Discovery of $\text{bla}_{\text{OXA-199}}$, a Chromosome-Based $\text{bla}_{\text{OXA-48}}$-Like Variant, in *Shewanella xiamenensis*

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Abstract

**Introduction:** $\text{bla}_{\text{OXA-48}}$ is a globally emerging carbapenemase-encoding gene. The progenitor of $\text{bla}_{\text{OXA-48}}$ appears to be a *Shewanella* species. The presence of the $\text{bla}_{\text{OXA-48}}$-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 $\text{bla}_{\text{OXA-48-like}}$ genes and to obtain their complete sequences. A phylogenetic tree of the $\text{bla}_{\text{OXA-48-like}}$ genes was constructed. The genetic context of the $\text{bla}_{\text{OXA-48-like}}$ gene in strain WCJ25 was investigated by inverse PCR using self-ligated Asel- or Rsal-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 $\text{bla}_{\text{OXA-48-like}}$ variant, designated $\text{bla}_{\text{OXA-48b}}$, with four silent nucleotide differences from the $\text{bla}_{\text{OXA-48}}$ (designated $\text{bla}_{\text{OXA-48a}}$) found in the *Enterobacteriaceae* was identified in strain S4. Strain WCJ25 had a new $\text{bla}_{\text{OXA-48-like}}$ variant, $\text{bla}_{\text{OXA-199}}$, with five nucleotide differences from $\text{bla}_{\text{OXA-48}}$ and $\text{bla}_{\text{OXA-48b}}$. The OXA-199 protein has three amino acid substitutions (H37Y, V44A and D153G) compared with OXA-48. Both $\text{bla}_{\text{OXA-48b}}$ and $\text{bla}_{\text{OXA-199}}$ were found adjacent to genes encoding a peptidase (designated as orf), a protein of unknown function (*orf*), and an acetyl-CoA carboxylase-encoding gene downstream. In addition, the insertion sequence IS_Shes2 was found inserted downstream of $\text{bla}_{\text{OXA-199}}$ but not of $\text{bla}_{\text{OXA-48b}}$. The 26 bp sequences upstream and 63 bp downstream of $\text{bla}_{\text{OXA-48b}}$, $\text{bla}_{\text{OXA-48b}}$, and $\text{bla}_{\text{OXA-199}}$ were identical.

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

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Introduction

$\text{bla}_{\text{OXA-48}}$, 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 $\text{bla}_{\text{OXA-48-like}}$ variants have been identified recently, including $\text{bla}_{\text{OXA-102}}$ (GenBank Accession no. GU197550; one nucleotide different from $\text{bla}_{\text{OXA-48}}$), $\text{bla}_{\text{OXA-163}}$ (98.1% nucleotide identity with $\text{bla}_{\text{OXA-48}}$) [4], $\text{bla}_{\text{OXA-111}}$ (94.4% nucleotide identity with $\text{bla}_{\text{OXA-48}}$) [5], $\text{bla}_{\text{OXA-201}}$ (nucleotide sequence not available but encoding two amino acid substitutions compared with OXA-48) and $\text{bla}_{\text{OXA-232}}$ (nucleotide sequence not available but encoding a single amino acid substitution compared with OXA-111) [3]. The $\text{bla}_{\text{OXA-41}}$ gene was previously proposed as both derived from the chromosome-encoded $\text{bla}_{\text{OXA-54}}$ of *Shewanella oneidensis*, but the two genes have only 94% nucleotide identity [6]. Through analyzing the complete genome sequences of a few strains belonging to various *Shewanella* species available in the GenBank, the $\text{bla}_{\text{OXA-41}}$-like genes are present on the chromosome of several *Shewanella* species with at least 80% identity to $\text{bla}_{\text{OXA-41}}$. Thus, the actual progenitor of $\text{bla}_{\text{OXA-48}}$ 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 $\text{bla}_{\text{OXA-41-like}}$ 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 then used as a template for inverse PCR. The links between genetic elements were confirmed by overlapping PCR (Figure 2, primers listed Table 1).

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), and bla_{OXA-181} (JN205300) and those without assigned gene names on chromosomes of Shewanella spp., i.e., S. algae osaSH (AY066004), S. baltica BA175 (CP002767), S. baltica OS117 (CP002811), S. baltica OS155 (CP0000563), S. baltica OS185 (CP000753), S. baltica OS195 (CP0000891), S. baltica OS223 (CP001252), S. baltica OS678 (CP002383), S. lohica 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 of bla_{OXA-48} was investigated using inverse PCR. Genomic DNA of WCJ25, prepared using a commercial kit (Tiangen, Beijing, China), was restricted with Asel- or Rsal (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-48} 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 3730d 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. xiamensis 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. xiamensis 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 N173D) compared to OXA-48. During the process of this work, bla_{OXA-181} was identified in a S. xiamensis isolate from India [10]. However, bla_{OXA-181} was significantly divergent from bla_{OXA-48} (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}, bla_{OXA-48b}, bla_{OXA-181}, bla_{OXA-199} and bla_{OXA-181} were of a cluster from the chromosome-encoded bla_{OXA-48}-like genes of Shewanella species other than S. xiamensis. 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     | AGCAAGAGATTACCAATA          | bla_{OXA-48}-like genes, | Valenzuela JK |
| OXA48/54IR     | GGCATATCCATATTCATC          | screening       | unpublished |
| OXA48-up1      | ATTAAAGGAGGGATTTATG         | bla_{OXA-48}-like genes, | This study |
| OXA48-dw1      | GAGCATCAGGATTGTCGA          | complete sequences | This study |
| OXA48-R2       | GCAATACGCCGTGTATT           | bla_{OXA-48}-like genes | This study |
| OXA48-dw2      | GTTACGGGTATTGTGGTG          | Downstream of bla_{OXA-48} | This study |
| OXA199-up1     | TAAGGCTGAGCGCCCTAGAA        | Upstream of bla_{OXA-199} | This study |
| trnA_Shew-R1   | AATAGTTCCGAGGGGTGT          | trnA of ISShes2  | This study |
| orf25-R1       | ACCGCTAATTGTTTGTGG          | rsmE             | This study |
| orf25-R2       | CGCTCATGGAATCTCC            | rsmE             | This study |
| aceCoA-R2      | TGGGCAATAAAGCGGATAC         | acc              | This study |

