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MECHANISMS OF RESISTANCE

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

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ABSTRACT *Shewanella* spp. constitute a reservoir of antibiotic resistance determinants. In a bile sample, we identified three extended-spectrum-β-lactamase (ESBL)-producing bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, and *Shewanella* sp. strain JAB-1) isolated from a child suffering from cholangitis. Our objectives were to characterize the genome and the resistome of the first ESBL-producing isolate of the genus *Shewanella* and determine whether plasmidic exchange occurred between the three bacterial species. Bacterial isolates were characterized using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), standard biochemical tools, and antimicrobial susceptibility testing. *Shewanella* sp. JAB-1 and ESBL gene-encoding plasmids were characterized using PacBio and Illumina whole-genome sequencing, respectively. The *Shewanella* sp. JAB-1 chromosome-encoded OXA-48 variant was cloned and functionally characterized. Whole-genome sequencing (WGS) of the *Shewanella* sp. clinical isolate JAB-1 revealed the presence of a 193-kb plasmid belonging to the IncA/C incompatibility group and harboring two ESBL genes, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}2a}$. $bla_{CTX\text{-}M\text{-}15}$ gene-carrying plasmids belonging to the IncY and IncR incompatibility groups were also found in the *E. coli* and *K. pneumoniae* isolates from the same patient, respectively. A comparison of the $bla_{CTX\text{-}M\text{-}15}$ genetic environment indicated the independent origin of these plasmids and dismissed *in vivo* transfers. Furthermore, characterization of the resistome of *Shewanella* sp. JAB-1 revealed the presence of a chromosome-carried $bla_{OXA\text{-}535}$ gene, likely the progenitor of the plasmid-carried $bla_{OXA\text{-}436}$ gene, a novel $bla_{OXA\text{-}48}$-like gene. The expression of $bla_{OXA\text{-}535}$ in *E. coli* showed the carbapenem-hydrolyzing activity of OXA-535. The production of OXA-535 in *Shewanella* sp. JAB-1 could be evidenced using molecular and immunoenzymatic tests, but not with biochemical tests that monitor carbapenem hydrolysis. In this study, we have identified a CTX-M-15-producing *Shewanella* species that was responsible for a hepatobiliary infection and that is likely the progenitor of OXA-436, a novel plasmid-encoded OXA-48-like class D carbapenemase.

KEYWORDS CTX-M-15, OXA carbapenemase, WGS, plasmids, progenitor

Shewanella spp. are nonfermentative Gram-negative bacilli that are widely distributed throughout the world. They are found mainly in seawater in areas with warm climates (1, 2). Although *Shewanella* spp. are an unusual cause of infections in humans,

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the number of cases reported is increasing (1, 3–5). In most cases, this organism is cultured from samples of immunocompromised patients suffering from soft tissue infections after seawater exposure (3, 6). More recently, several cases of *Shewanella* infections have been reported in patients suffering from hepatobiliary diseases (3–5, 7). Human infections are most often caused by *Shewanella algae* and *Shewanella putrefaciens* (3). However, it is often difficult to identify the bacteria at the species level using conventional culture-based and biochemical tests. 16S rRNA gene sequencing improved the identification of *Shewanella* species as pathogenic organisms (3, 5).

Most *Shewanella* spp. are susceptible to piperacillin-tazobactam, ceftazidime, cefepime, imipenem, meropenem, gentamicin, tobramycin, amikacin, and ciprofloxacin, but susceptibility to penicillins is more varied (3, 7). Several naturally occurring β-lactamases have been identified in *Shewanella* spp., especially class D β-lactamases (8–11), also known as oxacillinases. For instance, *Shewanella oneidensis* strain MR-1 was found to naturally harbor the $bla_{\text{OXA-54}}$ gene, which is related to the $bla_{\text{OXA-48}}$ gene, a plasmid-mediated carbapenem-hydrolyzing class D β-lactamase (CHDL) gene involved in carbapenem resistance in *Enterobacteriaceae* (10). Moreover, OXA-55 was identified in *S. algae* and possesses biochemical properties similar to those of OXA-54, in particular the ability to hydrolyze carbapenems (11). *Shewanella xiamenensis* has been identified as the source of the $bla_{\text{OXA-181}}$ gene encoding another OXA-48-like variant with carbapenemase activity that is highly prevalent on the Indian subcontinent and increasingly reported in France (9, 12). Recently, next-generation sequencing enabled the identification of several $bla_{\text{OXA-48-like}}$ genes, such as $bla_{\text{OXA-199}}$, $bla_{\text{OXA-252}}$, $bla_{\text{OXA-514}}$, and $bla_{\text{OXA-515}}$, in different *Shewanella* species isolates, in food-producing animals, and in water samples (2). Furthermore, analysis of publicly available *Shewanella* species genomes revealed the presence of several uncharacterized $bla_{\text{OXA-48}}$ like genes, confirming the importance of *Shewanella* spp. as a OXA-48-like class D β-lactamase reservoir (13, 14).

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

In this study, we have characterized three CTX-M15-producing bacteria, including a *Shewanella* sp. isolate, obtained from a child suffering from cholangitis. Using antimicrobial susceptibility testing and whole-genome sequencing, we have characterized this *Shewanella* strain, investigated its resistome, and characterized the plasmids carrying the CTX-M15 determinant. Furthermore, we have cloned and expressed the naturally carried OXA-48-like gene ($bla_{\text{OXA-535}}$) and demonstrated its carbapenemase activity.

