UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF
Diagnostics and Public Health
Microbiology Section

GRADUATE SCHOOL OF
Life and Health Sciences

DOCTORAL PROGRAM IN
Applied Life and Health Sciences
Microbiology and Infectious Diseases
XXII Cycle Year 2016

MARINE MICROORGANISMS PRODUCING CARBAPENEMASES AND β-LACTAMASES AS ENVIRONMENTAL RESERVOIRS OF ANTIBIOTIC RESISTANCE GENES CONSTITUTING A RISK FOR HUMAN HEALTH

S.S.D. MED/07

Coordinator: Prof. Giovanni Malerba

Tutor: Prof.ssa Maria del Mar Lleo

PhD Student: Dr.ssa Vanessa Zago

Marine microorganisms producing carbapenemases and β-lactamases as environmental reservoirs of antibiotic resistance genes constituting a risk for human health

Vanessa Zago

Tesi di Dottorato

Verona, 21 Maggio 2020

Rare sono le persone che usano la mente,
poche coloro che usano il cuore e
uniche coloro che usano entrambi.

- RITA LEVI MONTALCINI -

RIASSUNTO

Le infezioni dovute a batteri multi-resistenti rappresentano ormai una vera e propria preoccupazione a livello globale, non solo in ambito nosocomiale ma anche e soprattutto nel settore veterinario, agroalimentare e ambientale. Ciò è dovuto alla capacità dei microrganismi esposti agli antibiotici di mettere in atto nuovi meccanismi di resistenza in tempi molto brevi e di diffonderli rapidamente alle altre specie batteriche che coesistono nella stessa nicchia ecologica attraverso sistemi di trasferimento per via orizzontale (HGT) o verticale. In particolar modo, l'utilizzo da parte delle cellule batteriche di elementi mobili genetici (MGEs) quali integroni, sequenze di inserzione, trasposoni, plasmidi ed elementi coniugativi ed integrativi (ICEs) facilitano questa operazione di diffusione. Le discipline omiche possono essere d'aiuto non solo nell'individuazione dei geni specifici coinvolti nell'antibiotico-resistenza, ma anche nel rilevare i possibili elementi mobili genetici coinvolti nella loro diffusione nel nostro territorio.

In questo contesto, nasce il mio progetto di dottorato che grazie all'utilizzo della tecnologia Next Generation Sequencing ha permesso di sequenziare l'intero genoma (Whole Genome Sequencing) di 12 ceppi appartenenti alla famiglia delle *Shewanellaceae* (n=10; *Shewanella algae*) e *Vibrionaceae* (n=2; *Vibrio anguillarum*, *Vibrio parahaemolyticus*) isolati in centri di acquacoltura italiani dislocati in differenti siti lungo la costa del Mar Adriatico. Lo scopo della tesi è stato quello di valutare il ruolo di questi microrganismi come possibili serbatoi di geni di antibiotico-resistenza nell'ambiente acquatico e la loro capacità di trasferire questi geni ad altre specie batteriche potenzialmente patogene per l'uomo che possono condividere temporaneamente e/o accidentalmente la stessa nicchia ecologica.

Il resistoma ha evidenziato la presenza di geni che conferiscono resistenza a diverse classi di antibiotici inclusi β-lattamici, chinoloni, tetracicline, macrolidi, polimixine, cloramfenicolo e anche ai metalli pesanti. Le resistenze coinvolte non sono solo di tipo enzimatico ma comprendono anche un sistema complesso di pompe di efflusso. In particolare, la resistenza ai β-lattamici è mediata dalla presenza dei geni codificanti per $bla_{\text{OXA-55-like}}$, bla_{AmpC} e *mexB-OprM* nei ceppi di *S. algae*, mentre per i vibrio sono stati identificati geni che codificano per bla_{AmpC} in *V. anguillarum* oltre che *mexA-OprM* e $bla_{\text{CARB-19}}$ in *V. parahaemolyticus*. La resistenza ai chinoloni è dovuta alla presenza di *qnrA3*, *qnrA7* e *qnrVC6*, di cui quest'ultimo trovato nel genoma di *V. anguillarum* e di

pompe di efflusso appartenenti alle famiglie MATE (Multidrug And Toxic compound Extrusion) e MFS (Major Facilitator System). Inoltre, la presenza di *macAB-TolC*, una pompa di efflusso del tipo ABC (ATP Binding Cassette) sembra essere responsabile della resistenza ai macrolidi. Tutti i ceppi analizzati risultano possedere l'*eptA*, ovvero la fosfoetanolammina transferasi che contribuisce alla resistenza alle polimixine, mentre la resistenza alle tetracicline è evidenziata dalla presenza di pompe di efflusso quali *TetR*, *Tet34* e *Tet35* in vibrio. Infine, la resistenza al cloramfenicolo è dovuta soprattutto alla presenza dei geni *cat* e *MdtL*. Per quanto riguarda i metalli pesanti sono stati individuati geni che conferiscono resistenza all'arsenico, rame, zinco, cobalto, cadmio, cromo, molibdeno, magnesio e nickel nella maggior parte dei ceppi analizzati.

L'analisi del mobiloma ha evidenziato la presenza di elementi mobili associabili all'integrone di classe I in tutti i ceppi di *S. algae* analizzati e nel ceppo di *V. anguillarum*. Inoltre, molte integrasi sono state identificate essere correlate filogeneticamente alle integrasi dell'integrone di classe II nelle shewanelle e all'integrone di classe IV nei vibrio. Per quanto riguarda le sequenze di inserzione (ISs) sono state individuate diverse famiglie, alcune presenti nelle shewanelle ed altri nei vibrioni. Di particolare interesse è il ceppo 353M di *S. algae* isolato in mare aperto e contenente degli elementi coniugativi ed integrativi coinvolti nella partizione (ParAB), replicazione (repAB, traI), traslocazione (traD), assemblaggio del sistema di secrezione di tipo IV (traC, traN) ed integrazione (XerD). Inoltre, in alcuni ceppi di *S. algae* (144bCP, 146bCP, 178CP, 353M, 38LV, 57CP, 83CP) sono presenti degli elementi di circa 200 kbp che contengono dei moduli genetici ben strutturati con il t-RNA-Phe, l'IHFα, una serie di geni coinvolti nel metabolismo, nella resistenza agli antibiotici e ai metalli pesanti, geni di virulenza (emolisina) ed infine l'IHFβ che fanno presupporre la presenza di altre ICEs non ben identificate durante l'assemblaggio e l'annotazione dei genomi. Infine, sono stati individuati alcuni elementi fagici completi ed incompleti. Tra i fagi completi sono stati identificati quelli appartenenti a *Aeromonas* phage phiO18P (NC_009542), *Escherichia* phage D108 (NC_013594), *Shewanella* sp. phage 1/44 (NC_025463), *Enterobacteria* phage phi92 (NC_023693), *Enterobacterial* phage mEp213 (NC_019720) e *Vibrio* phage VP882 (NC_009016).

L'analisi del viruloma ha evidenziato la presenza di batteriocine e di emolisine (hlyD, hemolysin III) presenti in tutti i ceppi di *S. algae*. Per quanto riguarda i ceppi di vibrio sono state individuate altre emolisine (tlh, hemolysin III), la rtxA e l'emoagglutinina nel *V. anguillarum*, mentre l'emolisina D e l'emolisina termostabile delta-VPH sono state

rinvenute nel *V. parahaemolyticus*. Infine, sono stati identificati i sistemi di secrezione di tipo I, II, III, IV and VI che possono essere utilizzati nella secrezione di fattori di virulenza.

Nella seconda parte del progetto si è proceduto con l'analisi filogenetica delle sequenze amminoacidiche predette di $bla_{\text{OXA-55-like,}}$ bla_{AmpC} e $bla_{\text{CARB-19}}$ al fine di valutare la similarità con le sequenze depositate in Genbank appartenenti ad altre specie di *Shewanella* spp., *Vibrio* spp. e di interesse clinico (*Enterobacteriaceae* e *Acinetobacter* spp.). I risultati ottenuti hanno evidenziato come OXA-55-like rappresenti un cluster filogeneticamente correlato alle altre varianti di OXA presenti nel genere *Shewanella*, ma di interesse è la sua stretta correlazione con OXA-20 ed OXA-21 individuate in ceppi clinici rispetto ad altre varianti isolate in *Acinetobacter* spp. Inoltre, la presenza di specifiche mutazioni in determinate posizioni all'interno delle sequenze di AmpC nei ceppi di *S. algae* ha permesso di suddividere i ceppi secondo i siti e le date di campionamento, indicando la presenza lungo la costa adriatica italiana di cloni contenenti mutazioni diverse della stessa beta-lattamasi, indice della capacità di questi microrganismi di rispondere alla pressione selettiva ambientale. Al contrario, l'analisi della sequenza di CARB-19 ha riportato alcune mutazioni puntiformi che non hanno mutato la sua sequenza amminoacidica. Infine, il clonaggio di bla_{AmpC} e $bla_{\text{CARB-19}}$ e la trasformazione in *Escherichia coli* TOP10 ha permesso di mobilizzare queste due beta-lattamasi e individuare come varia il livello di espressione in membri appartenenti alla famiglia delle *Enterobacteriaceae*. I risultati ottenuti hanno permesso di confermare alcune resistenze (amoxicillina e ticarcillina) e un aumento alla resistenza alla piperacillina per pCARB-19. Per quanto riguarda pAmpC si sono registrate una diminuzione delle MIC per gli antibiotici appartenenti alle ammino e carbossipenicilline e ureidopenicilline rispetto a *V. anguillarum* 28AD WT, mentre si è confermata la resistenza alle cefalosporine di prima generazione.

ABSTRACT

Nowadays, infections due to multi drug resistant bacteria represent a real global concern, not only in the nosocomial environment but also and above all in the veterinary, agri-food and environmental sectors. This is due to the ability of microorganisms, exposed to antibiotics, to implement new resistance mechanisms in a very short time and to spread them rapidly to other bacterial species that coexist in the same ecological niche through horizontal (HGT) or vertical transfer systems. In particular, the use of mobile genetic elements (MGEs) such as integrons, insertion sequences, transposons, plasmids and conjugative and integrative elements (ICEs) facilitate this operation in the four areas previously mentioned. For this purpose, the omic disciplines can help us not only in identifying the specific genes involved in antibiotic resistance, but also in detecting the possible mobile genetic elements involved in their diffusion in our territory.

It is in this context that my doctoral project was placed. The use of Next Generation Sequencing technology allowed to sequence the whole genome (Whole Genome Sequencing) of 12 strains belonging to the *Shewanellaceae* (n=10; *Shewanella algae*) and *Vibrionaceae* (n=2; *Vibrio anguillarum*, *Vibrio parahaemolyticus*) families isolated in Italian aquaculture centers located at different sites along the coast of the Adriatic Sea. The aim of the thesis was to evaluate the role of these microorganisms as possible reservoirs of antibiotic resistance genes in the aquatic environment. Moreover, the ability to transfer these genes to other potentially pathogenic bacterial species which can temporarily and/or accidentally share the same environment was investigated as well.

The resistome showed the presence of genes conferring resistance to different classes of antibiotics including β-lactams, quinolones, tetracyclines, macrolides, polymyxins, chloramphenicol and heavy metals. The mechanisms of resistance involved were not only enzymatic but a complex system of efflux pumps was also found. In particular, β-lactam resistance was mediated by the presence of genes coding for $bla_{\text{OXA-55-like}}$, bla_{AmpC} and *mexB-OprM* in *S. algae* strains, while for vibrios the genes coding for bla_{AmpC} in *V. anguillarum*, *mexA-OprM* and $bla_{\text{CARB-19}}$ in *V. parahaemolyticus* were identified. The resistance to quinolones was due to the presence of *qnrA3*, *qnrA7* and *qnrVC6*, of which this one was found in the genome of *V. anguillarum*. In addition, efflux pumps belonging to the families MATE (Multidrug And Toxic compound Extrusion) and MFS (Major Facilitator System) contributed as well to the phenotype. Furthermore, the presence of

macAB-TolC, an ABC-type efflux pump (ATP Binding Cassette) appeared to be responsible for the resistance to macrolides. All the analyzed strains showed to possess the *eptA*, the phosphoethanolamine transferase that contributed to the resistance to polymyxins, while the resistance to tetracyclines was mainly due to the presence of efflux pumps such as *TetR*, *Tet34* and *Tet35* in vibrios. Finally, the presence of genes such as *cat* and *MdtL* conferred resistance to chloramphenicol. Regarding heavy metals, genes conferring resistance to arsenic, copper, zinc, cobalt, cadmium, chromium, molybdenum, magnesium and nickel were detected in most of the analyzed strains.

The mobilome analysis showed the presence of mobile genetic elements that could be associated to the class I integron in all the analyzed *S. algae* and in the *V. anguillarum* strains. Regarding the insertion sequences (ISs), different families were detected in shewanellae and vibrios. Of particular interest was the 353M *S. algae* strain isolated in the open sea and containing the conjugative and integrative elements involved in the partition (ParAB), replication (repAB, traI), translocation (traD), the type IV secretion system assembly (traC, traN) and integration (XerD). Moreover, in some strains of *S. algae* (144bCP, 146bCP, 178CP, 353M, 38LV, 57CP, 83CP) some elements of about 200 kbp were detected. They contained well-structured genetic modules included t-RNA-Phe, IHFα, a series of genes involved in metabolism, resistance to antibiotics and heavy metals, virulence genes (hemolysin) and finally IHFβ, suggesting the presence of other ICEs not well identified during the genome assembly and annotation. In addition, some complete and incomplete phage elements were identified. Among the complete phages, those belonging to *Aeromonas* phage phiO18P (NC_009542), *Escherichia* phage D108 (NC_013594), *Shewanella* sp. phage 1/44 (NC_025463), *Enterobacteria* phage phi92 (NC_023693), *Enterobacterial* phage mEp213 (NC_019720) and *Vibrio* phage VP882 (NC_009016) were detected.

The virulome analysis showed the presence of bacteriocins and hemolysins (hlyD, hemolysin III) in all the *S. algae* strains. Regarding the vibrio strains, other hemolysins (tlh, hemolysin III), rtxA and hemagglutinin were detected in *V. anguillarum*, while hemolysin D and thermostable hemolysin delta-VPH were found in *V. parahaemolyticus*. Finally, type I, II, III, IV and VI secretion systems were identified.

In the second part of the project, I proceeded with the phylogenetic analysis of the predicted OXA-55-like, AmpC and CARB-19 amino acid sequences in order to evaluate their similarity with the sequences stored in Genbank belonging to other species of *Shewanella* spp., *Vibrio* spp. and of clinical interest (*Enterobacteriaceae* and

Acinetobacter spp.). The results showed that OXA-55-like represented a cluster phylogenetically related to the other OXA variants present in the genus *Shewanella*, but of interest was its close correlation with OXA-20 and OXA-21 identified in clinical strains if compared to other variants isolated in *Acinetobacter* spp. Furthermore, the presence of specific mutations in certain positions within the AmpC sequences in the *S. algae* strains allowed to group the strains according to the sampling sites and dates, indicating the presence along the Italian Adriatic coast of clones containing several mutations of the same beta-lactamase. This highlights the ability of these microorganisms to respond to environmental selective pressure. Conversely, CARB-19 sequence analysis reported some point mutations that did not change its amino acid sequence. Finally, bla_{AmpC} and $bla_{\text{CARB-19}}$ cloning in *Escherichia coli* TOP10 was performed. This has allowed to mobilize these two beta-lactamases to study how their expression level vary on *Enterobacteriaceae* family members. The results confirmed the resistance to amoxicillin and ticarcillin with an increase MIC values for piperacillin in pCARB-19. Conversely, regarding pAmpC, a decreasing MIC values for amino- and carboxypenicillins as well as for ureidopenicillins respect to *V. anguillarum* 28AD WT were obtained. Resistance to first generation cephalosporins was confirmed.

