIA (17 members) and SonIntIA-like (70 members).

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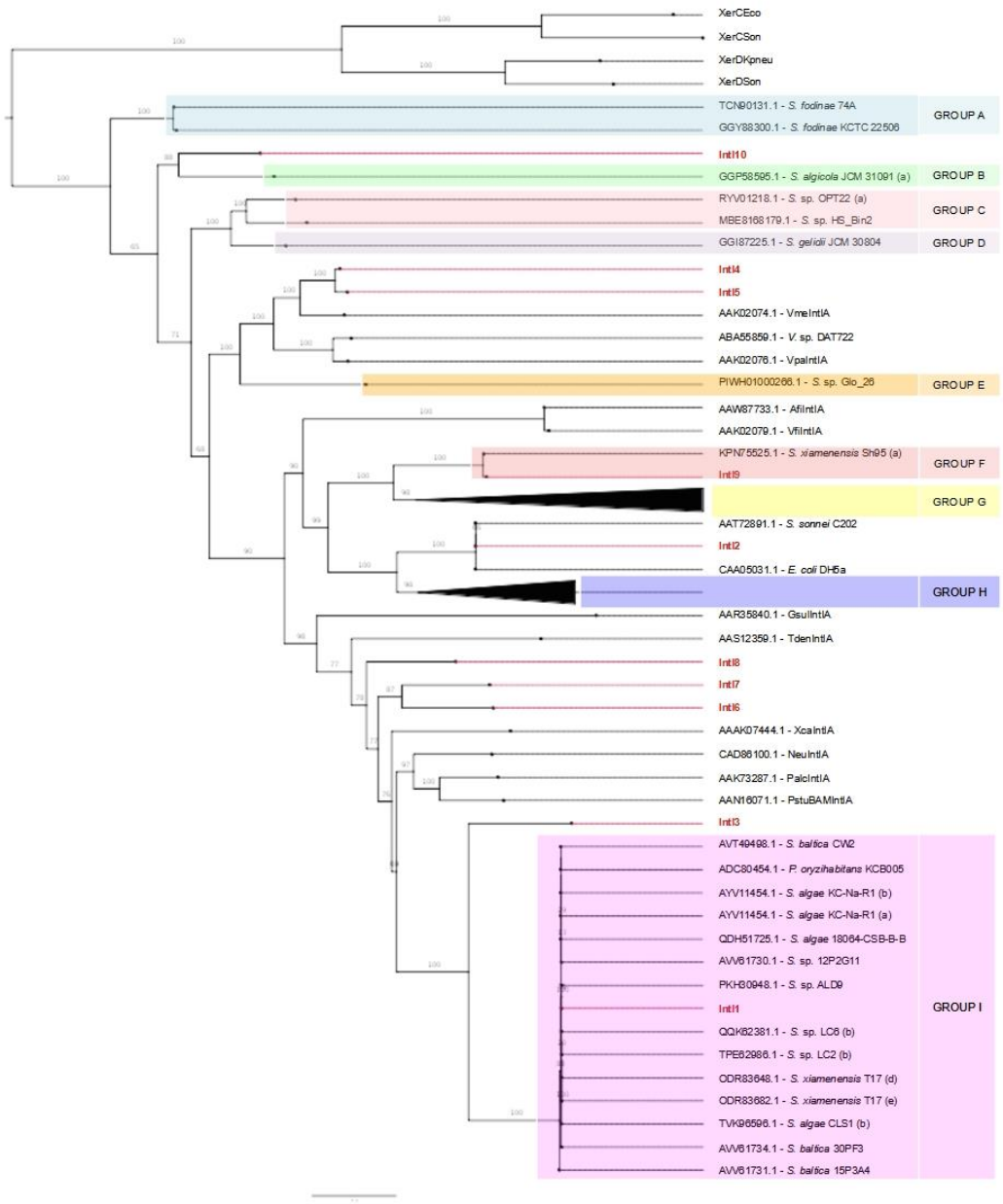

Figure 3. Phylogenetic tree of integron integrases found *Shewanella* spp.. Tree construction was done using the maximum-likelihood method with model LG+I+G4 and a bootstrap of 1000 replicates. IntI1 to IntI10 integron integrases are depicted in red. Groups G, H and I are collapsed, a detailed description of each group can be found in Figure S1 and Table S3.

