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Genetic analysis of a PER-2 producing *Shewanella* spp. strain harboring a variety of mobile genetic elements and antibiotic resistant determinants

Running Title: PER-2 producing *Shewanella* spp. strain

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Highlights

- Description of a novel multidrug-resistant *Shewanella* spp. recovered from a patient.
- *bla*$_{PER-2}$ producing *Shewanella* spp. strain harboring a novel complex class 1 integron
- HGT could influence in the survival and adaptability to hospital environment

ABSTRACT

Objective

The goal of this work is to investigate the molecular mechanisms that explain the multidrug resistant phenotype found in a novel clinical *Shewanella* spp. strain (Shew256) recovered from a diabetic patient.

Methods

The whole-genome shotgun sequencing was obtained with Illumina MiSeq-I and Nextera XT DNA library. De novo assembly was performed with SPADES. RAST was used to predict the open reading frames and the predictions were confirmed using BLAST. Further genomic analysis was carried out using average nucleotide identity (ANI), ACT (Artemis), OrthoMCL, ARG-ANNOT, ISFinder, PHAST, tRNAscan-SE, plasmidSPAdes, PlasmidFinder and MAUVE. PCR reactions and plasmid extraction were also performed.

Results

The genomic analysis revealed a total of 456 predicted genes, which are unique to Shew256 when compared with the other *Shewanella* genomes. Moreover, the presence of different resistance genes, including the presence of a *bla*$_{\text{PER-2}}$, were found. A complex class 1 integron containing the IS*CR1* gene, disrupted by two putative transposase genes were identified. Furthermore, other resistance genes, a transposon containing *aph(3')*, insertion sequences, phages, and non-coding RNAs were also found.

Conclusion

Evidences of acquisition of resistance genes and mobile elements that could explain the multidrug resistance phenotype were observed. This *Shewanella* species represents a prime example of how antibiotic resistance determinants can be acquired by uncommon pathogens.

1. Introduction

Shewanella spp. is an environmental bacterium typically isolated from the aquatic environment [1,2], which is also known for its versatility and plastic genome. It contains several mobile elements and acts as a reservoir for *bla*$_{\text{OXA-48}}$ carbapenemase. This genus is comprised of at least 50 species with a wide range of functions, but most notable is its use in bioremediation [1]. However, there have been an increased number of reports exposing the pathogenic capability of *Shewanella*. Among others, this genus is responsible for skin and soft tissue infections, biliary tract infections, bacteremia, endocarditis, arthritis, peritonitis and ventilator-associated pneumonia, being *Shewanella*

algae and *Shewanella putrefaciens* the most commonly reported pathogens [3,4]. Notwithstanding, multidrug resistance in *Shewanella* has also been reported [5].

Antibiotic resistance has steadily become an increasing threat to public health with the resistance to β-lactam antibiotics among gram-negative bacteria a great concern. The occurrence and spread of extended-spectrum β-lactamases (ESBLs) among pathogens have a direct impact in the resistance to β-lactam antibiotics observed in the past years [6].

Here, we present a novel multidrug resistant *Shewanella* spp. strain from the nosocomial niche harboring β-lactamases including *bla*$_{\text{PER-2}}$ which is usually associated to HGT among other antibiotic resistant determinants.

2. MATERIALS AND METHODS

2.1. Clinical case, clinical strains and susceptibility assays

In 2015, a 65-year-old male with Type 2 Diabetes mellitus was admitted to the trauma unit of a hospital in Argentina with prior trauma to the left knee in which the joint was immobilized with a full leg cast in the emergency room two weeks before admission. At the trauma unit the patient was diagnosed with a compound fracture of the left tibial plate where the cast was removed. Ecchymosis and edema of the posterior aspect of the distal third of the thigh and the proximal third of the left calf was observed. The patient had limited mobility of the knee without compromised neurovascular function. The left knee x-ray confirmed a compound fracture of the distal and medial tibial plate of the left leg. The patient was hospitalized for surgical intervention.

Peripheral venous blood sample took on admission showed the following laboratory findings: white blood cell count, 6,300/mm^3; hematocrit, 24.9%; hemoglobin count, 8.1 g/dl; platelet count, 229,000/mm^3. The serum biochemistry results were as follows: [Na], 128 meq/liter; [K], 5.3 meq/liter; blood urea nitrogen level, 1.19 g/l; creatinine level, 2.89 mg/dl, and glycemia 280 mg/dl.

