**Universidade de Aveiro** Departamento de Biologia
**2015**

**Maria do Carmo Oliveira Vendas**

# Fontes de resistência a antibióticos no ambiente

# Sources of antibiotic resistance in the environment

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Isabel da Silva Henriques investigadora auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Marta Cristina Oliveira Martins Tacão investigadora em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro

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“Enfim duma escolha faz-se um desafio
Enfrenta-se a vida de fio a pavio
Navega-se sem mar, sem vela ou navio
Bebe-se a coragem até dum copo vazio
E vem-nos à memória uma frase batida
Hoje é o primeiro dia do resto da tua vida”, Sérgio Godinho

Aos meus pais.

**o júri**

presidente

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**palavras-chave**

Resistência a antibióticos de último recurso; carbapenemos; OXA-48; *Shewanella*

**resumo**

A descoberta dos antibióticos foi um grande avanço na medicina. No entanto, pouco depois da sua introdução na clínica, bactérias resistentes foram detetadas. Nos dias de hoje, a resistência aos antibióticos constitui um grave problema de saúde pública. Em ambientes hospitalares os níveis de resistência são atualmente elevados, reduzindo drasticamente as opções terapêuticas.

Os carbapenemos são antibióticos de último recurso utilizados em Portugal, unicamente em ambiente hospitalar, para o tratamento de infeções graves. A resistência a esta classe de antibióticos tem aumentado nos últimos anos. Em bactérias Gram-negativas a produção de carbapenemases é um mecanismo comum de resistência. OXA-48 é uma carbapenemase da classe D de Ambler e representa uma grande preocupação para a saúde humana sendo frequentemente detetada em isolados clínicos de Enterobacteriaceae. Existem alguns estudos que sugerem que os genes que codificam para variantes de OXA-48 foram originados em genes presentes no cromossoma de membros do género *Shewanella*, tendo sido disseminados posteriormente para membros de Enterobacteriaceae e associados a elementos genéticos móveis.

O objetivo deste estudo foi caracterizar estirpes de *Shewanella* isoladas de diferentes fontes ambientais para confirmar o papel do género como reservatório de OXA-48. Para isso, a afiliação filogenética de 33 estirpes de *Shewanella* foi realizada por sequenciação dos genes 16S rDNA e *gyr*B. As espécies mais comuns foram *S. hafniensis* e *S. xiamenensis* mas foram também identificadas *S. aestuarii*, *S. baltica*, *S. indica, S. haliotis, S. putrefaciens, S. algidipiscicola, S. irciniae, S. algae* e *S. fodinae.*.

O gene *bla*$_{\text{OXA-48-like}}$ foi detetado em 21 isolados: *S. hafniensis* (8/8), *S. xiamenensis* (5/5), *S. baltica* (4/4), *S. algae* (1/1), *S. fodinae* (1/1), *S. putrefaciens* (1/2) and *S. algidipiscicola* (1/2). Análises de sequenciação revelaram a presença de genes que codificam para enzimas idênticas a OXA-48, OXA-181 e OXA-204 mas também foram detetadas novas variantes diferentes de OXA-48 de 2 a 81 aminoácidos. A análise do contexto genético revelou o gene C15 a montante e o gene *LysR* a jusante, contexto este idêntico ao já descrito para *bla*$_{\text{OXA-48-like}}$ em *Shewanella* spp.

A avaliação da suscetibilidade a antibióticos foi realizada para todos os isolados, usando o método de difusão em agar com discos de antibióticos. Em geral, observou-se uma grande suscetibilidade a todos os antibióticos, exceto amoxicilina e aztreonam. A multirresistência foi detetada em apenas 1 isolado. Outros genes de resistência e a presença de integrões não foram identificados. A presença de plasmídeos foi detetada em 30.3% dos isolados (10 /33).

Estes resultados reforçam o papel de *Shewanella* spp. como origem de genes *bla*$_{\text{OXA-48-like}}$.

**keywords**

Resistance of last-resort antibiotics; carbapenems; OXA-48; *Shewanella*

**abstract**

The discovery of antibiotics was a major breakthrough in medicine. However, short after their introduction in clinical practice resistant bacteria were detected. Nowadays, antibiotic resistance constitutes a serious public health problem. In hospital settings, with high resistance levels, reducing drastically the therapeutic options.

Carbapenems are last-resort antibiotics used in Portugal, only in hospitals, to treat serious infections. Bacterial resistance towards this class of antibiotics has increased during last years. In Gram-negative bacteria the production of carbapenemases is a common resistance mechanism. OXA-48 is a carbapenemase of Ambler class D and represents a major concern for human health. It is frequently detected in clinical isolates of Enterobacteriaceae. There are few studies suggesting that genes encoding for OXA-48 variants originated from genes present in the chromosome of members of genus *Shewanella*, and have disseminated to Enterobacteriaceae members, associated with mobile genetic elements.

The aim of this study was to characterize strains from different sources of *Shewanella* to confirm its role as OXA-48 progenitor. For this, the phylogenetic affiliation of 33 strains of *Shewanella* was performed by 16SrDNA and *gyrB* sequencing. The most common species were *S. hafniensis* and *S. xiamenensis,* but also *S. aestuarii*, *S. baltica, S. indica, S. haliotis, S. putrefaciens, S. algidipiscicola, S. irciniae, S. algae and S. fodinae* were identified.

*bla*$_{\text{OXA-48-like}}$ genes were detected in 21 isolates: *S. hafniensis* (8/8), *S. xiamenensis* (5/5), *S. baltica* (4/4), *S. algae* (1/1), *S. fodinae* (1/1), *S. putrefaciens* (1/2) and *S. algidipiscicola* (1/2). Sequence analysis revealed that genes encoded enzymes identical to OXA-48, OXA-181 and OXA-204 but also new variants differing from OXA-48 from 2 to 81 aminoacids. Genetic context analysis revealed the C15 gene upstream and *lysR* gene downstream, identical to what has been identified so far flanking *bla*$_{\text{OXA-48-like}}$ genes in *Shewanella* spp. The assessment of antibiotic susceptibility was performed for all isolates using the disk diffusion method. In general, it was observed a great sensitivity for all antibiotics except to amoxicillin and aztreonam. Multidrug resistance was detected in only 1 isolate. Other resistance genes and the presence of integrons were not identified. Plasmids were detected in 30.3% isolates (10/ 33). These results reinforce the role of *Shewanella* spp. as origin of *bla*$_{\text{OXA-48-like}}$ genes.

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**INDEX OF ABREVIATIONS**

μL: micro litter

μM: micro molar

AMC: Amoxicilin

AML: Amoxicilin/clavulanic acid

ARGs: Antibiotic resistance genes

AZT: Aztreonam

BOX-PCR: BOX elements – polymerase chain reaction

bp: basepare

BLAST: Basic Local Alignment Search Tool

C: Chloramphenicol

CAN: Kanamycin

CAZ: Ceftazidime

CIP: Ciprofloxacin

CLSI: Clinical and Laboratory Standards Institute

CTX: Cefotaxime

$dH_2O$: distilled water

DNA: deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ESBL: Extended-Spectrum β-lactamase(s)

FEP: Cefepime

g: grams

GATC: DNA sequencing and Bioinformatics

GEN: Gentamicin

HGT: Horizontal Gene Transfer

IPM: Imipenem

KF: Cefalotin

LB: Luria Bertani Broth

mg: milligram

mM: micro molar

mL: milliliter

min: minutes

M: Molar

MGEs: Mobile Genetic Elements

NAL: Nalidixic acid

ng: nanograms

ºC: degrees Celsius

OXA: oxacillin-hydrolysing

PBP: Protein Binding Protein

PCR: Polymerase Chain Reaction

pmol: picomole

s: seconds

SXT: Sulfonamide + trimethoprim

TE: Tetracycline

TE: Tris-EDTA

WHO: World Health Organization

WWTPs: Wastewater treatment plants

# I. INTRODUCTION

## 1. Antibiotics

An antibiotic, synthetic or natural, is defined as a molecule that inhibits or kills microorganisms, and is used for treatment of infectious diseases (Davies et *al*. 2010). Antibiotics are considered one of medicine's greatest achievements, alleviating human morbidity and mortality (Baquero *et* al. 2008; van Hoek *et* al. 2011).

Alexander Fleming, who won a Nobel Prize for his discovery of penicillin, warned in an interview, in 1945, that the misuse of these drugs could result in selection for resistant bacteria (Rosenblatt-Farrell *et* al. 2009). So, although antibiotics are a good response to bacterial infections, their misuse decreases their effectiveness (van Hoek *et* al. 2011).

### 1.1. Classes of antibiotics and their mechanisms of action

Numerous classes of antibiotics with different mechanisms of action are now available, as shown in table I.1. (Byarugaba *et* al. 2009; van Hoek *et* al. 2011).

**Table I.1** Examples of antibiotics classes and mechanisms of action

| Antibiotic Class | Mode of action | Examples |
|---|---|---|
| Beta-lactams | Inhibit the synthesis of bacterial cell wall (peptidoglycan biosynthesis) | Carbapenems (imipenem), Penicillins (amoxicillin), Cephalosporins (cefalotin, cefotaxime, ceftazidime), Monobactams (aztreonam) |
| Quinolones | Inhibition of synthesis or metabolism of nucleic acids | Ciprofloxacin, nalidixic acid |
| Aminoglycosides | Inhibit protein synthesis | Gentamicin, kanamycin |
| Sulfonamides | Modify the energy metabolism of the cell | Sulfonamide |
| Phenicols | Inhibit protein synthesis | Chloramphenicol |
| Tetracyclines | Inhibit protein synthesis | Tetracycline |
| Pyrimidines | Modify the energy metabolism of cell | Trimethoprim |

### 1.1.1 β-lactams

Penicillin was the first antibiotic to be described in the β-lactams class, which includes several clinically relevant antibiotics with a long history in the treatment of infectious diseases (Fuda *et* al. 2004; Poole *et* al. 2004).

These antibiotics inhibit bacteria cell wall synthesis by binding to PBPs (penicillin-binding proteins); weakening cell wall and leading to cytolysis.

The β-lactams class includes penicillins, carbapenems, cephalosporins, monobactams and the inhibitors of β-lactamases.

#### 1.1.1.1 Carbapenems

Carbapenems are broad spectrum antibiotics. They are considered last-resort antibiotics, applied to treat severe infections (Sousa 2005; Wallace *et* al. 2011). Examples of antibiotics in this class are imipenem, meropenem and ertapenem.

#### 1.1.1.2 Penicillins and derivates

Penicillins and derivates are susceptible to hydrolysis by bacterial enzymes and have a reduced spectrum of activity, as for example amoxicillin (Sousa, 2005).

#### 1.1.1.3 Cephalosporins

Cephalosporins are semi-synthetic antibiotics that may be divided in first, second, third, fourth and fifth generation according to their spectrum of activity and their introduction in therapeutic practice. It is noted an improvement in efficacy since the first generation until the most recent cephalosporins (Sousa, 2005). Examples of antibiotics in this class are cefalotin and cefotaxime.

#### 1.1.1.4 Monobactams

Monobactams only have activity against Gram-negative bacteria. These are compounds in which the β-lactam ring is not fused to any other ring (Sousa, 2005). Example: Aztreonam.

#### 1.1.1.5 Inhibitors of β-lactamases

The β-lactamase inhibitors are used in combination with various antibiotics for efficacy increase. These inhibitors can be classified as irreversible or reversible. The irreversible are more effective and can result in the destruction of enzymatic activity of β-lactamases (Neu *et* al. 1992). Examples: clavulanic acid, tazobactam and sulbactam.

## 2. Antibiotic Resistance

The discovery of antibiotics was a turning point in human history. However, the use of these compounds led to rapid appearance of resistant strains. This resistance has been promoted by the large-scale use of antibiotics. The antibiotic resistance is a serious public health concern and therefore it is important to understand how antibiotic resistance develops and disseminates, to minimize the threat of bacterial infections (Bennet *et* al. 2008).

Over the years, as shown in Figure I.1, it has been found that for some antibiotics the emergence of resistant strains is fast (Davies *et* al. 2010; Liu *et* al. 2008).

There are many factors that promote the dissemination and emergence of antibiotic resistance:

- Inadequate prescription;
- Food products circulation;
- Water pollution;
- Antibiotic use in agriculture and household chores;
- Overpopulation;
- Increasing national and international travel;
- Easy access to antibiotics;
- Absence of infection-control practices.

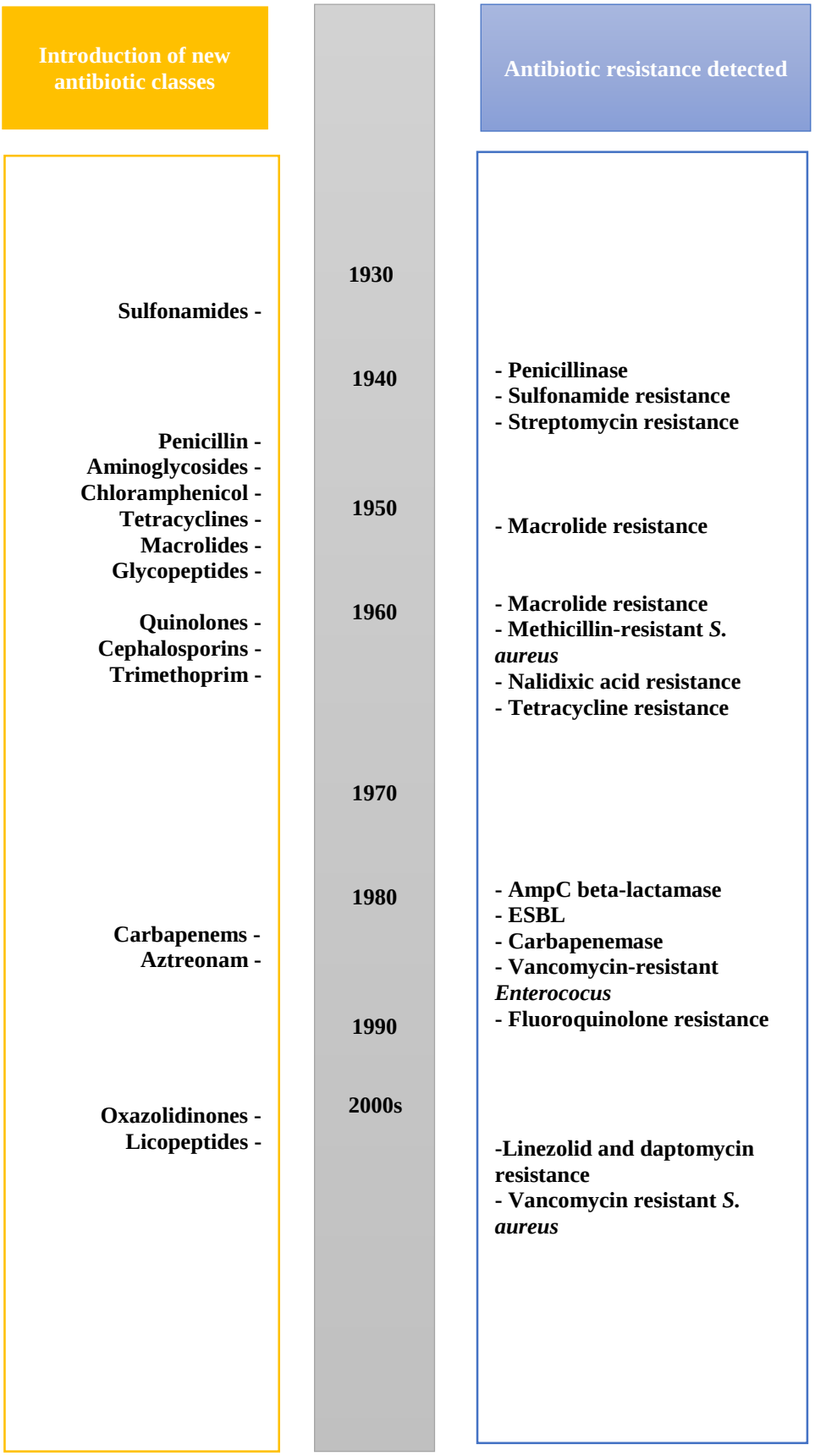


**Figure I.1:** Timeline displaying the introduction of some antibiotics commonly used in clinical and the first resistance registered towards those antibiotics (adapted from ECDC 2013).