RESULTS

Case report. A female child was directly transferred from a hospital in central Africa to a hospital pediatric department in France for biliary surgery. During her previous hospitalization, she underwent a cholecystectomy that was complicated with a leak from the common bile duct forming an abdominal effusion. A drain was then introduced to constitute an external bypass. Upon admission at the hospital, she had fever with abdominal pain, and cholangitis was diagnosed. The liquid collected from the drain was addressed to the microbiology laboratory, and antibiotic treatment with piperacillin and tazobactam was started. The culture revealed a polymicrobial infection due to numerous Gram-negative bacteria (*Pseudomonas putida*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Stenotrophomonas maltophilia*, and a *Shewanella* sp.) and to *Enterococcus avium*. Antimicrobial susceptibility testing revealed the presence of extended-spectrum β-lactamases (ESBL) in *Shewanella* sp., *E. coli*, and *K.*

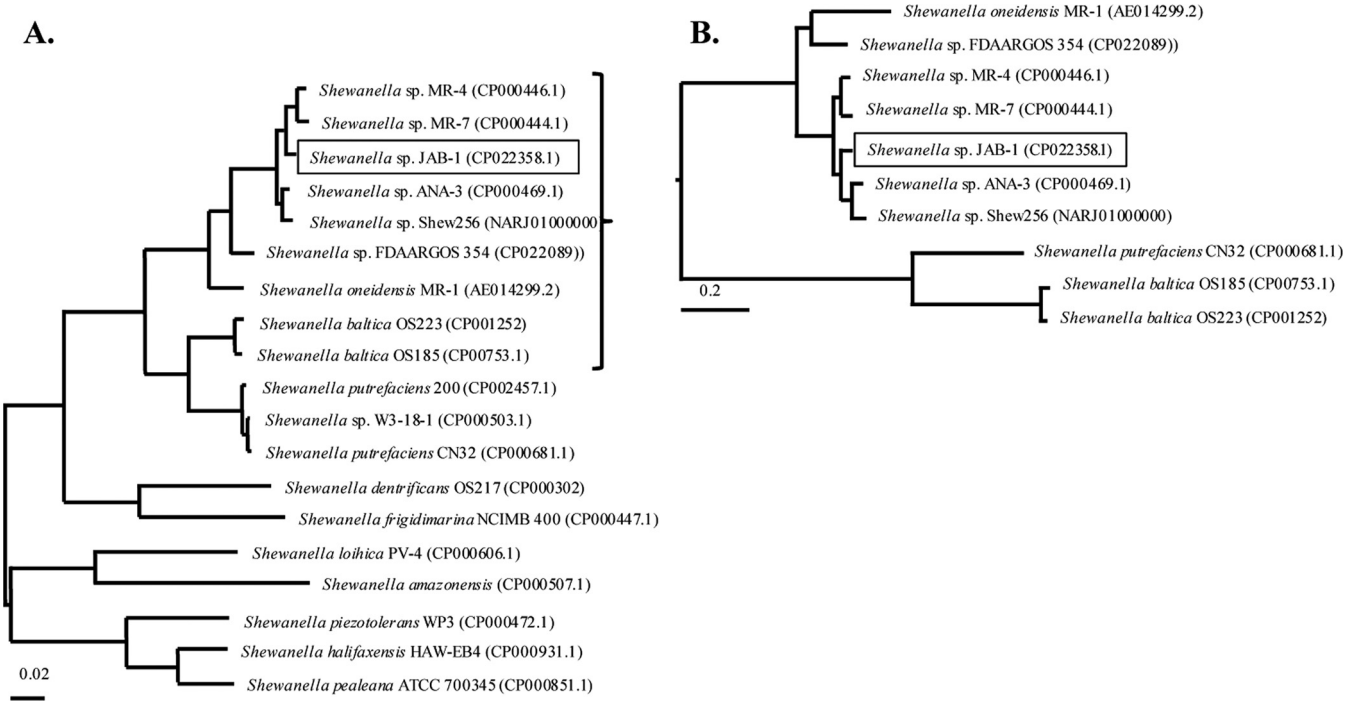

FIG 1 Phylogenetic analysis of representative *Shewanella* spp. (A) Unrooted phylogenetic tree based on *gyrB* and *rpoB* genes. The tree was constructed by maximum likelihood method with Tamura-Nei model using MEGA program (MEGA7.0). The tree is drawn to scale, with branch lengths representing the evolutionary distances. (B) A focus on *Shewanella* spp. close to *Shewanella* sp. JAB-1 was made, and the phylogenetic tree was based on whole-genome sequences. It was constructed by maximum likelihood method with Jukes-Cantor model, using the Parsnp software. The tree is drawn to scale based on the single nucleotide polymorphisms (SNPs) of the alignment. Accession numbers are shown in parentheses next to each organism name.

pneumoniae isolates. One week after admission, the patient underwent a laparotomy to perform a Roux-en-Y biliary bypass procedure and to introduce an internal-external biliary drain to protect the anastomosis. Five days after the surgery, the patient became feverish. A blood sample and a bile sample collected from the drain revealed the presence of an ESBL-producing *K. pneumoniae* isolate. Antibiotic therapy was switched to imipenem for 15 days. The biliary drain was withdrawn 1 month after the surgery, and no other complications occurred.

Identification of *Shewanella* sp. JAB-1. After 24 h of growth on a blood agar plate, the *Shewanella* sp. JAB-1 colonies were small, smooth, and oxidase and catalase positive. The API32GN system was unable to identify *Shewanella* sp. even at the genus level. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was used for species identification, yielding *S. putrefaciens*, with a best score value of 2.0, *Shewanella profunda* with a score of 1.7, and *Shewanella baltica* with a score of 1.5. Since MALDI-TOF MS discrepancies for the identification of *Shewanella* spp. are known, sequencing of 16S rRNA gene was performed. The 1,406-bp 16S rRNA gene sequence was 98% identical to *Shewanella seohaensis* S7-3T GU944672, making this strain the closest relative based on patristic distances (18). As the 16S rRNA gene sequence may not be discriminatory enough to differentiate closely related *Shewanella* species (9), we used whole-genome sequencing (WGS) data to dive deeper into the identification of this isolate. Genomes of representative *Shewanella* species were collected, and *rpoB-gyrB* concatemer sequences were extracted for phylogenetic analysis (Fig. 1A). The analysis revealed that *Shewanella* sp. JAB-1 did not belong to any known *Shewanella* species. Phylogeny based on the whole genome revealed that *Shewanella* sp. JAB-1 was closely related to four other isolates, namely, MR-4, MR-7, ANA-3, and Shew256, with average nucleotide identity (ANI) values of 94.6%, 94.35%, 94.32% and 93.90%, respectively (Fig. 1B).