TABLE OF CONTENTS

1. ABBREVIATIONS

AMR/AR, Antimicrobial Resistance

ARG, Antimicrobial Resistance Gene

AST, Antimicrobial Susceptibility Testing

HGT, Horizontal Gene Transfer

MDR, Multi Drug Resistant

ESBL, Extended Spectrum beta-lactamase

OXA, Oxacillinase

CARB, Carbenicillinase

CHDL, Carbapenem hydrolyzing class D beta-lactamase

MGEs, Mobile Genetic Elements

NGS, Next Generation Sequencing

WGS, Whole Genome Sequencing

ISs, Insertion Sequences

TEs, Transposable Elements

TE, Tris EDTA

SDS, Sodium Dodecyl Sulfate

SEVAG, chloroform:isoamyl alcohol

BCT, Blue Carba Test

PBP, Penicillin Binding Protein

CPE, Carbapenemase Producing *Enterobacteriaceae*

TSA, Tryptic Soy Agar

TSB, Tryptic Soy Broth

TCBS, ThiolSulphate-Citrate-Bile-Sucrose

MHA, Mueller Hinton Agar

PMQR, Plasmid-Mediated Quinolone Resistance

QRDR, Quinolone Resistance-Determining Region

ICEs, Integrative and Conjugative Elements

WT, Wild Type

SOB, Super Optimal Broth

2. RATIONAL & OBJECTIVE OF THE THESIS

In the nosocomial setting, the increasing resistance to last resort drugs such as carbapenems, tigecycline and colistin is currently an important concern. The environment is a determining factor in the spreading of antibiotic resistance and it is known that particularly the aquatic environment can play as antimicrobial resistance gene reservoir. This reservoir facilitates the exchange of mobile genetic elements carrying antimicrobial resistance genes (ARGs) not only among species belonging to the natural microbiota but also among the occasional ones including species potentially pathogens for humans. Moreover, the abuse and misuse of drugs in veterinary medicine and, in particular, in aquaculture, can stimulate the appearance of new resistant microbial populations that might spread along the coastal areas contributing to this genetic reservoir.

In order to evaluate the contribution of aquaculture centers to the emergence of antibiotic resistant bacterial strains, the aim of my thesis has been to investigate, using the Next Generation Sequencing technology, the resistome, mobilome and virulome of a Multi Drug Resistant marine bacteria collection isolated in several aquaculture centers located along the coast of the Adriatic Sea in Italy.

The data obtained allowed to decipher a number of genes and mobile genetic elements involved in the resistance to several classes of antibiotics found in the analyzed marine species belonging to *S. algae*, *V. anguillarum* and *V. parahaemolyticus*. In particular, it is worthwhile to highlight the plasticity of their genomes and the presence, within them, of genetic elements that can derive from other aquatic or pathogenic bacteria confirming the interchange of ARGs among the microbial population.

The collaboration with the Medical and Molecular Microbiology Unit led by Prof. Nordmann at the University of Fribourg in Switzerland has allowed to *in vitro* mobilize bla_{AmpC} and $bla_{\text{CARB-19}}$ beta-lactamase encoding genes present in *V. anguillarum* and in *V. parahaemolyticus* chromosomes respectively, by cloning. This process has occurred easily between *Vibrio* spp. and *Escherichia coli*.

Finally, this work has also contributed into the analysis of the point mutations present in the amino acid sequences of OXA-55-like, AmpC and CARB-19 found in *S. algae*, *V. anguillarum* and *V. parahaemolyticus*, in order to understand the mutation rate within these beta-lactamases and how it varies among the two genera analyzed.

PART I

WHOLE GENOME SEQUENCING ANALYSIS TO INVESTIGATE THE RESISTOME, MOBILOME AND VIRULOME OF *SHEWANELLA ALGAE* AND *VIBRIO* SPP. STRAINS ISOLATED IN AQUACULTURE CENTERS IN ITALY

3. INTRODUCTION

3.1 Antimicrobial resistance in the environment: an ancient concern for public health

Antimicrobial resistance is increasing worldwide and is becoming a threat to human health. This is a natural evolutionary response to antimicrobial exposure documented by an ECDC (European Center for Disease Control) study reporting about 33.000 deaths occurred in 2015 in Europe as a direct consequence of an infection due to bacteria resistant to antibiotics (Cassini *et al*., 2019). Considering this dramatic situation, due mainly to the misuse and abuse of antibiotics, a predominant role is played by livestock and agriculture. In these sectors, antibiotics are used to support animal and plant health contributing for between one third and two thirds of total antibiotic sales and including use of critically important antibiotics (CIAs) which comprise antibiotics deemed vital to maintain human health included aminoglycosides, macrolides, penicillins, quinolones, sulfonamides and tetracyclines (Ledingham, WHO, 2019; Santos and Ramos, 2018). It is generally acknowledged and heavily debated that the irresponsible and excessive use of antibiotics for the control of infections in animals, combined with the administration at sub-therapeutic doses (for prophylaxis or as growth promoters) in healthy animals through feed and water, has, through the years, contributed to the increased resistance of some pathogens that can propagate to humans (Ferri *et al*., 2017). In this scenario, a great contribution to maintain antibiotic reservoir in the environment is given by aquaculture sector where veterinary medicines are widely used (FAO, 2018). A consequence of the excessive use of antibiotics in food producing animals is the excretion of these unmetabolized drugs directly into the environment where they can persist creating an opportunity for the selection of resistant strains within exposed bacterial population (Rosenblatt-Farrell, 2009). ARGs are found in many environments such as 30.000-year-old permafrost, isolated caves, Alaskan soil and glaciers as Pal and coworkers stated (2016), suggesting the ancienty of the issue. This is not a surprise, considering that most antimicrobial drugs are naturally produced by microorganisms, including environmental fungi and saprophytic bacteria, or are synthetic modifications of them, with only a few drugs (eg, sulfonamides and fluoroquinolones) being wholly synthetic (Holmes *et al*., 2016). In particular, aquatic environment is increasingly being recognized as an important reservoir of ARGs able to retain and transfer resistance (Lloyd *et al*., 2018). Exposure to contaminants, drug residues and metals originating from anthropogenic activities, included aquaculture, can trigger a process called co-selection contributing in the

maintenance of the resistant microorganisms in the environment. This, facilitate the spread of ARGs from human pathogens to the natural microbiota and viceversa when they coexist in the same ecological niche. In addition to that, the natural microflora itself is a reservoir of ARGs which more often are located in the bacterial chromosome constituting an intrinsic resistance to several classes of antibiotics.

Although the role of the environment as an important source and dissemination route of resistance has been increasingly recognized (Bondarczuk *et al*., 2015; Ashbolt *et al*. 2013; Finley *et al*.2013; Pruden *et al*.2013; Wright, 2010; Martinez, 2008), the knowledge about its contribution is still limited. This is due to the fact that we cannot consider the environment alone but as a collection of the contributions in AMR coming from human, animal and agricultural sectors in a One-Health approach as described in Figure 1.

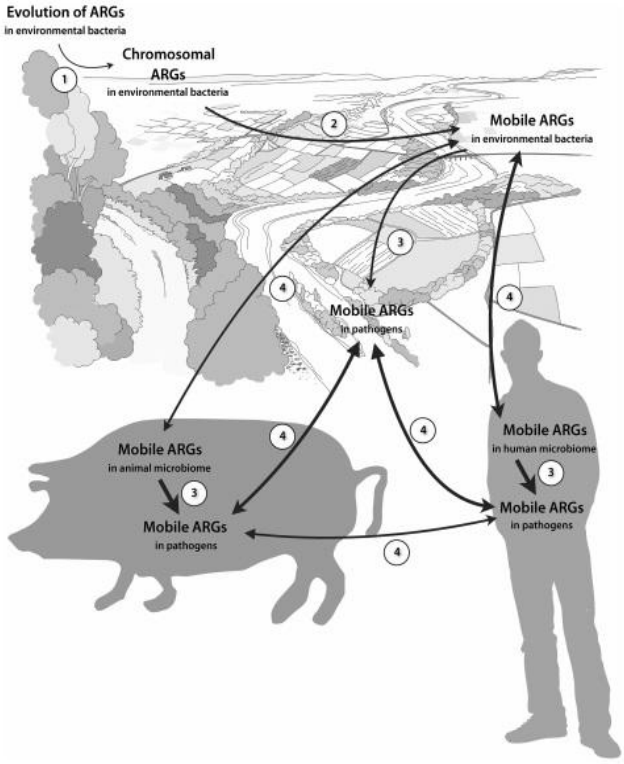

Figure 1. Overview of how external environment, livestock and humans can act in disseminating and developing AMR (Bengtsson-Palmer *et al*., 2018). The antibiotic exposure in the environment can select for AMR bacteria with novel ARGs (1), that can survive in the environment and spread in it through MGEs (2). Human pathogens can interact with the environment resistome constituted by the microflora of a specific ecological niche and acquire new ARGs through their mobilization (3). Then these ARGs are disseminated in the human microbiome (4).

3.1.1 AMR in aquaculture and its consequences in the aquatic environment

The growth of aquaculture as a commercial activity has been rapid since 1970s (Diana *et al*., 2013). Fish and shellfish, reared under crowded and stressful conditions, are prone to bacterial infections. Increases in industrialisation of aquaculture has been paralleled by increased prophylactic and therapeutic use of antimicrobials and chemical disinfectants to prevent and treat these infections (Cabello *et al*., 2013). This is why this sector is particularly touched by the problem of AMR due to the extensive, unregulated, and indiscriminate use of antibiotics in much of the world. Despite there is much less data available on the public health impact of antimicrobial usage in aquaculture compared to other sources, it seems that the use of antimicrobials might contribute to the emergence and spread of AMR through the water, pond sediments, fish, shellfish and finally present a risk to public health (Heuer *et al*., 2009; Miranda and Zemelman, 2001). In Europe and in Italy, the antibiotics authorized for use in aquaculture are amoxicillin, ampicillin, tetracycline, oxytetracycline, chlortetracycline, florfenicol, thiamfenicol, oxolinic acid, enrofloxacin, flumequin, erythromycin, and sulfonamides (potentiated with trimethoprim) (OIE, 2018; EEC Regulation No 2377/90). Furthermore, it is easily understood that aquacultural antibiotic doses can be proportionately higher than those used in terrestrial animal farming (Santos and Ramos, 2018). Also the integrated fish farming with the use of animal manure as fertilizers seems to favor antimicrobial-resistant bacteria in the pond environment (Petersen *et al*., 2002). Although the total quantities of antibiotics employed in aquaculture are estimated to be smaller than those used in land animal husbandry, there is much greater use of antibiotic families that are also used in human medicine (e.g., quinolones, beta-lactams, tetracyclines) (Marshall and Levy, 2011). Moreover, the presence of ineffective vaccines against the most common fish pathogens and the crowded conditions of fish farming, see the use of antimicrobials as a need in this field. This can select for aquatic antimicrobial-resistant bacteria that can contaminate aquaculture products marketed for human consumption (Santos and Ramos, 2018; Cabello *et al*., 2016). These bacteria and antimicrobial residues can reach human comsumers and alter their microbiome (Blaser, 2014; Chen *et al*., 2013; Love *et al*., 2011). Furthermore, heavy metals and disinfectants used in aquaculture can also increase AMR in the aquacultural environment (Pal *et al*., 2015; Seiler *et al*., 2012). Antimicrobials used in aquaculture are largely administered in feed, occasionally by bath. Up to 80% of the antimicrobials are deposited in water and sediments close to sites of application in the absence of collectors under pens to catch uneaten medicated feed (Figure 2). Ingested but not absorbed antimicrobials, together with their metabolites in

stools, urine, and other secretions sediment to these same areas with their antimicrobial activity largely intact (Figure 2). Generally, antimicrobials in aquatic environments are rapidly transported from sites of application and diluted, but sometimes antimicrobials (e.g. tetracyclines) and their metabolites can remain active in aquatic sediments for months at sufficiently high concentrations to exert selective effects on aquatic bacterial diversity in these environments (Hektoen *et al*., 1995; Samuelsen *et al*., 1992; Björklund *et al*., 1991).

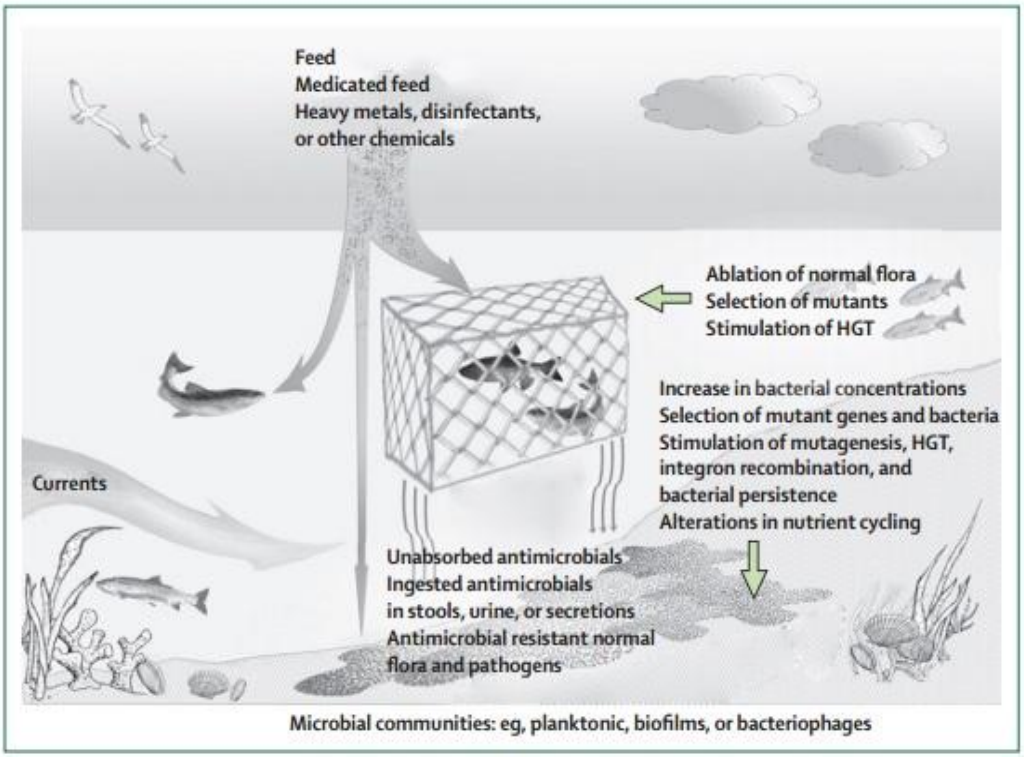

Figure 2. AMR in the aquacultural environment (Cabello *et al*., 2016). The antimicrobials reach the farmed and wild fish through feed with deep consequences in their microbiomes. In addition, the unmetabolized antibiotics and their residues are released in the aquatic environment and sediment exerting a selection pressure on the autochthonous microflora. In this way, ARGs can be spread by HGT in the microbial community.

The composition of microbiota in the aquatic environment can be altered by the microbiota of stools and secretions of fish and shellfish treated with antimicrobials through the addition of high levels of commensals and potential pathogens containing ARGs (Romero *et al*., 2014; Cantas *et al*., 2012; Navarrete *et al*., 2008). The aquatic environment also contains high concentrations of bacteriophages and other gene transfer

agents able to mediate and stimulate HGT by transduction and generate naked DNA for transformation by bacterial lysis (Cabello *et al*., 2013; Srinivasiah *et al*., 2008). Such naked DNA can be taken up by many bacterial species such as by animal pathogens like vibrios (Domingues *et al*., 2012). Biofilms as well, being common in the aquatic environment, can mediate HGT and persistence (Balcázar *et al*., 2015).

3.1.2 Mechanisms of antibiotic resistance in Gram-negative bacteria

Bacteria have a remarkable genetic plasticity that allows them to respond to a wide array of environmental threats, including the presence of antibiotic molecules that may jeopardize their existence (Munita and Arias, 2016). They can be intrinsically resistant to one or more antimicrobials due to the selective pressure during antibiotic exposure. But, in most cases the AMR can be acquired in a bacterial population previously susceptible to a specific antimicrobial compound. However, the two major genetic strategies that bacteria can use to defend from antibiotics are mutations in gene(s) often associated with the mechanism of action of the compound and acquisition of foreign DNA coding for resistance determinants through HGT. Generally, mutations alter the antibiotic action through modifications of the antimicrobial target decreasing the affinity for the drug (Figure 3). Moreover, the ability to create biofilms is another strategy to escape to the drug action.

