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Discovery of $bla_{OXA-199}$, a Chromosome-Based bla_{OXA-48}-Like Variant, in *Shewanella xiamenensis*

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Abstract

Introduction: bla_{OXA-48} is a globally emerging carbapenemase-encoding gene. The progenitor of bla_{OXA-48} appears to be a *Shewanella* species. The presence of the bla_{OXA-48}-like gene was investigated for two *Shewanella xiamenensis* strains.

Methods: Strain WCJ25 was recovered from post-surgical abdominal drainages, while S4 was the type strain of *S. xiamenensis*. Species identification for WCJ25 was established by sequencing the 16S rDNA and *gyrB* genes. PCR was used to screen the bla_{OXA-48}-like genes and to obtain their complete sequences. A phylogenetic tree of the bla_{OXA-48}-like genes was constructed. The genetic context of the bla_{OXA-48}-like gene in strain WCJ25 was investigated by inverse PCR using self-ligated AseI- or RsaI-restricted WCJ25 DNA fragments as template, while that in strain S4 was determined by PCR mapping using that in WCJ25 as template.

Results: A new bla_{OXA-48} variant, designated $bla_{OXA-48b}$, with four silent nucleotide differences from the bla_{OXA-48} (designated $bla_{OXA-48a}$) found in the *Enterobacteriaceae* was identified in strain S4. Strain WCJ25 had a new bla_{OXA-48}-like variant, $bla_{OXA-199}$, with five nucleotide differences from bla_{OXA-48}a and $bla_{OXA-48b}$. The OXA-199 protein has three amino acid substitutions (H37Y, V44A and D153G) compared with OXA-48. Both $bla_{OXA-48b}$ and $bla_{OXA-199}$ were found adjacent to genes encoding a peptidase (indicated as orf), a protein of unknown function (*sprT*), an endonuclease I (*endA*), and a ribosomal RNA methyl transferase (*rsmE*) upstream and to transcriptional regulator gene *lysR* and an acetyl-CoA carboxylase-encoding gene downstream. In addition, the insertion sequence IS*Shes2* was found inserted downstream of $bla_{OXA-199}$ but not of $bla_{OXA-48b}$. The 26 bp sequences upstream and 63 bp downstream of $bla_{OXA-48a}$, $bla_{OXA-48b}$ and $bla_{OXA-199}$ were identical.

Conclusions: $bla_{OXA-48a}$, $bla_{OXA-48b}$ and $bla_{OXA-199}$ might have a common origin, suggesting that the $bla_{OXA-48a}$ gene found in the *Enterobacteriaceae* could have originated from the chromosome of *S. xiamenensis*.

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Introduction

bla_{OXA-48}, encoding the carbapenem-hydrolyzing enzyme OXA-48, was initially found in *Klebsiella pneumoniae* from Turkey [1] and has now been spread to other *Enterobacteriaceae* species in a few countries [2,3]. Several bla_{OXA-48}-like variants have been identified recently, including $bla_{OXA-162}$ (GenBank Accession no. GU197550; one nucleotide different from bla_{OXA-48}), $bla_{OXA-163}$ (98.1% nucleotide identity with bla_{OXA-48}) [4], $bla_{OXA-181}$ (94.4% nucleotide identity with bla_{OXA-48}) [5], $bla_{OXA-204}$ (nucleotide sequence not available but encoding two amino acid substitutions compared with OXA-48) and $bla_{OXA-232}$ (nucleotide sequence not available but encoding a single amino acid substitution compared with OXA-181) [3]. The bla_{OXA-48} gene was previously proposed as been derived from the chromosome-encoded bla_{OXA-54} of *Shewanella oneidensis*, but the two genes have only 84% nucleotide identity [6]. Through analyzing the complete genome sequences of a few strains belonging to various *Shewanella* species available in the GenBank, the bla_{OXA-48}-like genes are present on the chromosome of several *Shewanella* species with at least 80% identity to bla_{OXA-48}. Thus, the actual progenitor of bla_{OXA-48} may rather lie within a *Shewanella* species other than *S. oneidensis*. A *Shewanella* clinical strain previously isolated and characterized [7] and the type strain of *Shewanella xiamenensis* [8] were investigated for the presence of a bla_{OXA-48}-like gene.