In surgery, the fracture was reduced and osteosynthesis was done in the left tibia, a debridement was performed. Bone and soft tissue samples were taken for culturing. In the three soft tissue samples, *Shewanella* spp. developed along with *Klebsiella pneumoniae* producing KPC, and *Enterococcus faecium* resistant to vancomycin (EVR). The patient demonstrated no signs of a surgical wound infection. During his hospitalization, he developed an intra-hospital pneumonia requiring mechanical ventilation for 24 hours and antimicrobial treatment. This past clinical history could explain the isolation of EVR and *K. pneumoniae* harboring KPC from the wound. The three microorganisms isolated from the tissues of the patient were considered colonizers of the surgical wound. The patient received only empirical treatment before surgery (cefazolin 1 gr IV every 6 hours) and was discharged six days after surgery.

Considering the need of molecular techniques to correctly identify *Shewanella* isolates, sequencing of the 16S rDNA gene was performed showing 99% of identity with *Shewanella* sp. ANA-3 (CP000469) and *Shewanella decolorationis* DRK1 (KJ522792).

The antibiotic susceptibility test was performed using the VITEK 2. The minimum

inhibitory concentration (MIC) results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) categories for non-*Enterobacteriaceae* [7].

2.2. Whole-genome sequence of Shew256 clinical strain

Whole-genome shotgun sequencing was performed using Illumina MiSeq- I. De novo assembly was performed with SPADES assembler version 3.1.0 [8], using a pre-assembly approach with Velvet [9]. RAST server was used to predict open reading frames [10] and BLAST (version 2.0) software was utilized to confirm the predictions. tRNAscan-SE was used to predict tRNA genes [11]. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NAJR00000000. The version described in this paper is version NAJR00000000.

2.3. Genome Sequences for comparative genomics

For comparative analysis 56 *Shewanella* genomes and 28 genomes from the *Alteromonadales* order were downloaded via ftp from the NCBI site (ftp.ncbi.nih.gov). The final dataset for comparative analysis comprised 85 genomes, distributed in 10 genera, that is the downloaded data plus the Shew256 draft genome reported here (Supplementary Table S1).

2.4. Clustering of homologous genes and phylogenetic analysis

Identification of homologs among the analyzed genomes was carried out using the OrthoMCL method [12] implemented in the get_homologous software, version 1.3 [13]. Protein sequences were aligned using Clustal Omega v1.2.0 [14]. Phylogenetic trees were inferred using the maximum-likelihood method with an amino acid LG+G (8 categories) model by means of Phyml version 3.1 [15]. The default SH-like test was used to evaluate branch supports in each analysis [16] and a consensus tree was inferred using the sumtrees.py script [17].

2.5. Average nucleotide identity

The average nucleotide identity score (ANI) between Shew256 and closely related genomes were estimated. The ANI score is used to delineate species using genome sequence data [18]. Two-way ANI (reciprocal best hits based comparison) was estimated by means of the ani.rb script available at enveomics.blogspot.com.

2.6. Genomic comparison, gene content and sequences analysis

Comparative genome analysis was performed with the open-source MAUVE aligner

version 2.3.1 [19]. ARG-ANNOT and ISfinder softwares were used to identify antibiotic resistance genes and insertion sequences, respectively [20,21]. Phage and prophage sequences were identified using PHAST [22]. PlasmidFinder was used to detect the presence of *Enterobacteriaceae* plasmids [23] and plasmidSPAdes software, which distinguishes plasmid sequences via the read coverage of contigs, was also used [24]. Prediction of small non-coding RNAs was done using RNA families from the Rfam database and the software Infernal [25].

2.7. General molecular techniques

PCR reactions using GoTaq enzyme (Promega, Madison, USA) were carried out to link contigs and to confirm the genetic environment of relevant resistance determinants (Supplementary Table S2). Plasmid DNA extraction was performed using the alkaline lysis [26], QIAfilter Midi prep Kit (QIAGEN, Hilden, Germany) and the PureLink™ HiPure Plasmid Midi/Maxiprep Precipitators (ThermoFisher Scientific).