Bacteria can become resistant to antibiotics through a number of different resistance mechanisms (Giedraitienė *et* al. 2011; Marti et al. 2014; Taylor et al. 2011):

(i) exclusion of the antibiotic by the cell membrane;

(ii) intracellular modification and/or deactivation of the antibiotic;

(iii) reduction in sensitivity of the cellular target;

(iv) extrusion from the cell; and

(iv) intracellular sequestration

Several resistance mechanisms are known for each antibiotic class, as shown in table I.2. The production of β-lactamases that hydrolyze β-lactams is the most common mechanism of resistance towards antibiotics in this class (Bush *et* al. 2010).

**Table I.2:** Common resistance mechanisms by antibiotic class.

| Antibiotic Class | Resistance mechanisms |
|---|---|
| β-lactams | Target modification, hydrolysis, efflux pumps, alteration porins |
| Quinolones | Target modification, acetylation, efflux pumps |
| Aminoglycosides | Target modification, efflux pumps, acetylation, phosphorylation |
| Sulfonamides | Efflux pumps, target modification, hydrolysis |
| Phenicols | Target modification, efflux pumps, acetylation |
| Tetracyclines | Target modification, efflux pumps, monooxygenation |
| Pyrimidines | Target modification, efflux pumps |

Bacteria developed other strategies that allow them to survive in the presence of these compounds, as for example the formation of biofilms: bacteria are organized in community in exopolysaccharide matrix becoming more resistant to the effects of drugs (Mah and Toole, *et* al. 2001).

A major problem of antibiotic resistance is its constant spread, mainly due to the overuse and misprescription of antibiotics. The World Health Organization (WHO) estimates that this problem is the cause of 15 million deaths per year and that infectious diseases are among the first 10 causes of death all over the world (WHO 2014a; WHO 2014b).

## 2.1 Intrinsic and acquired resistance

Bacterial resistance to antibiotics can be intrinsic or innate and/or acquired. Intrinsic resistance refers to an innate characteristic of a given species, frequently encoded in the chromosome, and that is common to most (or all) strains of that species. Acquired resistance refers to a resistance mechanism that was acquired by a strain and that is uncommon among strains of the same species. Susceptible bacteria may become resistant to antibiotics through mutations or through the acquisition of resistance genes.

## 2.2 Mechanisms of antibiotic resistance transfer

Horizontal gene transfer may occur through three mechanisms:

a) transformation, in which a cell takes up free DNA molecules from the surrounding medium;

b) conjugation, which involves the direct transfer of DNA from one cell to another;

c) transduction, in which the transfer is mediated by bacteriophages.

These events are principal causes for the acquisition of resistance mechanisms among species (Lupo *et* al. 2012; Moura *et* al. 2011).

Mobile genetic elements (MGEs) are genetic platforms that share the ability to move within a genome and/or to other bacterial cells. It has been described that antibiotic resistance genes (ARGs) are spread by MGEs like plasmids, insertion sequences, insertion sequence common region elements, transposons, integrons, genomic islands, integrating conjugative elements and bacteriophages (Alves *et* al. 2013; Marti *et* al. 2013).

Conjugative plasmids are elements that move from one bacteria to other bacterial cell. They are molecules that replicate autonomously in a host cell and have double-stranded deoxyribonucleic acid (DNA) (Couturier *et* al. 1988; Bennet *et* al. 2008).

Integrons are important due to their role in the spread of antibiotic resistance too, particularly among clinically-relevant Gram-negative bacteria. These genetic elements contain a site specific recombination system which facilitate the integration, excision and rearrangements of gene cassettes (Gillings *et* al. 2014; Moura *et* al. 2011). Integrons are divided in five classes, where class 1 has greater clinical importance (Gillings *et* al. 2014).

When discussing antibiotic resistance dissemination, it is easy to conclude that bacteria have great capacity of adaptation, by acquiring different ARGs. This represents a serious public health problem, since the number of microorganisms becoming extremely resistant to the existing antibiotics has been increasing (Alves, 2013).

### 2.3 Mechanisms of resistance to β-lactams

β-lactams are the most used antibiotics for the treatment of infections caused by Gram-negative bacteria (Bush *et* al. 1999). These antibiotics act inhibiting synthesis of bacterial cell wall. Bacterial resistance to β-lactams includes target modification, hydrolysis, alteration of membrane porins and efflux pumps. β-lactamases play a major role in intrinsic and acquired resistance to β-lactams in bacteria, predominantly in Gram-negative bacteria. These enzymes inactivate the antibiotics by hydrolyzing the β-lactam ring (Lakshmi *et* al. 2014; Li *et* al. 2009; Livermore *et* al. 1995).

β-lactamases can be classified according to their functional characteristics in four goups (Bush-Jacoby groups; Bush *et* al. 1995); or according to their molecular characteristics in four molecular classes (the Ambler classes A to D; Ambler 1980). β-lactamases from classes A, C and D have serine at their active site while class B β-lactamases are metallo-enzymes that require zinc for their catalytic activities (Li *et* al. 2009; van Hoek, Mevius *et* al. 2011).

β-lactamases can be divided according with their spectrum of activity in broad spectrum β-lactamases and extended-spectrum β-lactamases (ESBLs). The first confer resistance to older cephalosporins and penicillins and the last confer resistance to first, second and third generation cephalosporins, penicillins and monobactams (van Hoek, Mevius *et* al. 2011).

In the last decade many new enzymes clinically relevant have been described and associated with disease outbreaks. One example is CTX-M, a class A ESBL with increased prevalence and number of variants described recently. This enzyme confers resistance for example to penicillins and 3rd generation cephalosporins. CTX-M encoding genes have been associated to different MGEs, as insertion sequences IS*26* and IS*Ecp1* (Cantón and Coque *et* al. 2006), which promote their dissemination. CTX-M-producers are phylogenetically diverse (e.g. Enterobacteriaceae, *Pseudomonas* spp., *Aeromonas* spp.) and have been identified worldwide. Other example of a β-lactamase with high clinical relevance is NDM. This carbapenemase was described for the first time in India in 2008 and has been associated to different plasmids (for example IncA/C, IncF) and a wide range of hosts (for example Enterobacteriaceae members and *Aeromonas* spp.) (Nordman *et* al. 2011). Nowadays, it has been detected in all continents and there are more than 15 variants described (http://www.lahey.org/). Highly relevant is the fact that NDM-producers showed high rates of multiresistance, including resistance to tetracyclines, aminoglycosides and quinolones (Nordman *et* al. 2011; van Hoek, Mevius *et* al. 2011; Yong *et* al. 2009).

Among the class D β-lactamases, the OXA enzymes represent a numerous and diverse family of enzymes with clinical relevance (Tacão *et* al. 2014; van Hoek, Mevius *et* al. 2011). Most OXA enzymes confer resistance against amino and ureidopenicilin, having high level hydrolytic activity against cloxacilin, oxacilin and methicillin (Lakshmi *et* al. 2014).

### 2.3.1 Mechanisms of resistance to carbapenems

Carbapenems as ertapenem, meropenem and imipenem are often used as "antibiotics of last resort" in cases of infections caused by multiresistant bacteria. In Portugal some of these antibiotics are only used in hospitals (Henriques *et* al. 2012). Increasingly, Gram-negative bacteria and Gram-positive bacteria are becoming resistant to most clinically available carbapenems and this pattern poses a major public health threat (Braddley *et* al. 1999; Wallace *et* al. 2011).

The mechanisms of resistance to carbapenems include the production of carbapenemases. Additionally, mutations that alter the function and/or expression of porins and PBPs and efflux pumps have also been described as carbapenem resistance mechanisms.

The combinations of these mechanisms cause, in certain bacterial species such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* high levels of resistance to carbapenems but also to antibiotics included in other classes besides β-lactams (Wallace *et* al. 2011).

Carbapenem resistance is often mediated by intrinsic or acquired carbapenemase genes. Some Gram-negative bacteria acquire carbapenemase genes by horizontal gene transfer. These strains represent a great concern when associated to certain infections (Bush *et* al. 2013).

Carbapenemases include metallo-beta-lactamases (Ambler class B) and serine carbapenemases (Ambler class A and D) (Nordman *et* al. 2011).

Among class A carbapenemases, the KPC family is the most clinically important. This enzyme was first detected in a *K. pneumoniae* clinical isolate in the USA in 1996 (Yigit *et* al. 2011). Currently, KPC variants have been detected worldwide in *Pseudomonas* spp., Enterobacteriaceae and *Acinetobacter* spp. (Patel and Bonomo *et* al. 2013).

Metallo-beta-lactamases present a strong carbapenemase activity and are not inhibited by beta-lactamase inhibitors. Nowadays, these enzymes have been detected worldwide, especially in *Pseudomonas* spp., *Acinetobacter* spp. and Enterobacteriaceae (Bush 2010; Nordman *et* al. 2011). In terms of medical importance, the most relevant enzymes are the ones included in IMP-, NDM- and VIM-families (Patel and Bonomo *et* al. 2013).

Class D carbapenemases include the OXA family and have been associated to serious outbreaks of carbapenem-resistant bacteria such as *Pseudomonas* spp. (e.g. OXA-50), *Acinetobacter* spp. (e.g. OXA-23) and Enterobacteriaceae (e.g. OXA-48) (Evans *et* al. 2014).

#### 2.3.1.1 Mechanisms of resistance to carbapenems: OXA-48

*bla*$_{\text{OXA-48}}$ encoding the carbapenem-hydrolyzing enzyme OXA-48, was first described in Turkey in 2001 in a *Klebsiella pneumoniae,* and since then it has been found in other Enterobacteriaceae members in many countries all over the world, as shown in Figure I.2 (Potron *et* al. 2013; Zong *et* al. 2012). These enzymes hydrolyze carbapenems and penicilins but not 3$^{rd}$ generation cephalosporins (Poirel *et* al. 2012a).

There are more than 12 variants of OXA-48 identified so far differing in 1 to 5 amino acids: OXA-48, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-370 and OXA-405 (Dortet *et* al. 2015; Poirel *et* al. 2012; Sampaio *et* al. 2014; Tacão *et al*. 2014).

*bla*$_{\text{OXA-48-like}}$ genes have been associated with many outbreaks, in several countries (Poirel *et* al. 2012). In clinical settings these genes have been mainly identified in Enterobacteriaceae (e.g in *Citrobacter freundii* and *Escherichia coli*; Poirel et al. 2012b). Besides clinical settings, also *bla*$_{\text{OXA-48-like}}$ genes have been identified in Enterobacteriaceae environmental isolates as for example in *Escherichia coli* isolates from wastewaters in Austria (Galler *et* al. 2013) and *Serratia marcescens* isolates from rivers in Morocco (Potron *et* al. 2011).

A few studies suggest *Shewanella* spp. as possible origin of OXA-48-like genes (Poirel *et* al. 2012). Until now, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-199,}}$ *bla*$_{\text{OXA-181}}$ and *bla*$_{\text{OXA-204}}$ have been detected in *Shewanella* spp. (Evans *et* al. 2014; Tacão *et* al. 2013; Zong *et* al. 2012). *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{OXA-204}}$ were detected in *Shewanella xiamenensis* strains isolated from river water in Portugal (Tacão *et* al. 2013) and Morocco (Potron *et* al. 2011).

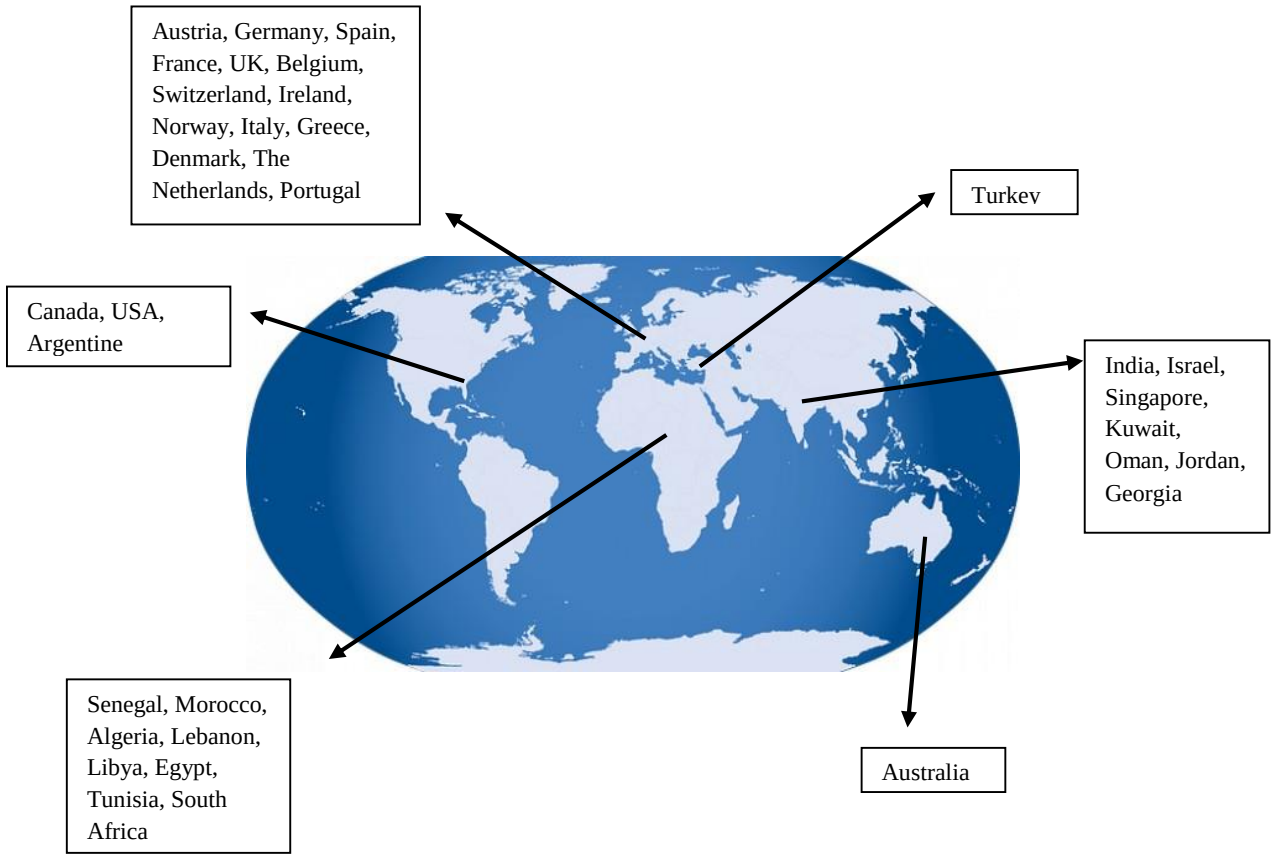


**Figure I.2** Distribution all over the world of the detection of OXA-48-like enzymes.

## 3. Environmental spread of antibiotic-resistant bacteria

The excessive use of antibiotics for treatment of human and animal infections resulted in an extensive spread of resistant bacteria, especially in aquatic environment, as shown in figure I.3. These environments are constantly impacted with a diverse mixture of compounds including antibiotics, detergents and metals, but also antibiotic resistant bacteria and ARGs that come from different sources as hospital wastes, aquaculture discharges and agricultural runoffs (Alves *et* al. 2013; Marti *et* al. 2013; Matyar *et* al. 2004). These are ideal conditions for rapid dissemination and also evolution of antibiotic resistance (Baquero *et* al. 2008).