Methods

Strains

Shewanella isolate WCJ25 was recovered from post-surgical abdominal drainages of a patient with pancreatitis and was identified as *S. xiamenensis* based on the close identity (99.6% for 16S rDNA gene and 98.5% for *gyrB*) between WCJ25 and the *S. xiamenensis* type strain S4 [7]. The *S. xiamenensis* type strain S4 was provided by Prof. Zhang Xiaobo, Zhejiang University.

Screening for bla_{OXA-48}-like Genes

PCR was used to screen bla_{OXA-48}-like genes and to obtain the complete sequence of the bla_{OXA-48}-like gene with primers listed in Table 1. PCR was conducted using the ExTaq mix (Takara, Dalian, China) with the conditions being 94°C for 5 min, 30 cycles (94°C for 30s, 52°C for 45 s, 72°C for 1 min) and a final elongation step at 72°C for 7 min. The amplicons were purified using the OMEGA Cycle Pure kit (Norcross, GA, USA) and sequenced.

Phylogenetic Analysis of the bla_{OXA-48}-like Genes

Sequences of bla_{OXA-48}-like genes were retrieved from GenBank. The bla_{OXA-48}-like genes and their accession numbers are bla_{OXA-48} (AY236073), bla_{OXA-54} (AY500173), bla_{OXA-55} (AY343493), $bla_{OXA-162}$ (GU197550), $bla_{OXA-163}$ (HQ700343), $bla_{OXA-181}$ (JN205800) and those without assigned gene names on chromosomes of *Shewanella* spp., i.e., *S. algae* oxaSH (AY066004), *S. baltica* BA175 (CP002767), *S. baltica* OS117 (CP002811), *S. baltica* OS155 (CP000563), *S. baltica* OS185 (CP000753), *S. baltica* OS195 (CP000891), *S. baltica* OS223 (CP001252), *S. baltica* OS678 (CP002383), *S. loihica* PV-4 (CP000606), *S. oneidensis* MR-1 (AE014299), *S. putrefaciens* CN-32 (CP000681), *S. putrefaciens* 200 (CP000681), *Shewanella* sp. ANA-3 (CP000469), *Shewanella* sp. MR-4 (CP000446), *Shewanella* sp. MR-7 (CP000444) and *Shewanella* sp. W3-18-1 (CP000563). A phylogenetic tree of the bla_{OXA-48}-like genes was constructed using the MEGA 4.0 program [9] using the neighbour-joining method and bootstrapping (value 100) (Figure 1).

Study on Genetic Context

The genetic context study of $bla_{OXA-199}$ was investigated using inverse PCR. Genomic DNA of WCJ25, prepared using a commercial kit (Tiangen, Beijing, China), was restricted with AseI- or RsaI (Figure 2), self-ligated with T4 DNA ligase (New England Biolabs, Ipswich, NY, USA) and then used as a template for inverse PCR. The links between genetic elements were confirmed by overlapping PCR (Figure 2, primers listed Table 1). The genetic context of bla_{OXA-48} in the strain S4 was characterized by PCR mapping using that of $bla_{OXA-199}$ as the template (Figure 2). Primers were designed based on available sequences using the primer3 software (http://frodo.wi.mit.edu/primer3/) with the default settings. Inverse PCR, overlapping PCR and PCR mapping were also conducted using the ExTaq mix with the conditions being 94°C for 5 min, 30 cycles (94°C for 30s, 55°C for 45 s, 72°C for 5 min) and a final elongation step at 72°C for 7 min.

Amplicons were sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) at the Beijing Genomics Institute (Beijing, China). Sequences were assembled using the SeqMan II program in the Lasergene package (DNASTAR Inc, Madison, WI) and similarity searches were carried out using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/).