3. RESULTS AND DISCUSSION

3.1. Shew256 genome sequence, features and comparative genomic analysis

3.1.1. Genome features, orthologous genes, phylogeny and ANI

The whole genome sequence of Shew256 produced a total of 7,015,616 high quality paired-end reads. The draft genome had a N_{50} of 404,243 bp with a maximum contig length of 662,530 bp and a G+C content of 47.9%. A total of 4,892 open reading frames were predicted using the RAST server. The genome of Shew256 was compared to other genomes of the order *Alteromonadales*. Fifty-six available *Shewanella* genomes in GenBank, up to August 2016, were included in the analysis.

One hundred eighteen orthologous genes were identified, translated and aligned, independently. Phylogenetic analysis was done for each cluster and subsequently a consensus tree was built. The *Shewanella* genus clustered as a monophyletic group with 86% of support. Five lineages could be recognized within the genus and are numbered arbitrarily according to the position in Fig.1. This study agrees with previous results for the genus [27], but included an updated phylogenetic analysis of the available genomes of *Shewanella.*

Shew256 is located in the fourth monophyletic cluster, this includes genomes of five different identified species; *S. baltica, S. putrefaciens, S. oneidensis, S. xiamenensis,* and

S. decolorationis. Shew256 likely represents a different species together with *Shewanella* sp. ANA-3 as suggested by the ANI score equal to 95.7 (SD +/- 3) (Supplementary Table S3).

3.1.2. Panmatrix genome

Clusters of 47,436 homologous genes were identified when considering all the analyzed genomes. Among them, a total of 29,139 homologous clusters compromised the pangenome of *Shewanella* spp.. The core genome of the genus includes 410 clusters of homologous genes, while the soft-core genome is 1,784 (at least in 95% of the genomes) (Supplementary Fig.S1). The latter is a better estimation of the conserved core when considering how many draft genomes we are working on.

In relation to Shew256, 456 predicted genes by RAST were unique to this genome, among them 346 were annotated as hypothetical proteins, 9 as phage-related proteins and 13 as mobile elements. These unique genes are distributed in 16 clusters into 9 contigs, considering clusters of at least 10 genes of unique or rare genes within the genus (less than 10% of the *Shewanella* genomes). Many of these genes were assigned to plasmids and/or were candidates to be the result of recent HGT acquisition.

Comparative genome analysis using MAUVE showed a strong synteny and closely relatedness between Shew256 and *Shewanella* sp. ANA-3 genomes, which correlates with ANI values.

3.2. Antibiotic Resistance determinants found in Shew256

Susceptibility tests revealed that Shew256 was resistant to ampicillin, ampicillin-sulbactam, cefotaxime, ceftazidime, cefepime, ciprofloxaxin and trimethropim-sulfamethoxazole, an unusual resistance profile for this bacterial species. The antimicrobial susceptibility to β-lactam antibiotics was confirmed by the broth microdilution method following the CLSI recommendations [7] (Table 1).

To determine the repertoire of resistant genes present in Shew256, the ARG-ANNOT database was utilized. A total of twelve resistance gene sequences were identified. Among them, we found twelve antimicrobial resistance genes encoding β-lactamases (*bla*$_{\text{PER-2,}}$ *bla*$_{\text{OXA-48-like}}$), aminoglycoside modification enzymes (*aadA16*, *aph(3'))*, rifampicin resistance (*arr3)*, *qnrVC1* conferring resistance to fluorquinolones, a sulfonamide-resistant dihydropteroate synthase (*sul1)*, a quaternary ammonium

compound-resistance protein (*qacEdelta1*), *dfrA27* conferring resistance to trimethoprim, the tetracycline resistance protein *tet*(C), and a macrolide efflux protein and phosphotransferase (*msrE* and *mphE*).

In order to analyze the organization and context of the β-lactamase genes, in-depth analysis of Shew256 genome sequences was performed.

3.2.1. Analysis of bla$_{\text{PER-2}}$ *genetic context*

bla$_{\text{PER-2}}$ gene was flanked by an incomplete IS*Pa12* upstream and followed by a gene coding for a glutathione S-transferase (GST) downstream. These genes were also associated with *bla*$_{\text{PER-2}}$ in a previous description in *Citrobacter freundii* (NG_036403). Moreover, downstream of the *gst* a putative ABC-transporter followed by a transposase with 99% of identity to a transposase reported in *Aeromonas media* SW (CP007567) were found.