Genomic characteristics of *Shewanella* sp. JAB-1. To reconstruct the genome of *Shewanella* sp. JAB-1, long-read PacBio-based sequencing was performed. The genome

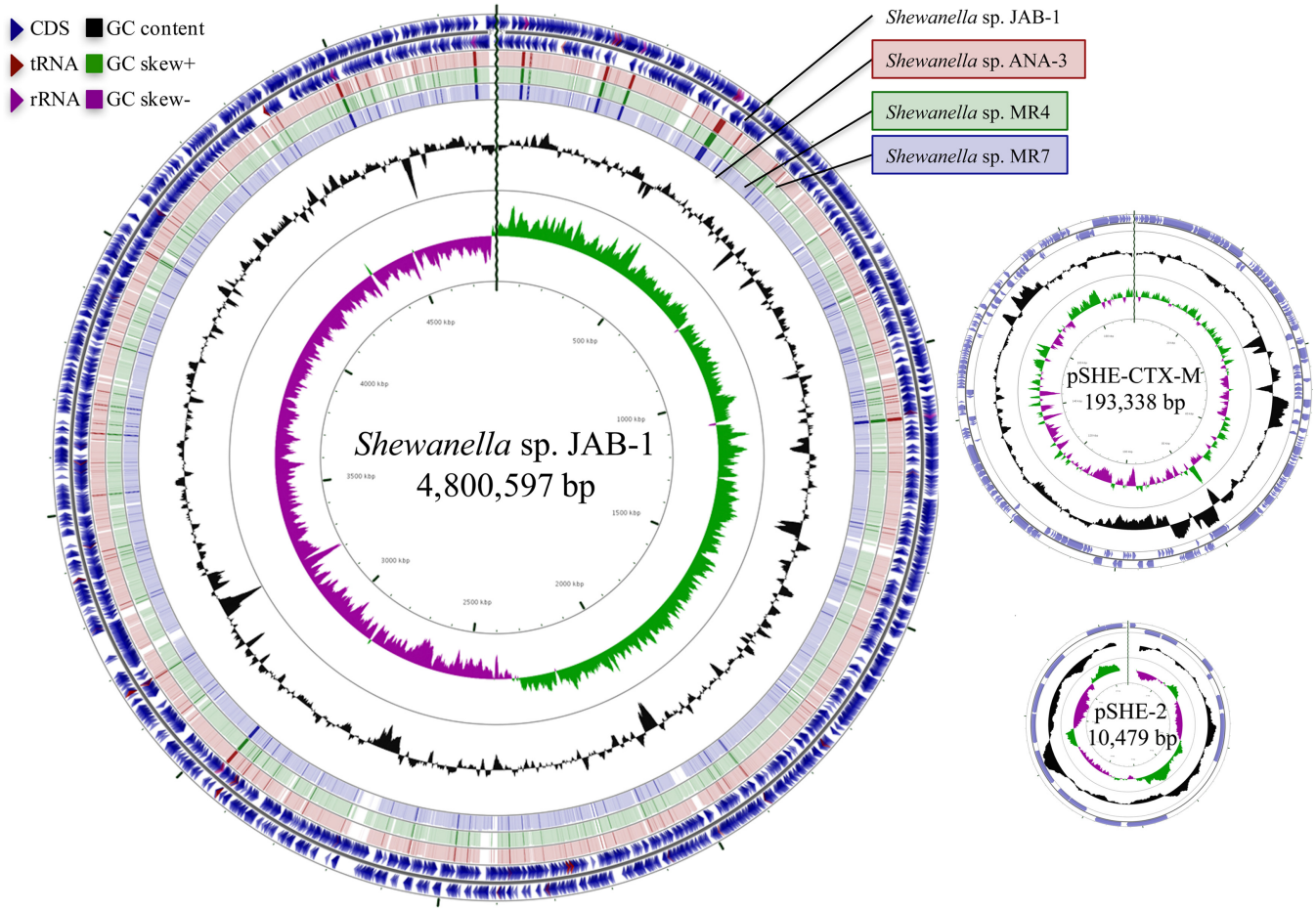

FIG 2 Genome representation of *Shewanella* sp. JAB-1 and its plasmids. The representation was performed using the CGview server. The outermost two rings show features extracted from the *Shewanella* sp. JAB-1 genome. The next three rings show the positions of BLAST hits detected through BLASTn comparisons of *Shewanella* sp. JAB-1 genome against the three closest genomes of *Shewanella* (MR-4, MR-7, and ANA-3, represented by red, green, and blue circles, respectively). Darker arc indicates high percent identity of the hit. The black circle displays the GC content, and inner circles display GC skew.

was 4,800,597 bp, with an average G+C content of 48%. It was composed of 4,161 coding sequences (CDS), 9 copies of rRNA operons, and 102 tRNA genes (Fig. 2). PacBio assembly revealed the presence of two plasmids of 10.5 and 193 kb in size (Fig. 2).