Last, Group I contained all IntI1 integrases (n=13) with a strong sequence conservation (identity >99.1%) (**Table S2 and Figure S1**). A few integrases were excluded from the analysis, i.e., integrase genes found in *S. xiamenensis* T17 (NGZL01000175.1_1) and *S. xiamenensis* CC4-7 (ALD16294.1), which were interrupted by a Tn-tet transposon [64] or a Tn-tet-like respectively, resulting in the interruption of IntI C-term end (Table S3), and those found in *S.* sp. Shew256 (NAJR01000050.1) and *S. putrefaciens* SA70 (ODR83671.1) that were fragmented at the end of the respective contigs (**Table S3**).

Our search revealed the presence of 22 integrase types, 17 of which have not been described before.

3.2. Unique distribution of integrase genes among Shewanella spp. genomes

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Most *Shewanella* spp. isolates encoded a single integrase, however we found several genomes with 2 or more homologues (20/158; 12.65%) belonging to different groups (**Figure S1 and Table S3**). The most frequent combinations corresponded to i) *intI1* with Son*intIA* or Son*intIA*-like, ii) integrase genes exclusively from Group G, or iii) integrase genes exclusively from Group H. It is important to mention that the higher frequency observed for these combinations may be due to a higher incidence of integrase genes from these 3 groups in our dataset. Furthermore, we noticed that the co-existence of these integrase genes within a host can occurr between those from MIs and CIs, as well as between CI integrase genes. This coexistence was observed in *S. algae* CLS1, *S. xiamenensis* Sh95, *S.* sp. LC6 and LC2 and *S. baltica* OS195, carrying each a CI and a MI. Various isolates had 2 CIs in their genomes (i.e., *S. algicola* JCM31091, *S. vesiculosa* LMG 24424, *S. amazonensis* SB2B, *S. woodyi* ATCC 51908, *S. japonica* KCTC 12235, *S. putrefaciens* 200, *S. sedimentimangrovi* FJAT-52962, *S. donghaensis* LT17, *S. baltica* OS185, *S.* sp. OPT22, *S.* sp. SG41-3 and *S.* sp. FDAARGOS_354), whereas *Shewanella* sp. SR44-3 encoded 3 genes located in different loci in its chromosome (Sve*intIA*, Sact*intIA*-like, Son*intIA*-like). Surprisingly, one isolate, *S. xiamenensis* T17, had 5 complete and 1 partial integron genes corresponding to 2 *intI1* and 3 Son*intIA* (excluding the above mentioned *intI1* gene interrupted by Tn-tet) [64].

Regarding the correlation between the different integrase types and *Shewanella* species, we observed two different patterns. One pattern was consistent with an heterogeneous dissemination of integrase genes throughout different species, as seen for *intI1*, which was found in *S. algae*, *S. xiamenensis* and *S. baltica* (**Figure S2**, Group I), or Sves*intIA* found in *S. vesiculosa*, *S. frigidimarina* and *S. baltica* (**Figure S2**, Group G). Other integrase gene types from Groups G and H were also found in different *Shewanella* species (**Table S3 and Figure S2**). Our analysis suggests that LGT events may have participated in the dissemination of integrase genes to different species.

Moreover, we wondered whether this lateral dissemination may have also occurred between different genera. Thus, we looked for all integrase types (n= 22) in other bacterial species and we found close homologues with high identity sequence (% ID > 95) in bacteria from *Alteromonadales*, *Vibronales*, *Chromatiales*, *Oceanospirillales* and *Enterobacterales* families (**Table S4**). As expected, MI integrases IntI1 and IntI9-like showed high identity and coverage values (> 99%), which reflect the inherent nature of their genes to spread by way of MGEs. Moreover, 4 homologues to integrases from Groups E, G, and H were found in a wide variety of *Vibrio* spp., that correlate to genes *SglointIA*, *SveintIA*, *SchointIA*, and *SonintIA*. Noteworthy, *SglointIA* homologue genes were found exclusively in *Vibrio* spp. showing a conserved distribution. Additionally, other integrase homologue genes seemed to have found an alternative host in which to adapt and co-evolve, such as Sact*intIA* homologues from *Pseudoalteromonas* spp. (**Table S4**). Among complete genomes, most integrase homologue genes were located within the host chromosome. Excluding MI integrase genes, only one homologue with high sequence identity (> 93% ID) was found in a plasmid. This gene corresponded to a *SalintIA* homologue found in the megaplasmid pPBSW1 of *Pseudoalteromonas* sp. Bsw20308 (CP013139.1).