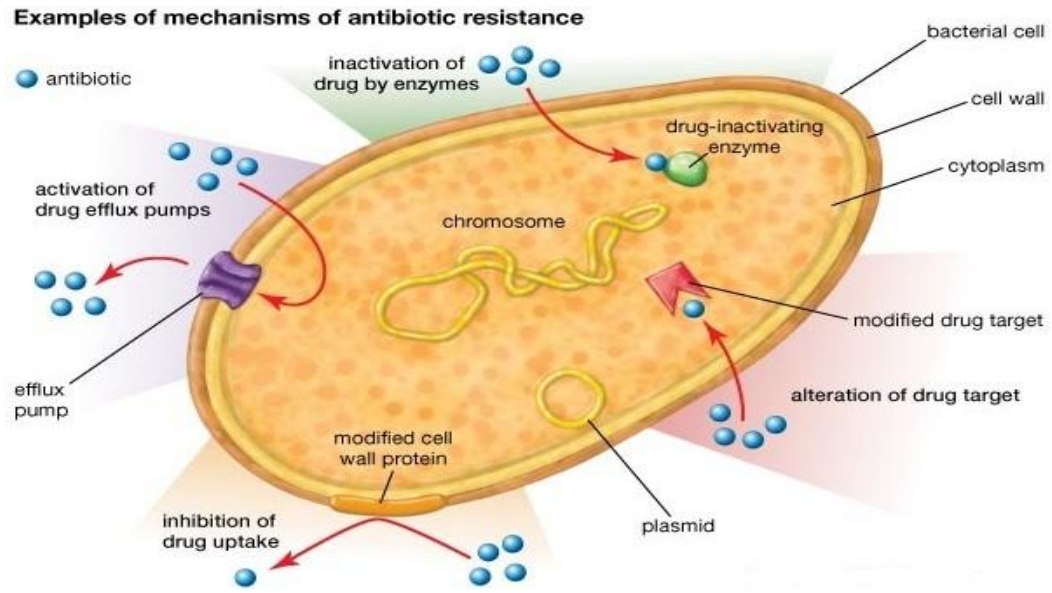

Figure 3. Schematic illustration of the antibiotic resistance mechanisms occurring in bacteria (Khameneh *et al*., 2016).

Concerning the modification of the antimicrobial compound, bacteria are able to produce specific enzymes capable of introducing chemical changes to the antimicrobial molecule. This is a well known acquired mechanism of antibiotic resistance. Many types of modifying enzymes have been described. The main example of the drug molecule distruction is described in a specific class of antibiotics such as beta-lactams. Here, the production of beta-lactamases able to destroy the amide bond of the β-lactam ring can render these molecules ineffective. The spread of these enzymes are a severe global health concern because of the emerging of new ones that are specific to last-resort beta-lactams such as carbapenems. This cause the rapid inefficacy of these drugs with dramatic consequences in the therapeutic treatment, in particular in MDR Gram-negative infections occurring in the nosocomial setting.

Another mechanism of AMR, very common in Gram-negative bacteria, is represented by the ability to decrease the uptake of the drug through porin alterations. Importantly, changes in permeability through any of these mechanisms frequently result in low-level resistance and are often associated with other mechanisms of resistance, such as increased expression of efflux pumps (Nikaido, 2003). These systems may be substrate-specific or with broad substrate specificity, which are usually found in MDR bacteria (Poole, 2005). Efflux pumps represent the first line of defense against antibiotics as they decrease the intracellular level of drugs while the bacterial cell activates the various other levels of protection such as the production of enzymes (Rahman *et al*., 2017). This mechanism of resistance affects a wide range of antimicrobial classes including protein synthesis inhibitors, fluoroquinolones, β-lactams, carbapenems and polymyxins. The genes encoding efflux pumps can be located in MGEs or in the chromosome. Efflux pumps are classified into five families (Figure 4): the Small Multidrug Resistance (SMR) family, the Multidrug And Toxic compound Extrusion (MATE) family, the Major Facilitator Superfamily (MFS), the ATP-Binding Cassette (ABC) family and the Resistance-Nodulation-cell Division (RND) family (Poole, 2007; Anes *et al*., 2015). These mechanisms of resistance are very common in Gram-negative bacteria both in human pathogens and in the marine bacteria belonging to *Shewanellaceae* and *Vibrionaceae* families.

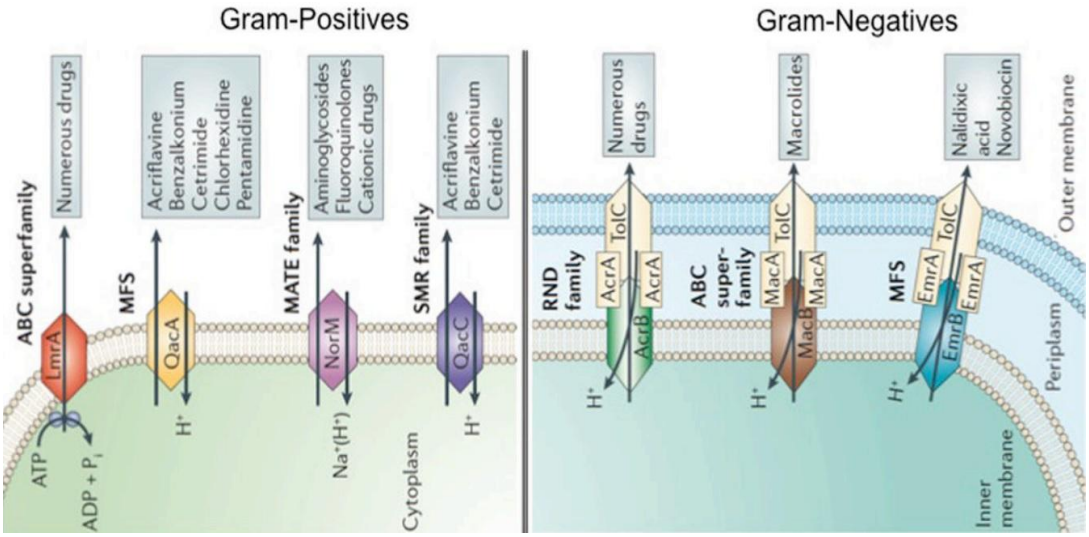

Figure 4. Comparison of all the efflux pumps families present both in Gram-positive and in Gram-negative bacteria according to their source of energy and their drugs or substrates extruded (Munita and Arias, 2016).

Another common strategy for bacteria to develop antimicrobial resistance is to avoid the action of the antibiotic by interfering with their target site. Although some of the genetic determinants coding for proteins that mediate target protection have been found in the bacterial chromosome, most of the clinically relevant genes involved in this mechanism of resistance are carried by MGEs. Examples of drugs affected by this mechanism include tetracycline (TetM and TetO) and fluoroquinolones (Qnr) (Aldred *et al*., 2014; Li *et al*., 2013; Dönhöfer *et al*., 2012; Rodríguez-Martínez *et al*., 2011; Martinez-Martinez *et al*., 1998).

3.1.3 MGEs involved in ARGs spread in the environment

The term MGEs is used to refer to elements that promote intracellular and intercellular DNA mobility (e.g., from the chromosome to a plasmid or between plasmids) (Partridge *et al*., 2018). To date, several MGEs are known to have an important role in ARGs dissemination in the microbial population present in a definite environment. Among them, ISs, transposons, plasmids, ICEs and integrons are involved in cell-cell contact mobilization whereas bacteriophages do not require cell contact (Figure 5).

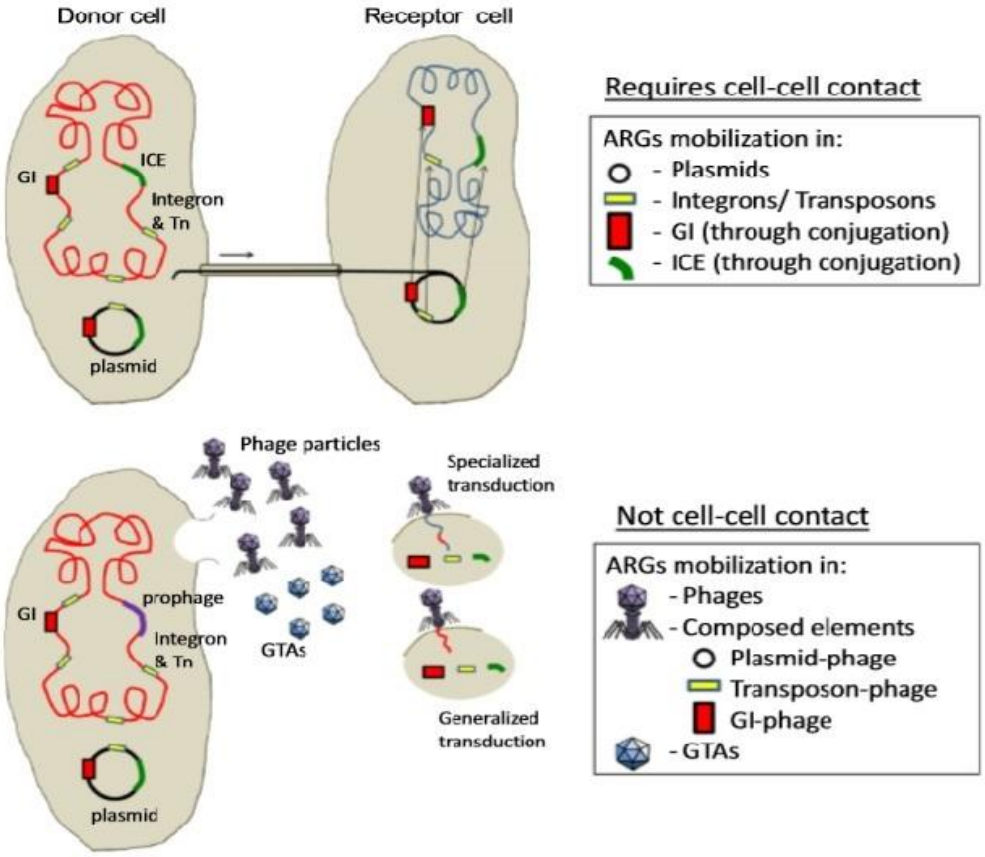

Figure 5. Schematic representation of how MGEs can act in disseminating ARGs in the environment through mechanisms that require cell-cell contact (such as plasmids, integrons, transposons, genomic islands (GIs) and ICEs) and other that do not (phages and phage-related elements, Gene Transfer Agents (GTAs)).

ISs or simple transposons are the smallest and most numerous autonomous TEs. ISs differ from transposons as they lack cargo genes but they only contain genes that are involved in the catalysis and regulation of the TE movement. Anyway, they move resistance genes as part of a composite (also called composed) transposon, a region bounded by two copies of the same or related IS that can move as a single unit (Partridge *et al*., 2018). Traditionally, ISs contain one or sometimes two transposase genes and can be divided into groups based partly on active site motifs in Tnp, designated by key amino acids DDE (Asp, Asp, and Glu) but also DEDD and HUH (two His residues separated by a large hydrophobic amino acid) (Hickman and Dyda, 2015).The most common ISs found in prokaryote genomes including marine strains such as shewanellae and vibrios belong to several families such as IS*3*, IS*4*, IS*5*, IS*30*, IS*110*, IS*200*/IS*605*, IS*630*, IS*L3* and IS*As1*, the latter one restricted only to Gram-negative bacteria (Mahillon and Chandler, 1998).

Chromosomal resistance can also often be captured in a transposon (Chancey *et al*., 2012; Quintiliani and Courvalin, 1996). The way in which transposons are integrated into and excised from the chromosome depends on whether they are "autonomous" or "non-autonomous". Autonomous transposons move by themselves; while non-autonomous transposons require the presence of other transposons to move. Integration is dependent on the presence of a transposase that recognizes direct repeats, but the main point is that transposons per se are not transferred horizontally.

Integrons are similar to transposons to some extent; they may also harbor antibiotic resistance and the main difference is that the enzyme that allows their integration is an integrase. This enzyme catalyzes the capture of discrete genetic elements known as gene cassettes.(Cambray *et al*., 2010). Integrons are usually part of larger transposons, which in turn are often part of conjugative plasmids or ICEs. Integrons are classified on the basis of their integrase sequence (*IntI*) with class I being the first reported and most common in antibiotic-resistant clinical isolates. Class II integrons, associated with Tn*7* and variants, often have a nonfunctional *IntI2* gene due to an internal stop codon and, probably as a consequence, house a limited variety of cassettes (Ramirez *et al*., 2010a). Class III integrons are more similar to class I integrons and also appear to be associated with Tn*402*-like transposons (Collis *et al*., 2002). Only a few examples have been identified, mostly carrying cassettes that encode beta-lactamases. Class IV was previously used to refer to an integron found in the *Vibrio cholerae* chromosome. "Mobile" integron types, now designated class IV and class V integrons (Escudero *et al*., 2015), appear to be rare and have not been identified in the species of clinical interest.

ICEs constitute a diverse group of mobile elements found in both Gram-negative and Gram-positive bacteria and have been reviewed recently (Delavat *et al*., 2017). Like plasmids, ICEs are self-transmissible by conjugation, but they integrate into the host chromosome and are replicated as part of it, although replication of excised ICEs has now been demonstrated (Carraro *et al*., 2015). ICE typically consist of a backbone (containing phage-like integration/excision functions, plasmid-like conjugation/maintenance components, and a regulation module) into which accessory genes are inserted (Figure 6). Excision of the ICE as a circular form and integration of this circle (at low frequency), usually into a unique attB site in the host chromosome, are catalyzed by the ICE-encoded site-specific integrase (Int). Plasmid R391, originally called IncJ, is now classified as one of the archetypes of the SXT/R391 family of ICE. These elements integrate into an attB site in the 5' end of the chromosomal *prfC* gene by site-specific recombination with their

attP site, catalyzed by the IntSXT tyrosine recombinase. They have an IncA/C-related conjugation region, encoding a MOB_{HI} family relaxase, and the regulation region includes a *mobI* gene and allows activation by the SOS response (Delavat *et al*., 2017). The excised form of these elements is able to replicate, and they carry a partitioning system (Carraro *et al*., 2015; Carraro and Burrus, 2014). Different insertions are found at certain positions in a shared 47-kb backbone, and hybrid elements have also been found (Burrus *et al*., 2006). SXT/R391 family elements are also able to mobilize adjacent sequences, including some genomic islands that have an oriT (Delavat *et al*., 2017). SXT carries resistance genes and can be found in *E. coli* but is mainly associated with *Vibrio* spp.

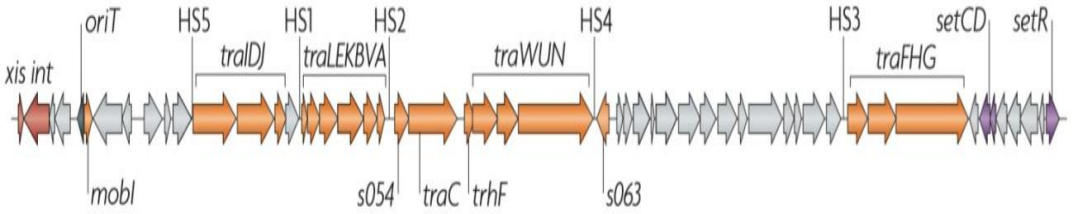

Figure 6. Genetic scheme of SXT-R391 family of ICE (Wozniak and Waldor, 2010). It contains four modules: *int-xis* is the integration and excision module; *mob* is the DNA mobilization and processing module; *mpf* is the mating pair formation module with *tra* genes and *reg* is the regulation module including *setCDR* genes. In orange are reported genes involved in the conjugation and gene transfer, purple genes are involved in regulation whereas red genes in integration/excision. Grey genes are accessory or unknown functions. The presence of five hotspots (HS1-HS5) allows DNA insertion. Xis, excisionase; int, integrase; oriT, origin of transfer; tra, conjugal transfer.