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

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

In agreement with this phenotype, we identified in the genome of *Shewanella* sp. four β-lactamase genes: two ESBL genes, $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-2a}}$; the narrow-spectrum oxacillinase gene $bla_{\text{OXA-1}}$; and a novel $bla_{\text{OXA-535}}$ gene. The $bla_{\text{OXA-535}}$ gene codes for an oxacillinase that is 91.3% identical to the carbapenem-hydrolyzing class D (CHDL) β-lactamase OXA-48, 98% identical to OXA-436 and OXA-48-like of *Shewanella* sp. strain ANA-3 (3 amino acids difference), and 99% to OXA-48-like in *Shewanella* sp. strains MR-4 and MR-7 (1 and 2 amino acids difference, respectively). OXA-436 is a

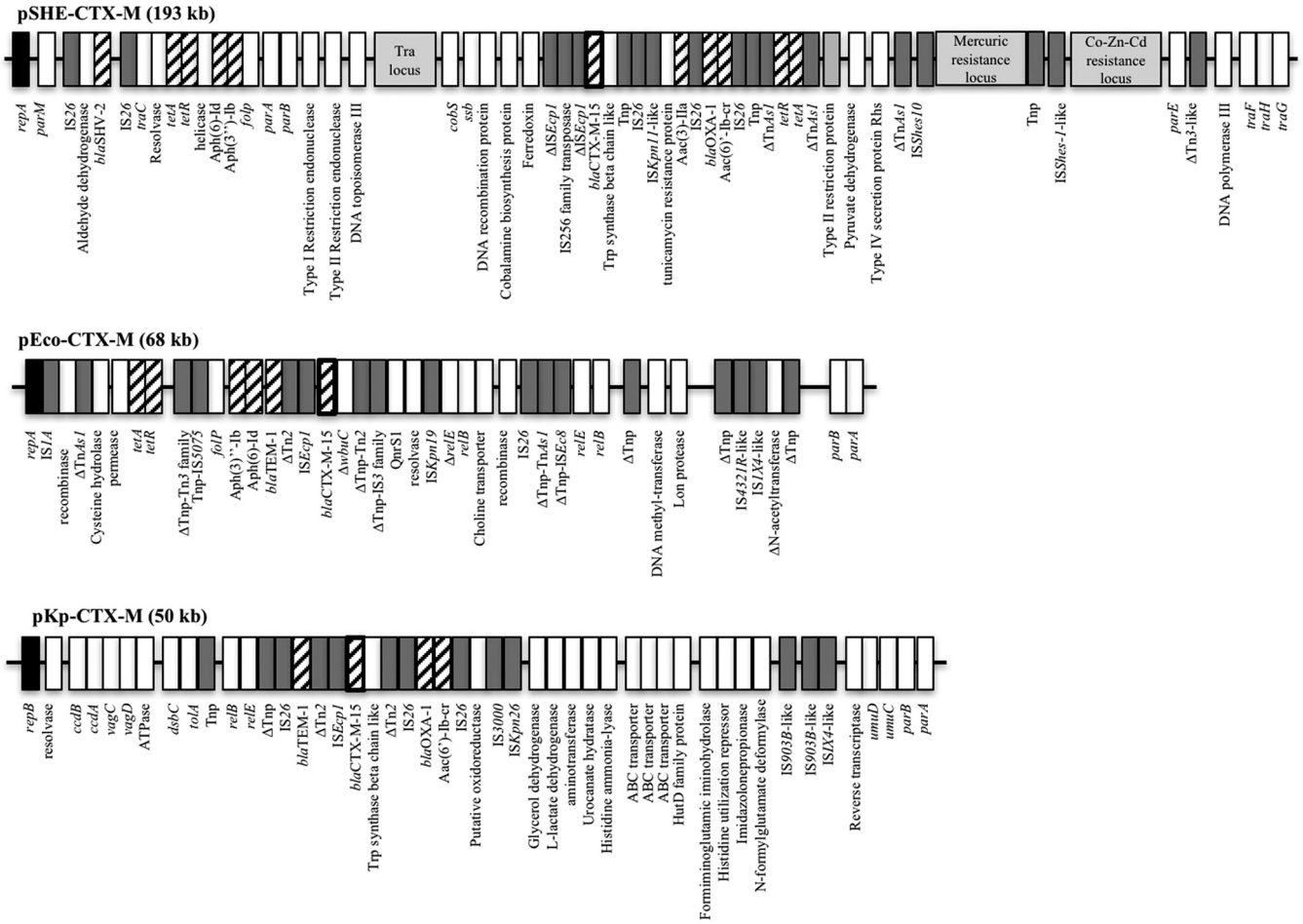

FIG 3 Schematic representation of the three ESBL-encoding plasmids. Main features are represented. Antimicrobial resistance-associated genes are indicated by hatched boxes. Transposon-related genes and insertion sequences are indicated by dark grey boxes. Replicase genes are indicated by black boxes. *tra* locus and heavy-metal resistance loci are indicated with light grey boxes. △ indicates a partial protein. Tnp, transposase.

plasmid-encoded OXA-48 variant detected in several species responsible for an outbreak in Denmark (O. Samuelsen, F. Hansen, B. Aasnaes, L. Jakobsen, P. Littauer, L. M. Soes, B. J. Holzknecht, L. P. Andersen, M. Stegger, A. S. Paal, and A. M. Hammerum, unpublished data; GenBank BioProject PRJNA297498). This outbreak involved OXA-436-producing *Citrobacter freundii*, *K. pneumoniae*, and *Enterobacter asburiae*.

Shewanella sp. JAB-1 is resistant to aminoglycosides (gentamicin, tobramycin, and netilmicin) due to the production of two acetyltransferase genes [*aac*(*6′*)-*Ib-cr* and *aac*(*3*)-*IIa*] and two phosphotransferase genes [*aph*(*3″*)-*Ib* and *aph*(*6*)-*Id*]. The resistance to ciprofloxacin is likely due to the acquisition of the *aac*(*6′*)-*Ib-cr*, known to confer reduced susceptibility to this fluoroquinolone by *N*-acetylation of its piperazinyl amine (19). Amikacin and co-trimoxazole are two antibiotics that remained active *in vitro*.