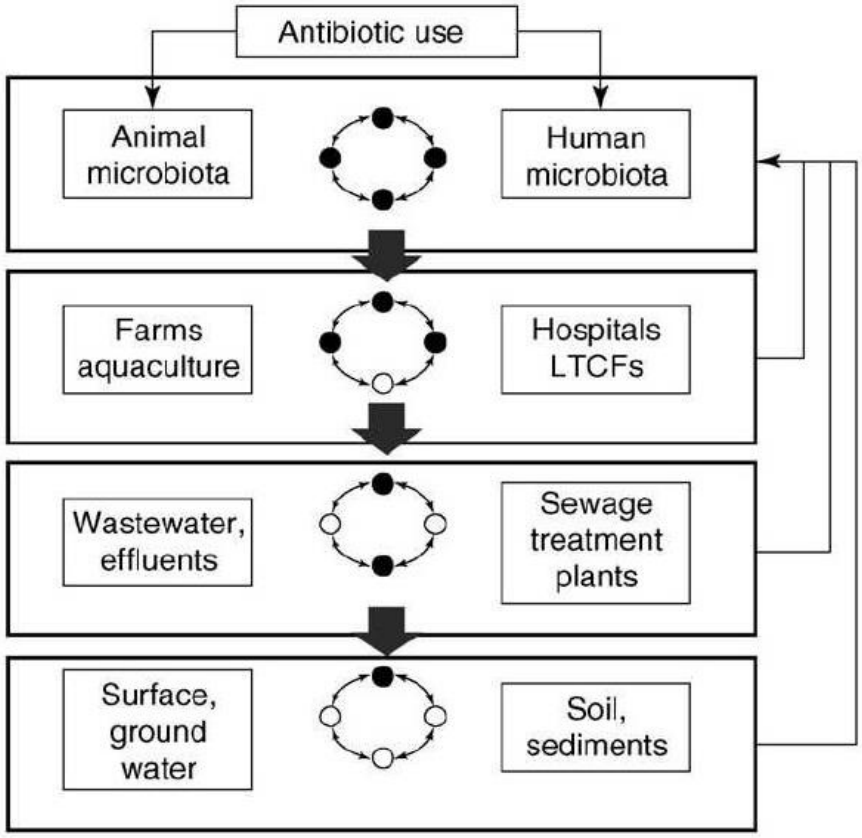


**Figure I.3**. Schematic representation of the environmental compartments contributing to antibiotic resistance dissemination and evolution (Baquero *et* al. 2008).

Several studies indicated that some clinically relevant resistance mechanisms have an environmental origin. For example, the widely disseminated *qnrA* and $bla_{CTX\text{-}M}$ genes have their origin in environmental strains, as *Shewanella* spp. (Kim *et* al. 2011) and *Kluyver*a spp. (Poirel *et* al. 2002), respectively.

### 3.1 Aquatic settings as reservoirs of ARGs

Aquatic systems are frequently impacted by anthropogenic activities, providing ideal settings for the acquisition and dissemination of antibiotic resistance. Disinfectants and heavy metals are launched in water and exercise a selective pressure that results in selection of antibiotic resistant bacteria. ARGs and antibiotic resistant bacteria can enter into aquatic environments through direct discharging of untreated wastewater or wastewater collection systems and can be transferred to soils (Baquero *et* al. 2008; Lupo *et* al. 2012; Tacão *et* al. 2012; Yang and Carlson *et* al. 2003; Zhang *et* al. 2009).

ARGs have a geographical distribution all around the world especially in aquatic environments. In Europe, ARGs have been frequently detected in aquatic environments of some countries, including Portugal (Alves *et* al. 2014; Araujo *et* al. 2014; Moura *et* al. 2007; Tacão *et* al. 2012; Zhang *et* al. 2009).

Wastewater treatment plants (WWTPs) are considered reservoirs for ARGs associated with human pathogens. Untreated sewage contains a variety of ARGs encoding resistance to all classes of antibiotics because it encloses bacteria exposed to antibiotics from hospitals and private households. Biofilm formation and environmental conditions can allow horizontal transfer of ARGs from one host to another (Schlüter *et* al. 2007b; Tennstedt *et* al. 2003; Zang *et* al. 2009).

## 4. The genus *Shewanella*

The genus *Shewanella* is mostly identified in aquatic systems. Microrganisms included in this genus are oxidase and catalase-positive, non-fermenters and Gram-negative. Relevant species of the genus *Shewanella* are *S. algae* and *S. putrefaciens*. These two species are more frequently described in literature associated with human infections (Janda *et* al. 2014).

Several studies were performed and nowadays there are 63 species described (http://www.bacterio.net/). Since 2000, a great explosion in the description of *Shewanella* species has occurred, which is clearly a byproduct of the affordability of gene sequencing technology and the general interest in the biodiversity of this genus, as shown in figure I.4 (Janda *et* al. 2014).

*Shewanella* species exist in nature in several different states: for example in a free-living form or in symbiotic relationships with marine organisms (Hau and Gralnick et al. 2007). *Shewanella* species can be found in a number of extreme environments such as the Antartic Continent or as piezophile, in energy rich sedimentation environments and in niches where redox potentials may change rapidly (Hau & Gralnick *et* al. 2007; Janda *et* al. 2014).

Clinically the genus is considered as a secondary or opportunistic pathogen. Most clinical cases documented in the literature refer to infections caused by *S. putrefaciens* and *S. algae*. For example, Brink and coworkers reported 28 infection cases by *S. putrefaciens* (Brink et al. 1995) in adults and children over a four-year period, in South Africa. Strains were isolated from blood samples. Other study performed by Holt and co-investigators (Holt *et* al. 1997) described an outbreak infection for 6 months in Denmark caused by *S. algae*. All strains were isolated from patients with ages between 3 and 15 years. In general, *Shewanella*-associated syndromes can be divided in 5 categories:

a) Infections of skin and soft-tissue;
b) Invasive disease;
c) Hepatobiliary illness;
d) Otitis;
e) Infections diverse.

Due to its relative abundance in aquatic environments, interacting closely with humans, infection acquisition may be facilitated. Currently, there are more than 150 reported cases of *Shewanella* infections (Janda *et* al. 2014).

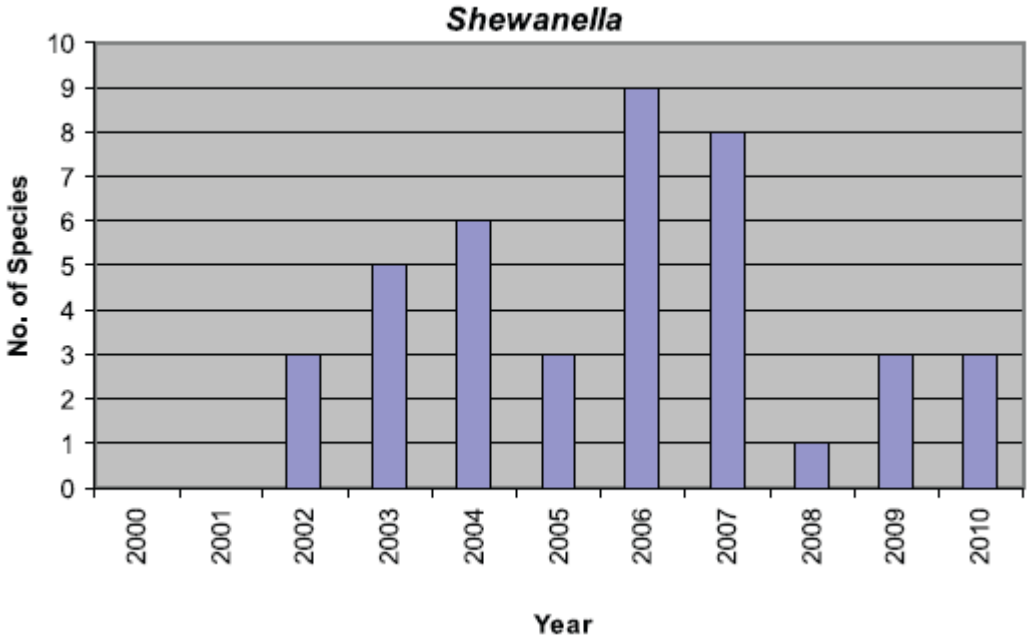


**Figure I.4** Expansion of species of *Shewanella* (2000-2010) (Janda *et* al. 2014).

### 4.1 *Shewanella* as the origin of OXA-48-like-genes

Bacteria of the genus *Shewanella* have been appointed as reservoir of antibiotic resistance determinants. In this genus, β-lactamases of classes B and D have been identified (Patel *et* al. 2013; Potron *et* al. 2011).

Recently it has been suggested that the actual progenitors of *bla*$_{\text{OXA-48-like}}$ may be *Shewanella* species (Poirel *et* al. 2004; Poirel *et* al. 2012; Tacão *et* al. 2013; Zong *et* al. 2012). However only few studies contribute with evidences to support this hypothesis. *bla*$_{\text{OXA-48-like}}$ genes were detected in *Shewanella xiamenensis* isolated from river water (Tacão *et* al. 2013). The analysis of these isolates revealed the presence of different variants namely *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{OXA-204}}$ (Tacão *et* al. 2013). Also, the analysis of available genome sequences of *Shewanella* spp. demonstrated the presence of *bla*$_{\text{OXA-48-like}}$ genes in their chromosome with at least 80% identity to *bla*$_{\text{OXA-48}}$ (Zong *et* al. 2012).

## 5. Hypothesis and goals of dissertation

This study was designed to contribute with data which support the hypothesis that genes encoding for carbapenemases OXA-48-like have originated in *Shewanella* strains. Considering this, the major aim of this work is to characterize 43 environmental *Shewanella* strains, obtained in previous studies, to confirm the role of this genus as progenitor of OXA-48-like genes. Specific aims are:

1) To type and identify the selected isolates at the species level;
2) To determine the *Shewanella* strains antimicrobial susceptibility profiles;
3) To detect and sequence $bla_{\text{OXA-48-like}}$ genes;
4) To analyze the genomic context of such genes;
5) To assess the presence of other antibiotic resistance genes;
6) To detect and characterize mobile genetic elements (e.g. integrons and plasmids).

# II. MATERIAL AND METHODS

## 1. Bacterial strains and molecular typing

In this investigation, we used 43 *Shewanella* isolates. These isolates were obtained in previous studies from different environmental sources (Table II.1). The 43 *Shewanella* isolates were typed by BOX-PCR with specific primer (Versalovic *et* al. 1994) (5′-CTACGGCAAGGCGACGCTGACG-3′). The PCR mixtures (25 µL total volume) consisted of 6.25 µL NZYTaq 2× Green Master Mix (2.5 mMgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase) (NZYtech, Portugal), 15.75 µL of ultrapure water, 2 µL of a 10 µM primer and 50–100 ng purified DNA. The PCR reactions were performed in a MyCycler Thermal Cycler (Bio-Rad, USA) and amplification were carried out as follows: initial denaturation (95ºC for 7 min); 30 cycles of denaturation (94º for 1min), annealing (53º for 1min) and extension (65º for 8min); and a final extension (72º for 16min). PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Overall, 30 isolates of *Shewanella* displaying unique BOX profiles were selected for further analysis.

**Table II.1** Bacterial isolates and sources.

| Strains References | Sources |
|---|---|
| **C1** | Salt marsh plant (endophytic) |
| **C14** | Salt marsh plant (endophytic) |
| **C17** | Salt marsh plant (endophytic) |
| **C18** | Salt marsh plant (endophytic) |
| **B39** | Salt marsh plant (endophytic) |
| **B54** | Salt marsh plant (endophytic) |
| **E74** | Salt marsh plant (endophytic) |
| **E80** | Salt marsh plant (endophytic) |
| **E81** | Salt marsh plant (endophytic) |

| | |
|---|---|
| **BR3** | Salt marsh plant (epiphytic) |
| **BR4** | Salt marsh plant (epiphytic) |
| **BR10** | Salt marsh plant (epiphytic) |
| **MAH-21.1** | Cockle |
| **M3H10** | Cockle |
| **M1H3** | Cockle |
| **M1M5** | Cockle |
| **M2M2** | Cockle |
| **M3H13** | Cockle |
| **M1M9** | Cockle |
| **E1M6** | Cockle |
| **E2M3** | Cockle |
| **E2M4** | Cockle |
| **E2B3** | Cockle |
| **GNNN3-I** | Estuarine water |
| **GNNP8-I** | Estuarine water |
| **GNDN5-I** | Estuarine water |
| **GCDN3-I** | Estuarine water |
| **GNDP6-I** | Estuarine water |
| **GCDP8-I** | Estuarine water |
| **ENDN9-I** | Estuarine water |
| **GNNN2-II** | Estuarine water |
| **GNDN6-II** | Estuarine water |
| **GCDN9-II** | Estuarine water |
| **ENDN4-II** | Estuarine water |
| **ENDN5-II** | Estuarine water |

| | |
|---|---|
| **ENDN10-II** | Estuarine water |
| **ECDP4-II** | Estuarine water |
| **GNDP2-III** | Estuarine water |
| **ECNP9-III** | Estuarine water |
| **GCDN4-III** | Estuarine water |
| **Sh31** | River water |
| **Sh32** | River water |
| **Sh33** | River water |

## 2. DNA isolation

DNA isolation was performed according to Silica Bead DNA Gel Extraction Kit #513 from MBI Fermentas (Vilnius, Lithuania) with adapted instructions. Finally, DNA extracted was analyzed by electrophoresis on a 0.8% agarose gel and stained with ethidium bromide.