When we focused our analysis on each *Shewanella* spp., we were able to observe a more conserved pattern for *S. algae*. Forty-five out of 87 genomes of *S. algae* from our dataset encoded a Son*IntIA*-like integrase gene (51.7%), while the remaining did not contain a homologous gene (**Table S3**). In addition to carrying Son*intIA*-like gene, strain CLS1 also had an *intI1* gene; whereas strains KC-Na-R1and 18064-CSB-B-B solely coded for the *intI1* gene. It is possible to assume that the acquisition of integrase genes related to Son*intIA*-like may have occurred before speciation of *S. algae*, with the ulterior loss of this gene in some lineages.

Taken together our results suggest that the evolutionary history of *Shewanella* integrase genes is a complex network that include genome co-evolution in *S. algae*, and several processes of gain and loss in other species showing limited associations of these genes with a specific host.

3.3. Integrase genes from Shewanella spp. are not niche dependent

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We wondered whether the distribution of integrase genes was related to the habitat from which each *Shewanella* isolate was recovered. Therefore we evaluated their association between the source of each isolate and the presence of an integrase gene. We first noticed that most integrase genes were found in bacteria recovered from sediments or aquatic niches (74/158; 46.8%), whereas it was less frequent to find integrase genes in bacteria isolated from human-associated niches (clinical samples, hospital environments or other impacted niches; n=44) (**Table S5**). Bacteria from aquatic or sediment habitats together with those from animal samples showed the widest variety of integrase gene types (Figure S4, blue and green bars respectively). A more limited distribution was noticed in bacteria recovered from clinical samples, which contained integrase genes *intI9*-like, Son*intIA* (or Son*intIA*-like) and/or *intI1*.

Regarding the distribution of integrase types throughout different ecosystems, we observed that only two integrase gene types showed a broader spread, Son*intIA* Group H and *intI1* (Group I). While the dissemination of *intI1* can be simply explained by LGT events, Son*intIA* and Son*intIA*-like are mostly CI integrase genes that seem to be established in the host chromosome. An explanation for the presence of CI integrase genes in different species may be related to the ability of *Shewanella* to thrive in a variety of habitats.

Last, it is worth to mention that *intI1*-bearing bacteria were mostly isolated from human-associated samples (6/13), however we also found that 4/13 were recovered from aquatic animals, which provides additional evidence of the role of animal reservoir and/or source in MDR bacteria evolution. Notwithstanding, the impact of animal reservoirs was also seen for Son*intIA* and Son*intIA*-like integrase genes, where 12 out of 81 were found in animal-associated bacteria.

3.4. Analysis of integron/cassettes systems in Shewanella genomes

Integrase genes are commonly adjacent to a variable region (VR) containing different gene cassette arrays. We first analyzed all genes and gene cassettes located downstream of integrase genes found in complete genomes and contigs with > 1000bp surrounding those genes in *Shewanella* spp. (n= 97) and assessed whether they had the VR. Several genomes harbored more than one integrase gene resulting in 121 integron systems. *attC* curation allowed us to define the VR of each integron and to identify all gene cassettes. Each potential gene cassette was analyzed by looking for key *attC* features, such as size (> than 59 bp), presence of core site (CS) and an inverse core site (ICS) sequences (GTTRRRY and RYYYAAC, respectively), and its secondary structure. Bottom strand of each *attC* site was folded in order to confirm the presence of a canonical DNA secondary structure [65]. This allowed us to detect several structures that could correspond to *attC* or *attC*-like site structures, not identified with IntegronFinder software [16].

Integrons harboring gene cassettes belonged to all defined Groups, which suggests that all groups contained active integrases capable of gaining or losing gene cassettes contributing to *Shewanella* evolution. Furthermore, analysis of each system showed that 58 of them had a VR with different gene cassettes, whereas 63 had an *intI* gene with no cassettes in the vicinity, and thus they were classified as In0 (**Figure S2**).