Plasmid characterization and genetic context of $bla_{CTX\text{-}M\text{-}15}$ genes in the three ESBL-producing isolates. Plasmid extraction of *Shewanella* sp. JAB-1 and subsequent electroporation and mating-out assay into *E. coli* TOP10 conferred resistance to penicillin and expanded-spectrum cephalosporins, with double-disk synergy images consistent with an ESBL phenotype. Amplification of the $bla_{CTX\text{-}M\text{-}15}$ gene on the *E. coli* transformants and transconjugants, as well as coresistance to aminoglycosides, indicated that both the $bla_{CTX\text{-}M\text{-}15}$ gene and *aac*(*6′*)-*Ib-cr* gene were located on the largest plasmid, namely, pSHE-CTX-M, of ca. 193 kb. pSHE-CTX-M was fully sequenced using PacBio technologies. The overall structure of the plasmid indicated that it belonged to the IncA/C incompatibility group (Fig. 3). The plasmid backbone shares features of

IncA/C-type plasmid, i.e., partitioning, replication, and conjugation apparatus. In the variable region, several antibiotic resistance genes have been identified in addition to $bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}2a}$, and $bla_{OXA\text{-}1}$ β-lactamase genes, the four aminoglycoside-modifying enzyme genes [*aph*(3″)-*Ib*, *aph*(6)-*Id*, *aac*(6′)-*Ib-cr*, and *aac*(3)-*IIa*], the tetracycline resistance gene (*tetA*), the sulfonamide resistance gene (*sul2*), and the chloramphenicol resistance gene (*catB3*).

E. coli JAB-1 carried three plasmids, as observed following Kieser extraction (data not shown). Electroporation in *E. coli* TOP10 and selection on ticarcillin conferred an ESBL phenotype as well as resistance to co-trimoxazole. The transformant carried a plasmid of 68 kb, namely, pECO-CTX-M, which was fully sequenced. Several antibiotic resistance genes were found on this IncY plasmid (Fig. 3). The ESBL-encoding $bla_{CTX\text{-}M\text{-}15}$ gene and the narrow-spectrum β-lactamase-encoding $bla_{TEM\text{-}1}$ gene, *dfrA14*, encoding a trimethoprim-insensitive dihydrofolate reductase variant, *sul2*, encoding a dihydropteroate synthase that is not inhibited by sulfonamide, *tetA* (tetracycline efflux protein) and aminoglycoside resistance genes [*aph*(6)-*Id* and *aph*(3″)-*Ib*] were present on the plasmid. Finally, the plasmid-carried fluoroquinolone resistance gene *qnrS1* was responsible for reduced susceptibility to ciprofloxacin.

Kieser extraction showed that *K. pneumoniae* JAB-1 carries three plasmids (data not shown). Electroporation into *E. coli* TOP10 and selection on ticarcillin conferred an ESBL phenotype, and the transformant carried a plasmid of 60 kb, called pKP-CTX-M, which belonged to the IncR incompatibility group and was fully sequenced (Fig. 3). We identified in this plasmid five resistance genes encoding three β-lactamases ($bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1b}$, and $bla_{OXA\text{-}1}$), an aminoglycoside acetyltransferase gene [*aac*(6′)-*Ib-cr*], and the chloramphenicol resistance gene *catB3*.

A comparison of the three $bla_{CTX\text{-}M\text{-}15}$ gene-harboring plasmids revealed major differences in size, backbone, and incompatibility group, thus ruling out *in vivo* plasmid exchanges between these three species (Fig. 3). There was no plasmid deposited in the NCBI database that shared the same structure as pSHE-CTX-M and pKP-CTX-M, but we found one plasmid called tig00003056 (accession no. CP021681.1) very close to pECO-CTX-M (A. Jousset, personal communication). To gain further insights into the acquisition of the $bla_{CTX\text{-}M\text{-}15}$ gene by these plasmids, we analyzed the genetic environment of the ESBL gene (Fig. 4). It revealed that the $bla_{CTX\text{-}M\text{-}15}$ gene was part of an IS*Ecp1*-borne transposon, as previously described (20). In pKP-CTX-M and pECO-CTX-M, the IS*Ecp1*-borne transposon was inserted in the transposase *tnpA* gene of Tn*2*, generating the same target site duplication (TSD; TCACA; Fig. 4A and B). This is in favor of the idea of the acquisition of $bla_{CTX\text{-}M\text{-}15}$ gene occurs by homologous recombination of this region rather than by a transposition event. This structure was also identified in other $bla_{CTX\text{-}M\text{-}15}$ gene-harboring plasmids, such as the archetypal IncFII-type plasmid pC15-1A circulating in Canada in the early 2000s (21). In pSHE-CTX-M, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15}$ is also inserted within the Tn*2* transposase gene, but this insertion differs by two different features: (i) the DNA fragment upstream of Tn*2* transposase is missing and (ii) an insertion sequence (IS) belonging to the IS*256* family with a TSD of 8 bp was identified (CATTTTGG) within the IS*Ecp1* transposase gene. Our data suggest a common ancestor for these structures, but they have evolved differently and are carried on different plasmids. Despite the insertion of an IS*256*-like gene in IS*Ecp1* in pSHE-CTX-M, the expression of CTX-M-15 did not seem to be impacted based on the MICs measured for third-generation cephalosporins (Table 1). Accordingly, the promoter sequences provided by IS*Ecp1* (−35 TTGAAA and −10 TACAAT) remained intact.

