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CTX-M-15-Producing *Shewanella* sp Clinical Isolate Expressing OXA-535, a Chromosome-Encoded OXA-48 Variant, Putative Progenitor of the Plasmid-Encoded OXA-436

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2 **CTX-M-15-producing *Shewanella* sp. clinical isolate expressing OXA-535, a**
3 **chromosome-encoded OXA-48 variant, putative progenitor of the plasmid-encoded**
4 **OXA-436**

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34

ABSTRACT

36
37 *Shewanella spp.* constitute a reservoir of antibiotic resistance determinants. In a bile sample,
38 we have identified three Extended Spectrum β -lactamase (ESBL)-producing bacteria
39 (*Escherichia coli*, *Klebsiella pneumoniae* and *Shewanella sp.* JAB-1) isolated from a 10-year-
40 old child suffering from cholangitis. Our objectives were to characterize the genome and the
41 resistome of the first ESBL-producing isolate of the genus *Shewanella* and determine whether
42 plasmidic exchange occurred between the three-bacterial species. Bacterial isolates were
43 characterized using MALDI-TOF, standard biochemical tools and antimicrobial susceptibility
44 testing. *Shewanella sp.* JAB-1 and ESBL gene-carrying plasmids were characterized using
45 PacBio and Illumina whole genome sequencing, respectively. The *Shewanella sp.* JAB-1
46 chromosome-encoded OXA-48-variant was cloned and functionally characterized.
47 Whole genome sequencing (WGS) of the *Shewanella sp.* clinical isolate JAB-1 revealed the
48 presence of a 193-kb plasmid belonging to IncA/C incompatibility group and harboring two
49 ESBL genes: *bla*_{CTX-M-15} and *bla*_{SHV-2a}. *bla*_{CTX-M-15} gene carrying plasmids belonging to IncY
50 and IncR incompatibility groups were also found in the *E. coli* and *K. pneumoniae* isolates
51 from the same patient, respectively. Comparison of the *bla*_{CTX-M-15} genetic environment
52 indicated the independent origin of these plasmids and dismissed *in vivo* transfers.
53 Furthermore, characterization of the resistome of *Shewanella sp.* JAB-1 revealed the presence
54 of a chromosome-encoded *bla*_{OXA-535} gene, likely the progenitor of the plasmid-encoded
55 *bla*_{OXA-436} gene, a novel *bla*_{OXA-48}-like gene. Expression of *bla*_{OXA-535} in *E. coli* showed the
56 carbapenem-hydrolyzing activity of OXA-535. The production of OXA-535 in *Shewanella*
57 *sp.* JAB-1 could be evidenced using molecular and immuno-enzymatic tests, but not with
58 biochemical tests that monitor carbapenem-hydrolysis. In this study, we have identified a
59 CTX-M-15-producing *Shewanella* species that was responsible of an hepatobiliary infection

60 and that is likely the progenitor of OXA-436, a novel plasmid-encoded OXA-48-like class D
61 carbapenemases.

62

INTRODUCTION

63 *Shewanella spp.* are non-fermentative Gram-negative bacilli that are widely
64 distributed throughout the world. They are found mainly in sea water in areas with warm
65 climates (1, 2). Although *Shewanella spp.* are an unusual cause of infections in humans, the
66 number of cases reported is increasing (1, 3–5). In most cases, this organism is cultured from
67 samples of immunocompromised patients suffering from soft tissue infections after sea water
68 exposure (3, 6). More recently, several cases of *Shewanella* infections have been reported in
69 patients suffering from hepatobiliary diseases (3–5, 7). Human infections are most often
70 caused by *Shewanella algae* and *Shewanella putrefaciens* (3). However, it is often difficult to
71 identify the bacteria at the species level using conventional culture-based and biochemical
72 tests. 16S rRNA gene sequencing improved identification of *Shewanella* species as
73 pathogenic organisms (3, 5).

74 Most *Shewanella spp.* are susceptible to piperacillin/tazobactam, ceftazidime,
75 cefepime, imipenem, meropenem, gentamicin, tobramycin, amikacin and ciprofloxacin, but
76 susceptibility to penicillins is more variable (3, 7). Several naturally-occurring β -lactamases
77 have been identified in *Shewanella spp.*, especially class D β -lactamases (8–11) also known
78 as oxacillinases. For instance, *Shewanella oneidensis* strain MR-1 was found to naturally-
79 harbor the *bla*_{OXA-54} gene, which is related to *bla*_{OXA-48} gene, a plasmid-mediated carbapenem-
80 hydrolyzing class D β -lactamase (CHDL) involved in carbapenem resistance in
81 Enterobacteriaceae (10). Moreover, OXA-55 was identified in *S. algae* and possesses
82 biochemical properties similar to those of OXA-54, in particular the ability to hydrolyze
83 carbapenems (11). *Shewanella xiamenensis* has been identified as the source of *bla*_{OXA-181}
84 gene encoding another OXA-48-like variant with carbapenemase activity that is highly
85 prevalent on the Indian subcontinent and increasingly reported in France (9, 12). Recently,
86 next-generation sequencing enabled to identify several *bla*_{OXA-48-like} genes, such as *bla*_{OXA-199},

87 *bla*_{OXA-252}, *bla*_{OXA-514} and *bla*_{OXA-515} in different *Shewanella* spp. isolates, in food-producing
88 animals and water samples (2). Furthermore, analysis of publicly available *Shewanella* spp.
89 genomes revealed the presence of several uncharacterized *bla*_{OXA-48} like genes, confirming the
90 importance of *Shewanella* spp. as OXA-48-like class D β -lactamase reservoir (13, 14).

91 Extended-spectrum β -lactamases (ESBLs) are being increasingly reported worldwide
92 in Enterobacteriaceae (15) but have never been described in *Shewanella* spp yet. ESBLs
93 belong to the Ambler class A β -lactamases (16). ESBLs are usually described as acquired β -
94 lactamases that are encoded mostly by plasmid-located genes. There are three major types of
95 ESBLs: TEM, SHV and CTX-M (16). CTX-Ms are now the most prevalent ESBLs
96 worldwide (17). CTX-M-producing Enterobacteriaceae are not only responsible for
97 nosocomial infections and hospital outbreaks, but they are now also considered to be true
98 community pathogens (15).