Several gene cassettes had two (n=18) or three (n=4) *orfs* in tandem associated to a single *attC* in their VR (i.e., *S. fodinae* strains 74A and KCTC 22506 in Group A, *S. gelidii* JCM 30804 in Group D, *S. japonica* UCD-FRSSP16_17 and *S.* sp. Actino-trap-3 in Group G, *S. amazonensis* SB2B, *S. khirikhana* TH2012, *S. xiamenensis* T17, *S.* sp. strains YLB-06, Scap07, LC6, LC2, WE21 and *S. algae* strains 150735, A59, CECT 5071, ATCC 51192, G1, A292, CCUG-789 in Group H; **Figure S2**; blue triangle). In addition, one integron system contained a gene cassette lacking an *orf* suggesting that they may express a non-coding RNA (i.e., *S. inventionis* CGMCC 1.15339 in Group G; **Figure S2**, green triangle). The presence of two *orfs* in tandem in a single gene cassette or no *orf* in it has been previously reported for other integrons [30, 66]; however they have not been reported in previously characterized integrons from *Shewanella* spp..

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Array analysis revealed that *Shewanella* integron systems may contain up to 9 gene cassettes (**Figure S2**). Sequence analysis showed that their functions were quite diverse, encoding antimicrobial resistance mechanisms, as well as proteins involved in stress resistance, DNA repair, and cellular regulation, whereas, most gene cassettes coded for proteins of unknown function (n=141) (**Figure S2 and Figure S5)**. Regarding the AMR gene cassettes, all of them were located in the VR of class 1 integrons, except for *aadA24*. This gene cassettes conferred resistance to different antibiotic families, i.e., aminoglycosides (*aadA1*, *aadA1e*, *aadA2*, *aadA6*, *aadA16*, *aadA24*, *aadB*, *aacC1*, *aac(6')-Ib7Δ*), trimethoprim (*dfrA12*, *dfrA15*, *dfrA27*), rifampicin (*arr-2*, *arr-3*), chloramphenicol (*cmlA5*), beta-lactams (bla_{VEB-1}, bla_{OXA-10}), or quaternary ammonium salts (*qacE* and *qacH*).

We also found group II introns (GII), which are ribozymes capable to retrotransposing to new regions in a genome, inserted at the *attC* site of gene cassettes *dfrA15*, *qacE*, *aadA24* and *aadA1e*. Invasion of AMR gene cassettes by these retroelements were previously reported for class C-attC group II introns [67-68] (**Figure S2**). C-attC GII introns were found in gene cassettes inserted in integron systems from groups A, F, G, H, and I, suggesting that independent recombination events may have occurred. In addition, two integrons contained other GII introns corresponding to chloroplast Cl1 and bacterial E classes (i.e., strains *S. putrefaciens* 200 and *S. saliphila* JCM 32304, **Figure S2**). While some GII introns invaded AMR gene cassettes (i.e., *dfrA15* from strain Sh95), most ribozymes were inserted within gene cassettes encoding proteins of unknown function (i.e., in strain *S. fodinae* 74A from group A, *S. baltica* NCTC10737, *S. livingstonensis* LMG 19866, *S. saliphila* JCM 32304 from Group G and *S.* sp. strains LC6, LC2, FDAARGOS_354, *S. putrefaciens* NCTC12093, *S. xiamenensis* T17 (a,c) from Group H; **Figure S2**). C-attC GII introns are known for inserting into the target DNA in opposite orientation to gene cassettes; however, we noticed that the ribozyme located in the genome of *S. algae* KC-Na-R1 was in the same orientation, which suggests that retrotransposition might have followed a different invasion process **(Figure S2**, *S. algae* KC-Na- R1 (b), Group I).

The variable region of several integron systems was also interrupted by a wide variety of ISs, which belonged to families: IS*91* (IS*91*-like, IS*Shvi3*), IS*1595* (IS*1595*-like, IS*Sod11*), IS*110* (IS*Sde13*, IS*110*-like) (**Figure S2**). IS are common MGEs invading integrons, which may have deleterious effects or contribute to the diversity of variable regions. Most IS interrupts coding regions, however, it has been reported that IS*1111* elements are capable of invading the *attC* site of gene cassettes similarly to GII introns [69]. Although there were no IS*1111*, we found an IS*91*-like element, named IS*Shvi3*, in the variable region of the integron from *S.* sp. Choline-02u, which was adjacent to an *attC* site yielding a new gene cassette structure.