Characterization of OXA-535. To determine whether OXA-535 can hydrolyze carbapenems, the $bla_{OXA\text{-}535}$ gene was cloned into pTOPO together with its surrounding regions. *E. coli* TOP10 expressing the recombinant plasmid (pTOPO-OXA-535) displayed a class D carbapenemase phenotype with resistance to temocillin, resistance to penicillins that was not restored by the addition of clavulanic acid, and susceptibility to third-generation cephalosporins (Table 1). In addition, *E. coli* TOP10(pTOPO-OXA-535) had reduced susceptibility to carbapenems compared to *E. coli* TOP10. The Carba

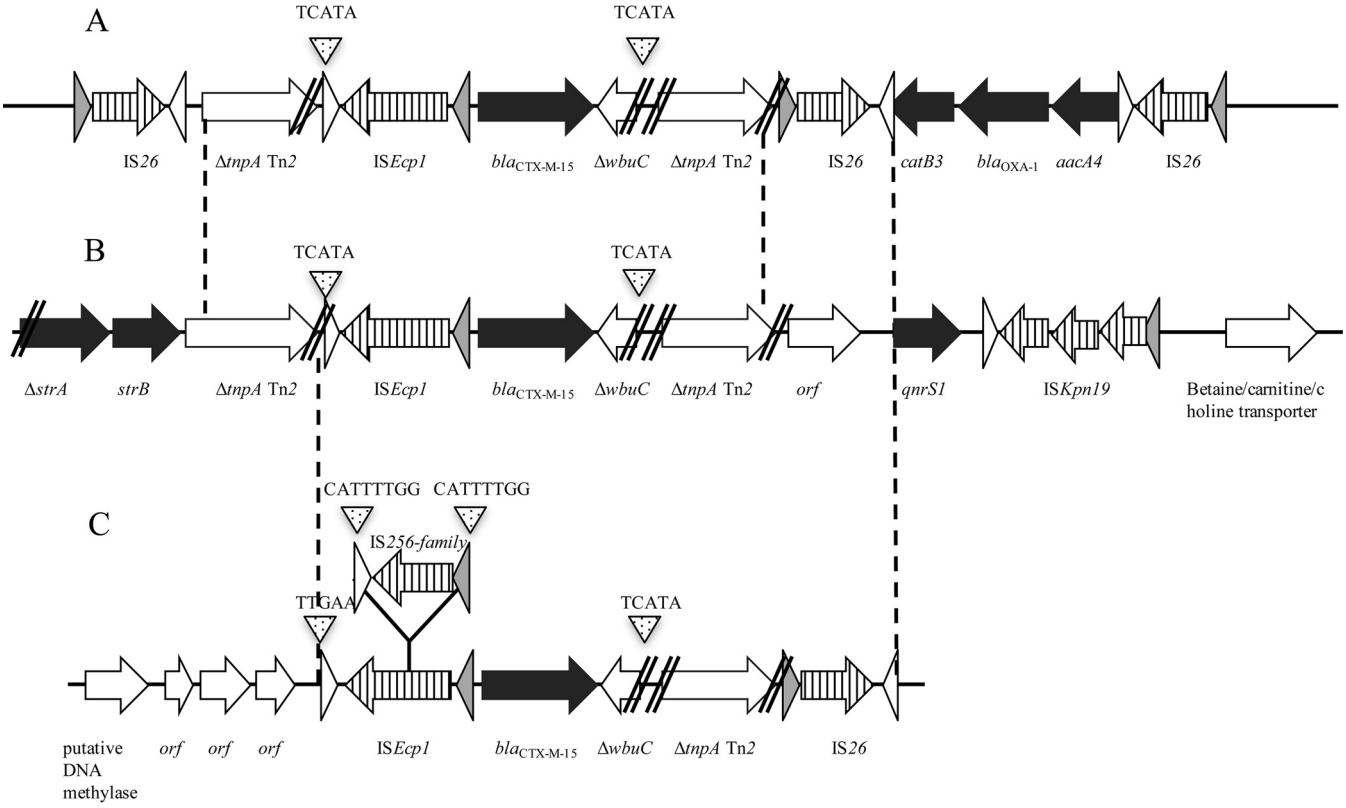

FIG 4 Genetic environment of the $bla_{CTX\text{-}M\text{-}15}$ gene of *K. pneumoniae* JAB-1 (A), *E. coli* JAB-1 (B), and *Shewanella* sp. JAB-1 (C). Antimicrobial resistance genes are indicated with black arrows. Open reading frames within insertion sequences (IS) are represented with striped arrows. Target site duplications are indicated with small dotted triangle. Inverted repeats IR_R and IR_L are represented by white and light gray arrows, respectively. Parallel lines indicate truncated genes.

NP test performed on the recombinant strain was positive, suggesting that the OXA-535 enzyme hydrolyzes carbapenems. Furthermore, specific activity (SA) measured with a culture extract of *E. coli* TOP10(pTOPO-preOXA-535) showed that this strain hydrolyzes imipenem significantly (SA = 270 mU · mg · 1iter of protein), similar to that of *E. coli* TOP10(pTOPO-OXA-48), which was used as a control (SA = 130 mU · mg · 1iter of protein). The MICs for β-lactams for OXA-535 and OXA-48 were similar, confirming that these enzymes possessed similar resistance patterns (Table 1).

Interestingly, when performed directly on the *Shewanella* sp. JAB-1, the Carba NP test and the β Carba test were negative. Since tests based on carbapenem hydrolysis can lack sensitivity to detect isolates expressing β-lactamase with low carbapenemase activity, such as OXA-48-like enzymes, an immunochromatographic test known to detect OXA-48-like enzymes with high sensitivity was performed (22). Indeed, the OXA-48 *K*-SeT gave a positive result on colonies of *Shewanella*. Therefore, it seems that

TABLE 1 MIC values of *Shewanella* spp. and transformants

Antibiotic	MIC (μg/ml)[a]						
	S. putrefaciens (OXA-181 chr)	*S. xiamenensis* CIP 67.65	*S. algae* CIP 103562	*Shewanella* sp. JAB-1 (OXA-535 chr)	*E. coli* TOP10(pTOPO-OXA-535)	*E. coli* TOP10(pTOPO OXA-48)	*E. coli* TOP10
Temocillin	0.38	0.75	0.19	0.25	>1,024	>1,024	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

[a]chr indicates the presence of a natural oxacillinase in the chromosome.

the OXA-535 enzyme is expressed at a low level but sufficiently to be detected by the immunochromatographic test. At last, a molecular test routinely used in clinical microbiology lab, the Xpert Carba-R test, was performed directly on the *Shewanella* sp. JAB-1 colonies and yielded a positive PCR for OXA-48-like carbapenemase.