The genus *Shewanella* has not been previously reported as carrier of *bla*$_{\text{PER-2}}$. Most of the reports were members of the *Enterobacteriaceae* family and also in *Vibrio cholerae, Pseudomonas aeruginosa* and *Acinetobacter* spp. [28,29,30].

Evidence of the role of *Shewanella* species as reservoirs of a class D β-lactamases, called OXA-48, was previously documented [31]. Several OXA-48-like variants have been described being OXA-48 extensively spread, not only among *Shewanella* spp. but also in many *Enterobacteriaceae*, such as *K. pneumoniae* [32,33]. This supports the potential interchange of genetic material between *Shewanella* and other gram-negative bacilli. Moreover, Antonelli *et al*. 2015 [34], described the case of intestinal carriage of *Shewanella xiamenensis* in a pediatric patient, who had previously been colonized and infected by an OXA-48–producing *K. pneumoniae.* The authors suggested a potential mobilization of *bla*$_{\text{OXA-48–like}}$ genes in the “intestinal” environment. Based on our data, we can also suggest that Shew256 could have acquired *bla*$_{\text{PER-2}}$ from an *Enterobacteriaceae* or another bacterial species that could have shared the same niche.

3.2.2. Analysis of bla$_{\text{OXA-48-like}}$ *genetic context*

Protein analysis of the OXA enzyme revealed a 99% identity to an OXA-48 family class D β-lactamase previously described in *Shewanella* sp. MR-7 (WP_011627184) and *Shewanella* sp. MR-4 (WP_011621491). Moreover, it also showed a 98% identity to the OXA-436 enzyme that was described in *C. freundii* AMA 948 (WP_058842180). The

bla$_{\text{OXA-48-like}}$ was found between the transcriptional regulator (*lysR*) and a HP coding gene, which is the same locus reported for other OXA-48-like in several *Shewanella* isolates [33]. Taking all of this into consideration, we can suggest that this OXA enzyme is present in Shew256 chromosome.

3.3. Identification of mobile genetic elements in Shew256 genome

3.3.1. Insertion sequences and transposon distribution in Shew256 genome

The Shew256 genome contains a variety of insertion sequence (IS) elements. A total of 10 complete ISs and 5 partial ISs were identified (Supplementary Table S4).

A transposon containing *aph(3')* within IS*26* was found. This latter structure has previously been described in some species such as *K. pneumoniae* NY9 (CP015386).

*3.3.2. A complex class 1 integron containing a truncated IS*CR1 *was found in Shew256 genome*

A complex class 1 integron, containing the resistance gene-cassettes *arr-3*, *dfrA27*, and *aadA16*, were found within the variable region 1 (VR1). A very similar VR1 was previously described in *K. pneumoniae* Kp791 (KJ668705). The 3'-CS conserved region containing *qacEΔ1*/*sul1* genes was present. An IS*CR1*, which was disrupted by two putative transposase genes, was also found (Fig.2). Downstream of it, an incomplete *parE* gene was observed, followed by a putative transposase gene and *qnrVC1*. Two putative transposase genes were found downstream of this later gene. Surprisingly, the second 3'-CS2 conserved region, which is typical in complex class 1 integrons, was not found.

Through bioinformatic analysis, we found that *Shewanella seohaensis* (KX923814) possessed a complex class 1 integron with an intact IS*CR1* and a similar (Fig.2). However, the second variable region (VR-2) contains *sdr* followed by *qnrB6*. In sum, our results show that this region has been subjected to further recombination events resulting in a non-previously described genetic structure with a disrupted IS*CR1*.

3.3.3. Presence of plasmid-related sequences in Shew256 genome

Putative plasmid sequences were predicted by plasmidSPAdes software [24]. These sequences were re-annotated resulting in 564 coding sequences. 97.3% of them were already annotated as part of the Shew256 genome (Supplementary Table S5). According to the plasmidSPAdes prediction any of the genes related with antimicrobial resistance nor the complex class 1 integron were predicted to be located in plasmids.

To further investigate the presence of plasmids, plasmid extraction by three different methods were performed with negative results. Bioinformatic prediction suggested that plasmid-related sequences might be present. However, this prediction implied that the complex class 1 integron and the antimicrobial resistance gene, including *bla*$_{PER-2}$, were most likely integrated into the chromosome.