Previous studies have shown that gene cassettes are not only located within the variable regions, but also they can form clusters of *attC* sites lacking integron-integrases (also known as CALINs) [16]. In this regard, we observed a peculiar amount of *attC* sites that were found in distal regions of integrase genes. These CALINs were present in most *Shewanella* spp. strains. A detailed analysis showed that complete genomes that carried *intI* genes and CALINs (n=33) were more frequent than genomes with CALINs without *intI* genes (n=26). Genomes containing CALINs (n=59) had 2 (n=47), 3 (n=7), 4 (n=2), 5 (n=1) and up to 6 (n=1) *attC* sites, each adjacent to an *orf* (**Figure 4** and **Table S6**). Furthermore, we found single orphan gene cassettes (n=115) classified as SALINs (single *attC* sites lacking integron-integrases; Alonso et al., in revision), scattered around several genomes (n=41). CALIN arrays seem to have more *attC* sites in the presence of integrase genes.

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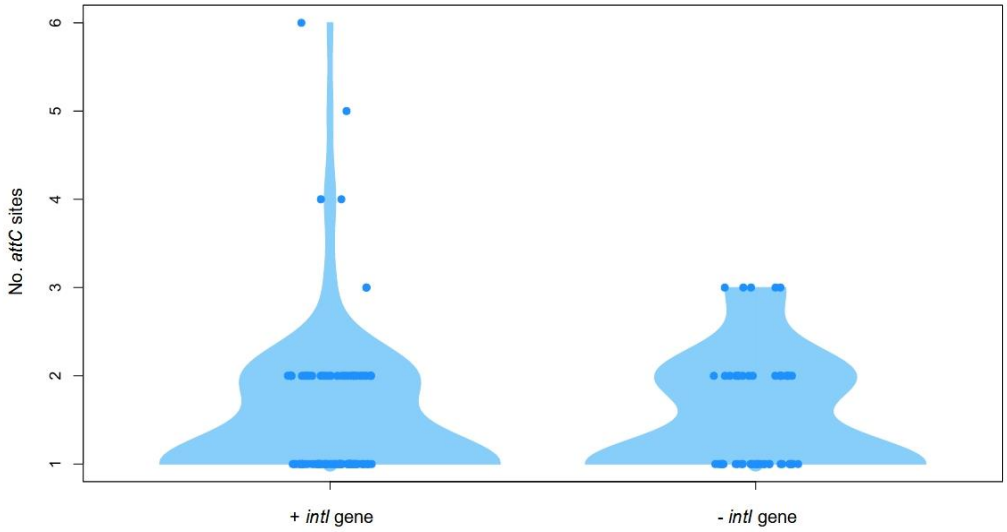

Figure 4. *attC* sites found in complete genomes of *Shewanella* spp.
attC sites in CALINs and SALINs found in *Shewanella* spp. complete genomes with (left) and without (right) the *intI* integrase genes.

3.5. Genetic context of Shewanella spp. integron systems

We then studied the genetic context of the integron systems found in *Shewanella* spp.. First, we looked up all genes located upstream of the integrase gene in all complete genomes and in contigs with enough information that allowed us to assess the genetic background (length > 1000bp). Overall we analyzed 121 integron sequences, found in 47 complete and 74 draft genomes (**Figure S2**). At a first glance, we noticed genes encoding proteins with a wide variety of functions and low loci conservation (**Figure S2**). Genetic contexts were very diverse even within the same *intI* group, i. e., Sve*intIA* genes were adjacent to hypothetical proteins PMH88747.1, PMI02144.1, VEF25666.1 and ABX51800.1, as well as to the peptidase M28 (MBB1477376.1) and NADH dehydrogenase (ABE54417.1). In a few instances, we found some conservation where homologous *intIA* genes from the same group were adjacent to the same gene (Sfo*intIA* from Group A; Sfri*intIA* and Sact*intIA* from Group G and Son*intIA*-like from *S. algae* and *chilikensis* from Group H). Interestingly, we also found different integrase genes from different groups located downstream of the same gene. For example, Spie*intIA* from *S. piezotolerans* WP3, Scho*intIA* from *S.* sp. Choline-02u-19 (Group G) and Sgu*intIA* from *S.* sp. KX20019 (Group H) were located downstream of a gene coding for the subunit α of the tryptophan synthase $\alpha 2\beta 2$ (TrpA; WP_202285266) (**Figure S2)**.