DISCUSSION

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

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

Next-generation sequencing enabled us to study the complete resistome of the *Shewanella* sp. JAB-1 as well as the features of the three ESBL-encoding plasmids (pSHE-CTX-M, pECO-CTX-M, and pKP-CTX-M). Even if the three strains shared the same clinical environment, they did not share their plasmid, since they belong to different incompatibility groups. Plasmid continuous sequences allowed us to determine the genetic environment of $bla_{\text{CTX-M-15}}$. Indeed, distinct episodes of homologous recombination and of transpositions seem to have occurred rather than direct plasmid conjugation between strains in the patient.

The $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-2a}}$ genes are two ESBL genes present on an IncA/C plasmid of 193 kb in *Shewanella* sp. JAB-1. ESBL production has, to date, never been reported in any *Shewanella* species. IncA/C plasmids are known to efficiently spread many resistance genes, in particular, $bla_{\text{CMY-like}}$ Ambler class C β-lactamase genes (24). They have been detected worldwide, and carbapenemase genes, such as $bla_{\text{NDM-1}}$ and $bla_{\text{VIM-4}}$, have been identified on that plasmid scaffold (25, 26). Recently, characterization of ESBL-encoding plasmids in clinical isolates of *K. pneumoniae* in Taiwan revealed that the most common replicon type was incompatibility group IncA/C (27). Coexpression of $bla_{\text{CTX-M}}$ and bla_{SHV} is not rare on this scaffold (27). Therefore, IncA/C plasmids seem to efficiently spread ESBL genes among *Enterobacteriaceae* and non-*Enterobacteriaceae*.

By sequencing the *Shewanella* sp. JAB-1 genome, we identified a new oxacillinase variant called OXA-535. According to the enzymatic activities, OXA-535 can be considered a new carbapenem-hydrolyzing β-lactamase. OXA-535 presented only 3 amino acids difference with the plasmidic carbapenemase OXA-436. Moreover, we could identify a 7.3-kb fragment in the chromosome of *Shewanella* sp. JAB-1 including the $bla_{\text{OXA-535}}$ gene, which presented 96% nucleotide identity with the plasmid-carried $bla_{\text{OXA-436}}$ gene region. Considering the high similarities between these structures, we can speculate that this 7.3-kb fragment has been mobilized on a plasmid and that the $bla_{\text{OXA-535}}$ gene is likely the precursor of the plasmid-carried $bla_{\text{OXA-436}}$ gene.

Interestingly, antimicrobial susceptibility testing, the Carba NP test, the β Carba test, and OXA-48 *K*-SeT indicated that $bla_{\text{OXA-535}}$ gene is barely expressed in *Shewanella* sp. JAB-1. Accordingly, we did not identify any insertion element (IS) upstream of the $bla_{\text{OXA-535}}$ gene that could provide strong promoter sequences, unlike the plasmid-encoded OXA-181 and OXA-48, where IS*Ecp1* and IS*1999*, respectively, allow strong carbapenemase expression. Like in other sequenced *Shewanella* spp., we identify a gene coding for a peptidase C15 family upstream of $bla_{\text{OXA-535}}$ and a *lysR* gene coding for a putative LysR transcriptional regulator downstream (13).

The Xpert Carba-R test performed on the colonies could amplify the $bla_{OXA-535}$ gene (data not shown). We can speculate that if this test would have been performed directly on a rectal swab of this patient, as is recommended for screening of high-risk patients (28), it might have been falsely positive for the detection of a carbapenemase-producing *Enterobacteriaceae*. A false-positive molecular screening test has already been reported by Antonelli et al., likely due to the presence in the human gut of *S. xiamenensis* carrying $bla_{OXA-416}$ in its chromosome (29). The rate of fecal carriage of *Shewanella* spp. in the general population is currently unknown, as well as the bacterial concentration sufficient to yield a positive molecular test result. Nevertheless, the analytical limit of detection of the Xpert Carba-R assay is quite low (ranging from 1.1×10^2 to 1.2×10^3 CFU/swab depending on the carbapenemase gene [30]). Given these data, false-positive molecular test results remain troublesome, since hepatobiliary diseases have been proposed as a risk factor for infections (or colonization) due to *Shewanella* spp. and can therefore contribute to regularly inoculate the gastrointestinal tract.

MATERIALS AND METHODS

Bacterial strains. *Shewanella* sp. JAB-1, *Escherichia coli* JAB-1, and *Klebsiella pneumoniae* JAB-1 clinical isolates were from the Bicêtre Hospital, Le Kremlin-Bicêtre, France. They have been identified with MALDI-TOF MS using the Bruker MS system (Bruker Daltonics, Bremen, Germany), according to the manufacturer's instructions. In addition, sequencing of 16S rRNA using universal primers 16S 8-27 and 16S 1512-1491 was performed for the *Shewanella* isolate, as described previously (9). Biochemical features were studied using API32GN and API20E (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Electrocompetent *E. coli* TOP10 (Invitrogen, Éragny, France) was used as a recipient for electroporation experiments. *E. coli* J53 RIF^r, which is resistant to azide and rifampin (RIF^r), was used for conjugation experiments. *E. coli* 50192 was used as a reference strain for plasmid extraction (31). *S. algae* CIP 103562, *S. putrefaciens* CIP 67.65, and *S. xiamenensis* S12 (9) were used as references for susceptibility testing and determination of biochemical features.