**Silica Bead DNA Gel Extraction Kit #513 (MBI Fermentas)**

1. Strains were grown overnight in LB broth or Marine Broth (compositions are described on appendices).
2. One mL of cell culture was centrifuged during 10 minutes at 13000 rpm and the pellet was ressuspended in 100µl of buffer B1 (50 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 0.5% Tween 20; 0.5% Triton X-100).
3. One hundred µL of mastermix (previously prepared: per mL B1 add 100 mg/mL RNase A, 100 mg/mL Lysozym, Protease in 20mg/mL) were added to each cell suspension.
4. The suspension was mixed and incubated for 30 minutes at 37ºC. Then, 70µL of buffer B2 (3M Guanidine Hydrochloride / 20% Tween 20) were added, followed by incubation at 50ºC for 30 minutes.

5. Following, 500µL of Binding Solution were added and mixed.
6. Silica Suspension (Fermentas DNA Gel Extraction Kit #513) was ressuspended for 1 minute and 5µL were added to each cell suspension.
7. The solution was gently inverted and incubated at 55ºC during 5 minutes. In final, was centrifuged for 5 sec.
8. The supernatant was eliminated and 500µL of Wash Buffer were added and the solution was centrifuged at 13000 rpm during 5sec. This step was repeated twice.
9. The pellet was centrifuged and all traces of supernatant were removed. Then, all tubes were left to dry for 10 minutes.
10. The DNA was ressuspended in 60 µL of DNA Elution Buffer (TE) and stored at -20ºC.

## 3. PCR products purification and Sequencing

The PCR products were purified with NZYGelpure (Nzytech, Portugal) following the instructions described below.

**NZYGelpure:**

1. Four volumes of Binding Buffer were added up to 1 volume of a PCR sample. After mixing, the sample was transferred to a Nzytech Spin Column placed in a 2 mL Receiver Tube and then centrifuged at >12000 x g for 30s - 1min.
2. The column was re-inserted in the empty Receiver Tube and 600ul Wash Buffer were added, and then centrifuged at <12000 x g for 30s - 1min.
3. The flow-through was discarded and the column was placed in the same but empty Receiver Tube, and centrifuged again at >12000 x g for 1 min.
4. The column was placed in to a 1.5 mL microcentrifuge tube. 25 µL of sterile water was added to the column and centrifuged at >12000 x g for 2 min.
5. Purified DNA was stored at -20ºC.

## 4. Phylogenetic affiliation *of Shewanella* strains

Phylogenetic affiliation of strains was performed based on 16S rDNA gene and *gyr*B amplification and sequencing with specific primers shown in table II.2. PCR reactions were carried out with either *Taq* polymerase and buffers from Promega (Madison,USA) or *Taq* polymerase and buffers from Nzytech (Portugal). The PCR reaction mixtures (25 ul) contained: 1) when using Promega reagents - 1 X PCR buffer, 200 μM of each nucleotide, 25 mM MgCl2, 7.5 pmol of each primer, 1U of Taq polymerase, and 50–100 ng purified DNA or 2) when using Nzytech reagents - 6.25 μL NZYTaq 2× Green Master Mix (2.5 mMgCl2; 200 μM dNTPs; 0.2 U/μL DNA polymerase) (NZYtech, Portugal), 15.75 μL of ultrapure water, 1 μL of a 10 μM of each primer (forward and reverse) and 50–100 ng purified DNA.

The PCR reactions were performed in a MyCycler Thermal Cycler (Bio-Rad, USA). Amplifications were carried out as follows: 1) for the *16S* rRNA gene amplification - initial denaturation (94ºC for 5 min); 30 cycles of denaturation (94º for 30 s), annealing (54º for 30 s) and extension (72º for 1 min); and a final extension (72º for 7 min); 2) for the *gyr*B amplification - initial denaturation (94ºC for 5 min); 35 cycles of denaturation (94º for 30 s), annealing (54º for 30 s) and extension (72º for 1 min); and a final extension (72º for 7 min). PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Amplicons were purified, according to the protocol described above, and sequenced by the company GATC Biotech (Konstanz, Germany). The sequence similarity search was performed using the BLAST program and Eztaxon program. Phylogenetic affiliation was confirmed by constructing phylogenetic trees. For this, nucleotide sequences were aligned by Clustal Omega and phylogenetic trees were obtained using MEGA, version 6.0.

**Table II.2** PCR primers used for phylogenetic determination.

| **Target** | **Amplicon Size (bp)** | **Primers Sequence (5'-3')** | **Tº annealing** | **References** |
|---|---|---|---|---|
| 16S rDNA gene | 1400 | 27F: AGAGTTTGATCCTGGCTCAG<br>1492R: GGTTACCTTGTTACGACTTT | 54º | Lane *et* al. 1991 |
| *gyr*B | 740 | SW_GyrB_F: GAAGTGGCKATGCAGTGGAA<br>SW_GyrB_R: CGCCRAATACCACAGCCRAG | 54º | Antonelli *et* al. 2015 |

## 5. Antimicrobial Susceptibility

The antibiotic susceptibility of the *Shewanella* isolates was tested against 15 antibiotics (Table II.3) by the disk diffusion method according to the procedure described next and established by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). *E. coli* ATCC25922 was used as a quality control strain. Results were analyzed following the Clinical and Laboratory Standards Institute (CLSI) guidelines.

**Agar antibiotic disk diffusion technique:**

1. Prepare a bacterial suspension in sterile saline solution with turbidity equivalent to 0.5 McFarland scale (3-4 colonies);
2. Inoculate the suspension in plates with Mueller Hinton Agar (Merck, Darmstadt, Germany) with a swab;
3. After dry apply the disks with antibiotic;
4. Incubate at 37ºC, 24 hours;
5. Measure the diameters of the inhibition zones for each antibiotic;
6. Classify each strain as sensitive, intermediate or resistant comparing measured diameters with the criteria established by CLSI.

**Table II.3** Antibiotics used for the susceptibility testing, antibiotics concentration in the disk and abbreviation according to the supplier (Oxoid Ltd, Basingstoke, UK).

| **Classes** | **Antibiotics** | **Oxoid abr.** | **Disk (µg)** |
|---|---|---|---|
| Penicillins | Amoxicillin | AML | 10 |
| | Amoxicillin + Clavulanic acid | AMC | 30 |
| 1st. gen. cephalosporins | Cefalotin | KF | 30 |
| 3rd. gen. cephalosporins | Cefotaxime | CTX | 30 |
| | Ceftazidime | CAZ | 30 |
| 4th. gen. cephalosporins | Cefepime | FEP | 30 |
| Aminoglycosides | Gentamicin | GEN | 10 |
| | Kanamycin | CAN | 30 |
| Carbapenems | Imipenem | IPM | 10 |
| Quinolones | Nalidixic Acid<br>Ciprofloxacin | NAL<br>CIP | 30<br>5 |
| Tetracycline | | TE | 30 |
| Chloramphenicol | | C | 30 |
| Sulfamethoxazole + Trimethoprim | | SXT | 25 |
| Monobactams | Aztreonam | AZT | 30 |

## 6. Amplification of *bla*$_{\text{OXA-48-like}}$ gene fragments and determination of genomic context by PCR

*bla*$_{\text{OXA-48-like}}$ gene fragments were amplified from each isolate using four *bla*$_{\text{OXA-48-like}}$ specific primer sets described in table II.4. The genomic context was assessed with specific primers described in table II.4 (*c15* and *lysR* together with one of the primers used for detection of *bla*$_{\text{OXA-48-like}}$ gene). PCR reaction mixtures prepared with NZYTaq 2X Green Master as described in point 4 of this section. PCR reactions were performed in MyCycler Thermal cycler (Bio-Rad, USA). Program used was: initial denaturation (94ºC for 5 min); 35 cycles of denaturation (94º for 30 s), annealing (temperature indicated on table II.4 for each primer for 30 s) and extension (72º for 1 min); and a final extension (72º for 7 min). Positive and negative controls were included in each PCR reaction. *Shewanella xiamenensis* (Tacão *et* al. 2013) and *Shewanella baltica* (unpublished) strains carrying a *bla*$_{\text{OXA-48}}$ gene were used as positive controls and water was used as negative control. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Amplicons were purified and sequenced by the company GATC Biotech (Konstanz, Germany). Amino acid sequences were deduced from nucleotide sequences using the Translate Expasy tool (http://web.expasy.org/translate/) and similarity searches against the GenBank database were performed using the BLASTp tool.

**Table II.4** PCR primers used for amplification of *bla*$_{\text{OXA-48}}$ and context of *bla*$_{\text{OXA-48}}$.

| Target | Amplicon Size (bp) | Primers Sequence (5'-3') | Tº annealing | References |
|---|---|---|---|---|
| *bla*$_{\text{oxa-48}}$ set1 | 743 | OXA48_F: GCGTGGTTAAGGATGAACAC OXA48_R: CATCAAGTTCAACCCAACCG | 55º | Poirel *et* al. 2004 |
| *bla*$_{\text{oxa-48}}$ set2 | 571 | OXA48 54I_F: AGCAAGGATTTACCAATAAT OXA4854I_R: GGCATATCCATATTCATC | 50º | Zong *et* al. 2012 |
| *bla*$_{\text{oxa-48}}$ set3 | 800 | OXA-48_All_Fwd: GCGTGTATTAGCCTTATCGGC OXA-48_All_R: CTAGGGAATAATTTTTTCCTGTTTG | 50º | unpublished |

| | | | | |
|---|---|---|---|---|
| $bla_{oxa-48}$ set4 | 866 | Sb_OXA_Fwd: GCCATATCGACTGTGTTG Sb_OXA_R: ACGTGTTCCAGTTTTAA | 50º | unpublished |
| *c15* | - | C15_fwd: TTACGGCCTGGGAAGTGTTC | | Tacão *et* al. 2013 |
| *lys R* | - | Lys_R: AAGGGATTCTCCCAAGCTGC | | Tacão *et* al. 2013 |

## 7. Screening for other antibiotic resistance genes (ARGs)

The presence of other resistance genes ($bla_{SHV;}$ $bla_{TEM;}$ *qnrA*) was inspected by PCR according with results of antimicrobial susceptibility tests.

Reaction conditions to each target were performed with same conditions described in point 4 of this section and primers are described in table II.5. PCR reactions were performed in a MyCycler Thermal cycler (Bio-Rad, USA) as described above and conditions used were: initial denaturation (94ºC for 5 min); 30 cycles of denaturation (94º for 30 s), annealing (temperature indicated in Table II.5 for 30 s) and extension (72º for 1 min); and a final extension (72º for 7 min). In case of *qnrA* we used another program, as well as the program presented above: initial denaturation (93ºC for 2 min); 40 cycles of denaturation (93º for 30 s), annealing (53º for 30 s) and extension (68º for 1 min); and a final extension (68º for 5 min).

**Table II.5** PCR primers used for amplification of ARGs.

| **Target** | **Amplicon Size (bp)** | **Primers Sequence (5'-3')** | **Tº annealing** | **Control strains** | **References** |
|---|---|---|---|---|---|
| $bla_{SHV}$ | 304 | SHV_F: GCGAAAGCCAGCTGTCGGGC SHV_R:GATTGGCGGCGCTGTTATCGC | 62º | E. coli A7 | Alves *et* al. 2014 |
| $bla_{TEM}$ | 425 | TEM_F: AAAGATGCTGAAGATCA TEM_R: TTTGGTATGGCTTCATTC | 44º | E. coli A7 | Moura *et* al. 2014 |
| *qnrA* | 521 | qnrA_F: TTCTCACGCCAGGATTTG qnrA_R: CCATCCAGATCGGCAAA | 53º | - | Guillard *et* al. 2011 |

## 8. Integron screening and characterization

Screening was performed for the presence of the integrase genes *intI*1 and *intI*2 as previously described (Henriques *et* al. 2006). Primers used are described in table II.6. Reaction conditions and PCR reactions are the same conditions described in point 4 of this section.

**Table II.6** PCR primers used for the amplification of integrons.

| Target | Amplicon Size (bp) | Primers Sequence (5'-3') | Tº annealing | Control strains | References |
|---|---|---|---|---|---|
| *intI*1 | 280 | Int1_F: ACATGCGTGTAAATCGTC<br>Int1_R:CTGGATTTCGATGACGGCACG | 55º | *E. coli* A4 | Moura *et* al. 2014 |
| *intI*2 | 233 | Int2_F: ACGGCTACCCTCTGTTAT<br>Int2_R: TTATTGCTGGGATTAGGC | 50º | *E. coli* A33 | Moura *et* al. 2014 |
| *variable region* | variable | 5'-CS:GGC ATC CAA GCA GCA AG<br>3'-CS: AGG CAG ACT TGA CCT GA | 58.5º | *E. coli* A2 100.1 | Allen *et* al. 2009 |

## 9. Plasmid screening and characterization

For characterization of plasmids, isolates were cultivated in 800mL Luria Bertani, Luria Bertani/seasalt and Marine Broth tubes overnight at 30ºC, 12000rpm. Extraction of plasmids was performed with NZYMiniprep (Nzytech, Portugal) or the EZNA Plasmid Mini Kit II (Omega Bio-tek, GA, USA), following the instructions described below. The results were visualized by electrophoresis on a 0.8% agarose gel at 40V for 150 minutes.

**NZYMiniprep:**

1. **Cultivate and harvest bacterial cells**

   Strains were grown overnight in LB broth and Marine Broth (compositions are described on appendices). Centrifuge 8ml of culture for 30s. Discard supernatant.

2. **Cell Lysis**

   Re-suspend cell pellet in 250 μL Buffer A1 (supplied in the kit and kept at 4ºC) and then vigorous vortex. Add 250 μL of Buffer A2 (supplied in the kit) and mix gently by inverting the tube 6-8times and incubate at room temperature for a maximum of 4 minutes. In the final, add 300 μL Buffer A3 (supplied in the kit) and mix gently by inverting the tube for 6-8times.

3. **Clarification of lysate**

   Centrifuge for 10 minutes.

4. **Bind DNA**

   Place Nzytech spin column in a 2 mL collecting tube and load the supernatant from step 3 onto the column, centrifuge for 1min at 11.000xg. In the final, discard flow-through.

5. **Wash silica membrane**

   Add 500 μL of Buffer AY (supplied in the kit), centrifuge for 1 minute and discard flow-through. Then, add 600 μL of Buffer A4 (supplied in the kit and with ethanol added), centrifuge for 1 minute and discard flow-through.

6. **Dry silica membrane**

   Re-insert the spin column into the empty 2 mL collecting tube and centrifuge for 2 minutes.