An association between ISs or MGE-related genes and integrons was seen (**Figure S2**, depicted with orange arrows). Incidence of these elements was higher in Group I, which encompass all class 1 integrons embedded in transposons that most likely are harbored in different plasmids (**Figure S2**).

Surprisingly, we found 2 integrase genes, Son*intIA* and Sve*intIA* located in plasmids. Both integrase genes lacked gene cassettes and were classified as In0. Son*intIA* (QWY79362.1; Group H) was encoded in the megaplasmid pNi1-3 (CP076856.1) from *S. decolorationis* Ni1-3, and Sve*intIA* (ABX51799.1; Group G) was encoded in plasmid pS19502 from *S. baltica* OS195 (CP000893.1). While Son*intIA* was in the vicinity of an IS*Pa33*, Sve*intIA* was surrounded by IS*Sod12* downstream of the integrase gene and IS*Psy42*-like upstream of it (**Figure 5).** Comparative analysis of plasmids harboring these integrase genes showed that they do not have sequence conservation nor similar IS elements. This data suggests that each integrase gene was transferred from chromosome to a plasmid after independent recombination and LGT events. The association of CI with plasmids and the presence of MGEs near integrase genes reveals the evolution of CIs into MI integrases.

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Figure 5. Genetic context of integrase genes found in plasmids from *Shewanella* spp.. **A.** Integrase gene Sve*intIA* within plasmid pS19502 from *S. baltica* OS195 (CP000893.1). **B.**Integrase gene Son*intIA* within megaplasmid pNi1-3 from *S. decolorationis* Ni1-3 (CP076856.1).

4. Discussion

The study of integron systems in the genus *Shewanella* revealed unique features about these genetic platforms that contribute to increasing our knowledge regarding their pattern of dissemination and their relevance in nature. Our *in silico* approach provides a detailed analysis of their genetic surroundings that widen our understanding on integron evolution towards MI in MDR clinical strains, as well as their consequent role in the adaptation of this genus to new niches.

Our analysis led us to the identification of a surprising amount of integrase gene types within a single genus. In addition to the already reported integrase genes in *Shewanella* [48, 52], we found 16 new types clustered in different groups (**Table S3**). To properly identify all candidate integrases, we analyzed the extensive work of previous groups and established an effective criterion [19, 25, 30], which allowed us to detect a wide variety of integrases within this genus. Novel integrase genes from *Shewanella* spp. showed key differences at the protein sequence level, such as the highly variable ALER motif, with up to 21 differences, which might directly impact on the enzyme activity [24-25, 30].

The richness of integrase genes in the environment has been previously reported; however, most studies used culture-independent techniques, and cannot be assigned to a specific bacterial species or genus, except for *Vibrio* spp. [38, 50, 70-74]. In *Shewanella*, the various integrase gene types were found scattered around the different species. All detected integrase genes were grouped in 9 clusters (from A to I) most of them encompassing CI integrase genes, except for MI integrase genes *intI1* and *intI9*-like (**Figure S2**). Moreover, some strains of *S. algae* and *S. xiamenensis* encoded up to 5 integrase genes, revealing the co-existence of several CIs and MIs, which has not been commonly found among bacteria bearing integrons. Interestingly, both species are frequently detected in clinical samples, evidencing their role as reservoir and vector for dissemination of AMR gene cassettes from the environment to the nosocomial niche, and vice versa. In addition to evidencing the ability of *Shewanella* to acquire MI integrases, such as *intI1* and *intI9*, we also observed signs of lateral genetic transfer of the novel integrase genes from Groups G and H. These are the cases for Sal*intIA*, Sglo*intIA*, Sve*intIA* and Son*intIA*, which had more than 95% identity with their counterparts found in other genera including *Vibrio* spp. **(Table S4**).