99 In this study, we have characterized three CTX-M15-producing bacteria, including a
100 *Shewanella* sp. isolate, isolated from a 10-year-old child suffering from cholangitis. Using
101 antimicrobial susceptibility testing and whole genome sequencing, we have characterized this
102 *Shewanella* strain, investigated its resistome and characterized the plasmids carrying the
103 CTX-M15-determinant. Furthermore, we have cloned and expressed the naturally-encoded
104 OXA-48-like gene (*bla*_{OXA-535}) and demonstrated its carbapenemase activity.

105

106

RESULTS

107 **Case report.** A 10-year-old child was directly transferred from a hospital in Niger to
108 the Bicêtre hospital pediatric department, Ile-de-France, France, for biliary surgery. During
109 her previous hospitalization, she underwent a cholecystectomy that was complicated with a
110 leak from the common bile duct forming an abdominal effusion. A drain was then introduced
111 to constitute an external bypass. Upon admission at the Bicêtre hospital, she had fever with

112 abdominal pain and cholangitis was diagnosed. The liquid collected from the drain was
113 addressed to the microbiology laboratory and an antibiotic treatment with piperacillin and
114 tazobactam was started. The culture revealed a polymicrobial infection due to numerous
115 Gram-negative bacteria (*Pseudomonas putida*, *Citrobacter freundii*, *K. pneumoniae*, *E. coli*,
116 *Stenotrophomas maltophilia* and a *Shewanella* sp.) and to *Enterococcus avium*. Antimicrobial
117 susceptibility testing revealed the presence of extended-spectrum β -lactamases (ESBL) in
118 *Shewanella* sp., *E. coli* and *K. pneumoniae* isolates. One week after the admission, the patient
119 underwent a laparotomy to perform a Roux-en-Y biliary bypass procedure and to introduce an
120 internal-external biliary drain to protect the anastomosis. Five days after the surgery, the
121 patient became feverish. A blood and a bile sample collected from the drain revealed the
122 presence of an ESBL-producing *K. pneumoniae* isolate. Antibiotic therapy was switched to
123 imipenem for 15 days. The biliary drain was withdrawn one month after the surgery and no
124 other complication occurred.

125 **Identification of *Shewanella* sp. JAB-1.** After 24h of growth on blood-agar plate, the
126 *Shewanella* sp. JAB-1 colonies were small, smooth, oxidase and catalase positive. Api32GN
127 was unable to identify *Shewanella* sp. even at the genus level. MALDI-TOF MS was used for
128 species identification yielding *S. putrefaciens* with a best score value of 2.0, *S. profunda*
129 (score 1.7) and *S. baltica* (score 1.5). Since MALDI-TOF discrepancies for *Shewanella*'s
130 identification are known, sequencing of 16S rRNA gene was performed. The 1,406 bp 16S
131 rRNA gene sequence was 98% identical to *Shewanella seohaensis* T GU944672 making this
132 strain the closest relative based on patristic distances (18). As the 16S rRNA gene sequence
133 may not be discriminatory enough to differentiate closely related *Shewanella* species (9), we
134 used WGS data to dive deeper into the identification of this isolate. Genomes of
135 representative *Shewanella* species were collected and *rpoB/gyrB* concatemer sequences were
136 extracted for phylogenetic analysis (Fig. 1A). It revealed that *Shewanella* sp. JAB-1 did not

137 belong to any known *Shewanella* species. Phylogeny based on whole genome revealed that
138 *Shewanella* sp. JAB-1 was closely related to four other isolates, namely MR-4, MR-7, ANA-3
139 and Shew256 with Average Nucleotide Identity (ANI) of 94,6%, 94,35%, 94,32% and
140 93,90% respectively (Fig. 1B).

141

142 **Genomic characteristics of *Shewanella* sp. JAB-1.** To reconstruct the genome of the
143 *Shewanella* sp. JAB-1, a long read PacBio-based sequencing was performed. The genome
144 was of 4,800,597 bp with an average GC% of 48%. It was composed of 4161 CDS, 9 copies
145 of rRNA operons and 102 tRNAs genes (Figure 2). PacBio assembly revealed the presence of
146 two plasmids of 10.5 and 193-kb in size (Figure 2).

147 A function-based comparison using RAST server revealed unique features in
148 *Shewanella* sp. JAB-1, with 90 and 100 unique CDS with known functions compared to MR-
149 4 and MR-7 respectively. These CDS include widely distributed functions notably involved in
150 amino acid and carbohydrate metabolism, gene regulation and resistance to antibiotic
151 compounds (Figure 2).

152 **Susceptibility testing and detection of resistance genes.** *E. coli*, *K. pneumoniae* and
153 *Shewanella* sp. JAB-1 shared the same phenotype regarding susceptibility to β -lactams. They
154 were resistant to all penicillins tested and association with clavulanic acid did not fully restore
155 penicillins activity. They were resistant to expanded-spectrum cephalosporins but susceptible
156 to cefoxitin and to piperacillin-tazobactam combination. Finally, double-disk synergy tests
157 revealed ESBL activity in the three strains.

158 In agreement with this phenotype, we identified in the genome of *Shewanella* sp. four
159 β -lactamases genes: two ESBL genes, (*bla*_{CTX-M-15} and *bla*_{SHV-2a}), the narrow spectrum
160 oxacillinase *bla*_{OXA-1}, and a novel *bla*_{OXA-535} gene. It codes for an oxacillinase 91.3% identical
161 to the carbapenem-hydrolyzing class D (CHDL) β -lactamase OXA-48, 98% to OXA-436 and

162 OXA-48-like of *Shewanella* sp. ANA-3 (3 amino-acids of difference) and 99% to OXA-48-
163 like in *Shewanella* sp. MR-4, MR-7 (one and 2 amino-acids of difference, respectively).
164 OXA-436, is a plasmid encoded OXA-48-variant detected in several species responsible of an
165 outbreak in Denmark (unpublished, genbank bioproject PRJNA297498). This outbreak
166 involved OXA-436-producing *Citrobacter freundii*, *K. pneumoniae* and *Enterobacter*
167 *asburiae*.

168 *Shewanella* sp. JAB-1 is resistant to aminoglycosides (Gentamicin, Tobramycin and
169 Netilmicin) due to the production of two acetyltransferases (*aac(6')*-Ib-cr and *aac(3)*-IIa) and
170 two phosphotransferases (*aph(3'')*-Ib and *aph(6)*-Id). The resistance to ciprofloxacin is likely
171 due to the acquisition of the *aac(6')*-Ib-cr, known to confer reduced susceptibility to this
172 fluoroquinolone by *N*-acetylation of its piperazinyl amine (19). Amikacin and cotrimoxazole
173 are two antibiotics that remained active *in vitro*.