Plasmids are important vehicles for the carriage of other MGEs and acquired antimicrobial resistance genes associated with these elements in both Gram-negative and Gram-positive genera. They vary in size from less than a kilobase to several megabases (Shintani *et al*., 2015). Plasmids are extrachromosomal DNA molecules able to replicate in cells autonomously and they contain partition gene systems to their stable inheritance in the same host cell line .Conjugation or mobilization functions may also be encoded by plasmids allowing them to spread horizontally. Together the genes encoding these functions form a "backbone" (Thomas, 2000) to which can be added "accessory" nicheadaptive activities that might benefit the host cell (and hence the plasmid itself) in a particular environment. In resistance plasmids, these accessory regions are typically made up of one or more resistance genes and associated mobile elements of the types described

above (IS, Tn, and/or In). ARGs can be conjugally transferred to cells from a broad range of organisms; in Gram-negative bacteria the process is mediated by the type IV secretion system (T4SS) (Cabezón *et al*., 2015). Some plasmids can also be inserted into the bacterial chromosome and mobilized vertically (Boccard *et al*., 1989).

The most suitable vehicle for transfer between noncontiguous cells are bacteriophages. Recent reports indicate that phage implication in the transfer of ARGs could be more important than previously thought, particularly in the environment. Recent studies show the presence of ARGs in phage particles in the viral fraction of contaminated water bodies (Balcázar, 2014), human gut (Quirós *et al*., 2014; Modi *et al*., 2013; Minot *et al*., 2011), human lung (Fancello *et al*., 2011) or waste water treatment plant sludge (Calero-Cáceres *et al*., 2014; Parsley *et al*., 2010). During the maturation process of the phage particle, specific viral enzymes can cleave bacterial DNA allowing its mobilization and package in the phage capsid (Brown-Jaque *et al*., 2015). In this way, the mature phage can infect other bacterial cells. In addition, phages can disseminate ARGs during the lytic step of their infective cycle. The lysis of the bacterial cell has as consequence the release in the environment of the bacterial DNA, included ARGs, that can be acquired as naked DNA through transformation mechanism by the microbial population.

3.2 The Shewanella genus

Shewanella is a unique genus of the *Shewanellaceae* family, which includes non-fermentative Gram-negative, motile rods with positive oxidase and catalase reaction. It is composed of more than 70 species that are widely distributed in nature, especially in aquatic environments such as sea- and freshwater, as well as in marine organisms (Lemaire *et al*., 2020; Satomi, 2014). Moreover, several members of this genus have been identified that could potentially play key roles in environmental processes such as bioremediation of toxic elements and heavy metals and serving as microbial fuel cells. In contrast to this beneficial role, shewanellae are increasingly being implicated as human pathogens in persons exposed through occupational or recreational activities to marine niches (Janda and Abbott, 2014). In such venues *Shewanella* can either serve as a secondary or opportunistic pathogen. The two species mostly involved in causing human infections are *S. algae* and *S. putrefaciens*. These infections are now being reported at increased frequency apparently due to better diagnostic capabilities in the clinical microbiology laboratory (Huang *et al*., 2018; Potter *et al*., 2017; Baruah and Grover,

2014; Jacob-Kokura *et al*., 2014). They are described in immunocompromised patients with renal failure, neutropenia, hepatobiliary disease, diabetes or those involved in trauma accidents (Yousfi *et al*., 2017; Jousset *et al*., 2017; Muñoz-Gallego *et al*., 2016; Jacob-Kokura *et al*., 2014; Janda and Abbott, 2014). However, the most common clinical manifestations due to *Shewanella* infection are skin and soft-tissue infections (SSTIs) (Mohr *et al*., 2016). Usually, the antibiotic therapy adopted includes beta-lactams, aminoglycosides and quinolones. They are generally susceptible to third and fourth generation cephalosporins, carbapenems, beta-lactamase inhibitor combinations, aminoglycosides, chloramphenicol, erythromycin, aztreonam and quinolones (Yousfi *et al*., 2017; Satomi, 2014; Janda and Abbott, 2014). By the way, resistance to these drugs is increasing due to the presence in their chromosome of class D beta-lactamase encoding genes (bla_{OXA}) conferring resistance to carbapenems, class C beta-lactamase (bla_{AmpC}) genes which decrease the susceptibility to cephalosporins and qnr genes responsible for resistance to quinolones (Huang *et al*., 2018; Jousset *et al*., 2017; Ceccarelli *et al*., 2017; Cimmino *et al*., 2016; Potron *et al*., 2011; Lascols *et al*., 2008; Kim *et al*., 2006; Poirel *et al*., 2005a; Poirel *et al*., 2004). The variants of qnrA detected in *S. algae* include *qnrA2*, *qnrA3*, *qnrA4*, and *qnrA5* (Poirel *et al*., 2005a). A study of seven *S. algae* and three *S. putrefaciens* clinical isolates revealed that *dfrA1* and *aadA1* gene cassettes conferring resistance to trimethoprim and aminoglycosides, respectively, were present in the variable regions of class I integrons in both species. Class II integrons were also associated with these genes and were detected only in *S. algae*. The *sul1* and *sul2* genes conferring resistance to sulfonamides were detected in *S. algae* and *S. putrefaciens*, and the $bla_{\mathrm{OXA\text{-}48}}$-like gene was detected only in *S. putrefaciens* isolates (Ramirez *et al*., 2010b). Furthermore, resistance to colistin, a last-resort antibiotic in human medicine, has been reported as well due to the presence of *eptA* gene (Cimmino *et al*., 2016; Telke and Rolain, 2015; Holt *et al*., 1997). This genus is regarded as a reservoir and vehicle of different antibiotic resistance genes owing to the several antibiotic resistance determinants identified in this genus. However, the genetic characterization of antimicrobial resistance in *Shewanella* remains limited. Reported studies mostly characterize phenotypic resistance for prescription purposes in clinical diagnostics. Regarding virulence-associated factors, hemolytic activity is reported (Cimmino *et al*., 2016; Kashe and Janda, 1998). Furthermore, the presence of MGEs associated with resistance genes in this genus supports the hypothesis that *Shewanella* may have a role in the transmission and dissemination of antimicrobial resistance.

3.3 The Vibrio genus: a focus on the human pathogen V. parahaemolyticus and the fish pathogen V. anguillarum

Vibrio is a genus of ubiquitous bacteria found in a wide variety of aquatic and marine habitats; of the >100 described *Vibrio* spp., about 12 cause infections in humans. Vibrios are motile, curve-shaped bacilli able to cause severe or mild gastroenteritis (Ahmed *et al*., 2018; Jacobs-Slifka *et al*., 2017). In particular, *V. cholerae* can cause cholera, a severe diarrhoeal disease that can be quickly fatal if untreated and is typically transmitted via contaminated water and person- to-person contact. Non-cholera *Vibrio* spp. (for example, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*) cause vibriosis, infections normally acquired through exposure to sea water or through consumption of raw or undercooked contaminated seafood. *V. parahaemolyticus* is a ubiquitous inhabitant of temperate and tropical coastal areas around the world (Baker-Austin *et al*., 2018). The pathogenicity of *V. parahaemolyticus* is usually associated with the presence of two principal virulence genes; the thermostable direct hemolysin (*tdh*) and/or TDH-related hemolysin (*trh*) (Elbashir *et al*., 2018). Among fish pathogenic vibrios, *V. anguillarum* is the most important, considering its implications in causing a deadly haemorrhagic septicaemic disease affecting various marine and fresh/brackish water fish, bivalves and crustaceans (Frans *et al*., 2011). Cases of vibriosis are typically self-limiting (for example, *V. parahaemolyticus*- associated gastroenteritis), with few notable long- term quality- of-life issues associated with these infections. Very rarely, medical intervention is required, although antibiotic therapy is sometimes used if infections do not resolve or progress to systemic infections. Tetracyclines, aminoglycosides, third generation cephalosporins, fluoroquinolones and folate pathway inhibitors are generally used as antibiotic therapy in human vibriosis outbreaks (Ceccarelli *et al*., 2016; Bier *et al*., 2015). Regarding *V. anguillarum*, antibiotic treatment is necessary and it relies on oxolinic acid and florfenicol (Frans *et al*., 2011). But, unfortunately, several cases of multidrug resistant vibrios are reported in literature whose resistance is described even to the last-resort antibiotics used in human medicine such as carbapenems, tetracycline, ceftazidime, cefotaxime, ciprofloxacin and kanamycin (Di *et al*., 2019; Ahmed *et al*., 2018; Lee *et al*., 2018; Chowdhury *et al*. 2016; Mangat *et al*., 2016; Aberkane *et al*., 2015; Bier *et al*., 2015). Moreover, resistance to quinolones is due to the presence of plasmid-mediated quinolone resistance (PMQR) mechanisms in *V. parahaemolyticus* whereas resistance to ampicillin and chloramphenicol is reported as well (Matamp and Bhat, 2019; Zhang *et al*., 2019; Elbashir *et al*., 2018). SXT/R391 ICE elements, class I integrons and plasmids are the main mobile elements found in these aquatic bacteria able to contribute to the

spread of resistance genes in the environment (Fang *et al*., 2018; Carraro and Burrus, 2014). Phage treatment has been proposed in the form of a novel alternative biocontrol agent for antibiotic resistant pathogens due to their bactericidal activity, host specificity, and safety for human applications (Brüssow, 2005).

3.4 When Next Generation Sequencing meets the microbiology laboratory

Despite technological advances in laboratory diagnostics, the clinical microbiology laboratory continues to rely heavily on traditional methods, including culture, phenotypical, and biochemical tests, to identify microorganisms present in clinical specimens. This is due, in part, to the complexity and variability of specimens received by the clinical laboratory (Buchan and Ledeboer, 2014). However, molecular methods, including PCR, microarray, and nucleic acid sequencing, have taken a prominent place in the clinical laboratory. This is due to the sensitivity and specificity of these methods in the microorganisms or genetic polymorphisms identification through amplification and detection of specific nucleic acid targets. In addition to that, the investment- and running-cost of NGS have decreased dramatically during the last decade (Dark, 2013; Sboner *et al*., 2011), allowing this technology to be more and more used in the clinical microbiology laboratory.

3.4.1 NGS platforms

Currently, a number of NGS platforms are available and the most important ones are represented in Figure 7, below. These platforms rely on different technologies such as Illumina sequencers, for example, that perform sequencing by synthesis of fluorescent, reversible terminators, whereas ThermoFisher sequencers use semiconductor sequencing that measure a change in pH during the incorporation of nucleotides. Pacific Biosciences use fluorescent nucleotides in their single molecule real-time (SMRT) technology whereas Oxford Nanopore platforms use ionic current sensing, in which DNA is guided through nano-pores thereby changing the current in a way that is specific for the type of nucleotide (Deurenberg *et al*., 2017).

Company	Equipment	Output/run (Gb)	Maximum read length (bp)	Reads ($x10^6$)	Running time
Illumina	MiniSeq	0.6–7.5	2 x 150	25	4–24 h
Illumina	Miseq	0.3–15	2 x 300	25	5–55 h
Illumina	NextSeq	20–120	2 x 150	130/400	12–30 h
Illumina	HiSeq 3000	125–700	2 x 150	2500	<1–3.5 days
ThermoFisher	Ion PGM™	0.03–2	200–400	0.4–5.5	2–7 h
ThermoFisher	Ion 5S™	0.6–15	200–400	3–80	2.5–4 h
ThermoFisher	Ion 5S™ XL	0.6–15	200–400	3–80	<24 h
Oxford Nanopore	MinION	21–42	230,000–300,000	2.2–4.4	1 min–48 h
Pacific Biosciences[a]	Sequel	0.75–1.25	>20,000	370,000	30 min–6 h
Pacific Biosciences[a]	RSII	0.5–1	>20,000	55,000	30 min–4 h

[a] The Pacific Biosciences data are per smart cell; both the Sequel and the RSII can run 1–16 smart cells in one run.

Figure 7. List of NGS platforms and their main properties (Deurenberg *et al*., 2017).

3.5 The Whole Genome Sequencing to investigate the bacterial resistome, mobilome and virulome

NGS allows sequencing of the whole genome of numerous pathogens in one sequence run, either from bacterial isolates of (different) patients, or from multiple species present in patient material from one individual (metagenomics) (Deurenberg *et al*., 2017). It is becoming a powerful tool in microbiology. In fact, it can help in revealing the presence of ARGs or genes that are involved in virulence or pathogenicity included their genetic mechanisms (Rossen *et al*., 2018). In particular, Whole Genome Sequencing has already found numerous applications in AMR ranging from the development of novel antibiotics, surveillance systems both in human and veterinary medicine, the study of the evolution of resistance in real-time under a variety of conditions, to the development of diagnostic tests (Köser *et al*., 2014). In addition, this technology can help in outbreak management, molecular case finding, characterization and surveillance of pathogens, rapid identification of bacteria using the 16S-23S rRNA region, taxonomy, metagenomics approaches on clinical samples, and the determination of the transmission of zoonotic microorganisms from animals to humans (Deurenberg *et al*., 2017).

3.5.1 General workflow for the Whole Genome Sequencing of bacterial cells

The most common workflow (for example, using Illumina technology) to be performed for WGS of bacterial strains rely on several steps: sample collection and preparation, nucleic acid extraction and fragmentation, NGS library preparation, sequencing, data analysis and data storage (Motro and Moran-Gilad, 2017). In particular, the nucleic acid fragmentation step produce fragments ranging from 100 to 1000 bp with the exception for the third generation sequencers that can generate larger fragments. This is usually performed by library preparation kits which have the advantage to combine the nucleic acid fragmentation and the fusion of specific adaptors to the generated fragments. After sequencing, the sequenced fragments, called reads, must be assembled (genome assembly). The genome assembly can use a reference organism to improve this step or not (*de novo* assembly). In the latter case, the reads are aligned against each other. To help in this process, several softwares are available (Figure 8). Genome assembly relies on softwares such as SPAdes and Velvet (Bankevich *et al*., 2012; Zerbino and Birney, 2008). Then, genome annotation allows to predict genes and proteins of the microorganism analyzed. For this purpose, PROKKA which contains Prodigal tool in its suite and RAST are the most common used (Overbeek *et al*., 2014; Seemann, 2014). Gene identification is performed by NCBI BLAST. Regarding the detection of resistance genes and virulence factors, specific softwares like ResFinder, the Comprehensive Antimicrobial Resistance Database (CARD), Antimicrobial Resistance Database (ARDB), ABRicate and the Antibiotic Resistance Gene ANNOTation (ARG-ANNOT) can be used (Jia *et al*., 2017; Gupta *et al*., 2014; Kleinheinz *et al*., 2014). While VirulenceFinder and Virulence Factor Database (VFDB) are useful databases for detecting virulence factors. Concerning MGEs, INTEGRALL (Moura *et al*., 2009) and ISFinder databases (Siguier *et al*., 2006) are used to reveal integrons and ISs, whereas VR profile and ICEBerg for ICEs detection (Liu *et al*., 2019). PHASTER web server is usually used for phage detection (Arndt *et al*., 2016) whereas Recycler tool for circular elements such as plasmids identification (Rozov *et al*., 2017).