We also found a wide variety of gene cassettes; however only a few of them encoded known functions (AMR, repair and cellular functions). Solely one antimicrobial resistance gene cassette, *aadA24*, was found in SonIntIA CI (Group H) in two different *Shewanella* species (*S.* sp. FDAARGOS_354 and *S. putrefaciens* NCTC12093). In addition, we noticed that a few gene cassettes had unique features, i.e., they were interrupted by GII introns, contained fused genes or contained a non-coding gene. Our analysis exposes the wide diversity of gene cassettes that exists in a single genus, which is consistent on a smaller scale with the reports about the gene cassette pool in nature [26, 28]. The significant amount of hypothetical proteins encoded within gene cassettes, which has also been reported for other CIs [75, 34, 63], reflects the vast unawareness of the benefits that these traits provide to the host, which combines with the ability of *Shewanella* spp. to thrive in

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different habitats. Accordingly, the presence of different species carrying various integrase gene types in diverse niches, as seen in our data (**Figure S2**), could enhance the acquisition and spread of beneficial gene cassettes.

Regarding the variable region, we found that they contained on average one and up to 9 gene cassettes (**Figure S2**). This characteristic resembles the VR size of MIs and CIs from *Xanthomonas, Nitrosomonas* or *Geobacter*, among others, but it is in contrast with the CI arrays from *Vibrio* spp., which usually contain around 100 gene cassettes [38, 51, 55, 76]. The differences in the composition of the VR in integrons with identical integrase genes suggests that either their respective enzymes are active or that other integron integrases are carrying on the integration/excision of gene cassettes (**Figure S2**, *S.* sp. FDAARGOS_354 (b) and *S. putrefaciens* NCTC12093). Further studies are necessary to demonstrate the activity of the novel *Shewanella* integrases identified here [48,52]. We also found that the 52% (63/121) of integrons did not harbor any gene cassette in the variable region, evidencing also a unique feature of CIs in the genus *Shewanella*. Analysis of the array content did not show any particular association in regards to *Shewanella* species, integrase gene type or habitat, which indicate that these integrons followed an independent evolution. While CI gene cassette arrays from *Vibrio cholerae* are highly conserved within the species [77], this is not the behavior observed for those from *Shewanella* spp.

Although there are different ways to classify integrons [26, 28, 32-34], a hallmark of CIs is that they co-evolved with their host over long evolutionary periods allowing to identify its ubiquity within a given species or lineage [28, 33, 77], compared to MIs which have a dynamic mobilization by LGT events to several hosts. While CI are considered to be ubiquitous within a species such as the case of *intIA* from *V. cholerae* [28, 38], MI can be found in a wide variety of genera such as *intI1* in *Enterobacterales* and distant bacterial genus [16, 78]. In this regard, the distribution of integrase genes in the genus *Shewanella* followed different patterns. Integrase genes were not ubiquitous within any species and they seem to be strain-specific, evidencing continuous processes of gain and loss along time in each species (**Table S3 and Figure S2**). As an example of this, in *S. baltica*, where only 6 out of 10 complete genomes had an integrase gene, they were found at different loci, as seen for MIs such as *intI1* (**Table S3**). Also, an ample dissemination was seen for other integrase gene types, such as Sve*intIA* or Son*intIA* **(Table S3**). In addition to this pattern of distribution of integrase genes, we identified that Son*intIA-like* was found located in the same locus downstream of the S41 peptidase gene in several *S. algae* strains evidencing the possible co-evolution between integrase genes and this species as previously described for CI. The study of the genetic environments of the integrons also corroborates that they can be located at different sites throughout the *Shewanella* chromosome. Our results suggest that there are three different patterns of distribution of integrase genes in *Shewanella* spp. The first one corresponds to the typical CI co-evolving with the host with the integrase gene located at the same loci, such as the case for Son*intIA*-like in *S. algae*. Our phylogenetic studies show that SonIntIA-like from *S. algae* are likely to have evolved from a common ancestor. Since not all *S. algae* genomes encode this gene, it is possible to assume that these integrase genes have been subsequently lost after independent evolutionary events. This type of CIs with signs of sedentarism within the chromosome may be present in other species of *Shewanella*; however, due to the fact that in many species there are still very few genomes sequenced, we do not have enough data to confirm this hypothesis. The second group corresponds to CIs distribuited among different strains within *Shewanella* spp.. Our findings suggest that integrons in many *Shewanella* species may have been acquired independently by each strain and inserted in different locus. Although several CIs were adjacent to various insertion sequences, the mechanism of integron insertion or deletion is unknown. Likewise, LGT of integrons has also been proposed for *Vibrio fischeri, Shewanella denitrificans, Nitrosococcus mobilis*, and *Xanthomonas* spp. [28]. The third group corresponds to MIs. Some strains of *Shewanella* spp. have shown the capability to acquire different MGEs, such as plasmids and ICEs [9, 64], which can harbor class 1 and 9 integrons, revealing its worrisome potential to evolve towards an MDR phenotype. Altogether, *Shewanella* spp. provides a scaffold where different types of

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integrons can co-exist, thrive and evolve. To the best of our knowledge, the co-existence of chromosomal integrase genes in the same host has not been reported.