174 **Plasmid characterization and genetic context of *bla*_{CTX-M-15} genes in the three**
175 **ESBL-producing isolates.** Plasmid extraction of *Shewanella* sp. JAB-1 and subsequent
176 electroporation and mating-out assay into *E. coli* TOP10 conferred resistance to penicillin and
177 expanded-spectrum cephalosporins with double-disk synergy images consistent with an ESBL
178 phenotype. Amplification of the *bla*_{CTX-M-15} gene on the *E. coli* transformants and
179 transconjugants as well as co-resistance to aminoglycosides indicated that both of *bla*_{CTX-M-15}
180 gene and *aac(6')*-Ib-cr were located on the largest plasmid, namely pSHE-CTX-M of ca. 193
181 kb. pSHE-CTX-M was fully sequenced using PacBio technologies. The overall structure of
182 the plasmid indicated that it belonged to IncA/C incompatibility group (Fig. 3). The plasmid
183 backbone shares features of IncA/C-type plasmid i.e. partitioning, replication and conjugation
184 apparatus. In the variable region, several antibiotic resistance genes have been identified in
185 addition to *bla*_{CTX-M-15}, *bla*_{SHV-2a} and *bla*_{OXA-1} β -lactamase genes, the four aminoglycoside-
186 modifying enzymes (*aph(3'')*-Ib, *aph(6)*-Id, *aac(6')*-Ib-cr and *aac(3)*-IIa), the tetracycline

187 resistance gene (*tetA*), the sulfonamide resistance gene (*sul2*) and the chloramphenicol
188 resistance gene (*catB3*).

189 *E. coli* JAB-1 carried three plasmids as observed following Kieser extraction (data not
190 shown). Electroporation in *E. coli* TOP10 and selection on Ticarcillin conferred an ESBL
191 phenotype as well as resistance to cotrimoxazole. The transformant carried a plasmid of 68
192 kb, namely pECO-CTX-M that was fully sequenced. Several antibiotic resistance genes were
193 found on this IncY plasmid (Fig. 3). The ESBL encoding *bla*_{CTX-M-15} gene and the narrow-
194 spectrum β -lactamase encoding *bla*_{TEM-1} gene, *dfrA14*, encoding a trimethoprim-insensitive
195 dihydrofolate reductase variant, *sul2* encoding a dihydropteroate synthase that is not inhibited
196 by sulfonamide, *tetA* (tetracyclin efflux protein) and aminoglycosides resistance genes
197 (*aph(6)*-Id and *aph(3'')*-Ib) were present on the plasmid. Finally, the plasmid-encoded
198 fluoroquinolone resistance gene *qnrS1* was responsible for a reduced susceptibility to
199 ciprofloxacin.

200 Kieser extraction showed that *K. pneumoniae* JAB-1 carries three plasmids (data not
201 shown). Electroporation into *E. coli* TOP10 and selection on Ticarcillin conferred an ESBL
202 phenotype and the transformant carried a plasmid of 60 kb, called pKP-CTX-M that belonged
203 to IncR incompatibility group and was fully sequenced (Fig. 3). We identified in this plasmid
204 five resistance gene encoding three β -lactamases (*bla*_{CTX-M-15}, *bla*_{TEM-1b} and *bla*_{OXA-1}), an
205 aminoglycoside acetyl-transferase (*aac(6')*-Ib-cr) and the chloramphenicol resistance gene
206 *catB3*.

207 Comparison of the three *bla*_{CTX-M-15} gene harboring plasmids revealed major
208 differences in size, backbone and incompatibility group, thus ruling out *in vivo* plasmid
209 exchanges between these three species (Fig. 3). There was no plasmid deposited in the NCBI
210 database that shared the same structure as pSHE-CTX-M and pKP-CTX-M, but we found one
211 plasmid called tig00003056 (CP021681.1) very close to pECO-CTX-M (A Jousset, pers

212 comm). To gain further insights into the acquisition of *bla*_{CTX-M-15} gene by these plasmids, we
213 analyzed the genetic environment of the ESBL gene (Figure 4). It revealed that *bla*_{CTX-M-15}
214 gene was part of an *ISEcp1*-borne transposon as previously described (20). In pKP-CTX-M
215 and pECO-CTX-M, the *ISEcp1*-borne transposon was inserted in the transposase *tnpA* gene
216 of Tn2 generating the same target site duplication (TSD: TCACA; Figure 4A and B). This is
217 in favor of the acquisition of *bla*_{CTX-M-15} gene by homologous recombination of this region
218 rather than by a transposition event. This structure was also identified in other *bla*_{CTX-M-15}-
219 gene harboring plasmids such as the archetypal IncFII-type plasmid pC15-1A circulating in
220 Canada in the early 2000's (21). In pSHE-CTX-M, *ISEcp1-bla*_{CTX-M-15} is also inserted within
221 Tn2 transposase gene but this insertion differs by two different features; (i) the DNA fragment
222 upstream of Tn2 transposase is missing and (ii) an insertion sequence (IS) belonging to the
223 IS256 family with a TSD of 8 bp was identified (CATTTTGG) within *ISEcp1* transposase
224 gene. Our data suggest a common ancestor for these structures but they have evolved
225 differently and are carried on different plasmids. Despite the insertion of an IS256-like in
226 *ISEcp1* in pSHE-CTX-M, the expression of CTX-M-15 did not seem to be impacted based on
227 the MICs measured for third-generation cephalosporins (Table 1). Accordingly, the promoter
228 sequences provided by *ISEcp1* (-35 TTGAAA and -10 TACAAT) remained intact.

229 **Characterization of OXA-535.** To determine whether OXA-535 can hydrolyze
230 carbapenems, the *bla*_{OXA-535} gene was cloned into pTOPO together with its surrounding
231 regions. *E. coli* TOP10 expressing the recombinant plasmid (pTOPO-OXA-535) displayed a
232 class D carbapenemase phenotype with resistance to temocillin, to penicillins that was not
233 restored by clavulanic acid addition and susceptibility to third generation cephalosporins
234 (Table 1). In addition, *E. coli* TOP10 pTOPO-OXA-535 had reduced susceptibility to
235 carbapenems compared to *E. coli* TOP10. The Carba NP test, performed on the recombinant
236 strain was positive, suggesting that the OXA-535 enzyme hydrolyzes carbapenems.