Application	Software	Link	Note
Annotation	Prokka	www.vicbioinformatics.com	
	RAST	http://rast.nmpdr.org	
Assembly	BioNumerics	www.applied-maths.com	Commercial software
	CLC Genomic Workbench	www.clcbio.com	Commercial software
	SeqSphere	www.ridom.de	Commercial software
	SPAdes	http://bioinf.spbau.ru/spades	Unix-based
	Velvet	www.ebi.ac.uk/~zerbino/velvet	Unix-based
Data quality check	BaseSpace	https://basespace.illumina.com	Commercial software
	BioNumerics	www.applied-maths.com	Commercial software
	CLC Genomic Workbench	www.clcbio.com	Commercial software
	FastQC	www.bioinformatics.babraham.ac.uk	
Identification	K-merFinder	www.genomicepidemiology.org	
	NCBI BLAST	www.ncbi.nlm.nih.gov/blast	
Metagenomics	MEGAN	http://ab.inf.uni-tuebingen.de/software/malt	
Phylogeny	FastTree	www.microbesonline.org/fasttree	
	RAxML	http://sco.h-its.org/exelixis/software.html	
	SeqSphere	www.ridom.de	Commercial software
	SNPTree	www.genomicepidemiology.org	
Resistance	ARDB	https://ardb.cbcb.umd.edu	
	CARD	https://card.mcmaster.ca	
	ResFinder	www.genomicepidemiology.org	
SNP calling	BioNumerics	www.applied-maths.com	Commercial software
	CLC Genomic Workbench	www.clcbio.com	Commercial software
	Samtools	www.htslib.org	
	SeqSphere	www.ridom.de	Commercial software
Typing (wgMLST)	BIGSdb	http://bigsdb.readthedocs.io	
	BioNumerics	www.applied-maths.com	Commercial software
	CLC Genomic Workbench	www.clcbio.com	Commercial software
	EnteroBase	https://enterobase.warwick.ac.uk	
	SeqSpere	www.ridom.de	Commercial software
Virulence	VFDB	www.mgc.ac.cn/VFs	
	VirulenceFinder	www.genomicepidemiology.org	
Visualisation & comparative study	ACT	www.sanger.ac.uk/science/tools	
	Artemis	www.sanger.ac.uk/science/tools	
	BRIG	https://sourceforge.net/projects/brig/	
	ClustalW	www.genome.jp/tools/clustalw	
	DNA plotter	www.sanger.ac.uk/science/tools	
	WebACT	www.webact.org	

Figure 8. List of the most used softwares for the genome assembly, annotation, resistance and virulence determinant detection (Deurenberg *et al*., 2017).

3.5.2 Pros and cons of WGS analysis of MDR bacteria

Nowadays, high-throughput sequencing is not routinely performed in most hospital-based clinical microbiology laboratories (Rhoads, 2018). In fact, although WGS can be used to predict antimicrobial susceptibility from a single assay, it is difficult to assess genotypic-phenotypic concordance and to accreditate such kind of analysis (Rossen *et al*., 2018; Ellington *et al*., 2017). In addition to this, the workflow for WGS should be improved.

Undoubtedly, starting from the costs and the turnaround time from the library preparation and the runs on the WGS platforms (between 4 and 56 h to complete a single run for short read sequencers) (Schürch and van Schaik, 2017). Then, pipelines should be improved and developed in order to allow an easier use of softwares that are needed to obtain the final results. Another issue is the difficulty to have updated databases to find new ARGs, TEs and other genes involved in the pathogenicity of a specific bacterium. Moreover, these functional predictions are explicitly based only on sequence data and are vulnerable to false-positive predictions, particularly when functional assignments are based on annotations that have not themselves been directly validated biochemically or phenotypically (Crofts *et al*., 2017). In addition, the high error rate of long read sequencers should be decreased since their rapidity in sequencing. In particular, the benefit of the MinION sequencer is that sequencing data can be analyzed while they are being generated. This may lead to strain identification within 30 min and a prediction of the antibiotic-resistance profile of the strain 10 h after the start of a run. However, owing to the relatively high error rate, the technology does not currently allow the reliable identification of strains on the basis of their multi locus sequence type, which may limit the MinION's usefulness in mapping outbreaks of drug-resistant pathogens (Schürch and van Schaik, 2017). Then, data storage is another big issue. Anyway, although several difficulties are encountered in using WGS technology routinely, its use in culture-independent bacteria could speed up the clinical laboratory workflow, particularly to decrease the time to genotypic AST (Tagini and Greub, 2017).

The increasing outbreaks in the last years of human infections caused by shewanellae and vibrios due to global warming suggest the need of surveillance programs for these emerging and opportunistic bacteria. For this purpose, we used the NGS technology to investigate the resistome, mobilome and virulome of 12 Multi Drug Resistant *Shewanella algae* and *Vibrio* spp. strains isolated in several aquaculture centers in Italy in order to study the resistance determinants involved and how they spread in our territory with possible outcomes in public health.

4. MATERIALS & METHODS

4.1 Strain identification, phenotypical and biochemical analysis

A collection of bacterial strains (*Shewanellaceae* and *Vibrionaceae*) isolated in fish farms located along the coast of the Adriatic Sea in Italy (Labella *et al*., 2013) was screened for the presence of beta-lactamase and/or carbapenemase producing bacteria. Twelve MDR isolates were selected on the basis of their antimicrobial resistance profiles obtained by disk diffusion susceptibility test on MHA, their ability to hydrolyze imipenem in BCT (Pires *et al*., 2013) and their sampling sites (Table 1). They were submitted to WGS analysis to study and compare their resistomes, mobilomes and virulomes. Bacterial growth was carried out overnight on TSA supplemented with NaCl 1% at 37°C for shewanellae whereas for vibrios the growth was sustained on TCBS at 37°C in the case of *V. parahaemolyticus* VPE116 strain and at room temperature for *V. anguillarum* 28AD. Species identification was confirmed using BLAST analysis of 16S rRNA gene predicted from genome sequencing. The culture media used are listed in Annex I.

4.2 Biochemical analysis of the selected marine strains

The Blue Carba Test

The BCT is a modified biochemical test used for the rapid detection of carbapenemase-producing strains directly from bacterial cultures (Pires *et al*., 2013). The Carba NP test (Nordmann *et al*., 2012) relies on the use of a lysis buffer (TrisHCl) for bacterial protein extraction and detection of hydrolysis of the carbapenem β-lactam ring through the acidification of a phenol red solution used as color indicator. In the Blue-Carba test variant, bromothymol blue has been selected as the indicator, since it includes the optimal pH range (6.0 to 7.6) for most β-lactamases (pH = 6.8), which is a key factor for a direct colony approach. A commercially and widely available imipenem (Imipenem – cilastatin; Hikma Farmaceutica, Portugal) was used as the substrate for carbapenemases. The test solution consisted of an aqueous solution of bromothymol blue at 0.04% (Carlo Erba, Milano, Italy) adjusted to pH 6.0, 0.1 mmol/liter $ZnSO_4$, and 3 mg/ml of imipenem, with a final pH of 7.0. A negative-control solution (0.04% bromothymol blue solution, pH 7.0) was prepared. A loop (approximately 5 μl) of a pure bacterial culture recovered from

Mueller-Hinton agar (MicroBiol Diagnostici, Cagliari, Italy) was directly suspended in 100 µl of both test and negative-control solutions in a 1.5 ml eppendorf and incubated at 37°C with agitation (150 rpm) for 2 h for *S. algae* and *Vibrio* spp. strains. Carbapenemase activity was revealed when the test and negative-control solutions, respectively, were (i) yellow versus blue, (ii) yellow versus green, or (iii) green versus blue (Figure 9). Noncarbapenemase producers remained blue or green on both solutions. The test was performed in triplicate for all the isolates.

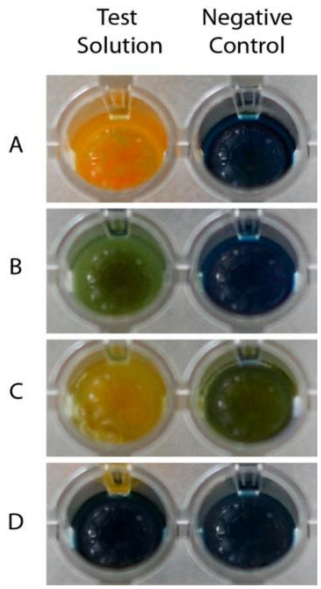

Figure 9. Representative results of the Blue-Carba test obtained from carbapenemase producers (A, B, and C) and non-carbapenemase producers (D) with test solution (left) and negative control solutions (right). (A) NDM-1-producing *E. coli*. (B) OXA-23-producing *Acinetobacter baumannii*. (C) OXA-48-producing *Klebsiella pneumoniae*. (D) *E. coli* ATCC 25922 (Pires *et al*., 2013).

4.3 Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted using the disk diffusion method in MHA plates modifying the EUCAST guidelines (Non Species Related and *Pseudomonas* spp. tables) since specific breakpoints for these emerging pathogens are not available. Resistance profiles were obtained when no zones of inhibition or alternatively zones of inhibition with small diameter sizes were detected. The antibiotics used in this study were amoxicillin (25 µg/ml), amoxicillin+clavulanic acid (30 µg/ml), ticarcillin (75 µg/ml), ticarcillin+clavulanic acid (85 µg/ml), piperacillin (75 µg/ml), piperacillin+tazobactam (85 µg/ml), ceftazidime (10 µg/ml), cefepime (30 µg/ml), temocillin (30 µg/ml),

cefoxitin (30 µg/ml), cefotaxime (30 µg/ml), cefepime (30 µg/ml), cephalothin (30 µg/ml), aztreonam (30 µg/ml), imipenem (10 µg/ml), ertapenem (10 µg/ml), meropenem (10 µg/ml), trimethoprim+sulfamethoxazole (25 µg/ml), colistin (50 µg/ml), chloramphenicol (30 µg/ml), sulfonamides (200 µg/ml), fosfomycin (200 µg/ml), tobramycin (10 µg/ml), kanamycin (30 µg/ml), gentamicin (15 µg/ml), amikacin (30 µg/ml), nalidixic acid (30 µg/ml), ciprofloxacin (5 µg/ml), tetracycline (30 µg/ml), tigecycline (15 µg/ml). MHA plates were incubated at 37°C overnight for *S. algae* strains and *V. parahaemolyticus* VPE116 whereas at room temperature for *V. anguillarum* 28AD.

4.4 Genomic DNA extraction and genome sequencing

Genomic DNA was extracted using CTAB method (described in Annex II) and 1 µg of gDNA was used for library preparation with Illumina TruSeq DNA PCR-free kit (Illumina, Milan, Italy). WGS was performed by Illumina NextSeq® 500 platform (Illumina, San Diego, CA, USA) using the paired-end strategy with 400X as coverage and producing 2 x 150 bp reads (average read length). The quality of generated reads was checked using FastQC software whereas adapters trimming was performed using Scythe v0.991 and Sickle v1.33. Then, genomes were assembled by SPAdes v3.10.1 assembler (Bankevich *et al*., 2012).

4.5 Genome annotation

The annotation was carried out using Prodigal v2.6, a tool provided by PROKKA v1.12 software (Seemann, 2014). Antimicrobial resistance genes were detected using several databases such as ResFinder, the Comprehensive Antibiotic Resistance Database (CARD), ABRicate and the Antibiotic Resistance Gene ANNOTation (ARG-ANNOT; Jia *et al*., 2017; Gupta *et al*., 2014; Kleinheinz *et al*., 2014). Plasmids and other mobile genetic elements such as integrons and transposons were investigated using INTEGRALL (Moura *et al*., 2009) and ISFinder databases (Siguier *et al*., 2006), VR profile v2.0 and ICEBerg v2.0 for ICEs detection (Liu *et al*., 2019) and PHASTER web server for phages (Arndt *et al*., 2016) as well as Recycler tool (Rozov *et al*., 2017). Virulence factors were detected using gene annotation and prediction performed by Prodigal.

5. RESULTS

5.1 Bacterial strain characterization

The strain identification was confirmed at species level blasting the 16S rRNA gene predicted by WGS and resulting in 10 *S. algae*, 1 *V. anguillarum* and 1 *V. parahaemolyticus*. The growth of the 12 strains was inhibited on ChromID ESBL medium. Regarding the BCT, 10 *S. algae* isolates were positive to the test with a pH decrease of the solution due to the hydrolysis of the imipenem and its change from blue to green. For *V. anguillarum* 28AD, a BCT doubtful result was obtained. *V. parahaemolyticus* VPE116 being ESBL and BCT negative was used as negative control strain (Figure 10).

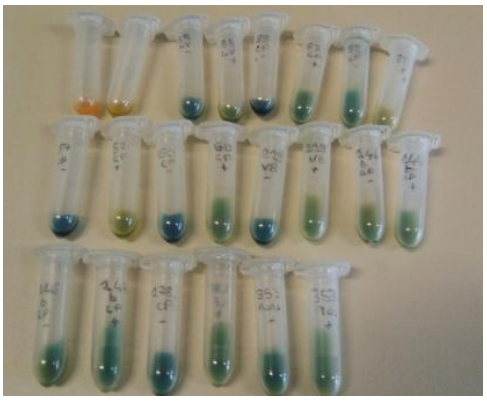

Figure 10. BCT results for the *S. algae* isolates.

Antimicrobial resistance profiles were obtained by standard antibiotic susceptibility testing performed following the modified EUCAST guidelines as described in Materials and Methods. Resistance towards β-lactams (including imipenem), colistin, sulfonamides and fosfomycin was reported in all the 10 *S. algae* isolates (Table 1). Only the 57CP and 38LV strains were resistant to tetracycline and tigecycline. Resistance to the same antibiotics detected in all the shewanellae was revealed also in *V. anguillarum* which was resistant to ticarcillin alone and combined with clavulanic acid, temocillin and amikacin as well. *V. parahaemolyticus* VPE116 showed resistance to aminoglycosides (tobramycin, kanamycin, amikacin and gentamicin), colistin and sulfonamides but it was susceptible to imipenem, cephalothin and cefotaxime.

5.2 Genome features

Genome sizes, total number of reads, contigs and scaffolds generated, %GC contents, number of predicted genes, proteins, hypothetical proteins and RNA encoding genes are reported in Table 2 for each isolates. A k-mer value of 97 was selected to obtain the best genome assembly for all the strains.

Table 1. List of the MDR marine isolates under study. Sampling dates, sources, species identification and antimicrobial resistance profiles are reported for each strain.

STRAIN	SAMPLING DATE	SOURCE	16S rRNA GENE IDENTIFICATION	ANTIMICROBIAL RESISTANCE PROFILE
353M	10/08/2010	Water Open sea	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS
178CP	13/06/2011	Water Veneto II station	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS
146bCP	13/06/2011	Water Veneto II station	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS
144bCP	13/06/2011	Water Veneto II station	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS
219VB	30/09/2010	Water Veneto I station	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS
82CP	15/04/2011	Water Veneto II station	*Shewanella algae*	CEF, FOX, IPM, CS, SUL, FOS
38LV	22/07/2011	Water Varano lake	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS, TET, TGC
57CP	15/04/2011	Water Veneto II station	*Shewanella algae*	CEF, FOX, IPM, CS, SUL, FOS, TET, TGC
60CP	15/04/2011	Water Veneto II station	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS
83CP	15/04/2011	Water Veneto II station	*Shewanella algae*	CEF, FOX, IPM, AMX, CS, SUL, FOS
28AD	01/02/2007	European seabass	*Vibrio anguillarum*	CEF, FOX, AMX, TIC, AMC, TCC, TEM, CS, SUL, AKN
VPE116	26/06/2007	Water Caleri lagoon	*Vibrio parahaemolyticus*	AMX, TIC, TMN, KMN, AKN, GMI, CS, SUL

AMC, amoxicillin-clavulanic acid; AMX, amoxicillin; AKN, amikacin; CEF, cephalothin; CS, colistin; CTX, cefotaxime; FOS, fosfomycin; FOX, cefoxitin; GMI, gentamicin; IPM, imipenem; KMN, kanamycin; SUL, sulfonamides; TCC, ticarcillin-clavulanic acid; TEM, temocillin; TET, tetracycline; TGC, tigecycline; TIC, ticarcillin; TMN, tobramycin.

Table 2. Summary of the genome sizes, %GC content, total number of reads, contigs, predicted proteins, hypothetical proteins and RNA encoding genes for each isolate under study.