7. **Elute highly pure DNA**

   Place the dried spin column into a clean 1.5mL microcentrifuge tube and add 50 μL of Buffer AE (supplied in the kit). Finally, incubate 1 minute at room temperature and centrifuge for 1 minute. Store the purified DNA at -20ºC

**E.Z.N.A. Plasmid DNA Mini Kit II Spin Protocol**

1. Strains were grown overnight in LB broth and Marine Broth (compositions are described on appendices). Centrifuge 8 mL of culture at 5,000 x g for 10 minutes at room temperature.
2. Decant or aspirate the medium and discard.
3. Add 500 µL Solution I/RNase A (previously prepared with RNase A and stored at 2-8ºC) and then vortex to mix thoroughly.
4. Transfer suspension into a new 2 mL microcentrifuge tube.
5. Add 500 µL Solution II (supplied in the kit) and then invert and gently rotate the tube several times to obtain a clear lysate.
6. Add 700 µL Solution III (supplied in the kit) and immediately invert several times until a flocculent white precipitate forms.
7. Centrifuge at maximum speed (>13.000 x g) for 10 minutes at room temperature.
8. Insert a HiBind DNA mini Column into a 2 mL Collection tube.
9. Centrifuge at maximum speed for 1 minute.
10. Discard the filtrate and reuse the collection tube and repeat steps 8-10 until all cleared lysate.
11. Add 500 µL HBC Buffer (previously prepared with addition of 32 mL Isopropanol). Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse collection tube.
12. Add 700 µL DNA Wash Buffer (previously prepared with 100 mL of ethanol). Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse the collection tube.
13. Centrifuge the empty HiBind DNA Mini Column for 2 minutes at maximum speed and then transfer HiBind DNA Mini Column to clean 1.5 mL microcentrifuge tube.
14. Add 80-100 µL Elution Buffer (supplied in the kit) and let sit at room temperature for 1 minute. Then centrifuge at maximum speed for 1 minute.
15. Store DNA at -20ºC.

## III. RESULTS

### 1. *Shewanella* isolates genotypic diversity

For this study, 43 *Shewanella* isolates were selected from different environmental sources. Clonal relationships were evaluated by BOX-PCR, as exemplified in figure III.1 and 33 isolates displaying unique BOX profiles were selected for further studies.

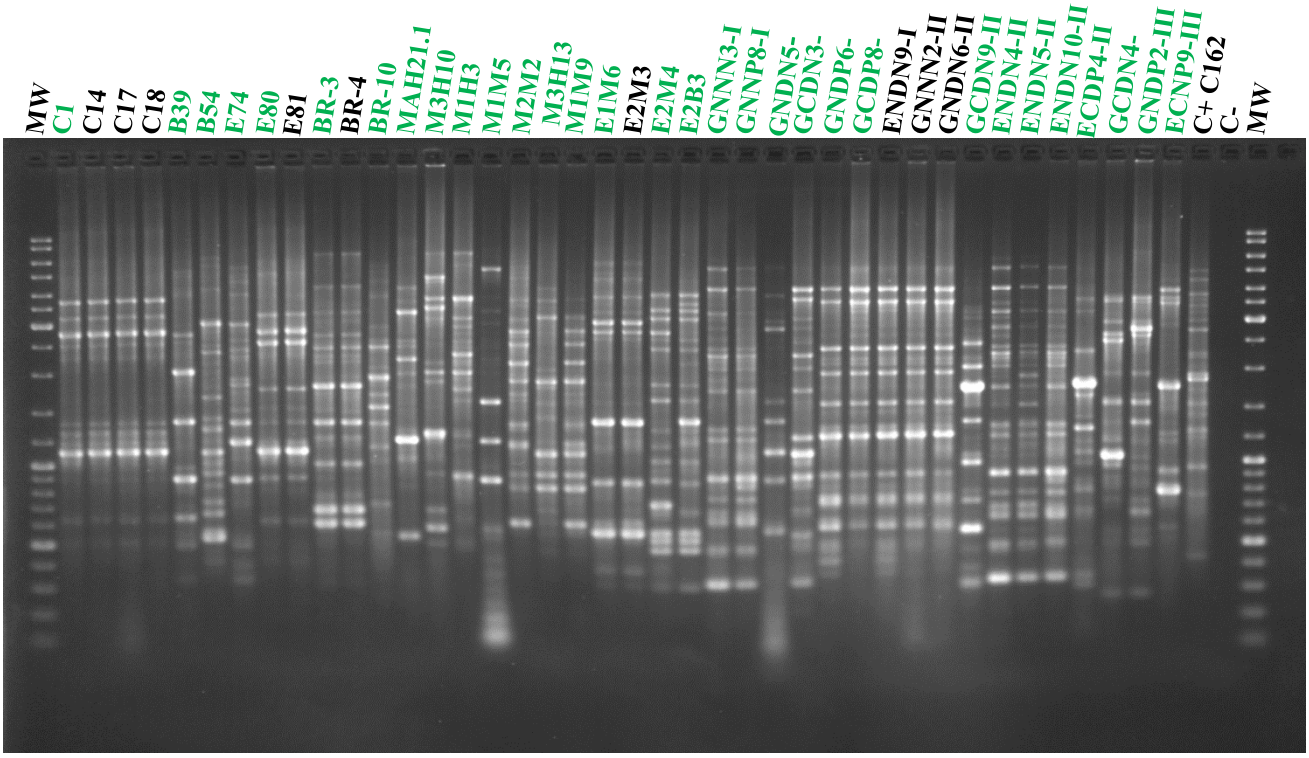


**Figure III.1 :** BOX-PCR gel from 40 isolates of *Shewanella*. Selected isolates with distinct BOX-PCR profiles are labeled with green colour.

Identical profiles were always from the same source.

The phylogenetic affiliation was performed based on 16S rDNA and *gyr*B sequences. First 16S rRNA gene closest sequences were inspected using the Eztaxon tool and the results are described in table III.1.

**Table III.1** Closest type strains based on a 16S rDNA sequence search against the EzTaxon database.

| Strains References | Sequence size | Closest relative strains | Similarity | Accession nº GenBank |
|---|---|---|---|---|
| **Sh1** | 1416 | *S. xiamenensis S4* | 100% | FJ589031 |
| **Sh2** | 1410 | *S. algae JCM 21037* | 99.91% | BALO01000089 |
| **Sh3** | 1418 | *S. fodinae JC15* | 99.43% | FM203122 |
| **Sh4** | 1417 | *S. haliotis JCM 14758* | 100% | BALL01000107 |
| **Sh5** | 1380 | *S. xiamenensis S4* | 100% | FJ589031 |
| **Sh6** | 1401 | *S. irciniae UST040317-058* | 96.12% | DQ180743 |
| **Sh7** | 1398 | *S. irciniae UST040317-058* | 94.99% | DQ180743 |
| **Sh8** | 1382 | *S. aestuarii SC18* | 98.62% | JF751044 |
| **Sh9** | 1411 | *S. baltica NCTC 10735* | 98.94% | AJ000214 |
| **Sh10** | 1390 | *S. hafniensis P010* | 98.92% | AB205566 |
| **Sh11** | 1416 | *S. aestuarii SC18* | 98.16% | JF751044 |
| **Sh12** | 1396 | *S. aestuarii SC18* | 98.64% | JF751044 |
| **Sh13** | 1059 | *S. aestuarii SC18* | 98.87% | JF751044 |
| **Sh14** | 1402 | *S. haliotis JCM 14758* | 100% | BALL01000107 |
| **Sh15** | 1404 | *S. indica KJW27* | 99.93% | HM016084 |
| **Sh16** | 1403 | *S. indica KJW27* | 100% | HM016084 |
| **Sh17** | 1408 | *S. hafniensis P010* | 98.93% | AB205566 |
| **Sh18** | 1393 | *S. hafniensis P010* | 98.93% | AB205566 |
| **Sh19** | 1381 | *S. baltica NCTC 10735* | 98.91% | KC969079 |
| **Sh20** | 1400 | *S. hafniensis P010* | 99.36% | AB205566 |
| **Sh21** | 1415 | *S. baltica NCTC 10735* | 99% | AJ000214 |
| **Sh22** | 1385 | *S. baltica NCTC 10735* | 99% | AJ000214 |

| | | | | |
|---|---|---|---|---|
| **Sh23** | 1423 | *S. putrefaciens LMG26268* | 99.65% | X81623 |
| **Sh24** | 1413 | *S. hafniensis P010* | 98.94% | AB205566 |
| **Sh25** | 1403 | *S. hafniensis P010* | 98.86% | AB205566 |
| **Sh26** | 1403 | *S. hafniensis P010* | 98.35% | AB205566 |
| **Sh27** | 1410 | *S. putrefaciens LMG 2628* | 99.72% | X81623 |
| **Sh28** | 1394 | *S. algidipiscicola S13* | 99.21% | AB205568 |
| **Sh29** | 1405 | *S. hafniensis P010* | 99.36% | AB205566 |
| **Sh30** | 1417 | *S. hafniensis P010* | 99.21% | AB205568 |
| **Sh31** | 1059 | *S. xiamenensis S4* | 99.24% | FJ589031 |
| **Sh32** | 1059 | *S. xiamenensis S4* | 99.24% | FJ589031 |
| **Sh33** | 1059 | *S. xiamenensis S4* | 99.24% | FJ589031 |

The phylogenetic affiliation was confirmed by constructing a phylogenetic tree shown in figure III.2.

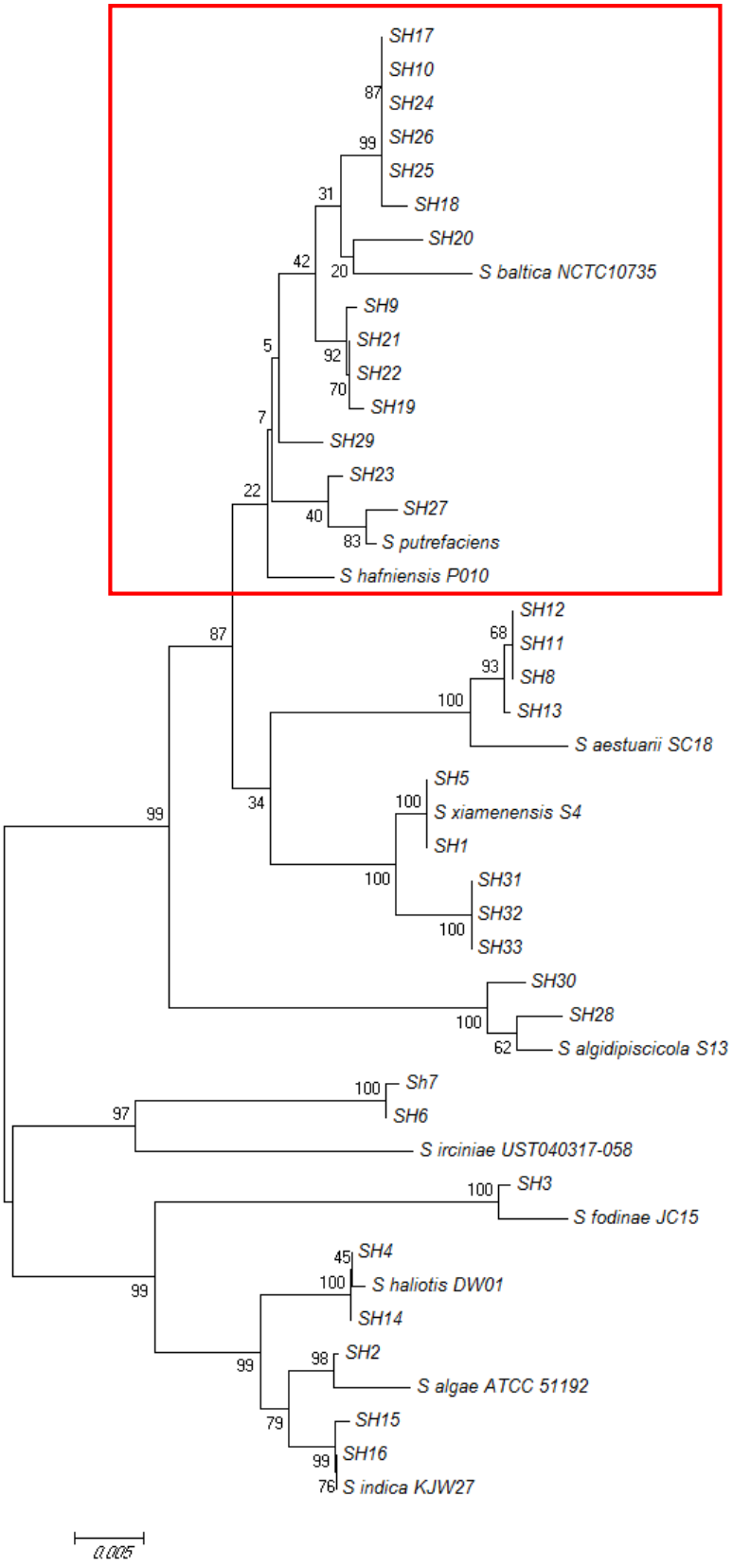


**Fig III.3:** Phylogenetic tree based on 16S rDNA gene sequences of *Shewanella* isolates obtained in this study together with closest type strains and representative sequences retrieved from GenBank database. The tree was generated using the neighbor-joining method tree with 500 bootstrap replicates. Bootstrap confidence are shown in %. The affiliation of the isolates included in the red box were further analysed by *gyr*B sequencing.

Taking into account these results, we can consider that in general there was agreement between the results obtained in closest relative sequences and the phylogenetic tree of 16S rDNA. However, for the isolates included in the red rectangle the phylogenetic affiliation was confirmed with *gyr*B gene sequencing. Results are shown in table III.2 and phylogenetic tree in figure III.3.