237 Furthermore, specific activity (SA) measured with a culture extract of *E. coli* TOP10-pTOPO-
238 preOXA-535 showed that this strain hydrolyzes imipenem significantly (SA=270 mU.mg⁻¹ of
239 protein) similar to that of *E. coli* TOP10-pTOPO-OXA-48 used as control (SA=130 mU.mg⁻¹
240 of protein). The MICs for β -lactams for OXA-535 and OXA-48 were similar, confirming that
241 these enzymes possessed similar resistance patterns (Table 1).

242 Interestingly, when performed directly on the *Shewanella* sp. JAB-1, the Carba NP test
243 and the β -CARBA testTM were negative. Since tests based on carbapenem hydrolysis can lack
244 of sensitivity to detect isolates expressing β -lactamase with low carbapenemase activity such
245 as OXA-48-like enzymes, an immunochromatographic test known to detect OXA-48-like
246 enzymes with high sensitivity was performed (22). Indeed, the OXA-48 K-Set gave a positive
247 result on *Shewanella*'s colonies. Therefore, it seems that the OXA-535 enzyme is expressed at
248 a low level but sufficiently to be detected by the immunochromatographic test. At last, a
249 molecular test routinely used in clinical microbiology lab, the Xpert Carba-R test, was
250 performed directly on the *Shewanella* sp. JAB-1 colonies and yielded a positive PCR for
251 OXA-48-like carbapenemase.

252

253 **DISCUSSION**

254 We report the case of a biliary infection that occurred in a 7-year-old child with a
255 novel *Shewanella* sp. belonging to an uncharacterized species. MALDI-TOF MS identified
256 this isolate at the genus level, whereas WGS provided high resolution to identify this isolate
257 as closely related to *Shewanella* sp. MR-4, MR-7, ANA-3 and Shew256 (Figure 2).
258 Phylogenetic analyses revealed that these strains belong to a unique lineage that likely
259 constitutes a novel species.

260 *Shewanella* spp. are increasingly reported as pathogens, especially in patients with
261 underlying hepatobiliary diseases. In a case series performed by Chen *et al.*, all patients with

262 bacteremia had underlying hepatobiliary disorders (23). Here, bacterial culture from the bile
263 of the patient identified in addition to the *Shewanella* sp. JAB-1, six other clinically-relevant
264 bacteria. In a case series performed by To *et al.* bile samples whose culture were positive for
265 *Shewanella* were also polymicrobial, along with other enteric bacteria (7). Since *Shewanella*
266 spp. are often isolated with other pathogens in clinical specimens, their clinical significance
267 might be difficult to assess (4).

268 Next Generation Sequencing enabled us to study the complete resistome of the
269 *Shewanella* sp. JAB-1 as well as the features of the three ESBL-carrying plasmids (pSHE-
270 CTX-M, pECO-CTX-M and pKP-CTX-M). Even if the three strains shared the same clinical
271 environment, they did not share their plasmid since they belong to different incompatibility
272 groups. Plasmid continuous sequences allowed us to determine the genetic environment of
273 *bla*_{CTX-M-15}. Indeed, distinct episodes of homologous recombination and of transposition seem
274 to have occurred rather than direct plasmid conjugation between strains in the patient.

275 The *bla*_{CTX-M-15} and *bla*_{SHV-2a} genes are two ESBLs present on an IncA/C plasmid of
276 193 kb in *Shewanella* sp. JAB-1. ESBL production has to date never been reported in any
277 *Shewanella* spp. IncA/C plasmids are known to efficiently spread many resistance genes, in
278 particular *bla*_{CMY-like} Ambler class C β -lactamases genes (24). They have been detected
279 worldwide, and carbapenemase genes such as *bla*_{NDM-1} and *bla*_{VIM-4} have been identified on
280 that plasmid scaffold (25, 26). Recently, characterization of ESBLs plasmids in clinical
281 isolates of *K. pneumoniae* in Taiwan revealed that the most common replicon type was
282 incompatibility group IncA/C (27). Co-expression of *bla*_{CTX-M} and *bla*_{SHV} is not rare on this
283 scaffold (27). Therefore, IncA/C plasmids seem to efficiently spread ESBL genes among
284 Enterobacteriaceae and non-Enterobacteriaceae.

285 By sequencing *Shewanella* sp. JAB-1 genome, we identified a new oxacillinase
286 variant called OXA-535. According to the enzymatic activities OXA-535 can be considered

287 as a new carbapenem-hydrolyzing β -lactamase. OXA-535 presented only 3 amino-acids of
288 difference with the plasmidic carbapenemase OXA-436. Moreover, we could identify a 7.3 kb
289 fragment in the chromosome of *Shewanella* sp. JAB-1 including the *bla*_{OXA-535} gene that
290 presented 96% of nucleotide identity with the plasmid carrying *bla*_{OXA-436} gene region.
291 Considering the high similarities between these structures, we can speculate that this 7.3 kb
292 fragment has been mobilized on a plasmid and that *bla*_{OXA-535} gene is likely the precursor of
293 the plasmid-encoded *bla*_{OXA-436} gene.

294 Interestingly, antimicrobial susceptibility testing, the Carba NP test, the β -CARBA testTM and
295 OXA-48 K-Set indicated that *bla*_{OXA-535} gene is barely expressed in *Shewanella* sp. JAB-1.
296 Accordingly, we did not identify any IS upstream the *bla*_{OXA-535} gene that could provide
297 strong promoter sequences, unlike to the plasmid encoded OXA-181 and OXA-48 where
298 *ISEcp1* and *IS1999*, respectively allow strong carbapenemase expression. Like in other
299 sequenced *Shewanella* spp., we identify a gene coding for a peptidase C15-family upstream
300 *bla*_{OXA-535} and a *lysR* gene coding for a putative LysR transcriptional regulator downstream
301 (13).

302 The Xpert Carba-R test performed on the colonies could amplify the *bla*_{OXA-535} gene
303 (data not shown). We can speculate that if this test would have been performed directly on a
304 rectal swab of this patient, as recommended for screening of high risk patients (30), it might
305 have been falsely positive for the detection of a carbapenemase-producing Enterobacteriaceae.
306 A false positive molecular screening test has already been reported by Antonelli *et al.* , likely
307 due to the presence in the human gut of *S. xiamenensis* carrying *bla*_{OXA-416} in its chromosome
308 (31). The rate of fecal carriage of *Shewanella* spp. in the general population is currently
309 unknown, as well as the bacterial concentration sufficient to yield a positive molecular test
310 result. Nevertheless, the analytical limit of detection of the Xpert Carba-R assay is quite low
311 (ranging from 1.1×10^2 to 1.2×10^3 CFU/swab depending on the carbapenemase gene (32)).