3.3.4. Identification of phage sequences in Shew256 genome

Considering that among the unique genes observed in Shew256 genome, 9 phage-related proteins were identified, we decide to analyze phage sequences using PHAST tool in more detail. Two intact prophages and two incomplete prophages were predicted. Sequence analysis of one of the intact prophages (1) revealed that it possessed high degree of identity to one prophage that was found in *S. baltica* OS223 (NC_011663). The genome size of prophage was predicted as 46,736 bp and a G+C content of 49.56% with 40 predicted open reading frames. The second intact prophage (2) was found interrupting a gene coding for an exodeoxyribonuclease I (EC 3.1.11.1). It contained 31,817 bp and a G+C content of 46.44% with 34 predicted open reading frames. Phages are major genetic factors promoting HGT between bacteria [35,36]. Their roles in bacterial genome evolution have been increasingly highlighted. In the work recently published by Keen et al. 2017 [35], they showed how a subset of the natural lytic phage population, which they called “superspreaders”, releases substantial amounts of intact, transformable plasmid DNA upon lysis, thereby promoting HGT by transformation. In our case we have identified two intact prophages in the Shew256 genome. This finding is further proof of DNA acquisition in this particular strain.

3.4. Presence of putative non-coding RNAs in Shew256 genome

We looked for non-coding RNAs using the Infernal software with 720 covariance models, which resulted in 39 candidates (riboswitches FMN, TPP, cobalamin, glycine, lysine, SAM_alpha and MOCO; thermoregulator CspA; small RNAs CsrC, CsrB, Spot-42, sroH, t44, RyeB, SraC_RyeA, sau-50; antisense RNAs RNA-OUT and HPnc0260, the antitoxin SymR; and the cis-regulatory RNAs yybP-ykoY, Alpha_RBS, traJ-II, SECIS_3, mini-ykkC, c-di-GMP-I). Four out of 39 regulatory RNAs were found associated to mobile genetic elements or antimicrobial resistance: RNA-OUT, mini-ykkC, traJ-II and SymR. The antisense element RNA-OUT is directly involved in the dissemination of Tn10-like

transposons [37]. The cis-regulatory element mini-ykkC is probably involved in the regulation of gene sugE, a quaternary ammonium resistance gene. The cis regulator traJ-II, is located in the conjugative transfer operon *tra*. And the antitoxin SymR, which is implicated in a type I toxin/antitoxin system and is in charge of the repression of translation of SymE, a toxic protein [38]. Since SymE and traJ-II were found alongside with genes involved in the partition, replication and transfer of plasmids, Shew256 chromosome probably contains some remnants or other integrative and conjugative elements [39,40]

CONCLUSION

In the present study we have delved into the genome of *Shewanella* sp. Shew256 and confirmed that this strain belongs to a different species. Phylogenetic analyses showed that Shew256 together with *Shewanella* sp. ANA-3 belongs to a unique lineage, most likely as a new species.

The genomic analysis revealed 456 predicted genes as unique to this genome within the genus. Twelve antimicrobial resistance genes were also identified, this being the first report of a *Shewanella* strain carrying $bla_{\text{PER-2}}$. $bla_{\text{OXA-48-like}}$ analysis showed the same genetic context described in other *Shewanella* isolates and supports the idea that this enzyme is present in Shew256 chromosome.

A wide variety of mobile genetic elements were found, including several IS, a transposon containing *aph(3')* within IS*26*, a unique complex class 1 integron and two intact prophages.

Recent reports of emerging pathogens that acquired exogenous DNA from other bacterial species and/or serve as reservoir of resistance genes are more common than was previously thought [4, 28, 30, 41]. In the present case, Shew256 showed a variety of mobile genetics elements that are known to play a key role in intra- and interspecies transfer of antimicrobial resistance determinants. HGT could have a direct influence in the evolution and adaptability of this strain to extreme environments, such as hospital settings.

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Ethical Approval: NA

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Legends to figure

Figure 1. Phylogenetic relationship of all the genomes used in this study. This consensus phylogram was inferred from 118 maximum likelihood (ML) trees, one from each cluster of orthologous proteins. The monophyletic *Shewanella* genus was indicated with a square bracket. Node support derived from the percentage of phylogenies in which a certain node appeared.