**Table III. 2:** Closest type strains based on a *gyr*B sequence search against the GenBank database.

| **Strains References** | **Sequence size** | **Closest relative** | **Similarity** | **Accession nº GenBank** |
|---|---|---|---|---|
| **Sh9** | 634 | *S. baltica OS638* | 98% | HM179266 |
| **Sh10** | 643 | *S. hafniensis P010* | 94% | AB208056 |
| **Sh17** | 639 | *S. hafniensis P010* | 99% | AB208056 |
| **Sh18** | 643 | *S. hafniensis P010* | 98% | AB208056 |
| **Sh19** | 657 | *S. baltica OS106* | 99% | HM204458 |
| **Sh20** | 640 | *S. hafniensis P010* | 98% | AB208056 |
| **Sh21** | 642 | *S. baltica OS106* | 99% | HM204458 |
| **Sh22** | 643 | *S. baltica OS106* | 99% | HM204458 |
| **Sh23** | 640 | *S. baltica WRW4* | 99% | KT183385 |
| **Sh24** | 656 | *S. hafniensis P010* | 98% | AB208056 |
| **Sh26** | 647 | *S. hafniensis P010* | 98% | AB208056 |
| **Sh27** | 675 | *S. putrefaciens 200* | 98% | CP002457 |
| **Sh29** | 639 | *S. hafniensis P010* | 98% | AB208056 |

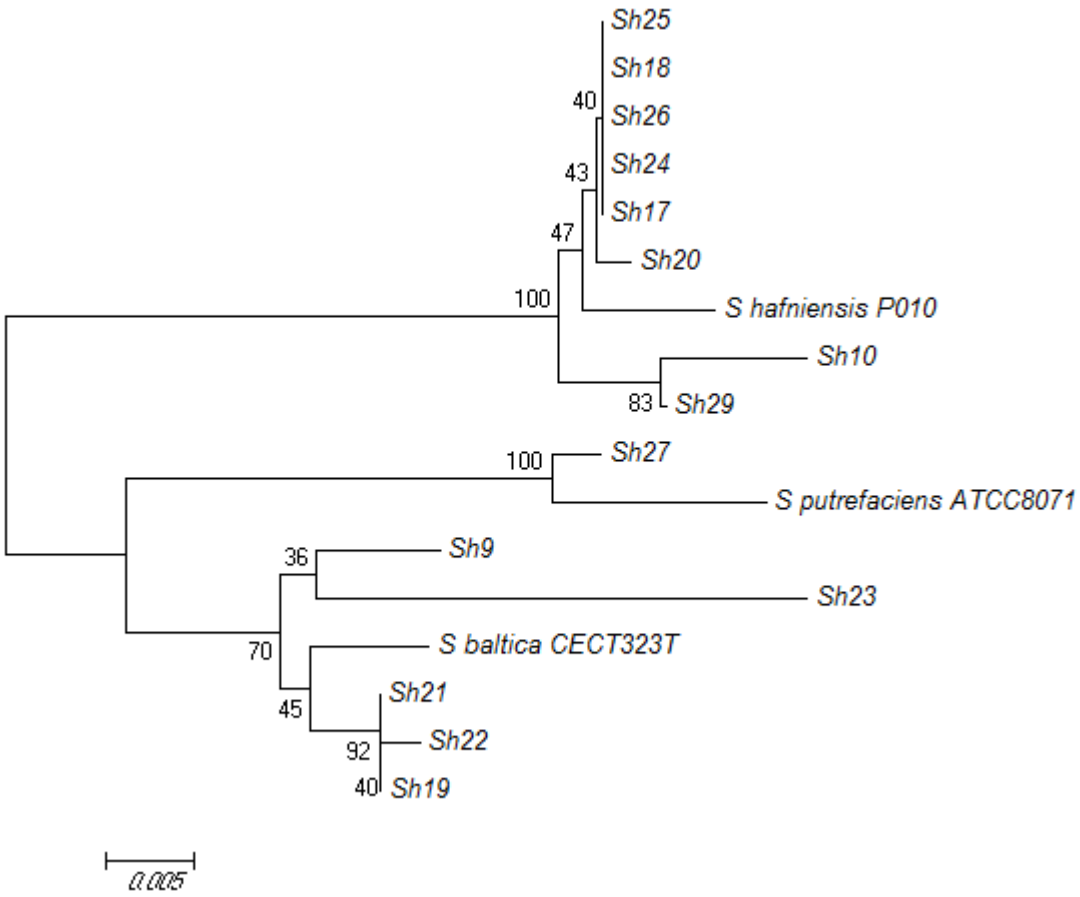


**Fig III.3:** Phylogenetic tree based on *gyr*B gene sequences of isolates that affiliated with S. baltica, S. hafniensis and S putrefaciens, together with closest type strains and representative sequences retrieved from GenBank database. The tree was generated using the neighbor-joining method tree with 500 bootstrap replicates. Bootstrap confidence are shown in %.

Taking in consideration the results presented above, our final collection was composed by 11 species of *Shewanella*. Most strains belonged to S. *hafniensis* (n=9), S. *xiamenensis* (n=5), S. *aestuarii* (n=4) and S. *baltica* (n=4). The least represented species were S. *haliotis* (n=2), S. *indica* (n=2), S. *putrefaciens* (n=2), S. *algidipiscicola* (n=1), *Shewanella* sp. (n=2), S. *algae* (n=1) and S. *fodinae* (n=1). In table III.3 are represented all species used in this work and corresponding source.

**Table III. 3:** Sources and species used in this study.

| Strains References | Species | Sources |
|---|---|---|
| **Sh1** | *S. xiamenensis* | Salt marsh plant (endophytic) |
| **Sh2** | *S. algae* | Salt marsh plant (endophytic) |
| **Sh3** | *S. fodinae* | Salt marsh plant (endophytic) |
| **Sh4** | *S. haliotis* | Salt marsh plant (endophytic) |
| **Sh5** | *S. xiamenensis* | Salt marsh plant (endophytic) |
| **Sh6** | *Shewanella* sp. | Salt marsh plant (epiphytic) |
| **Sh7** | *Shewanella* sp | Salt marsh plant (epiphytic) |
| **Sh8** | *S. aestuarii* | Cockle |
| **Sh9** | *S. baltica* | Cockle |
| **Sh10** | *S. hafniensis* | Cockle |
| **Sh11** | *S. aestuarii* | Cockle |
| **Sh12** | *S. aestuarii* | Cockle |
| **Sh13** | *S. aestuarii* | Cockle |
| **Sh14** | *S. haliotis* | Cockle |
| **Sh15** | *S. indica* | Cockle |
| **Sh16** | *S. indica* | Cockle |
| **Sh17** | *S. hafniensis* | Estuarine water |
| **Sh18** | *S. hafniensis* | Estuarine water |
| **Sh19** | *S. baltica* | Estuarine water |
| **Sh20** | *S. hafniensis* | Estuarine water |
| **Sh21** | *S. baltica* | Estuarine water |
| **Sh22** | *S. baltica* | Estuarine water |
| **Sh23** | *S. putrefaciens* | Estuarine water |
| **Sh24** | *S. hafniensis* | Estuarine water |

| | | |
|---|---|---|
| **Sh25** | *S. hafniensis* | Estuarine water |
| **Sh26** | *S. hafniensis* | Estuarine water |
| **Sh27** | *S. putrefaciens* | Estuarine water |
| **Sh28** | *S. algidipiscicola* | Estuarine water |
| **Sh29** | *S. hafniensis* | Estuarine water |
| **Sh30** | *S. hafniensis* | Estuarine water |
| **Sh31** | *S. xiamenensis* | River water |
| **Sh32** | *S. xiamenensis* | River water |
| **Sh33** | *S. xiamenensis* | River water |

## 2. Antibiotics susceptibility tests

Antimicrobial susceptibility was tested for 33 *Shewanella* isolates and the resistance frequencies to antibiotics are shown in figure III.4. Isolates were classified as susceptible, intermediate or resistant to antibiotics.

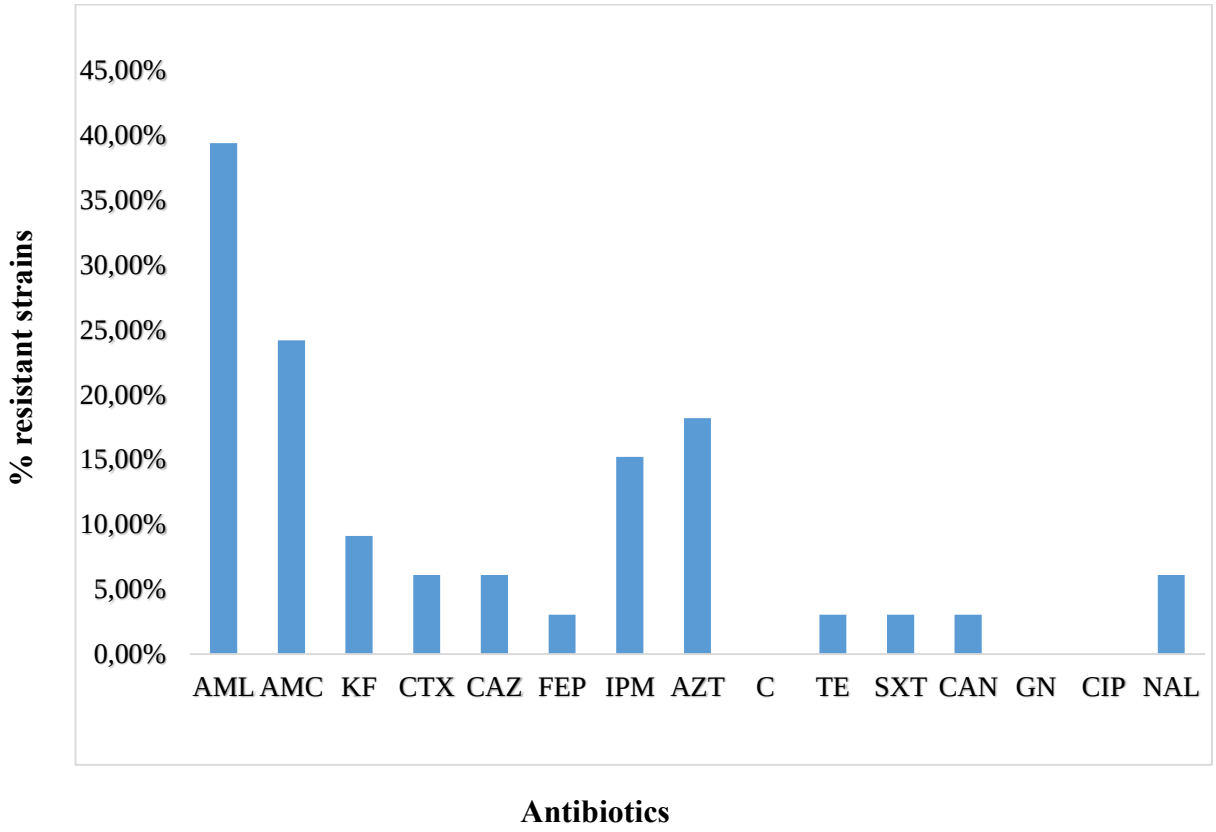


**Fig. III.4:** Antibiotic susceptibility of *Shewanella* isolates analysed during this study. (AML–amoxicillin; AMC–amoxicillin and clavulanic acid; KF-cephalotin; CTX–cefotaxime; CAZ–ceftazidime; FEP-cefepime; IPM–imipenem; AZT–aztreonam; C–chloramphenicol; TE–tetracycline; SXT-trimethoprim-sulphamethoxazole; CAN–kanamycin; GN–gentamicin; CIP–ciprofloxacin; NAL-nalidixic acid).

Taking into account the 33 isolates, amoxicillin is the antibiotic towards which there are greater levels of resistance (39.4%), followed by the combination of amoxicillin/clavulanic acid (24.2%), aztreonam (18.2%), imipenem (15.2%) and cefalotin (9.1%). Levels of resistance to ceftazidime, cefotaxime and nalidixic acid were identical (6.1%) and the same happens to cefepime, tetracycline, kanamycin and combination of

sulfamethoxazole/trimethoprim with 3.03% of the strains being resistant to these antibiotics. All the isolates were sensitive to chloramphenicol, ciprofloxacin and gentamicin.

Analysing resistance by species, it was possible to observe a great resistance to penicillins (amoxicillin) in all species but especially in S. *hafniensis* and S. *xiamenensis.* Resistance to cephalosporins was higher in *Shewanella* sp. (6,1% resistant to these antibiotics compared to 15,2% resistance level in collection) S. *algae* and S. *fodinae* (3,03% for each specie resistant to these antibiotics compared to 15,2% resistance level in collection). In general, resistance to carbapenems is low. Strains showing resistance to these antibiotics belong to S. *xiamenensis* and S. *haliotis* (3,03% for each specie resistant to these antibiotics compared to 6,06% resistance level in collection). Other classes as aminoglycosides, quinolones, monobactams and combination sulfamethoxazole with trimethoprim registered resistance levels in only one isolate of species S. *xiamenensis* and two isolates of S*hewanella* sp.. It was observed that one S. *xiamenensis* isolate presented resistance to quinolones and two *Shewanella* sp. isolates were resistant to monobactams, aminoglycosides (kanamycin) and combination sulfamethoxazole.

Generally, isolates from rivers (n=3 resistant at least to four antibiotics) and salt marsh plant (epiphytic) (n=2 resistant at least to five antibiotics) were more resistant than isolates from other sources.

Multiresistance (resistance to three or more classes of antibiotics) was present in one *Shewanella* sp. isolated from salt marsh plant (epiphytic) and it presented resistance to 3 classes of antibiotics.

## 3. Amplification of *bla*$_{\text{OXA-48-like}}$ gene fragments and determination of genomic context by PCR

### 3.1 Amplification of *bla*$_{\text{OXA-48-like}}$ gene fragments

Considering the total collection of *Shewanella* isolates (n=33), we tested the presence of the *bla*$_{\text{OXA-48-like}}$ gene with primers described in the section Material and Methods. Results are shown in table III.3.

**Table III.4** Results of amplification of *bla*$_{\text{OXA-48-like}}$ gene with different primers.

| | **Amplification of *bla*$_{\text{OXA-48-like}}$** | | | |
|---|---|---|---|---|
| **Strains** | **set1** | **set2** | **set3** | **set4** |
| *S. xiamenensis* Sh1 | - | + | + | nt* |
| *S. algae* Sh2 | - | - | + | nt |
| *S. fodinae* Sh3 | - | - | + | nt |
| *S. haliotis* Sh4 | - | - | - | - |
| *S. xiamenensis* Sh5 | - | - | + | nt |
| *Shewanella* sp. Sh6 | - | - | - | - |
| *Shewanella* sp. Sh7 | - | - | - | - |
| *S. aestuarii* Sh8 | - | - | - | - |
| *S. báltica* Sh9 | + | - | - | + |
| *S. hafniensis* Sh10 | - | - | - | + |
| *S. aestuarii* Sh11 | - | - | - | - |
| *S. aestuarii* Sh12 | - | - | - | - |
| *S. aestuarii* Sh13 | - | - | - | - |
| *S. haliotis* Sh14 | - | - | - | - |
| *S. indica* Sh15 | - | - | - | - |
| *S. indica* Sh16 | - | - | - | - |
| *S. hafniensis* Sh17 | - | - | - | + |
| *S. hafniensis* Sh18 | + | - | - | + |
| *S. baltica* Sh19 | - | - | + | - |

| | | | | |
|---|---|---|---|---|
| *S. hafniensis* Sh20 | - | - | - | + |
| *S. baltica* Sh21 | - | - | - | + |
| *S. baltica* Sh22 | - | - | - | + |
| *S. putrefaciens* Sh23 | - | - | - | + |
| *S. hafniensis* Sh24 | - | - | - | + |
| *S. hafniensis* Sh25 | - | - | - | + |
| *S. hafniensis* Sh26 | - | - | - | + |
| *S. putrefaciens* Sh27 | - | - | - | - |
| *S. algidipiscicola* Sh28 | - | - | - | - |
| *S. hafniensis* Sh29 | - | - | - | + |
| *S. hafniensis* Sh30 | - | - | - | + |
| *S. xiamenensis* Sh31 | + | + | + | nt |
| *S. xiamenensis* Sh32 | + | + | + | nt |
| *S. xiamenensis* Sh33 | + | + | + | nt |

* -: not detected; +: detected, nt: not tested

As we can see in table III.3, the presence of *bla*$_{\text{OXA-48-like}}$ genes was detected in 21 isolates: *S. hafniensis* (n=9), *S. xiamenensis* (n=5), *S. baltica* (n=4), *S. algae* (n=1), *S. fodinae* (n=1) and *S. putrefaciens* (n=1).