312 Given these data, false positive molecular test results remain troublesome since hepatobiliary
313 diseases have been proposed as a risk factor for infections (or colonization) due to *Shewanella*
314 spp. and can therefore contribute to regularly inoculate the gastrointestinal tract.

315

316 **MATERIAL AND METHODS**

317 **Bacterial strains.** *Shewanella* sp. JAB-1, *Escherichia coli* JAB-1 and *Klebsiella*
318 *pneumoniae* JAB-1 clinical isolates were from the Bicêtre hospital, Le Kremlin-Bicêtre,
319 France. They have been identified with MALDI-TOF MS using Bruker MS System (Bruker
320 Daltonics, Bremen, Germany), according to the manufacturer's instructions. In addition,
321 sequencing of 16S rRNA using universal primers 16S 8-27 and 16S 1512-1491 was
322 performed for the *Shewanella* isolate, as described previously (9). Biochemical features were
323 studied using API32GN and API20E (Bio-Mérieux, Marcy l'Etoile, France) according to the
324 manufacturer's instructions. Electrocompetent *E. coli* TOP10 (Invitrogen, Eragny, France)
325 was used as a recipient for electroporation experiments. *E. coli* J53 RIF^R, which is resistant to
326 azide and rifampin, was used for conjugation experiments. *E. coli* 50192 was used as a
327 reference strain for plasmid extraction (33). *S. algae* CIP 103562, *S. putrefaciens* CIP 67.65
328 and *S. xiamenensis* S12 (9) were used as reference for susceptibility testing and determination
329 of biochemical features.

330 **Susceptibility testing and MIC determinations.** Susceptibility testing was
331 performed by the disc diffusion method on Mueller–Hinton agar plates (Bio-Rad) incubated
332 for 18 h at 37°C. There are no recognized Clinical and Laboratory Standards Institute (CLSI)
333 MIC interpretative standards specified for *Shewanella* genus. We used the MIC breakpoints
334 of CLSI approved standard M100-S24 categories for other non-Enterobacteriaceae in
335 accordance with previous reports (3). MICs of carbapenems were determined using the E-test
336 (bioMérieux, La Balmes les Grottes, France).

337 **Resistance genes detection.** Genes coding for Ambler class A β -lactamases were
338 sought by PCR using primers specific for the *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes, as
339 previously described (34). PCR products were purified using Qiaquick PCR purification kit
340 (Qiagen, Courtaboeuf, France) and sequenced on both strands with an automated sequencer
341 (ABI 3100; Applied Biosystems, Foster City, Calif.).

342 **Cloning experiments.** Genomic DNA of *Shewanella* sp. JAB-1 isolate extracted
343 using the Qiagen DNAamp kit (Qiagen) was used as template for amplification of the *bla*_{OXA-}
344 ₅₃₅ gene with its surrounding region (290 nt before the ATG and 181 nt after the stop codon)
345 was performed with pre-OXA-535-For (5'- CGTTTGGGTTTGCTTCAT-3') and pre-OXA-
346 535-Rev (5'- GACTAGGCTTTTTGCGTT-3') and the PCR product was inserted into
347 pTOPO (Invitrogen, Eragny, France) resulting in pTOPO-preOXA-535. *E. coli* TOP10
348 carrying recombinant plasmids pTOPO-preOXA-535 was selected using Trypticase soy (TS)
349 agar plate containing Ticarcillin (50 mg/L) and Kanamycin (50 mg/L). The inserted DNA
350 fragment was verified by Sanger sequencing.

351 **Plasmid extraction, electroporation and mating-out assay.** Natural plasmids were
352 extracted using the Kieser extraction method (35) and subsequently analyzed by
353 electrophoresis on a 0.7% agarose gel. ESBL-carrying plasmids DNA of *Shewanella* sp. JAB-
354 1 (pSHE-CTX-M), *E. coli* (pEco-CTX-M) and *K. pneumoniae* (pKp-CTX-M) isolates were
355 extracted using the QIAGEN Plasmid Maxi Kit (QIAGEN, Courtaboeuf, France) and
356 analyzed by agarose gel electrophoresis (Invitrogen, Paris, France).

357 Transfer of the β -lactam resistance markers from *Shewanella* sp. JAB-1 was performed by
358 liquid mating-out assays at 37°C using *E. coli* J53 RIF^R as the recipient strain (33).

359 Recombinant plasmids (pTOPO-preOXA-48 and pTOPO-preOXA-535) were introduced by
360 electroporation into *E. coli* TOP10 using a Gene Pulser II (Bio-Rad Laboratories) (33).

361 **Detection of carbapenemase presence.** Bacterial colonies of *Shewanella* sp. JAB-1

362 recovered from trypticase soy agar were used to perform Xpert Carba-R test version 2
363 (Cepheid, Sunnyvale, CA, USA), OXA-48 K-SeT assay (Coris BioConcept, Gembloux,
364 Belgium), the Carba NP test and β -CARBA testTM, according to the manufacturer's
365 recommendations (22, 36, 37) or updated guidelines (38). The Carba NP test was also
366 performed on recombinant *E. coli* TOP10 carrying pTOPO-preOXA-535.

367 **Enzymatic activities.** The specific activities of the OXA-48 and OXA-535 β -
368 lactamases were determined using the supernatant of a whole-cell crude extract obtained from
369 an overnight culture of *E. coli* clones expressing those two β -lactamases (pTOPO-preOXA-48
370 and pTOPO-preOXA-535 in *E. coli* TOP10) with an Ultrospec 2000 UV spectrophotometer
371 (Amersham Pharmacia Biotech), as previously described (39). Imipenem was used as a
372 substrate at a concentration of 100 μ M.