STRAIN	16S rRNA GENE IDENTIFICATION	GENOME SIZE (bp)	%GC	NR OF TOTAL READS	NR OF TOTAL CONTIGS	NR OF PREDICTED PROTEINS	NR OF HYPOTHETICAL PROTEINS	NR OF RNA ENCODING GENES
219VB	*Shewanella algae*	4821382	53.08	8255755	40	3900	782	106
353M	*Shewanella algae*	4918582	52.90	7184343	52	3987	810	99
146bCP	*Shewanella algae*	4897215	53.03	8131593	57	3991	818	105
144bCP	*Shewanella algae*	4893423	53.02	8117126	52	3991	818	112
57CP	*Shewanella algae*	4822400	53.08	7527876	67	3867	738	114
82CP	*Shewanella algae*	4859761	52.96	8711104	67	3907	771	106
38LV	*Shewanella algae*	4782393	53.10	8333193	46	3846	738	106
83CP	*Shewanella algae*	4899381	53.00	8713510	55	3918	768	106
178CP	*Shewanella algae*	4895527	53.02	8758503	56	3991	817	109
28AD	*Vibrio anguillarum*	3868695	44.55	12908870	86	3180	365	99
60CP	*Shewanella algae*	4799216	53.07	13809993	72	3853	741	86
VPE116	*Vibrio parahaemolyticus*	5089623	45.45	13957982	63	4275	582	91

5.3 Resistome analysis

The resistome analysis revealed the presence of resistance genes active against several antibiotic classes including beta-lactams, quinolones, tetracyclines, macrolides, polymyxins, chloramphenicol as well as heavy metals. Several resistance mechanisms resulted to be involved, mostly by production of specific enzymes such as beta-lactamases and acetyltransferases as well as different types of multidrug efflux pump systems.

Resistance to beta-lactams was mainly due to the presence in the *S. algae* strains of $bla_{\text{OXA-55-like}}$, bla_{AmpC} and *mexB-OprM* genes which confer resistance to carbapenems, cephalosporins and penicillins. A bla_{AmpC} encoding gene was detected in *V. anguillarum* 28AD whereas *mexA-OprM* and $bla_{\text{CARB-19}}$ were found in *V. parahaemolyticus* VPE116 (Table 3).

The expression of this chromosome-encoded beta-lactamase is regulated by different transcriptional regulators. In particular, in all the *S. algae* strains the $bla_{\text{OXA-55-like}}$ expression is regulated by a member of LysR family transcriptional regulator as already reported in literature (Héritier *et al*., 2004) whereas bla_{AmpC} is located near *cat* (chloramphenicol acetyltransferase), HTH-type transcriptional regulator DmlR, *czcA* (cobalt-zinc-cadmium resistance protein), *OprM* (Outer membrane protein OprM) and *pqiAB* (paraquat-inducible protein AB) genes (Figure 11). Its induction is mediated by beta-lactams and regulated by AmpD, AmpE, AmpR, a LysR-type regulator and AmpG, a series of genes that have not been all found in our sequences. This is probably due to the difficulties encountered in short read sequencing, in which the generated reads can render the genome assembly very difficult leading to an incorrect gene annotation and prediction. Concerning the bla_{AmpC} of *V. anguillarum* 28AD, it is regulated by the same system described above but the *cat* gene is not reported in this case. *V. parahaemolyticus* VPE116 harbored the $bla_{\text{CARB-19}}$ gene located near *cusAB* genes which are involved in silver, copper and fosfomycin resistance.

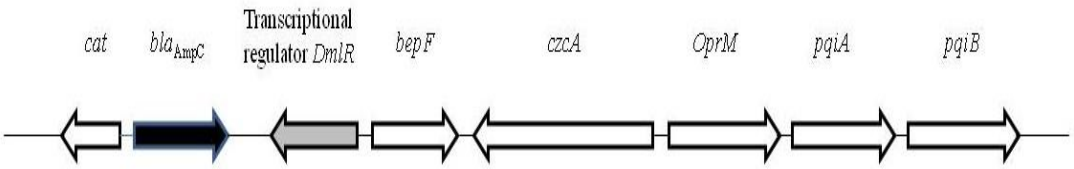

Figure 11. Genetic surrounding of bla_{AmpC} gene in the studied *S. algae* strains. The black arrow represents the bla_{AmpC} gene, the grey one the transcriptional regulator, whereas the white ones the other genes found in this module.

As regards the resistance to quinolones, QnrVC6, which is an integron-mediated quinolone resistance protein, was found only in *V. anguillarum* while QnrA7, a PMQR protein found in *S. algae*, was present only in some of the shewanellae strains studied here (Table 3). The strains 353M, 219VB, 38LV, 57CP, 60CP, instead, resulted positive for the presence of *qnrA3* gene encoding a quinolone resistant protein reported also in *E. coli* and *Salmonella enteritidis* (Poole, 2007; Anes *et al*., 2015). Mutations on gyrase A and ParC genes encoding for gyrase and topoisomerase IV respectively, were found in quinolone resistance-determining regions (QRDR). The gyrA protein sequences revealed the following substitutions Tyr50Phe, Gly78His, Ser80His, Ser88Thr, Glu133Gln whereas ParC the following ones: Glu51Arg, Ala56Met, Thr66Lys, Asp69Lys, Gly72Arg, Gly78Leu, Ser80Lys, Ser83Pro, Glu84His, Cys107Val, Ala108Asp, Phe115Ala, Ala141Ser (data not shown). In particular, the most important mutations involved in quinolone resistance, Ser83 and Asp87, were conserved in all the strains in gyrA but not in the parC protein sequences where a Ser83Pro was detected. Conversely, the gyrB and parE protein sequences did not show mutations of particular interest in QRDRs.

Bacterial MDR efflux pumps are classified into five different structural families: the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the multidrug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, and the resistance/nodulation/division (RND) superfamily. Of these efflux pump families all of them, excepting the SMR one, which is present only in Gram-positive bacteria, were detected in our *S. algae* and *Vibrio* spp. strains (Table 3). These efflux pumps are not only involved in resistance to antibiotics but also to bile salt derivatives and SDS. Regarding the resistance to fluoroquinolones efflux pumps belonging to the MATE and MFS families, such as MdtK, EmrAB-TolC, MepAB,

NorM, Bmr3, MdtH and the pentapeptide MfpA were detected. In particular, while MdtK, EmrAB-TolC and MepAB were present in all the analyzed strains, NorM, Bmr3, MdtH and MfpA were found only in the *Vibrio* spp. isolates (Table 3). Moreover, the regulation of the *EmrAB-TolC* system is described by the presence of an HTH-type transcriptional regulator DmlR (belonging to LysR family transcriptional regulator) which can regulate EmrAB expression followed by an acetyltransferase belonging to the GNAT family N-acetyltransferase, able to contribute to quinolone resistance in *S. algae* isolates.

A list of an important multidrug resistance efflux pump system belonging to RND conferring resistance to a variety of molecules was identified for each of every strain analyzed and reported in Table 3. Among them, *AcrAB-TolC*, *AcrE-TolC*, *MdtAC-TolC*, *emrD* (MFS family) and *MdtN* were found in all the *S. algae* strains. *EmrYK-TolC*, a member of MFS family, was found only in some *S. algae* (144bCP, 178CP, 146bCP, 83CP, 57CP, 60CP) and in the two vibrio strains. *DrrA* (doxorubicin and daunorubicin resistance), *Stp* (Spectinomycin Tetracycline efflux Pump), *bcr* (bicyclomycin resistance) genes were found in all the *S. algae* conferring resistance to several molecules such as norfloxacin, erythromycin, tetracycline, anticancer agents and chloramphenicol. In addition, *V. parahaemolyticus* VPE116 harbored *mdtG* (MFS family) and *mdlB* (ABC family) contributing to generate a resistance phenotype against fluoroquinolones and fosfomycin, respectively.

As concerns efflux pumps involved in tetracycline resistance, *tet34*, *tet35* as well as *tetR*, their regulator, were detected only in the two vibrios analyzed. Finally, an ABC-type tripartite multidrug efflux pump, *macAB-TolC*, is responsible for macrolide antibiotic resistance and polipeptide virulence factors while *MdtE-TolC* is another RND-type efflux pump that can contribute to macrolide and beta-lactam resistance. The first was detected in all the studied strains while the second one was found in *Vibrio* spp. genomes but not in the *S. algae* ones (Table 3).

A chromosomal encoding gene for phosphoethanolamine transferase (*eptA*) was detected in both the two genera causing a resistant phenotype towards polymyxins. Moreover, *Cat* and *MdtL* genes, involved in the chloramphenicol resistance, were found.

Resistance to heavy metals was also observed (Table 4). Some of the genes were found in all the 12 strains, namely all the genes involved in resistance to arsenic, copper, molybdenum and the genes *czcD* (cobalt-zinc-cadmium resistance protein), *corC* and *mgtE* involved in magnesium resistance. Other were typical of only vibrios such as *czcR*,

zur, *znuA*, *znuB*, *znuC* (cobalt, zinc and cadmium resistance) or of only *S. algae*, *chrA* for chromium, *corA* for magnesium and *nikR* for nickel resistance.

Table 3. List of the resistance genes found in the *S. algae* and *Vibrio* spp. grouped on the basis of their mechanisms and antimicrobial targets.

Antimicrobial	Pentapeptide protein	Enzyme	Efflux system	Pump family	Strain
Beta-lactams		OXA-55-like			All *S. algae*
		AmpC			All *S. algae*,*V. anguillarum* 28AD
			MexAB-OprM	RND	All *S. algae*, *V. parahaemolyticus* VPE116
Fluoroquinolones	QnrA3				353M, 219VB, 38LV, 57CP, 60CP
	QnrA7				144bCP, 178CP, 146bCP, 82CP, 83CP
	QnrVC6				*V. anguillarum* 28AD
			MdtK	MATE	All *S. algae*, *V. anguillarum* 28AD
			EmrAB-TolC	MFS	All *S. algae*, *V. parahaemolyticus* VPE116
	MepAB				All *S. algae*,*V. anguillarum* 28AD
			NorM	MATE	*V. anguillarum* 28AD, *V. parahaemolyticus* VPE116
			Bmr3	MFS	*V. anguillarum* 28AD
			MdtH	MFS	*V. anguillarum* 28AD
	MfpA				*V. anguillarum* 28AD
Multiple substrates			AcrAB-TolC	RND	All *S. algae*, *V. parahaemolyticus* VPE116
			AcrEF-TolC	RND	All *S. algae*, *V. parahaemolyticus* VPE116
			MdtABC-TolC	RND	All strains
			EmrD	MFS	All strains
			EmrYK-TolC	MFS	All strains excepting for 353M, 219VB, 82CP, 38LV
			MdtN	RND	All strains
			DrrA	ABC	All strains excepting for *V. anguillarum* 28AD

Antimicrobial	Pentapeptide protein	Enzyme	Efflux system	Pump family	Strain
Multiple substrates			Stp	MFS	All *S. algae*
			Bcr	MFS	All *S. algae*
			MdtG	MFS	*V. parahaemolyticus* VPE116
			MdlB	ABC	*V. parahaemolyticus* VPE116
Tetracyclines			TetR	MFS	*V. parahaemolyticus* VPE116
			Tet34	MFS	*V. anguillarum* 28AD, *V. parahaemolyticus* VPE116
			Tet35	MFS	*V. parahaemolyticus* VPE116
Macrolides			MacAB-TolC	ABC	All strains
			MdtE-TolC	RND	*V. anguillarum* 28AD, *V. parahaemolyticus* VPE116
Polymyxins		EptA			All strains
Chloramphenicol			MdtL	MFS	All strains
		Cat			All strains excepting for *V. anguillarum* 28AD

ABC, ATP Binding Cassette; MATE, Multidrug And Toxic compound Extrusion; MFS, Major Facilitator System; RND, Resistance-Nodulation-Cell Division

Table 4. List of the heavy metal and other antimicrobial compound resistance genes found in the *S. algae* and *Vibrio* spp. genomes.

Compound	Putative gene	Function	Strain
Arsenic	*ArsR*	Arsenic resistance transcriptional regulator	All
	ArsA	Arsenical pump-driving ATPase	All
	ArsC	Arsenate reductase	All
	acr3	Arsenical resistance protein *acr3*	All
Copper	*CusA*	Cation efflux system protein *cusA*	All
	CusB	Cation efflux system protein *CusB*	All
	CopA	Copper-exporting P-type ATPase A	All
	copR	Transcriptional activator protein CopR	All
Cobalt-zinc-cadmium	CzcA	Cobalt-zinc-cadmium resistance protein CzcA	All *S. algae*, *V. parahaemolyticus* VPE116
	CzcB	Cobalt-zinc-cadmium resistance protein CzcB	All *S. algae*, *V. parahaemolyticus* VPE116
	CzcC	Cobalt-zinc-cadmium resistance protein CzcC	All *S. algae*, *V. anguillarum* 28AD
	CzcD	Cobalt-zinc-cadmium resistance protein CzcD	All
	CzcR	Transcriptional activator protein CzcR	All *Vibrio* spp.
	Zur	Zinc uptake regulation protein Zur	All *Vibrio* spp.
	ZnuA	High-affinity zinc uptake system protein ZnuA	All *Vibrio* spp.
	ZnuB	High-affinity zinc uptake system membrane protein ZnuB	All *Vibrio* spp.
	ZnuC	Zinc import ATP-binding protein ZnuC	All *Vibrio* spp.
Chromium	*ChrA*	Chromate transport protein	All *S. algae*
	SrpC	Putative chromate transport protein	All *Vibrio* spp.
	ChrR	Chromate reductase	All *Vibrio* spp.
Molybdenum	*MoeA*	Molybdopterin molybdenum transferase	All
	MoeB	Molybdopterin-synthase adenylyltransferase	All
Magnesium	*CorA*	Magnesium transport protein CorA	All *S. algae*
	CorC	Magnesium and cobalt efflux protein CorC	All
	MgtE	Magnesium transporter MgtE	All

Compound	Putative gene	Function	Strain
Nickel	*NikR*	Putative nickel-responsive regulator	All *S. algae*
Bacteriocin	*LodA*	L-lysine 6-oxidase	All *S. algae*
	LodB	Putative FAD-dependent oxidoreductase LodB	All *S. algae*
	CvpA	Colicin V production protein	All *S. algae*

5.4 Mobilome analysis

The analysis of the mobile genetic elements using specific softwares revealed the absence of circular elements such as plasmids but other MGEs such as ICEs, class I integrons, ISs and bacteriophages were detected. Of interest was the presence in 353M, a strain isolated in the open sea, of several genetic elements associated to *int-xis* (integration and excision module), *mob* (DNA mobilization and processing module) and *mpf* (mating-pair formation module) machineries that are typical of ICEs and involved in the integration and conjugative transfer. The genome annotation and softwares like VR profile and ICEBerg allowed to detect the following genes: *traID, traC, traN, traG*, a tyrosine type XerD recombinase (integrase), a site-specific integrase and several other conjugal transfer proteins predicted as hypothetical proteins but containing domains involved in the conjugative process as confirmed by BLAST analysis (data not shown; Figure 12). In particular, Tra amino acid sequences showed a high similiarity (99-100%) to *Salmonella enterica* ICE transfer apparatus (TraC, TraG, TraI, TraN) whereas TraD revealed a percentage of similarity of 61.71% to the same protein detected in *Pseudoalteromonas* sp. GutCa3 (WP_101217907). Concerning the integration module, the site-specific integrase sequence protein was similar at 89.09% to that found in *S. putrefaciens* (WP_086903394.1) whereas the XerD recombinase showed a low similarity (41.03%) to the tyrosine-type recombinase/integrase found in the marine bacterium *Halofilum ochraceum* (WP_070988554.1). Furthermore, several genes involved in the partitioning such as ParAB, replication (RepA, DNA polymerase III, DNA topoisomerase III, DNA binding proteins, IncW-like replication protein), recombination (RecF, SbcC) and several other genetic elements associated to type II, III and IV secretion systems were revealed and they are always involved in the conjugal transfer. In addition, in 8 out of 10 *S. algae* strains we found a genetic element of about 200 kbp containing metabolism, virulence factor, antibiotic and heavy metal resistance encoding genes. Most of these resistance genes are efflux pumps. They are also flanked by integration and recombination genes such as integrases, endonucleases, transposases and phage-associated elements. They seem to be ICEs considering their large size and the presence of the genes mentioned above. To confirm this, we have also found in the surrounding of these elements a $tRNA^{Phe}$ encoding gene as possible insertion site in the host chromosome. Moreover, an integration host factor (*ihf*) gene was found to flank this region with the alfa subunit near to the $tRNA^{Phe}$ gene and the beta subunit at the opposite end of this genetic element. Conversely, these elements seem to be absent in the vibrios under study.