PCR products were sequenced and the amino acid sequence was deduced from the obtained nucleotide sequences. For three isolates it was not possible to obtain quality sequences. From two *S. xiamenensis* isolates from river water, a sequence 100% identical to OXA-48 was obtained. OXA-181 was detected in 2 isolates from salt marsh plants belonging to species *S. fodinae* and *S. xiamenensis*, with one amino acid substitution when compared to OXA-48. From 1 *S. xiamenensis* isolate from river water, it was detected a sequence 100% identical to OXA-204. Seven new variants were detected in 13 different strains varying from

80% to 99% similarity between them, and from 80% to 99% similarity to OXA-48. The amino acid substitutions when compared to the OXA-48 sequence are shown in table III.5.

**Table III.5** OXA-48-like amino acid deduced sequences in *Shewanella* strains analysed in this study.

| Source | Strains | Variant | % Similarity to OXA-48 | aa substitutions (comparing to OXA-48) |
|---|---|---|---|---|
| Salt marsh plant (endophytic) | *S. xiamenensis* – Sh1 | OXA-VAR1 | 99% | 2 ≠ aa |
| Salt marsh plant (endophytic) | *S. fodinae* – Sh3 | OXA-181 | 98% | 4 ≠ aa |
| Salt marsh plant (endophytic) | *S. xiamenensis* – Sh5 | OXA-181 | 98% | 4 ≠ aa |
| Cockle | *S. hafniensis* – Sh10 | OXA-VAR2 | 81% | 80 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh17 | OXA-VAR3 | 81% | 75 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh18 | OXA-VAR3 | 81% | 75 ≠ aa |
| Estuarine water | *S. baltica* – Sh19 | OXA-VAR4 | 80% | 80 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh20 | OXA-VAR5 | 80% | 77 ≠ aa |
| Estuarine water | *S. baltica* – Sh21 | OXA-VAR4 | 80% | 80 ≠ aa |
| Estuarine water | *S. baltica* – Sh22 | OXA-VAR4 | 80% | 80 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh24 | OXA-VAR3 | 81% | 75 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh25 | OXA-VAR3 | 81% | 75 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh26 | OXA-VAR7 | 81% | 75 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh29 | OXA-VAR6 | 81% | 76 ≠ aa |
| Estuarine water | *S. hafniensis* – Sh30 | OXA-VAR3 | 81% | 75 ≠ aa |
| River water | *S. xiamenensis* – Sh31 | OXA-48 | 100% | - |
| River water | *S. xiamenensis* – Sh32 | OXA-48 | 100% | - |
| River water | *S. xiamenensis* – Sh33 | OXA-204 | 99% | 2 ≠ aa |

Amino acid sequences representing each variant identified in this study were aligned with OXA-48 using the software Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the alignment is shown in figure III.5.

```
                  10                  20        30
                  *                   *         *
OXA-48       MRVLALSAVFLVAS----------IIGMPAVAKEWQENKSWNAHFTEHK-----------
OXA-181      ------------------------------------------------------------
OXA-204      ------------------------------------------------------------
Var1         ------------------------------------------------------------
Var4         --SF-I-T-LVMS-LLASSTIAAPTFAST-AKT----TR--D-I--Q-QVEPQQAKQQQA
Var2         --SF-I-T-LVMS-LLASSIIAAPTFAST--K-----TR--D-I--Q-QVEPQQAKQQQA
Var3         --SF-I-T-LVMS-LLASSIIAAPTFAST--K-----TR--D-I--Q-QVEPQQAKL---
Var7         --SF-I-T-LGMS-LLASSIIAAPTFAST--K-----TR--D-I--Q-QVEPQQAKL---
Var5         --SF-I-T-LVMS-LLASSIIAAPTFAST--K-----TR--D-I--Q-QVEPQQAKL---
Var6         --SF-I-T-LVMS-LLASSIIAAPTFAST--K-----TR--D-I--Q-QAEPQQAKL---

                  40        50        60        70        80        90
                  *         *         *         *         *         *
OXA-48       ---------SQGVVVLWNENKQQGFTNNLKRANQAFLPASTFKIPNSLIALDLGVVKDEH
OXA-181      ------------------------------------------------------------
OXA-204      ------------------------------------------------------------
Var1         --------------A---------------------------------------------
Var4         KPQKTKSQQAS-------------Y---------G---------------------------
Var2         KPQKTKSQQTS---A---------Y---------G---------------------------
Var3         --QKTKLQQTS---A---------Y---------G---------------------------
Var7         --QKTKLQQTS---A---------Y---------G---------------------------
Var5         --QKTKLQQTS---A---------Y---------G---------------------------
Var6         --QKTKLQQTS---A---------Y---------G---------------------------

                 100       110       120       130       140       150
                  *         *         *         *         *         *
OXA-48       QVFKWDGQTRDIATWNRDHNLITAMKYSVVPVYQEFARQIGEARMSKMLHAFDYGNEDIS
OXA-181      -------------A-----D-------------------------------------------
OXA-204      -------HR----------------------------------------------------
Var1         ------------------------------------------------------------
Var4         -------KS--------------G-----------------------IAS-----------
Var2         -------KS--------------G-----------------------IAS-----------
Var3         -------KS--------------G-----------------------IAS------T--
Var7         -------KS--------------G-----------------------IAS------T--
Var5         -------KS---V------S---G-----------------------IAS-----------
Var6         -------KS--------------G-----------------------IAS-----------

                 160       170       180       190       200       210
                  *         *         *         *         *         *
OXA-48       GNVDSFWLDGGIRISATEQISFLRKLYHNKLHVSERSQRIVKQAMLTEANGDYIIRAKTG
OXA-181      ----------------Q--A------------------------------------------
OXA-204      ------------------------------------------------------------
Var1         ----------------I-------------------------------------------
Var4         --L---------------D---R-----I-A-----------------S-----------
Var2         --L---------------D---R--Y--I-A-----------------S-----------
Var3         --L---------------D---R-----V-A-----------------S-----------
Var7         --L---------------D---R-----V-A-----------------S-----------
Var5         --L---------------D---R--Y--I-A-----------------S-----------
Var6         --L---------------D---R--Y--I-A-----------------S-----------
```

```
                  220       230       240       250       260
                    *         *         *         *         *
OXA-48       YSTRIEPKIGWWVGWVELDDNVWFFAMNMDMPTSDGLGLRQAITKEVLKQEKIIP
OXA-181      ------------------------------------------------------
OXA-204      ------------------------------------------------------
Var1         ------------------------------------------------------
Var4         -AV-A--S-----------N----------I-DAA--P-----------L-HV--
Var2         -AV-A--S-------I--------------I-DAA--P-----------L-HV--
Var3         -AV-A--S----------------------I-DAA--P-----------L-HV--
Var7         -AV-A--S----------------------I-DAA--P-----------L-HV--
Var5         -AV-A--S----------------------I-DAA--P-----------L-HV--
Var6         -AV-A--S-------I--------------I-DAA--P-----------L-HV--
```

**Fig III.5:** Deduced amino acid sequence alignment of OXA-48 and the other variants found. Dashes indicate identical residues among all the amino acid sequences. Amino acid motifs that are conserved among class D beta-lactamases are indicated by boxes in grey. Numbering is according to the class D beta-lactamase system (DBL).

Figure III.6 shows a phylogenetic tree of amino acid sequences obtained in this study and other OXA-48-like variants previously reported. Sequences obtained in this study seem to be grouped by species and affiliated with variants of OXA-48-like class D carbapenemases.

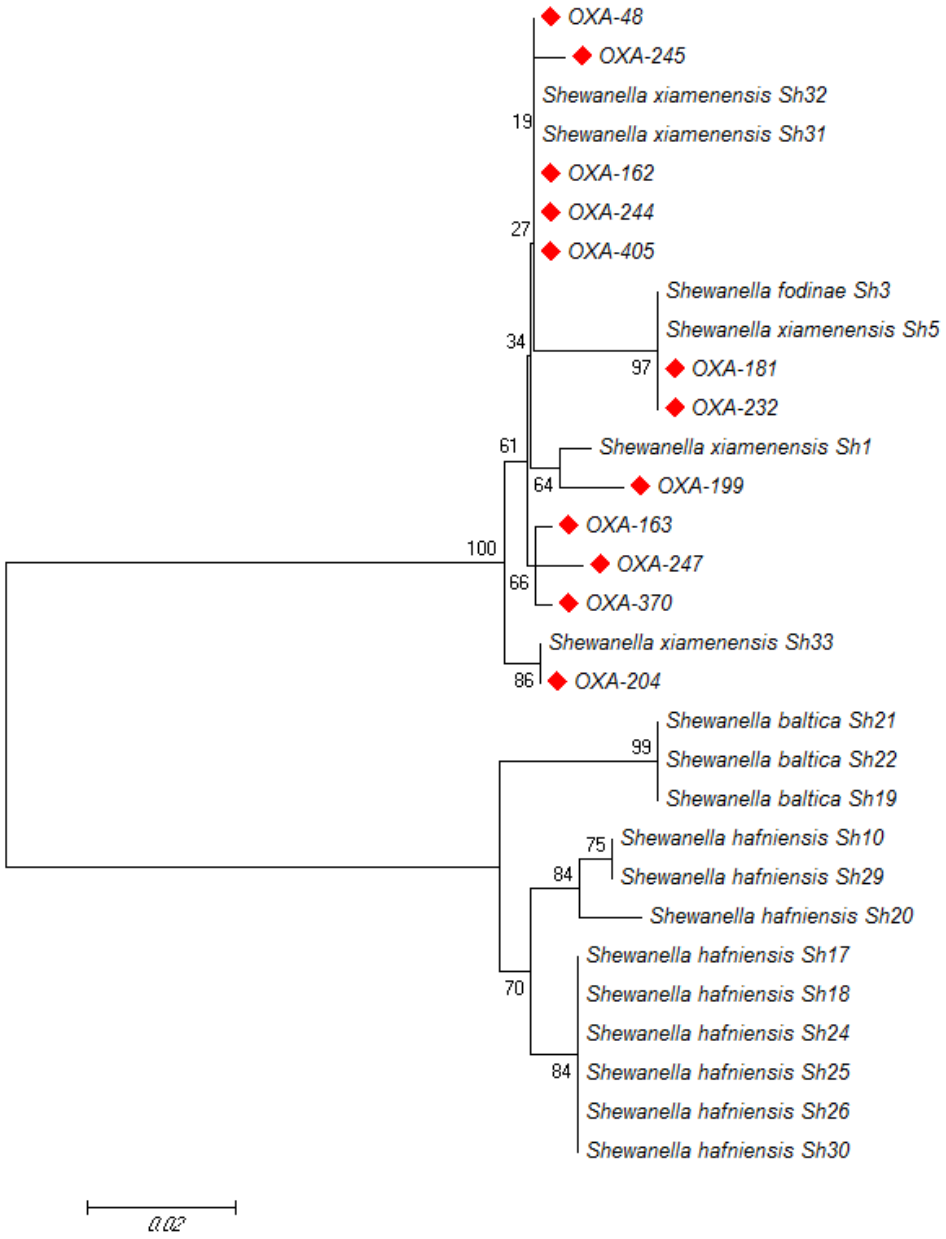


**Fig III.6:** Phylogenetic tree based on OXA sequences of *Shewanella* isolates obtained in this study together with closest matches and representative sequences retrieved from GenBank database. OXA beta-lactamase families searched in Gen Bank are marked in red. The tree was generated using the neighbor-joining method tree with 500 bootstrap replicates. Bootstrap confidence are shown in %.

To investigate the genetic context, primers were designed to regions commonly described as flanking *bla*$_{\text{OXA-48-like}}$ genes in *Shewanella* spp., as presented in figure III.7 (Tacão *et* al. 2013): upstream a gene encoding the peptidase *c15* and downstream the *lysR* gene (primers described in section Material and Methods). These results are shown in table III.6 below.


**Figure III.7** Schematic representation of the *bla*$_{\text{OXA-48}}$ genomic context reported in the literature (adapted from Zong *et* al. 2012).

**Table III.6** Context of OXA-48-like genes in *Shewanella* strains analysed in this study.

| | **Context of *bla*$_{\text{OXA-48-like}}$** | |
|---|---|---|
| | **Upstream** | **Downstream** |
| **Strains References** | **C15** | **LysR** |
| *S. xiamenensis* – Sh1 | √ | unknown |
| *S. fodinae* – Sh3 | √ | unknown |
| *S. xiamenensis* – Sh5 | √ | √ |
| *S. baltica* – Sh9 | unknown | √ |
| *S. hafniensis* – Sh10 | √ | √ |
| *S. hafniensis* – Sh17 | √ | √ |
| *S. hafniensis* – Sh18 | √ | √ |
| *S. baltica* – Sh19 | unknown | √ |
| *S. hafniensis* – Sh20 | √ | √ |
| *S. baltica* – Sh21 | √ | √ |
| *S. baltica* – Sh22 | √ | √ |
| *S. hafniensis* – Sh24 | √ | √ |

| | | |
|---|---|---|
| *S. hafniensis* – Sh25 | √ | √ |
| *S. hafniensis* – Sh26 | √ | √ |
| *S. hafniensis* – Sh29 | √ | √ |
| *S. hafniensis* – Sh30 | √ | √ |
| *S. xiamenensis* – Sh31 | √ | √ |
| *S. xiamenensis* – Sh32 | √ | √ |
| *S. xiamenensis* – Sh33 | √ | √ |

Amplified fragments were sequenced and analyzed. Taking into account the results shown in table III.6, sequencing of the amplified region revealed an identical context for OXA-181, OXA-204 and the OXA-48 presenting upstream and downstream, *c*15 and *lys*R respectively. The 13 strains that exhibit 5 new variants, 12 of them showed upstream *c*15 gene and downstream the *lys*R gene. For the others it was not possible to confirm the context.

## 4. Other antibiotic resistance genes and integrons screening

The presence of other resistance genes was screened in all isolates listed in table III.7.

**Table III.7** Prevalence of resistance genes detected in *Shewanella* isolates.

| **Antibiotics** | **Tested genes** | **Positive PCR results (% of the total number of strains)** |
|---|---|---|
| **Penicillins** (AML, AMC, OX)<br><br>&<br><br>**3rd generation cephalosporins** (CAZ, CTX) | *bla*$_{TEM}$<br><br>*bla*$_{SHV}$ | 0<br><br>0 |
| **Quinolones** (NAL, CIP) | *qnr*A | 23,3% |

All isolates were tested for the presence of *bla*$_{SHV}$ and *bla*$_{TEM}$ genes with specific primers: these genes were not detected with the primers and PCR conditions used. Also, isolates were tested for the presence of *qnrA* genes: PCR and sequencing results showed a percentage of 23,3% isolates with this gene.