GenBank accession number. The genetic context of $bla_{OXA-199}$ in WCJ25 and that of bla_{OXA-48} in the strain S4 have been deposited in GenBank as JN704570 and JX644945, respectively.

Results and Discussion

S. xiamenensis is a newly-recognized species originally found in the coastal sea sediment in Xiamen, China [8] and has also been recovered from gutters in India very recently [10]. The identification of *S. xiamenensis* in India and two distant parts of China suggested that this species might be an underrecognized member of *Shewanella* with a wide geographical distribution.

The bla_{OXA-48}-like gene of strain S4 was confirmed as a variant of bla_{OXA-48}, designated $bla_{OXA-48b}$ here, which had four silent nucleotide differences from the bla_{OXA-48} variant (AY236073), designated $bla_{OXA-48a}$ here, found in the *Enterobacteriaceae*. Strain WCJ25 harboured a novel bla_{OXA-48}-like gene, designated $bla_{OXA-199}$ by the β-lactamases numbering system available at www.lahey.org. The $bla_{OXA-199}$ gene had five nucleotide differences from both $bla_{OXA-48a}$ and $bla_{OXA-48b}$ (99.4% identity), specifying the OXA-199 protein with three amino acid substitutions (H37Y, V44A and D153G) compared to OXA-48. During the process of this work, $bla_{OXA-181}$ was identified in a *S. xiamenensis* isolate from India [10]. However, $bla_{OXA-181}$ was significantly divergent from $bla_{OXA-48b}$ (94.7% identity, 42 nucleotide differences), $bla_{OXA-48a}$ (94.4% identity, 45 nucleotide differences) and $bla_{OXA-199}$ (94.1% identity, 47 nucleotide differences). Based on a phylogenetic tree (Figure 1) constructed by the MEGA program, the results showed that the bla_{OXA-48}-like genes could be divided into three clusters among which $bla_{OXA-48a}$, $_{-48b}$, $_{-162}$, $_{-163}$, $_{-181}$ and $_{-199}$ were of a cluster different from the chromosome-encoded bla_{OXA-48}-like genes of *Shewanella* species other than *S. xiamenensis*. The bla_{OXA-48}-like genes of the same *Shewanella* species clustered together, suggesting

Table 1. Primers used.

Primer	Sequence 5′-3′	Target	Source
OXA48/54IF	AGCAAGGATTTACCAATAAT	bla_{OXA-48}-like genes,	Valenzuela JK
OXA48/54IR	GGCATATCCATATTCATC	screening	unpublished
OXA48-up1	ATTAAGCAAGGGGACGTTATG	bla_{OXA-48}-like genes,	This study
OXA48-dw1	GAGCATCAGCATTTTGTCCA	complete sequences	This study
OXA48-IR2	GCAACTACGCCCTGTGATTT	bla_{OXA-48}-like genes	This study
OXA48-dw2	GTTAGCGCGTATTTGTGTG	Downstream of $bla_{OXA-199}$	This study
OXA199-up1	TAAGCCTGAACGCCCTAGAA	Upstream of $bla_{OXA-199}$	This study
tnpA_Shewa-R1	AATAGTTTCGGCAGGGGTTT	*tnpA* of IS*Shes2*	This study
orfJ25-R1	ACGGCTAATGGTTGAGGTTG	*rsmE*	This study
Orf25-R2	CCGTCATAGCGATTTCTTCC	*rsmE*	This study
aceCoA-R2	TTGGGCAATAAAGCCGATAC	*acc*	This study