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

Resistance gene detection. Genes coding for Ambler class A β-lactamases were sought by PCR using primers specific for the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes, as previously described (32). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Courtabœuf, France) and sequenced on both strands with an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA).

Cloning experiments. Genomic DNA of the *Shewanella* sp. JAB-1 isolate extracted using the Qiagen DNAamp kit (Qiagen) was used as the template for amplification of the $bla_{OXA-535}$ gene with its surrounding region (290 nucleotides [nt] before the ATG and 181 nt after the stop codon) and was performed with pre-OXA-535-For (5′-CGTTTGGGTTTGCTTCAT-3′) and pre-OXA-535-Rev (5′-GACTAGGCT TTTTGCGTT-3′) primers, and the PCR product was inserted into pTOPO (Invitrogen, Éragny, France), resulting in pTOPO-preOXA-535. *E. coli* TOP10 carrying recombinant plasmid pTOPO-preOXA-535 was selected using a Trypticase soy (TS) agar plate containing ticarcillin (50 mg/liter) and kanamycin (50 mg/liter). The inserted DNA fragment was verified by Sanger sequencing.

Plasmid extraction, electroporation, and mating-out assay. Natural plasmids were extracted using the Kieser extraction method (33) and subsequently analyzed by electrophoresis on a 0.7% agarose gel. ESBL-encoding plasmid DNA of *Shewanella* sp. JAB-1 (pSHE-CTX-M), *E. coli* (pECO-CTX-M), and *K. pneumoniae* (pKP-CTX-M) isolates were extracted using the Qiagen plasmid maxikit (Qiagen, Courtabœuf, France) and analyzed by agarose gel electrophoresis (Invitrogen, Paris, France).

Transfer of the β-lactam resistance markers from *Shewanella* sp. JAB-1 was performed by liquid mating-out assays at 37°C using *E. coli* J53 RIF^r as the recipient strain (31).

Recombinant plasmids (pTOPO-preOXA-48 and pTOPO-preOXA-535) were introduced by electroporation into *E. coli* TOP10 using a Gene Pulser II (Bio-Rad Laboratories) (31).

Detection of carbapenemase presence. Bacterial colonies of *Shewanella* sp. JAB-1 recovered from Trypticase soy agar were used to perform Xpert Carba-R test version 2 (Cepheid, Sunnyvale, CA, USA), OXA-48 *K*-SeT assay (Coris BioConcept, Gembloux, Belgium), the Carba NP test, and the β Carba test, according to the manufacturer's recommendations (22, 34, 35) or updated guidelines (36). The Carba NP test was also performed on recombinant *E. coli* TOP10 carrying pTOPO-preOXA-535.

Enzymatic activities. The specific activities of the OXA-48 and OXA-535 β-lactamases were determined using the supernatant of a whole-cell crude extract obtained from an overnight culture of *E. coli* clones expressing those two β-lactamases (pTOPO-preOXA-48 and pTOPO-preOXA-535 in *E. coli* TOP10) with an Ultrospec 2000 UV spectrophotometer (Amersham Pharmacia Biotech), as previously described (37). Imipenem was used as the substrate at a concentration of 100 μM.

Whole-genome sequencing and bioinformatic analysis. Total DNA of *Shewanella* sp. JAB-1 was extracted using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. Total DNA and plasmid DNA (pSHE-CTX-M, pECO-CTX-M, and pKP-CTX-M) concentrations were measured using the Qubit double-stranded DNA (dsDNA) BR assay kit (Life Technologies, Carlsbad, CA, USA). The DNA libraries were prepared using the Nextera XT version 3 kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions, and then run on the HiSeq or MiSeq system (Illumina) to generate paired-end 150-bp reads. Total *Shewanella* DNA was also sequenced using PacBio long-read technology (Macrogen, Seoul, South Korea).

Illumina read *de novo* assembly was performed using CLC Genomics Workbench 9.0, according to the manufacturer's recommendations (Qiagen, Courtabœuf, France). PacBio subreads were assembled with both Canu (38) and the RS_HGAP_Assembly.3 protocol from the SMRT Analysis toolkit version 2.3, while consensus accuracy was further polished using Quiver (39), as was previously described (40). PCR sequencing was used to complete the assembly of pECO-CTX-M and pKP-CTX-M plasmids. A phylogenetic tree based on the *gyrB-rpoB* concatemer was drawn with the MEGA7 software using the maximum likelihood method based on the Tamura-Nei model (41). Phylogenetic analysis based on whole-genome sequences of *Shewanella* spp. close to *Shewanella* sp. JAB-1 was done using the Parsnp program from the Harvest Suite (42). tRNAs were detected by ARAGORN (http://mbio-serv2.mbioekol.lu.se/ARAGORN/) (43). Circular representation was performed by CGview (44). *Shewanella* sp. JAB-1 was compared with three closely related strains: *Shewanella* MR-4 (accession no. CP000446.1), MR-7 (accession no. CP000444.1), and ANA-3 (accession no. CP000469.1).

Accession number(s). The nucleotide sequence of the $bla_{OXA-535}$ gene of the assembled chromosome sequence of *Shewanella* sp. JAB-1 and of its 10.5-kb plasmid, with no antibiotic resistance features, have been deposited at the EMBL/GenBank nucleotide sequence database under the accession numbers KX828709, CP022358, and CP022360, respectively. The three assembled ESBL-encoding plasmids (pSHE-CTX-M, pECO-CTX-M, and pKP-CTX-M) have been deposited at the EMBL/GenBank nucleotide sequence database under the accession numbers CP022359, MF510423, and MF510424, respectively.

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