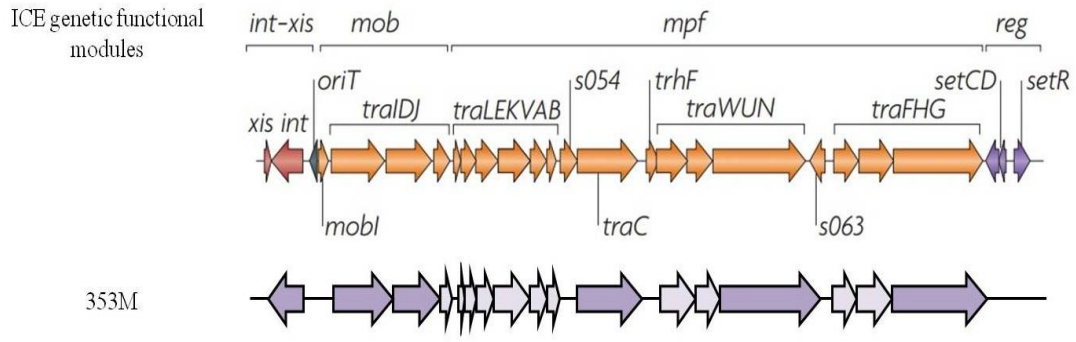

Figure 12. Description of the ICE detected in *S. algae* 353M. In the top of the picture are reported the genetic functional modules present in ICEs (Wozniak and Waldor, 2010); in the bottom the encoding genes found in the 353M strain (dark violet) belonging to *xis/int*, *mob* and *mpf* modules and the other conjugal proteins (light violet) predicted as hypothetical but containing protein domains involved in conjugation and transfer at BLAST analysis.

Class I integron elements were detected in all the *S. algae* and in *V. anguillarum* 28AD but not in *V. parahaemolyticus* VPE116 where only an *IntI* was found, whereas the same gene was detected in 5 out of 10 *S. algae* strain. These elements were predicted using INTEGRALL software but, due to the limits of short read sequencing, the genome annotation failed to detect the entire apparatus of these mobile genetic elements.

Regarding ISs, several families already described in *Enterobacteriaceae* and other aquatic bacteria such as *Aeromonas salmonicida*, *V. vulnificus*, *S. loihica*, *S. oneidensis*, *S. baltica*, *V. anguillarum*, *V. parahaemolyticus*, *V. splendidus* and *S. putrefaciens* were present in *S. algae* or *Vibrio* spp. isolates (Table 5). In particular, the following ISs families were found in *S. algae* strains IS*630*, IS*L3*, IS*3*, IS*110*, IS*200*/IS*605*, IS*481* and IS*4* whereas some ISs family subgroups such as IS*Slo2*, IS*Vvu3*, IS*Sod16*, IS*Sba6*, IS*Spu18* and IS*Spu8* were reported. Considering the two vibrios under study, *V. anguillarum* 28AD genome harbored IS*200*/IS*605*, IS*5*/IS*1182*, IS*As1*/IS*Spu13* and IS*30*/IS*Va6* whereas *V. parahaemolyticus* VPE116 carried IS*3*, IS*5*/IS*1182*, IS*5*/IS*Spu14*, IS*5*/IS*Vpa3* and IS*3*/IS*Visp3*.

The presence of bacteriophages and their related elements was investigated as well. As reported in Table 5, several prophages were detected also if incomplete. Interestingly, the presence of phages associated to other aquatic bacteria such as *Aeromonas* phage

phiO18P (NC_009542) and *Shewanella* sp. phage 1/44 (NC_025463) were showed in our *S. algae* strains. Moreover, phages derived from *Enterobacteriaceae* family members such as *Escherichia* phage D108 (NC_013594), *Enterobacteria* phage phi92 (NC_023693) and *Enterobacterial* phage mEp213 (NC_019720) were detected in the *S. algae* and in *V. parahaemolyticus* VPE116 strains. *Vibrio* phage VP882 (NC_009016) and bacteriophage phi 1.45 were found only in *V. parahaemolyticus* VPE116 whereas the 38LV strain did not harbor any phages. In addition to these elements also inovirus Gp2 encoding gene was found in 6 out of 12 strains (144bCP, 146bCP, 178CP, 38LV, 83CP, 28AD). This protein is associated to a genus of viruses able to infect Gram-negative and Gram-positive bacteria.

In addition, a defense system against bacteriophages was detected and belonging to CRISPR-Cas (clustered regularly interspersed short palindromic repeats-CRISPR-associated proteins) system in 3 out of 10 *S. algae* strains (219VB, 38LV, 82CP) and in *V. parahaemolyticus* VPE116.

Table 5. Summary of the mobile genetic elements found in the *S.algae* and *Vibrio* spp. genomes.

Strain	IS families	IS family subgroups	Integrons/Integrases	Bacteriophages
353M	IS*630*, ISL*3*, IS*3* IS*630* IS*3* IS*4*	 IS*Spu8* IS*Slo2* IS*Vvu3*	*IntI* Class I integron	Prophage-1 incomplete
219VB	IS*200*/IS*605*, IS*630*, ISL*3* IS*481* IS*110*, IS*3* IS*630* IS*4*	 IS*Spu18* IS*Spu8* IS*Vvu3*	 Class I integron	*Aeromonas* phage phiO18P (NC_009542)
144BCP	ISL*3* IS*481* IS*630*/IS*Spu8* IS*4* IS*3*, IS*630*, IS*110* IS*200*/IS*605* IS*110*, IS*4*	 IS*Spu18* IS*Vvu3* IS*Sod* IS*Sba*	 Class I integron	*Escherichia* phage D108 (NC_013594) *Shewanella* sp. phage 1/44 (NC_025463) Prophage-1 and Prophage-2 incomplete
178CP	ISL*3* IS*481* IS*630*/IS*Spu8* IS*4* IS*3*, IS*630*, IS*110* IS*200*/IS*605* IS*110*, IS*4*	 IS*Spu18* IS*Vvu3* IS*Sod* IS*Sba*	 Class I integron	*Escherichia* phage D108 (NC_013594) *Shewanella* sp. phage 1/44 (NC_025463) Prophage-1 and Prophage-2 incomplete
82CP	IS*630*, ISL*3*, IS*481*/IS*Spu18*, IS*110*, IS*3* IS*630*	 IS*Sod16*	*IntI* Class I integron	Prophage-1 *Aeromonas* phage phiO18P (NC_009542)

Strain	IS families	IS family subgroups	Integrons/Integrases	Bacteriophages
146BCP	ISL*3* IS*481* IS*630*/IS*Spu8* IS*4* IS*3*, IS*630*, IS*110* IS*200*/IS*605* IS*110*, IS*4*	 IS*Spu18* IS*Vvu3* IS*Sod* IS*Sba*	 Class I integron	*Escherichia* phage D108 (NC_013594) *Shewanella* sp. phage 1/44 (NC_025463) Prophage-1 and Prophage-2 incomplete
83CP	IS*200*/IS*605*, ISL*3*, IS*110* IS*630* IS*630* IS*4* IS*3*	 IS*Spu8* IS*Vvu3* IS*Sba6* IS*Slo2*	*IntI* Class I integron	Prophage-1 *Aeromonas* phage phiO18P (NC_009542)
38LV	ISL*3* IS*481* IS*110* IS*630*	 IS*Spu18* IS*Sod16*	*IntI* Class I integron	-
57CP	IS*200*/IS*605*, ISL*3*, IS*110* IS*3* IS*630* IS*630* IS*4*	 IS*Spu8* IS*Sod16* IS*Vvu3* IS*Sba6*	*IntI* Class I integron	*Enterobacteria* phage phi92 (NC_023693) Incomplete
60CP	IS*200*/IS*605*, ISL*3*, IS*110* IS*3* IS*630*	 IS*Slo2* IS*Sod16*	Class I integron	2 Unknown Prophages incomplete
28AD	IS*200*/IS*605*, IS*5*/IS*1182*, IS*As1* IS*30*	 IS*Spu13* IS*Va6*	*IntI* Class I integron	2 Unknown Prophages incomplete

Strain	IS families	IS family subgroups	Integrons/Integrases	Bacteriophages
VPE116	IS*5*/IS*1182*		*IntI*	*Enterobacterial* phage mEp213
	IS*5*	IS*Spu14*		(NC_019720)
	IS5	IS*Vpa3*		*Vibrio* phage VP882 (NC_009016)
	IS*3*	IS*Visp3*		

5.5 Virulome analysis

Virulence factor encoding genes were investigated both in the *S. algae* and in *Vibrio* spp. isolates. Hemolytic activity is predominant in these strains. In particular, hlyD, hlyD family secretion protein and hemolysin III were all found in *S. algae* strains as well as a complex machinery for the secretion of these factors such as type I, II, III, IV and VI secretion systems. Regarding *V. anguillarum* 28AD a thermolabile hemolysin, hemolysin III, hemagglutinin, rtxA and type I, II, III, IV and VI secretion systems were found. In addition to these factors, *V. parahaemolyticus* VPE116 harbored hemolysin D and the thermostable hemolysin delta-VPH as well. Furthermore, *lod*A, L-lysine 6-oxidase, *lod* B, a putative FAD-dependent oxidoreductase and *cvp*A, a colicin V production protein, are genes encoding bacteriocins that were detected in all the *S. algae* strains. In *V. anguillarum*, the gene *vabF*, encoding a hydrolase activity, was detected.

6. DISCUSSION

Antibiotics are widely used both in human medicine and food producing animals to prevent and treat infections. In fact, it is acknowledged that the improper and excessive use of antimicrobials for prophylaxis or as growth promoters in healthy animals through feed and water has contributed to the increased resistance of some pathogens that can propagate to humans (Ferri *et al*., 2017). Moreover, in the last decades, the aquaculture sector is increasing worldwide as an alternative and easier source of animal proteins compared to terrestrial animal farming that is climate and agriculture impacting. In this scenario, the use of antibiotics is not controlled due to the lack of legislation and regulation by the respective governments in much of the world (Topp *et al*., 2018; Watts *et al*., 2017). The most common classes of antibiotics used in Italian aquaculture include beta-lactams (amoxicillin, ampicillin), tetracyclines (chlortetracycline, tetracycline, oxytetracycline), amphenicols (florfenicol, thiamphenicol), macrolides (erythromycin), sulfonamides (all and trimethoprim+sulfonamide) and quinolones (oxolinic acid, flumequine, enrofloxacin) (OIE report, 2018; EEC Regulation No 2377/90). On the basis of the specific literature regarding AR in Italian aquaculture, it can be highlighted that most of the bacterial strains isolated in fish farms are resistant to beta-lactams, tetracycline, erythromycin and trimethoprim while in general they are susceptible to chloramphenicol, tobramycin and flumequine (Scarano *et al*., 2014). Many strains from *Aeromonas* spp. and *Vibrio* spp. showed multiple antibiotic resistance (Scarano *et al*., 2018; Ottaviani *et al*. 2013; Laganà *et al*., 2011; Ottaviani *et al*., 2001).

During my 3-year PhD project, the resistome, mobilome and virulome of 12 MDR marine strains previously isolated from different Italian fish farms located near the Italian coast of the Adriatic Sea was analyzed by NGS technology. New insights are provided in this study as regards the genes involved in the AMR, including mainly the production of beta-lactamases, other degrading enzymes and the involvement of multidrug efflux pumps belonging to different families and targeting multiple drugs. Moreover, the mobilome of these strains have been investigated deeply for the first time.

The WGS analysis allowed to detect the resistance determinants, the MGEs and virulence factors involved in these MDR strains. In fact, they resulted to be resistant to different families of antibiotics including beta-lactams, quinolones, tetracyclines, macrolides, polymyxins and chloramphenicol.

Regarding beta-lactams, the presence of class C and D beta-lactamases is already described in literature for *S. algae*, but few data are reported for *V. anguillarum* and *V. parahaemolyticus*. Here, we found that resistance to penicillins and first- and second-generation cephalosporins is due to the presence of a chromosome-encoded AmpC in *V. anguillarum* 28AD. On the other hand, a class A beta-lactamase (CARB-19) was detected in *V. parahaemolyticus* VPE116 conferring resistance to amino- and carboxypenicillins (in particular to amoxicillin and ticarcillin). However, the susceptibility to these antibiotics was restored if penicillins were associated to a beta-lactamase inhibitor such as clavulanic acid in *V. parahaemolyticus* VPE116 but not in the veterinary clinical strain *V. anguillarum* 28AD as reported in Table 1. Of interest was the genetic surrounding of these genes in which paraquat-inducible protein was present in the *S. algae* strains whereas *cusAB* genes which are involved in silver, copper and fosfomycin resistance were found in *V. parahaemolyticus* VPE116 confirming the contribution of heavy metal resistance encoding genes in the beta-lactam resistance phenotype in marine bacteria. Conversely, AmpC, in *V. anguillarum* 28AD strain, was flanked by its activator AmpR. MexAB-OprM pump was detected in the *S. algae* and *V. parahaemolyticus* strains. This efflux pump is documented to be involved in beta-lactam (carbapenem) resistance in *Pseudomonas aeruginosa*.

Particularly interesting is the resistance to fluoroquinolones, antibiotics that are frequently used in aquaculture. In our strains, MepAB was detected in all the isolates whereas MpfA was observed only in *V. anguillarum* 28AD. Furthermore, other pentapeptide proteins were detected in the analyzed strains and belonging to the Qnr group. Although these proteins are PMQR (except for QnrVC-like proteins), they are chromosome-encoded both in *Shewanella* and *Vibrio* genera as already reported by Poirel and coauthors (2012a). To be noted that some of the *S. algae* strains carrying the QnrA7 belong to a group of strains isolated from the same aquaculture center while the strains showing the QnrA3 protein were obtained in different sites and fish farms. Moreover, QnrVC6 determinant usually found in *V. cholerae* was detected here in *V. anguillarum* 28AD strain. It is not surprisingly to find QnrVC-like proteins in other *Vibrio* species, but the arising spread of the QnrVC6 determinant, is

alarming since it has been found in other Gram-negatives such as *P. aeruginosa*, *Pseudomonas putida* and *Citrobacter freundii* (Bado *et al*., 2018; Liu *et al*., 2018). The efflux pumps involved in quinolone resistance were detected in all the strains (MdtK and EmrAB-TolC) and in *V. anguillarum* (NorM, Bmr3 and MdtH). Interestingly, although the presence of these genes which should provide resistance to quinolones, the phenotypes of all our strains were susceptible to ciprofloxacin and nalidixic acid, the two fluoroquinolones tested for. We did not tested other quinolones, but maybe resistance can arise if exposed to other molecules such as flumequine, largely used in veterinary. Although, some mutations were found on their targets, gyrase A (gyrA) and topoisomerase IV (parC), the absence of specific amino acid substitutions in Ser83 and Asp87, the key positions in arising quinolone resistance, was evidenced. It is noted that PMQR determinants generally confer only low-level quinolone resistance that alone does not exceed the clinical breakpoint but they can favour the selection of additional resistance mechanisms (Correia *et al*., 2017). Conversely, all the *S. algae* strains provided a resistant phenotype to fosfomycin, but the WGS analysis failed to detect the genes possibly involved, indicating the presence of an unknown gene not yet identified that can be hidden as hypothetical protein in our results. Considering the data present in literature, very high MICs were reported by Torri and coworkers (2018) in clinical *S. algae* isolates during an Italian survey on shewanella-associated infections. Probably this is an intrinsic resistance present in *S. algae* strains whose gene is chromosomal-encoded considering the few presence of plasmids reported in these strains. In our case, the absence of a protein product conferring fosfomycin resistance can be due to the fact that this gene is still unknown and not reported on databases and for this reason, it can be hidden as hypothetical protein as final result. The main mechanism of resistance to macrolides, described and detected by WGS, was the efflux pump system. In particular, MacAB-TolC was found in all the strains whereas MdtE-TolC only in the two vibrios analyzed. Anyway, resistance to macrolides were not tested for during antimicrobial susceptibility testing.