373 **Whole Genome Sequencing & Bioinformatic analysis.** Total DNA of *Shewanella*
374 sp. JAB-1 was extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO
375 Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. Total DNA and
376 plasmid DNAs (pSHE-CTX-M, pECO-CTX-M and pKP-CTX-M) concentrations were
377 measured using Qubit dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA, USA). The
378 DNA libraries were prepared using the NexteraXT v3 kit (Illumina, San Diego, CA, USA),
379 according to the manufacturer's instructions, and then run on the HiSeq or MiSeq systems
380 (Illumina) for generating paired-end 150-bp reads. *Shewanella*'s total DNA was also
381 sequenced using PacBio long read technology (Macrogen, Seoul, South Korea).

382 Illumina reads *de novo* assembly was performed using CLC genomic workbench 9.0
383 according the manufacturer's recommandations (Qiagen, Courtaboeuf, France). PacBio
384 subreads were assembled with both Canu (40) and the RS_HGAP_Assembly.3 protocol from
385 the SMRT analysis toolkit v2.3, while consensus accuracy was further polished using Quiver
386 (41) as was previously described (42). PCR sequencing was used to complete the assembly of

387 pEco-CTX-M and pKp-CTX-M plasmids.
388 Phylogenetic tree based on *gyrB/rpoB* concatemer was drawn with MEGA7 software using
389 Maximum Likelihood method based on the Tamura-Nei model (43). Phylogenetic analysis
390 based on whole genome sequences of *Shewanella* sp. close to *Shewanella* sp. JAB-1 was done
391 using the Parsnp program from the Harvest Suite (44). tRNAs were detected by ARAGORN
392 (<http://mbio-serv2.mbioekol.lu.se/ARAGORN/>) (45). Circular representation was performed
393 by CGview (46). *Shewanella* sp. JAB-1 was compared with three closely related strains
394 *Shewanella* MR-4 (CP000446.1), MR-7 (CP000444.1) and ANA-3 (CP000469.1).

395 **Nucleotide sequence accession numbers.** The nucleotide sequence of the *bla_{OXA-535}*
396 gene of the assembled chromosome sequence of *Shewanella* sp. JAB-1 and of its 10.5 kb
397 plasmid with no antibiotic resistance feature have been deposited at the EMBL/GenBank
398 nucleotide sequence database under the accession numbers KX828709, CP022358 and
399 CP022360 respectively. The three assembled ESBL-carrying plasmids (pSHE-CTX-M,
400 pECO-CTX-M and pKP-CTX-M) have been deposited at the EMBL/GenBank nucleotide
401 sequence database under the accession numbers CP022359, MF510423, MF510424,
402 respectively.

403

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406 *S. algae* CIP 103562, *S. putrefaciens* CIP 67.65.

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413 **CONFLICT OF INTERESTS**

414 LD is co-inventor of the Carba NP Test, which patent has been licenced to bioMérieux (La
415 Balmes les Grottes, France).

416

417

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556

557

558 Table 1. MIC values of *Shewanella* spp.. and transformants

559

Antibiotic	MIC (µg/mL)						
	<i>S. putrefaciens</i> (OXA-181 chr*)	<i>S. xiamenensis</i> <i>CIP 67.65</i>	<i>S. algae</i> <i>CIP 103562</i>	<i>Shewanella</i> sp. JAB-1 (OXA-535 chr*)	<i>E. coli</i> TOP10 (pTOPO-OXA-535)	<i>E. coli</i> TOP10 (pTOPO OXA-48)	<i>E. coli</i> TOP10
Temocillin	0.38	0.75	0.19	0.25	>1024	>1024	4
Cefotaxime	0.094	0.064	0.032	>32	0.064	0.75	0.06
Ceftazidime	0.125	0.125	0.064	1.5	0.19	0.19	0.12
Imipenem	4	0.75	0.5	0.38	1	0.75	0.25
Ertapenem	0.064	1	0.023	1	0.25	0.25	0.003
Meropenem	0.125	0.5	0.023	0.38	0.19	0.25	0.016

560

561

562

*chr : indicated the presence of a natural oxacillinase in the chromosome

563 **LEGENDS TO FIGURES**

564

565 **Figure 1. Phylogenetic analysis of representative *Shewanella* spp.** (A) Unrooted
566 phylogenetic tree based on *gyrB* and *rpoB* genes. The tree was constructed by Maximum-
567 Likelihood method with Tamura-Nei model using MEGA program (MEGA 7.0). The tree is
568 drawn to scale with branch lengths representing the evolutionary distances. (B) A focus on
569 *Shewanella* sp. close to *Shewanella* sp. JAB-1 was made and the phylogenetic tree was based
570 on whole genome sequences. It was constructed by Maximum-Likelihood method with Jukes-
571 Cantor model, using Parsnp software. The tree is drawn to scale based on the Single
572 Nucleotide Polymorphisms (SNPs) of the alignment.

573

574 **Figure 2. Genome representation of *Shewanella* sp. JAB-1 and its plasmids.**

575 The representation was performed by CGview server. The outermost two rings show features
576 extracted from the *Shewanella* sp. JAB-1 genome. The next three rings show the positions of
577 BLAST hits detected through blastn comparisons of *Shewanella* sp. JAB-1 genome against
578 the three closest genomes of *Shewanella* (MR-4, MR-7 and ANA-3, represented by red, green
579 and blue circles respectively). Darker arc indicates high percent of identity of the hit. The
580 black circle display GC content and inner circles display GC skew.