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that the divergence of the blaOXA-48-like gene might reflect the phylogeny of Shewanella species.

Genetic contexts of blaOXA-48b and blaOXA-199 were shown in Figure 2. The 26 bp sequence upstream and 63 bp downstream of blaOXA-199 were identical to those of blaOXA-48a and blaOXA-48b, also suggesting a common origin of these genes. Both blaOXA-48a and blaOXA-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 blaOXA-48-like gene in shewanella spp (Figure 3). As mentioned above, genes located either upstream or downstream of the blaOXA-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 blaOXA-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 ISShes2 of the IS7 family in nucleotide sequences and had 25-bp inverted repeat sequences (IR) with 23 bp perfectly matched (Figure 2). The ISShes2 element has also been seen in several Shewanella strains whose complete genome sequences are available at GenBank.
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. oneidensis ATCC 700345 (CP000851) did not harbour IS\text{Shes2} but instead carried other insertion sequences sharing 65.7 to 85.9% nucleotide identity with IS\text{Shes2}.

Based on the significant similarity among contexts of \text{bla}\text{OXA-199}, \text{bla}\text{OXA-48b} and \text{bla}\text{OXA-48a}, it is reasonable to hypothesize that two copies of IS\text{1999}, one inserted at 26 bp upstream of a \text{bla}\text{OXA-199}-like gene and another inserted in \text{acc}, could move \text{bla}\text{OXA-199}-like-\text{lysR-acc}\text{D} from the chromosome of S. xiamenensis to a plasmid. Such plasmid could have been transferred to Enterobacteriaceae later on resulting in the emergence of \text{bla}\text{OXA-48a}-like genes. Of note, \text{bla}\text{OXA-48a} and \text{bla}\text{OXA-181} have always been found in distinct genetic contexts as \text{bla}\text{OXA-48a} is bracketed by two copies of IS\text{1999} while \text{bla}\text{OXA-181} is downstream of IS\text{Ecp1} [3]. In light of the distinct genetics and the significant nucleotide differences (94.4% identity) between \text{bla}\text{OXA-181} and \text{bla}\text{OXA-48b} or \text{bla}\text{OXA-199}, 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 \text{bla}\text{OXA-48a} might have originated from the \text{bla}\text{OXA} genes such as \text{bla}\text{OXA-48b} and \text{bla}\text{OXA-199} on the chromosome of certain S. xiamenensis strains. The significant nucleotide differences (<95% identity) between \text{bla}\text{OXA-181} and \text{bla}\text{OXA-48b} or \text{bla}\text{OXA-199} might represent the divergence of the chromosome-encoded \text{bla}\text{OXA-48a}-like genes between different S. xiamenensis strains in different geographical regions and could also suggest that \text{bla}\text{OXA-48a} and \text{bla}\text{OXA-181} were mobilized independently from different S. xiamenensis strains. The \text{bla}\text{OXA-48a} and \text{bla}\text{OXA-181} determinants

Figure 2. Genetic contexts of \text{bla}\text{OXA-199} and \text{bla}\text{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 \text{bla}\text{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 \text{bla}\text{OXA-199}, \text{bla}\text{OXA-48b}, and \text{bla}\text{OXA-48a} are illustrated by broken lines. IS\text{Shes2} is inserted between \text{bla}\text{OXA-199} and \text{lysR}, generating 3-bp DR (CCT). The \text{acc} gene was only partially sequenced. The gene encoding peptidase C15 is indicated as ‘orf’. Panel B, the genetic context of \text{bla}\text{OXA-48b} in S. xiamenensis strain S4. Panel C, the genetic context of \text{bla}\text{OXA-48a} in K. pneumoniae strain 11978 (AY236073). Two copies of IS\text{1999} formed a composite transposon and was inserted into the \text{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\text{Shes2}.

Figure 3. Genetic components surrounding \text{bla}\text{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 \text{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 \text{acc} sequence of strains WCJ25 and S4.
appeared to have distinct origins and the emergence of bla\textsubscript{OXA-48}-like genes in \textit{Enterobacteriaceae} thus probably can not be attributed to a single mobilization event in the species \textit{S. xiamenensis} but likely is a result of parallel or successive events occurring in multiple strains of \textit{S. xiamenensis}.

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\section*{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.