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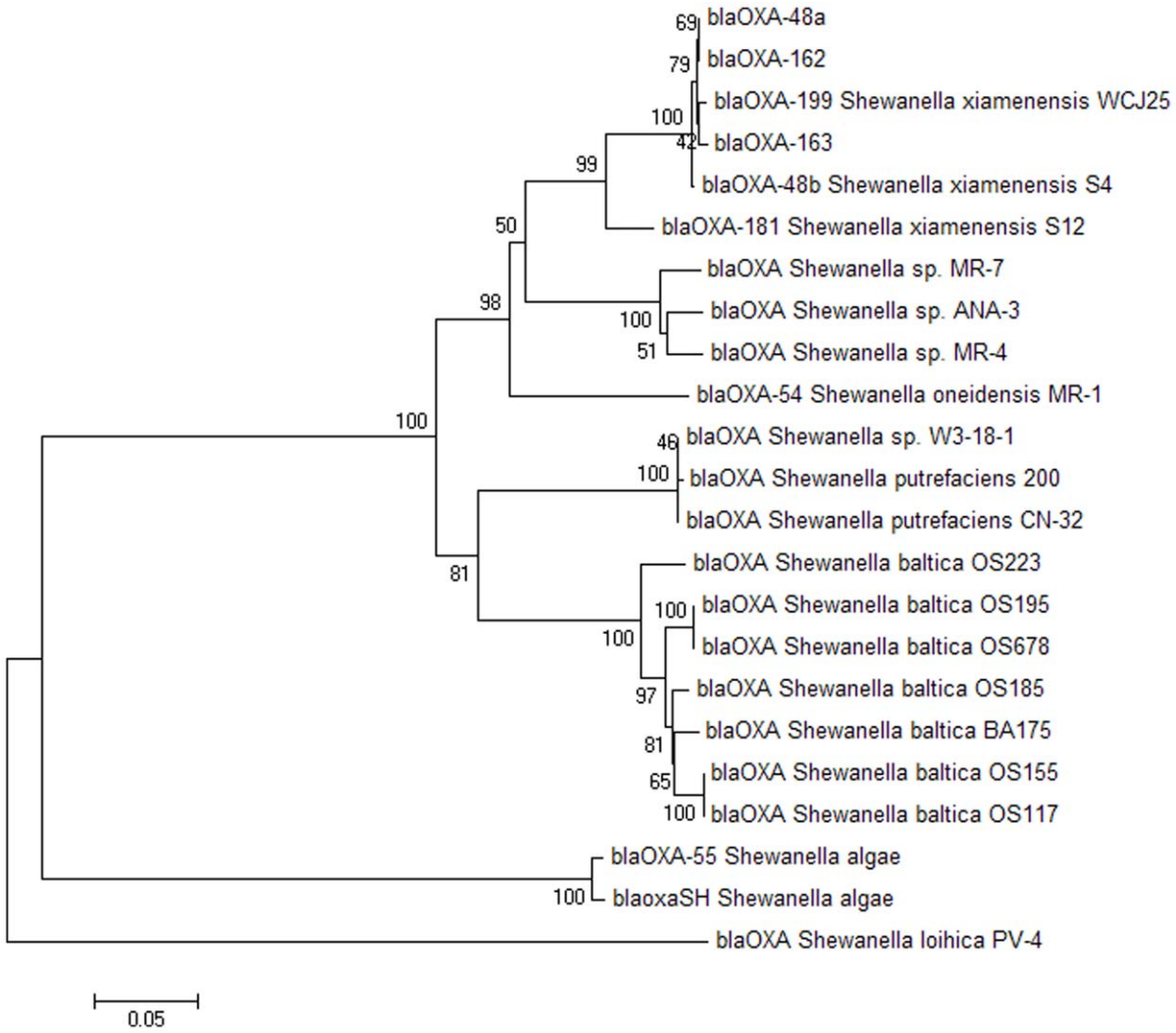

Figure 1. Neighbour-joining tree of *bla*$_{OXA-48}$-like genes. Constructed using the MEGA 4.0 program with bootstrap values and the bar of distance indicated. The host species and strains for the chromosome-encoded genes are indicated. Of note, *bla*$_{OXA-181}$ has also been found in the *Enterobacteriaceae* [10]. It appears that *bla*$_{OXA-48a}$, *bla*$_{OXA-162}$ and *bla*$_{OXA-163}$ have the *S. xiamenensis* origin.
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that the divergence of the *bla*$_{OXA-48}$-like gene might reflect the phylogeny of *Shewanella* species.