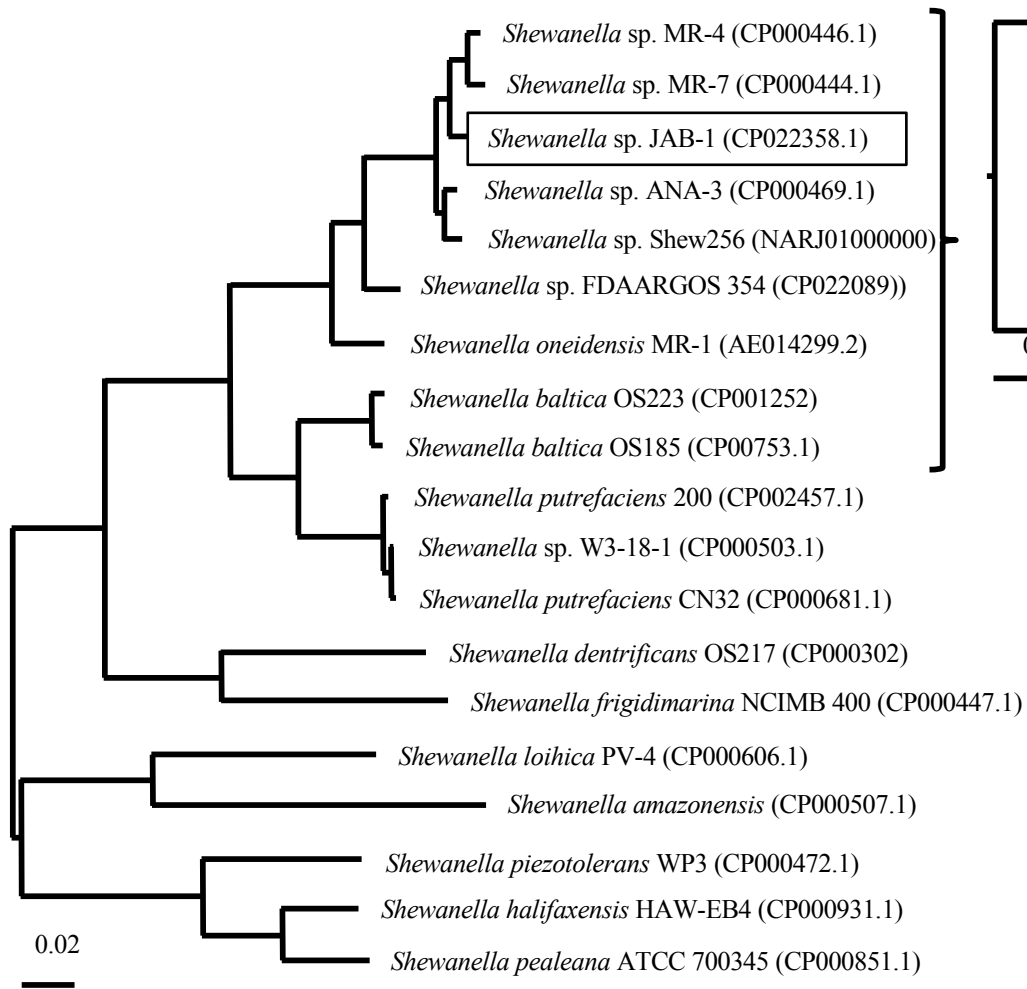
581

582 **Figure 3: Schematic representation of the three ESBL carrying plasmids.** Main features
583 are represented. Antimicrobial resistance associated genes are indicated by orange boxes.
584 Transposon related genes and insertion sequences are indicated by red boxes. Other genes are
585 indicated by boxes colored in violet (replicase genes) and in gray (restriction genes). *tra* locus
586 and heavy-metal resistance loci are indicated within blue boxes. Δ indicates a partial protein.
587 Tnp: transposase.

588

589 **Figure 4. Genetic environment of the *bla*_{CTX-M-15} gene of *K. pneumoniae* JAB-1 (A), *E.***
590 ***coli* JAB-1 (B), and of *Shewanella* sp. JAB-1 (C).** Antimicrobial resistance genes are
591 indicated with dark grey arrows. Open reading frames within insertion sequences (IS) are
592 represented with striped arrows. Target Site Duplications are indicated with small dotted
593 triangle. Inverted repeats IR_R and IR_L are represented by white and light grey arrows
594 respectively. Oblique lines indicate truncated genes.

A.



B.