Integrons were not identified with the primers and PCR conditions used.

## 5. Plasmids characterization

Plasmids were detected in 30.3% (10 out of 33) of isolates belonging to species *S. hafniensis* (n=3), *S. baltica* (n=1), *S. xiamenensis* (n=3), *Shewanella* sp. (n=1) and *S. aestuarii* (n=2). 6 out of 10 have $bla_{\text{OXA-48-like}}$ genes and 3 of which presenting identical plasmid profiles, as shown in figure III.6.

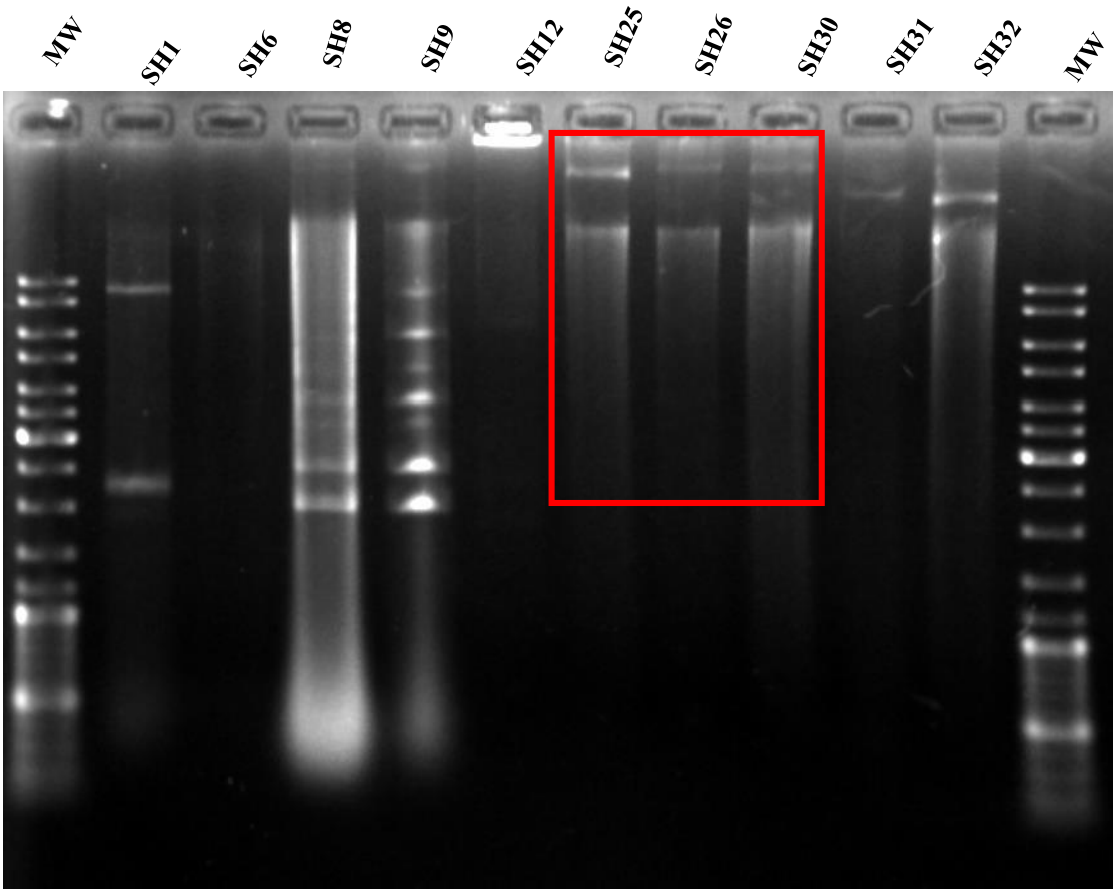


**Figure III.8 :** Detection of plasmids in isolates of *Shewanella*.

## IV. DISCUSSION

No doubt that the resistance to antibiotics is a serious public health problem and it has been increasing in recent years. The onset of clinical strains resistant to last resort antibiotics, such as carbapenems, has been addressed in many studies around the world and has worried the scientific community. The carbapenemase OXA-48 is now one of the greatest concerns in terms of antibiotic resistance. This enzyme and its variants have been detected in all continents. Studies indicate that *Shewanella* spp. can be the origin of OXA-48 encoding genes (Poirel *et* al. 2012; Tacão *et* al. 2013; Zong *et* al. 2012).

With this study, we confirmed that the origin of the OXA-48 encoding genes resides in environmental strains of the genus *Shewanella*. This conclusion is based on the analysis of a collection of strains (n=43) with great phylogenetic diversity.

The phylogenetic affiliation was performed using the 16S rDNA sequence. However, we verified that this information was not enough to identify some isolates at the species level. So we complemented this affiliation with the phylogenetic analysis of the sequence of the *gyr*B gene. Our collection included 11 different species of *Shewanella*, namely *S. hafniensis*, *S. xiamenensis*, *S. aestuarii*, *S. baltica*, *S. haliotis*, *S. indica*, *S. putrefaciens*, *S. algidipiscicola*, *S. algae* ,*S. fodinae* and two isolates showing similarity to its closest relative less than 95%, thus designated as *Shewanella* sp.. All isolates originated from different aquatic environments. The genus *Shewanella* is typical in aquatic ecosystems but was also found in clinical settings. For example in a study performed by To and colleagues in 2010 in hospitals from Hong Kong, a vast majority of *Shewanella* isolates were retrieved from patients with soft-tissue and intra-abdominal infections (To *et* al. 2010). Other case reported in literature refers that *S. xiamenensis* was described as a possible pathogen in cases of chronic pancreatitis (Zong *et* al. 2011). Literature indicates that *S. algae* and *S. putrefaciens* are the species more pathogenic to humans (Janda *et* al. 2014).

In this study, the susceptibility to antibiotics was tested for 15 different antibiotics following the guidelines of CLSI for the Enterobacteriaceae family, because this group is phylogenetically close to the genus *Shewanella*. The tested antibiotics are widely used in clinics for treatment of infections caused by Gram-negative bacteria (Sousa, 2005).

Higher levels of resistance were detected towards amoxicillin and the combination of amoxicillin with clavulanic acid. The β–lactam class is frequently used in human and veterinary medicine which results in highly disseminated resistance.

Information described in the literature indicates that genus *Shewanella* is more susceptible to aminoglycosides as gentamicin. Few isolates showed resistance resistant to other antibiotics, as imipenem (Janda *et* al. 2014; To *et* al. 2010). In general, our isolates were susceptible to carbapenems.

Since the first detection of $bla_{\text{OXA-48}}$ genes, the occurrence of this gene has been restricted to Enterobacteriaceae members and *Shewanella* species (Potron *et* al. 2011; Poirel *et* al. 2012; Sampaio *et* al. 2014). In our collection, we detected the presence of $bla_{\text{OXA-48-like}}$ genes in 21 isolates: $bla_{\text{OXA-48}}$ in 2 isolates of S. *xiamenensis*, $bla_{\text{OXA-204}}$ in 1 isolate of S. *xiamenensis*, $bla_{\text{OXA-181}}$ in 2 isolates of S. *xiamenensis* and S. *fodinae* and 7 new variants in 13 isolates of different species namely S. *hafniensis*, S. *baltica* and S. *algidipiscicola*. From the species identified in this study as $bla_{\text{OXA-48-like}}$ carriers, only S. *xiamenensis*, had been appointed as producer of OXA-48 in previous studies (Poirel *et* al. 2012; Tacão *et* al. 2013).

In general, as was it possible to observe by phylogenetic analysis of deduced amino acid sequences, the same species had the same variant or similar variants. This indicates that these genes are intrinsic of each species, probably evolving from a common ancestor and this evolution seems to have been parallel to the evolution of the genus. However, this gene is not present in all species of this genus; we can speculate that or the ancestral gene was acquired when the species having the gene had already been separated from other species, or the species that do not have the gene, lost it later. Given this, we can consider in our study that this mechanism of resistance is probably intrinsic of some species of *Shewanella*, as for example *S. xiamenensis, S. fodinae, S. baltica* and *S. hafniensis*.

When analyzing the phylogenetic tree based on OXA sequences of *Shewanella* isolates, we cannot exclude the possibility of horizontal gene transfer between species of *Shewanella*, for example between *S. xiamenensis* and *S. fodinae*. These species are phylogenetically distant but both carry the same $bla_{\text{OXA-48}}$ variant.

The detection of OXA-48 producers in the environment has already been reported in previous studies, for example in isolates of *Escherichia coli* and *Klebsiella pneumoniae* from wastewater (Galler *et* al. 2013) or isolates of *Serratia marcescens* (Potron *et* al. 2011) and *Shewanella xiamenensis* (Tacão *et* al. 2013) from river water. The fact that this gene has been

found in the environment is considered worrisome because can contribute to its dissemination in to the clinics.

Noteworthy is the fact that OXA-48 and some variants found in this study have been detected in clinical cases. For example, OXA-204 was detected in Tunisia in *Klebsiella pneumoniae* from urine specimens of a patient hospitalized (Potron *et* al. 2013) or OXA-181 detected in June 2010 in Japan in a man infected by *Klebsiella pneumoniae* (Kayama *et* al. 2015). This clearly shows a link between the variants we detected in environmental strains and the ones causing problems in hospital settings. If this correspondence between the clinics and the environment exists, so the new variants detected in this study can in the future appear associated to clinical cases, constituting a threat to human health.

Until now, only one case of $bla_{\text{OXA-48}}$ in clinical settings was reported in Portugal (Manageiro *et* al. 2014). The fact that these genetic determinants in Portugal are not detected may be due to carbapenems prescription policies (Henriques *et* al. 2012) or to a silent spread of these genes.

In fact, it is often difficult to detect OXA-48-like-producers due to the low levels of resistance conferred by these genes (Evans and Amyes *et* al. 2014). To overcome this difficulty, several new methods were described based on the use of specific antibiotic disks (e.g. temocillin disks and piperacillin–tazobactam disks; (Huang *et* al. 2014)), the use of alternative carbapenems (e.g. faropenem; (Day *et* al. 2013)) or medium supplements such as bicarbonates (Studentova *et* al. 2015). An alternative to these phenotypic methods might be the utilization of molecular methods based in PCR, using specific primers for these genes. For these methods to be efficient it is important to develop primers that assess the diversity of variants known. For this work, we used several sets of primers, some designed during this study. The sequence information provided added more relevant data to design new primers that may offer a more reliable detection of these genes in clinical isolates.

The genomic context tested in this work was based in the flanking regions reported by Zong in 2012 for *Shewanella xiamenensis* (Zong *et* al. 2012). This context appeared conserved in most isolates with $bla_{\text{OXA-48-like.}}$ This indicates, once again, that this gene is intrinsic of some species of *Shewanella*. However, as this context was not detected for all isolates carrying $bla_{\text{OXA-48-like}}$ genes, we cannot exclude the possibility that there are alternative genomic contexts.

The presence of other resistance genes was screened in all isolates. Detection of *bla*$_{TEM}$ were performed. It is important refer that *bla*$_{TEM-1}$ codes for TEM-1 β-lactamase the most common β-lactamase in Gram negative bacteria. However, this gene was not detected in our isolates. The same happened for the screening of *bla*$_{SHV,}$ despite the fact that this gene was indicated as one of the genes highly disseminated among clinical and environmental isolates in Portugal and Spain (Coque, Baquero et al. 2008).

Qnr-like genes, conferring resistance to quinolones, have been found in Gram-positive and Gram-negative bacteria. Few studies indicate that the origin of these genetic determinants resides in environmental Vibrionaceae members and *Shewanella,* more specifically in *Shewanella algae* (Kim *et* al. 2011; Poirel *et* al. 2005; Poirel *et* al. 2005a). The presence of the quinolone resistance gene *qnrA* was inspected and the results of amplification by PCR showed 23% of positive isolates. These isolates belong to species *S. algae* (n=1), *S. haliotis* (n=2), *S. baltica* (n=2) and *S. indica* (n=2). Our results suggest that, as for OXA-48, also in the case of *qnrA,* more studies are needed to establish the origin of this gene (by our results it seems to be not only *S. algae*) and to understand which different variants can be found in *Shewanella*.

As described above, OXA-48 has been described in Enterobacteriaceae, in either clinical isolates or in environmental isolates. Most probably, mobilization events allowed to transfer OXA-48 genes from *Shewanella* to Enterobacteriaceae. The gene has been detected in mobile genetic elements as for example IncA/C, Inc F-like and Inc L/M plasmids (Poirel *et* al. 2012). In our study plasmids were identified in 10 isolates and 7 of them carried OXA-48-like genes. However, these results were insufficient to establish some association between the presence of the OXA-48 gene and these mobile genetic elements.

## V. CONCLUSIONS

The prevalence of bacteria carrying the *bla*$_{\text{OXA-48}}$ gene or its variants in the environment may increase the risk of the emergence of these genetic determinants in clinical cases.

With this study it became clear that members of the genus *Shewanella*, present in aquatic environments, play a crucial role in the origin of *bla*$_{\text{OXA-48}}$ gene. We identified the species within this genus that are the origin of clinically-relevant variants of the gene. Besides we identified new variants, not yet found in clinical settings, adding new information on the diversity of these family of genes. These variants might be transferred to new hosts (e.g. Enterobacteriaceae), and emerge in clinics. Thus we can conclude that *Shewanella* is not only the origin of *bla*$_{\text{OXA-48-like}}$ genes but also a reservoir of new variants of this gene.

Besides this, the molecular methodology here applied added sequence information that might be relevant to design more specific and sensitive methods to detect these genes. These methods can be used in clinics.

In future work, it would be interesting to characterize the plasmids present in our isolates in order to search for links between these plasmids and the transfer of *bla*$_{\text{OXA-48}}$ to *Shewanella*; in parallel it would be important to inspect for the presence of insertion sequences previously linked to *bla*$_{\text{OXA-48-like}}$ genes in the genome of the *Shewanella* isolates. This would help to clarify the mechanisms responsible for *bla*$_{\text{OXA-48-like}}$ genes mobilization and transfer.

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## VII. APPENDICES

### Composition of Luria Bertani Broth

Peptone from casein 10.0g
Yeast extract 5.0g
Sodium chloride 10.9g (pH 7.0)

### Composition of Marine Broth

Peptone 5.0g
Yeast extract 1.0g
Ferric citrate 0.1g
Sodium chloride 19.45g
Magnesium chloride 5.9g
Magnesium sulfate 3.24g
Calcium Chloride 1.8g
Potassium chloride 0.55g
Sodium Bicarbonate 0.16g
Potassium Bromide 0.08g
Strontium Chloride 34.0mg
Boric Acid 22.0mg
Sodium Silicate 4.0mg
Sodium Fluoride 2.4mg
Amonium Nitrate 1.6mg
Disodium Phosphate 8.0mg (pH: 7.6)

### Composition of Mueller-Hinton (MH) Agar

Infusion from meat 2.0 g
Casein-hydrolysate 17.5 g
Starch 1.5 g
Agar-agar 17.0 g (pH 7.3)