Genetic contexts of *bla*$_{OXA-48b}$ and *bla*$_{OXA-199}$ were shown in Figure 2. The 26 bp sequence upstream and 63 bp downstream of *bla*$_{OXA-199}$ were identical to those of *bla*$_{OXA-48a}$ and *bla*$_{OXA-48b}$, also suggesting a common origin of these genes. Both *bla*$_{OXA-48b}$ and *bla*$_{OXA-199}$ genes were adjacent to several genes upstream, i.e. an orf encoding the peptidase C15, *sprT* encoding a SprT-like protein of unknown function, *endA* encoding the endonuclease I and *rsmE* encoding a ribosomal RNA small subunit methyltransferase. Variants of these genes are also present adjacent to the *bla*$_{OXA-48}$-like gene in *S. oneidensis* MR-1 (AE014299), *Shewanella* sp. MR-4 (CP000446), *Shewanella* sp. MR-7 (CP000444) and *Shewanella* sp. ANA-3 (CP000469). The nucleotide identities of these genes among *Shewanella* species are shown in Figure 3. The insertion sequence IS*Ecp1* has been found upstream of *bla*$_{OXA-181}$ [5] but was not detected upstream of *bla*$_{OXA-48b}$ and *bla*$_{OXA-199}$ using long-range PCR.

As seen in the contexts of *bla*$_{OXA-48a}$ in *K. pneumoniae* strain 11978 (AY236073) [1], a putative *lysR* transcriptional regulator gene was located downstream of *bla*$_{OXA-48b}$ and *bla*$_{OXA-199}$. The *lysR* gene was adjacent to an *acc* gene that encoded an acetyl-CoA carboxylase multifunctional enzyme at the other side. In *K. pneumoniae* 11978, the *acc* gene is truncated by the insertion of IS*1999* (an insertion sequence also called IS*10A*) and two copies of IS*1999* bracketing *bla*$_{OXA-48a}$-*lysR*-*accΔ* formed a composite transposon, which could mobilize *bla*$_{OXA-48a}$ to different locations [1,11]. The *lysR* and *acc* genes are commonly present downstream of *bla*$_{OXA-48}$-like genes on chromosomes of *Shewanella* spp (Figure 3). As mentioned above, genes located either upstream or downstream of the *bla*$_{OXA-48}$-like genes from different *Shewanella* spp. displayed variable degrees of identities, suggesting that these genes might have different mutation rates.

An insertion sequence was inserted between *bla*$_{OXA-199}$ and *lysR*, evidenced by the presence of 3 bp direct target repeats (DR) (Figure 2). This 1299-bp IS was 98.1% identical to IS*Shes2* of the IS*3* family in nucleotide sequences and had 25-bp inverted repeat sequences (IR) with 23 bp perfectly matched (Figure 2). The IS*Shes2* element has also been seen in several *Shewanella* strains whose complete genome sequences are available at GenBank,