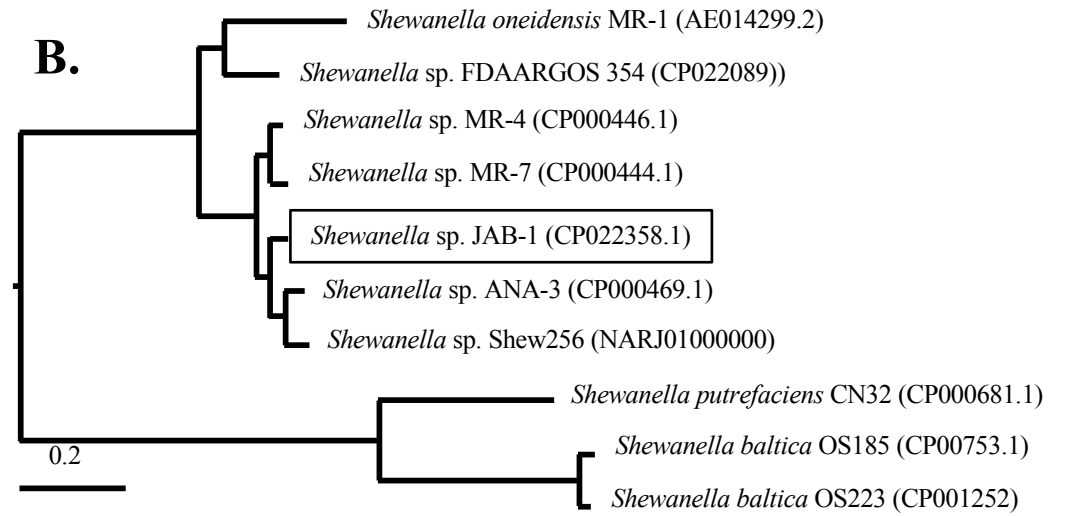


Fig.1

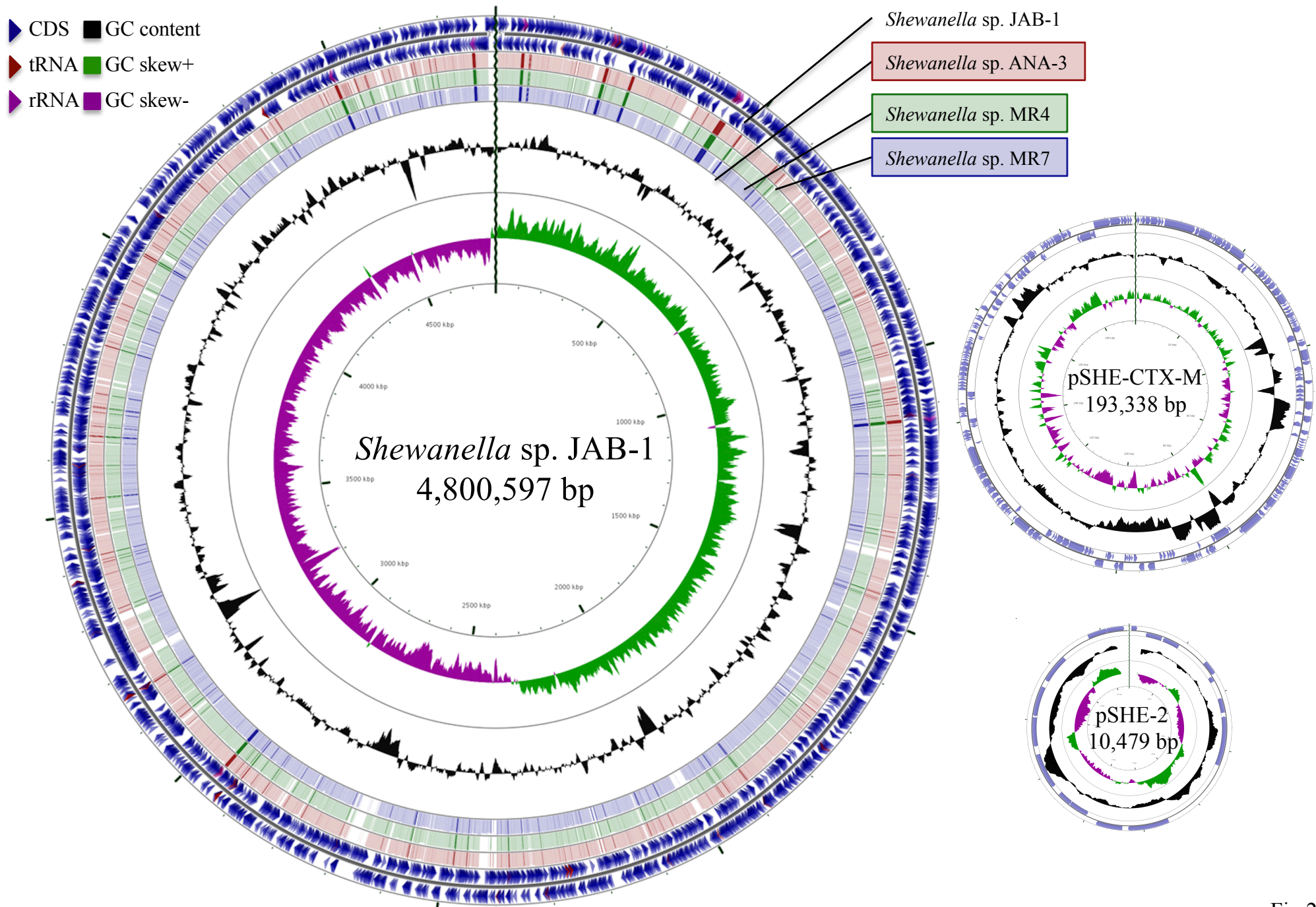


Fig.2

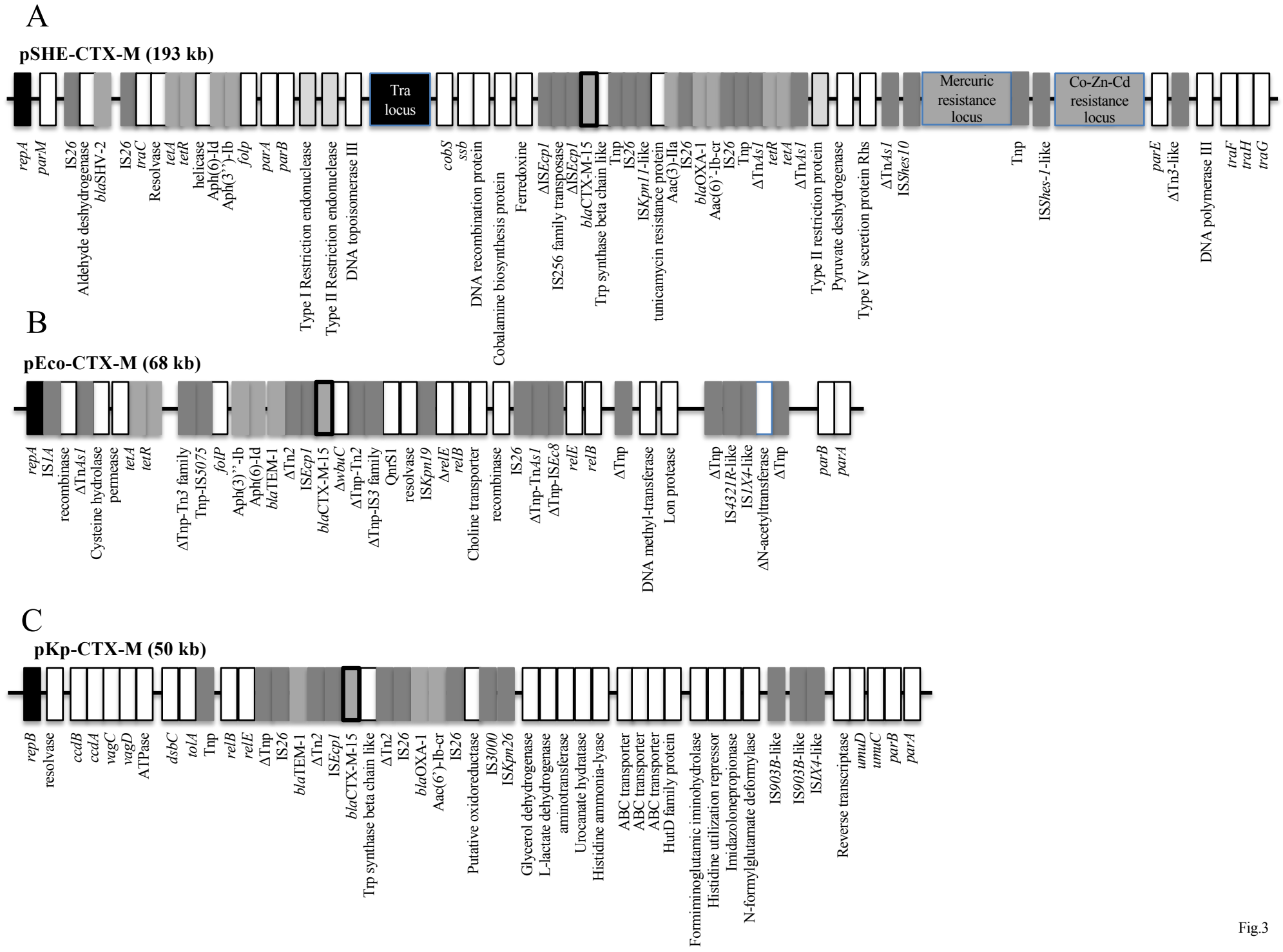


Fig.3