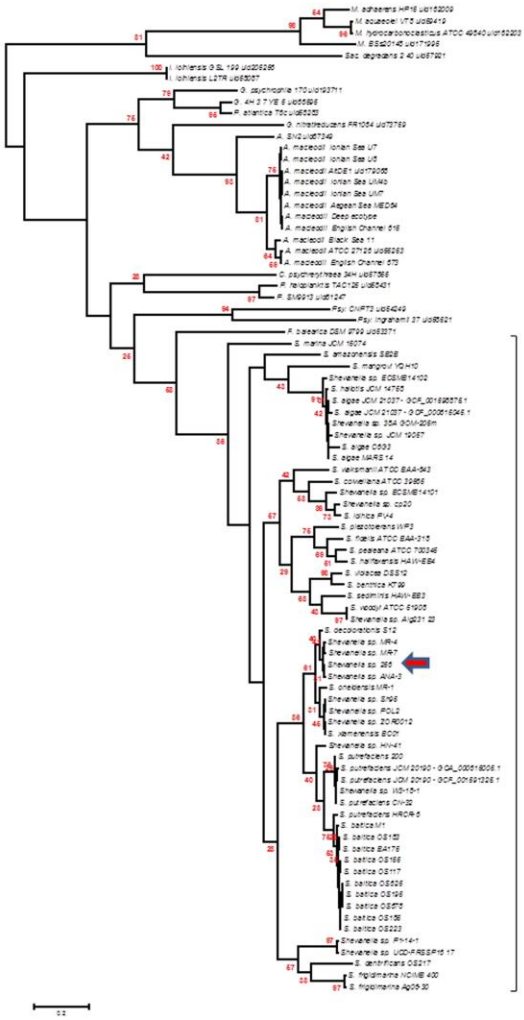

Figure 2. Schematic representation of the complex class 1 integron found in Shew256. The type 1 integrase gene and the 3-CS are shown by white arrow boxes. The attI1 site is shown as a white tall box. Genes of the variable region 1 (vr-1: *arr-3*, *dfrA27*, *aadA16*) are shown with striped arrow boxes. The ΔIS*CR1* is represented by two striped square boxes. The transposase genes are shown as grey arrow boxes and *parE* and *qnvrC1* genes are shown with striped arrow boxes. The grey vertical bars represent de identity between our sequences and the sequences described in *K. pneumoniae* and *Shewanella seohaensis*.

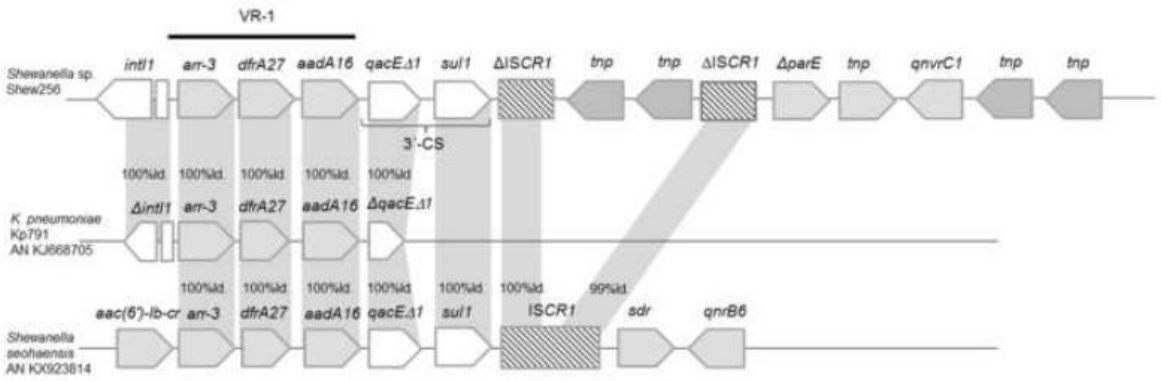

Table 1. Antibiotic susceptibility (MIC) of Shew256 clinical isolate.

Antimicrobial agent(s)	MIC (µg/ml)	Interpretation
Ampicillin	> 128	R
Ampicillin-sulbactam	> 128	R
Cephalotin	> 128	R
Piperacillin-tazobactam	2	S
Ceftriaxone	> 128	R
Ceftazidime	> 128	R
Cefepime	32	R
Imipenem	1	S
Meropenem	2	S