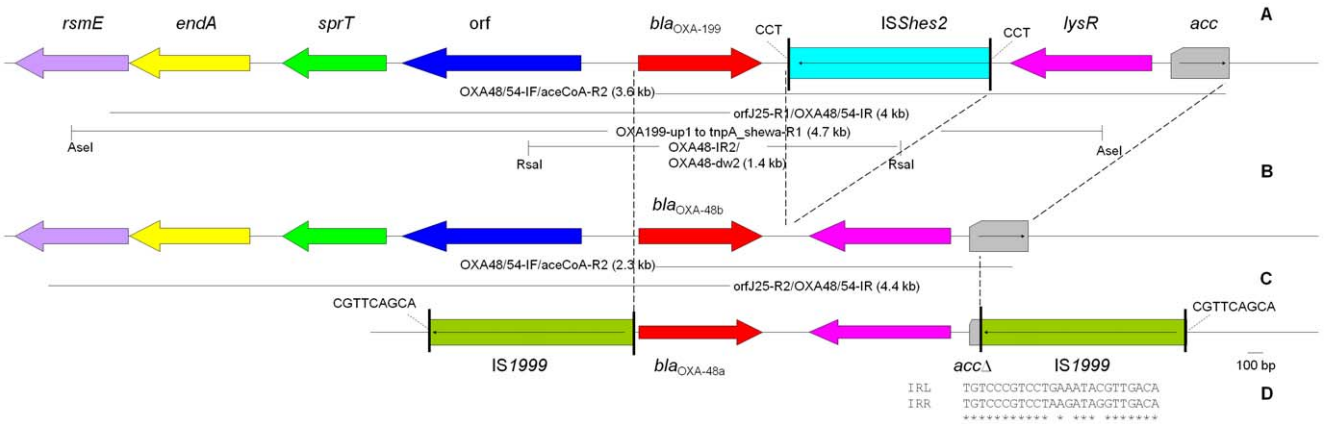

Figure 2. Genetic contexts of $bla_{OXA-199}$ and bla_{OXA-48}. The orientations of insertion sequences are indicated using arrows and the IRs are depicted as poles. Amplicons and sizes for PCR mapping are shown. Panel A, the genetic context of $bla_{OXA-199}$. Restriction sites of enzymes that were used to generate DNA fragments as templates for inverse PCR are indicated. Common structures in the contexts of $bla_{OXA-199}$, $bla_{OXA-48b}$ and $bla_{OXA-48a}$ are illustrated by broken lines. IS*Shes2* is inserted between $bla_{OXA-199}$ and *lysR*, generating 3-bp DR (CCT). The acc gene was only partially sequenced. The gene encoding peptidase C15 is indicated as 'orf'. Panel B, the genetic context of $bla_{OXA-48b}$ in *S. xiamenensis* strain S4. Panel C, the genetic context of $bla_{OXA-48a}$ in *K. pneumoniae* strain 11978 (AY236073). Two copies of IS*1999* formed a composite transposon and was inserted into the *tir* gene (responsible for transfer inhibition), which is part of the IncFII plasmid backbone, generating 9-bp DR (CGTTCAGCA). Panel D, the alignment of right-hand IR (IRR) and left-hand IR (IRL) of IS*Shes2*.
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including *Shewanella* sp. MR-4 (7 copies; CP000446), *Shewanella* sp. MR-7 (9 copies plus a truncated version; CP000444), *Shewanella* sp. ANA-3 (4 copies; CP000469), *S. baltica* OS195 (2 copies; CP000891), *S. baltica* OS678 (2 copies; CP002383) and *S. baltica* OS185 (1 copy; CP000753). Other *Shewanella* strains with complete genome sequences released, including *S. baltica* OS223 (CP001252), *S. baltica* BA175 (CP002767), *S. baltica* OS117 (CP002811), *S. baltica* OS155 (CP000563), *S. woodyi* ATCC 51908 (CP000961), *S. oneidensis* MR-1 (AE014299) and *S. pealeana* ATCC 700345 (CP000851) did not harbour IS*Shes2* but instead carried other insertion sequences sharing 65.7 to 85.9% nucleotide identity with IS*Shes2*.