Accordingly, our work also led to the identification of molecular features of integrons that could reflect the acquisition of novel MI within the genus. Two integrase genes commonly found in the host chromosome, Son*intIA* (from Group H) and Sve*intIA* (from Group G), were found in plasmids flanked by MGEs which may have contributed to their mobilization. Noteworthy, both integrase genes were also found in other distant genera, supporting the scenario of a possible transition from CIs to MIs. Spread of homologues of to Son*intIA* with > 93% identity and 100% coverage were found in the chromosome of *Vibrio plantisponsor* LMG 24470 and in diverse strains of *Pseudoalteromonas piscicida*, *Pseudoalteromonas sp.*, *Vibrio fluvialis* and *V. cholerae* (**Table S4**). Similarly, homologues to Sve*intIA* with 100% identity and coverage were found in the chromosome of *Vibrio metschnikovii* 07-2421, *Vibrio alginolyticus* VA181, *Vibrio sp. E4404* and in diverse strains of *V. fluvialis*. On the other hand, we found homologs to CI integrase genes, such as Sglo*intIA,* in different *Vibrio* spp. isolates (**Table S4**). This unique scenario of mobilization of integrase genes found within and from the genus *Shewanella* shows a panorama in which CI integrase genes may be shared among different bacteria in a niche, suggesting that there is probably a more extensive interchange of integrase genes than it was assumed so far. In some way, integrase genes that are part of MI from *Shewanella* spp. may be reproducing what happens in the clinical environment, where a particular mobile integrase, such as IntI1, is circulating between several genera giving each recipient strain the possibility of adapting to a new niche thanks to the possibility of accessing to the great pool of gene cassettes that exists in bacterial genomes.

Furthermore, integrase expression can be activated when bacteria encounter antimicrobial agents, giving rise to processes of excision and insertion of gene cassettes [79]. Recently, it has also been demonstrated that bacteria exposed to an increasing amount of the antimicrobial agent in the presence of a working integrase was able to reorganize useful AMR gene cassettes from the last position to a top position [80]. In this scenario, the spreading of Son*intIA* or Sve*intIA* or other novel MI to MDR clinical or environmental bacteria isolates that have AMR gene cassettes could seriously increase the burden raised today of antimicrobial resistance.

We believe that the diversity observed in this study is not unique to *Shewanella* and a similar approach should be employed in other integron-bearer bacteria, such as *Xanthomonas*, *Pseudomonas*, *Geobacter* or *Nitrosomonas* in order to improve our knowledge on the evolutionary pathways distribution of integrase genes in different taxa. It is obvious that the increase of sequenced bacterial genomes can provide a robust dataset that may lead to unveiling novel integrase genes within a genus.

As a hallmark of the genus, we observed that integrons in *Shewanella* spp. integrons followed complex evolutionary pathways. Our study shows this genus can acquire and disseminate integrase genes and lead to the emergence of novel MIs. The substantial diversity found in the variable regions, the genetic surroundings and their association with MGEs suggest a constant evolution and adaptation of the host, which probably responds to environmental niche changes and the composition of each microbial community. Furthermore, it must be taken in account that AMR gene cassettes found in hospital settings are recruited from the vast pool of gene cassettes of CI from the environment [34, 55]. In addition, since there is a clear link between the *intI1* gene and the dissemination of AMR in environmental and nosocomial niches, it is possible to assume that the considerable pool of integrase genes found in *Shewanella* spp. including novel MIs, pose a scenario in which a new integron system may emerge and contribute to the MDR threat. Active participants in these processes and dissemination may include *S. algae* and *S. xiamenensis* isolates, which are opportunistic pathogens that can cause serious infectious diseases.

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