Based on the significant similarity among contexts of $bla_{OXA-48a}$, $bla_{OXA-48b}$ and $bla_{OXA-199}$, it is reasonable to hypothesize that two copies of IS*1999*, one inserted at 26 bp upstream of a bla_{OXA-48}-like gene and another inserted in *acc*, could move bla_{OXA-48}-like-*lysR-accΔ* from the chromosome of *S. xiamenensis* to a plasmid. Such plasmid could have been transferred to *Enterobacteriaceae* later on resulting in the emergence of bla_{OXA-48}-like genes. Of note, $bla_{OXA-48a}$ and $bla_{OXA-181}$ have always been found in distinct genetic contexts as $bla_{OXA-48a}$ is bracketed by two copies of IS*1999* while $bla_{OXA-181}$ is downstream of IS*Ecp1* [3]. In light of the distinct genetics and the significant nucleotide differences (94.4% identity) between $bla_{OXA-48a}$ and $bla_{OXA-181}$, it seems unlikely that the two genes derived from each other through mutations but had different origins from two *Shewanella* strains [3].

Conclusions

From the phylogenetic analysis performed in this study, it appears that $bla_{OXA-48a}$ might have originated from the bla_{OXA} genes such as $bla_{OXA-48b}$ and $bla_{OXA-199}$ on the chromosome of certain *S. xiamenensis* strains. The significant nucleotide differences (<95% identity) between $bla_{OXA-181}$ and $bla_{OXA-48b}$ or $bla_{OXA-199}$ might represent the divergence of the chromosome-encoded bla_{OXA-48}-like genes between different *S. xiamenensis* strains in different geographical regions and could also suggest that $bla_{OXA-48a}$ and $bla_{OXA-181}$ were mobilized independently from different *S. xiamenensis* strains. The $bla_{OXA-48a}$ and $bla_{OXA-181}$ determinants

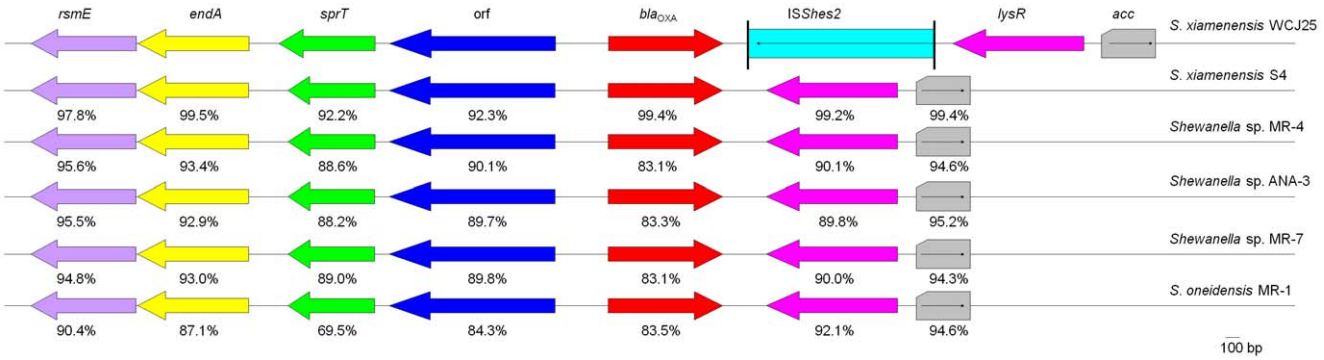

Figure 3. Genetic components surrounding bla_{OXA-48}-like genes in *Shewanella* strains WCJ25, S4, MR-1, MR-4 MR-7 and ANA-3. Variants of the same gene are depicted in the same colour with nucleotide identities compared to the counterparts of WCJ25 being indicated underneath. Of note, the *acc* genes of strains MR-1, MR-4 MR-7 and ANA-3 are complete with 4554 bp in length but only 333 bp were included into the analysis in parallel with the available partial *acc* sequence of strains WCJ25 and S4.
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appeared to have distinct origins and the emergence of bla_{OXA-48}-like genes in *Enterobacteriaceae* thus probably can not be attributed to a single mobilization event in the species *S. xiamenensis* but likely is a result of parallel or successive events occurring in multiple strains of *S. xiamenensis*.

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Author Contributions

Conceived and designed the experiments: ZZ. Performed the experiments: ZZ. Analyzed the data: ZZ. Contributed reagents/materials/analysis tools: ZZ. Wrote the paper: ZZ.

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