Regarding tetracycline resistance only vibrios harbored *tet34* or/and *tet35* encoding genes but they showed susceptible to this class of antibiotics. In particular, the *tet34* gene has been found in some Gram-negative genera (*Pseudomonas*, *Serratia*, and *Vibrio*) and it is unique to environmental bacteria (Miranda *et al*., 2003). In addition to that, also other efflux pumps like AcrAB-TolC and AcrEF-TolC can contribute to the tetracycline and tigecycline resistance overall in *Enterobacteriaceae* (Grossman, 2016). Surprisingly, two *S. algae*

strains, 38LV and 57CP, were resistant both to tetracycline and tigecycline, a recent commercialized antibiotic used against MDR pathogens to treat hospital-acquired pneumonia, ventilator-associated pneumonia and other infections (Grossman, 2016). Tigecycline is scarcely investigated in aquaculture because it is mainly used in human medicine. Here, for the first time in Italy, we report the presence of two resistant strains of *S. algae* isolated in two different aquaculture sites located in the Northern and Southern Adriatic Sea. The mechanism of resistance is still unknown, probably due to the presence of efflux pumps widely detected by WGS. Susceptibility to aminoglycosides such as amikacin, kanamycin, gentamycin and tobramycin is reported for most of the strains except for the two vibrios.

All the strains presented the *eptA* gene which contributed to the colistin resistance. This gene encodes for an enzyme that promotes the addition of a phosphoethanolamine moiety to the lipid A which is required for resistance to polymyxins. This is a chromosome-encoded gene that confers an intrinsic resistance to colistin in these marine bacteria. Finally, resistance to heavy metals have been reported suggesting an important role during the antimicrobial resistance gene expression and diffusion in the aquatic environment.

Globally, the data obtained for the resistome study seem to be in line with what reported for Italian aquaculture (Scarano *et al*., 2018; Scarano *et al*., 2014; Ottaviani *et al*. 2013; Laganà *et al*., 2011; Ottaviani *et al*., 2001). A great concern is the rising resistance to tigecycline found in two out of twelve isolates.

The mobilome analysis has revealed the presence of several MGEs that can contribute to the spread of these genes. Anyway, the analysis has been particularly difficult and laborious to perform, probably due to the Illumina technology which uses a mating pair and a short read sequencing. Thus, the genome assembly and annotation sometimes failed to predict the MGEs present in the genomes of our strains. Despite, these difficulties, we identified some integrative and conjugal elements that can be associated to the xis/int, mob and mpf modules of ICEs in the 353M strain isolated in the open sea. This is an interesting isolate considering its scarce relation to the aquaculture strains but potentially able to behave as reservoir of ARGs in the same environment facilitating the spread of these MGEs and of the genes carried on to other marine bacteria or human pathogens. Furthermore, the presence of large size elements in the *S. algae* strains and of specific insertion sites, recombinases, exonucleases,

integrases as well as genes involved in heavy metal and antibiotic resistance (especially efflux pumps), virulence factors and other genes involved in the bacterial fitness improvement has been highly documented in this study. These results underline a high incidence of these elements in aquaculture that has never been investigated before. The role of the detected bacteriophages has yet to be clarified. In fact, their involvement in the HGT of ARGs that can flank these elements during the excision process, packaging these bacterial ARGs in the mature virion particle and disseminating them in the genome of a new host cell is not to be excluded. Interestingly, both *S. algae* and *V. parahaemolyticus* VPE116 strains showed to be prone to be infected by bacteriophages belonging to different genera of marine and human pathogenic bacteria. We described the presence of specific bacteriophages belonging to *Shewanella*, *Aeromonas* and *Vibrio* genera as well as to *E. coli* and *Enterobacteriaceae*. Moreover, several site-specific integrases, *IntI* and class I integrons have been detected by softwares. In particular, resistance to sulfonamides, chloramphenicol and heavy metals might confirm their presence despite the lack of information through the genome annotation, thus having implications on the study of the gene cassette carried by these elements and the genes that are typically found in integrons. Finally, other transposable elements such as ISs family transposases were investigated. They are intracellular MGEs which need to be integrated in other mobile elements such as plasmids, bacteriophages or ICEs. ISs families are classified on the basis of the transposase type. Here, we reported the presence of transposases belonging to several groups depending on the chemistry of their active sites. In particular DDE (IS*3*, IS*4*, IS*5*, IS*630* IS30), DEDD (IS*110*), HUH (IS*200*/IS*605*) and orphan ISs such as ISL*3* and IS*As1* transposases were found. Moreover, several isoforms of these transposases were detected belonging to other aquatic and pathogenic bacteria indicating the ability of our strains to allocate TEs of different bacterial genera and species. Most of these ISs did not carry passenger genes such as transcription regulators, methyltransferases and antibiotic resistance, only 38LV carried a MFS transporter and an AraC family transcriptional regulator downstream the IS*481* family transposase, whereas the 82CP and 353M strains carried a LysR and an AraC family transcriptional regulators downstream the transposase genes, respectively.

Regarding the virulome, the analysis confirmed the hemolytic activity of the strains analyzed and the presence of bacteriocins and of several types of secretion systems (I, II, III, IV and VI) that can disseminate these virulence factors to other microbial cells. Furthermore, the

CRISPR-Cas system detected in some of these strains underlines the continuous arms race against the menaces coming from the environment around the bacteria.

The present study has allowed to improve the knowledge regarding the resistome and mobilome of these pathogens such as *S. algae*, *V. parahaemolyticus* and *V. anguillarum*. Although they are emerging and opportunistic pathogens, the number of human infections caused by these bacteria is increasing worldwide particularly in the immunocompromised people due to the global warming. As a consequence, the investigation of their phenotypes and genotypes has a crucial role to address clinicians to prescribe the correct chemotherapy. Moreover, these results help researchers to survey the antimicrobial resistance determinants and their mechanisms of transfer involved in the aquatic environment.

7. CONCLUSIONS

NGS technology is a useful mean for detecting and predicting ARGs, MGEs and virulence factors although their reliability is dependent upon the availability of updated and curated databases of reference sequences. Moreover, the genotype analysis sometimes does not correspond to the phenotype, letting the information achieved from this technology as a potential warning about the pathogenicity and the resistance of a microorganism. However, although transcriptome or proteome analysis should be more adequate in detecting the real presence of the products generated by the predicted ARGs, WGS can help in analyzing the surrounding of the ARGs, their regulation during the transcription and possible mobilization.

In conclusion, the improvement of the softwares and databases involved in the ARGs and MGEs will undoubtedly be able to predict new insights not only in the most common human pathogens but also in the emerging and opportunistic ones. Moreover, this will lead to a better understanding of the origins of the most involved antimicrobial resistance mechanisms.

PART II

ANTIMICROBIAL RESISTANCE PROFILES AND BETA-LACTAMASE PHYLOGENETIC ANALYSIS OF *SHEWANELLA* ALGAE AND *VIBRIO* SPP. STRAINS ISOLATED IN ITALIAN AQUACULTURE FARMS

8. INTRODUCTION

8.1 The beta-lactams world and their multiple use in the therapeutic treatment of infections

8.1.1 Mechanism of action

Beta-lactam antibiotics are currently the most used class of antibacterial agents in the infectious disease armamentarium. Because of their tolerance and efficacy, they are widely prescribed. Their mechanism of action is based on the inhibition of bacterial cell wall synthesis. In fact, the molecular structure of beta-lactam ring is sterically similar to the D-alanine-D-alanine of the *N*-acetylmuramic acid pentapeptide that constitutes together with the *N*-acetylglucosamine the units of the bacterial cell wall (Bonomo, 2017). The PBPs are the targets of beta-lactams. They are involved in the cell wall synthesis since the PBPs act as transpeptidases to catalyze the cross-linking of two D-alanine-D-alanine, the *N*-acetylmuramic acid pentapeptides (Fishovitz *et al*. 2015; Fisher and Mobashery 2014; Drawz and Bonomo 2010). PBPs can use the beta-lactams as substrates during the cell wall synthesis. This mistake generates the acylation of the PBP, which renders the enzyme unable to catalyze further carry out transpeptidation reactions. Beta-lactams are grouped in several classes on the basis of their functional groups while the main core constituted by the beta-lactam ring is conserved. They comprise penicillins, cephalosporins, carbapenems and monobactams.

8.2 Mechanisms of resistance to beta-lactams

Bacterial resistance to beta-lactams has historically compromised use of these agents in treating infectious disease and continues to do so, necessitating the continued development of new beta-lactams capable of overcoming this resistance. Although the production of beta-lactamases, especially in Gram-negative bacteria, is the most common mechanism of resistance, other can contribute to the resistant phenotype of bacteria.

Impermeability

The entry of hydrophilic antimicrobials such as beta-lactams into Gram-negative bacteria occurs via channels in the outer membrane formed by porins (Nikaido, 2000). Reduced outer membrane permeability to beta-lactams as a result of porin loss or changes in porin structure can promote resistance to these agents. Indeed, porin deficiency is a contributing factor to beta-lactam (including newer cephalosporin generation) resistance in a number of organisms, including *E. coli*, *Proteus* spp., *P. aeruginosa*, *Acinetobacter baumannii*, *Shigella dysenteriae*, *Neisseria gonorrhoeae*, *Serratia marcescens* and *Helicobacter pylori* (Clarke *et al*., 2003; Kwon *et al*., 2003; Quale *et al*., 2003; Weindorf *et al*., 1998) but also in *Klebsiella pneumoniae* and *Enterobacter* spp. (Gayet *et al*., 2003; Stürenburg *et al*., 2002) usually in conjunction with expression of a beta-lactamase. Porin deficiency is an important determinant, too, of carbapenem resistance/reduced susceptibility, particularly in *P. aeruginosa* where loss or mutation of the OprD porin is common in carbapenem-, especially imipenem-resistant strains (Pirnay *et al*., 2002).

Efflux pumps

Efflux has long been appreciated as a mechanism of antimicrobial resistance, with five families of bacterial efflux systems described to date and reported in the part I of this thesis. Members of the RND family, which are almost exclusively chromosomal and widely distributed in Gram-negative bacteria, appear to be the most significant as regards export conferring resistance to clinically important antimicrobials (Poole, 2004). In particular, MexAB-OprM, MexCD-OprJ and MexXY-OprM all demonstrated some ability to promote resistance to carbapenems, none of these had any effect on imipenem or biapenem resistance (Okamoto *et al*., 2002), and only MexAB-OprM has been implicated in carbapenem resistance in clinical strains.

Target site mutations: alterations in PBPs

Although beta-lactamases are associated with resistance to beta-lactams in Gram-negative organisms other non beta-lactamase mechanisms of resistance to these agents are seen mainly

due to alterations in PBPs. Resistance to carbapenems owing to changes in PBPs has been seen in *A. baumanni* and *Proteus mirabilis*. Production of altered PBPs with reduced affinity for beta-lactams is seen, too, in beta-lactam-resistant *S. dysenteriae*, *P. aeruginosa* and *H. pylori*.

Beta-lactamase production and classification

Beta-lactamases, the enzymes that inactivate beta-lactam antibiotics, serve as a major cause for resistance to this exceedingly valuable set of antibacterial agents (Gazin *et al*., 2012). They are the most common and important mechanism of resistance in Gram-negative bacteria. Beta-lactamases can be named on the basis of molecular characteristics or functional properties. Molecular classes A, B, C, and D define an enzyme according to amino acid sequence and conserved motifs. Functional groups 1, 2, and 3 are used to assign a clinically useful description to a family of enzymes, with subgroups designated according to substrate and inhibitor profiles (Bush, 2013; Figure 13). Moreover, classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B beta-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis (Bush and Jacoby, 2010).

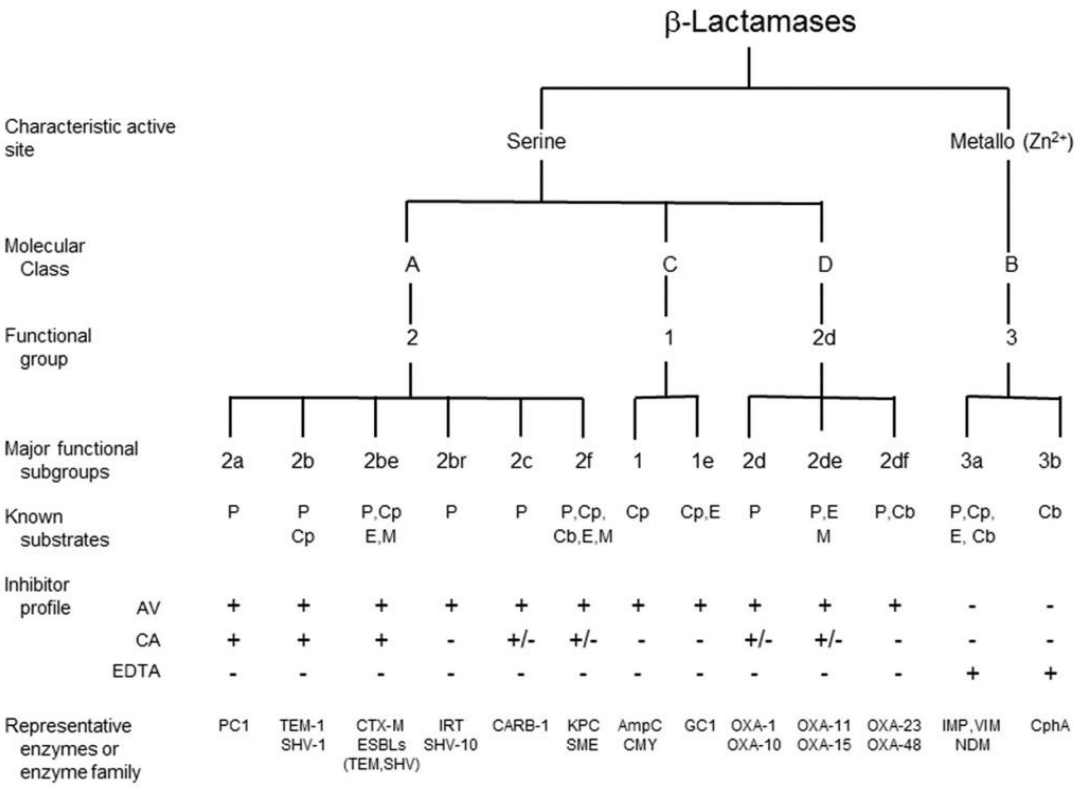

Figure 13. Schematic representation of Ambler's beta-lactamase classification (Bush, 2018). AV, avibactam; CA, clavulanic acid; Cb, carbapenem; Cp, cephalosporin; E, expanded-spectrum cephalosporin; M, monobactam; P, penicillin.

Class A beta-lactamases

Class A beta-lactamases are often plasmid-encoded, but can also be located on the bacterial chromosome. In general, class A enzymes are usually susceptible to inactivation by the clinically available beta-lactamase inhibitors: clavulanate, sulbactam, tazobactam, and avibactam. TEM, SHV, and CTX-M beta-lactamases are mostly found in *E. coli* and *Klebsiella* spp. Many class A beta-lactamases have substrate profiles that include expanded-spectrum cephalosporins, and ESBLs. The widespread distribution of CTX-M beta-lactamases, especially CTX-M-14 and CTX-M-15, in *E. coli* is responsible for the large part of the global advanced generation cephalosporin resistance seen in many clinical isolates. Other class A enzymes are encoded on integrons, for example, GES-1 from *K. pneumoniae* and VEB-1 in *P. aeruginosa* and *A. baumannii* (Poirel *et al*. 2012b). Few Ambler class A

beta-lactamases show carbapenem-hydrolyzing activity. The major class A serine carbapenemases include KPC and GES which are plasmid-encoded enzymes, whereas Nmc-A/ IMI, and SME are chromosomally-encoded beta-lactamases (Bonomo, 2017).

Class A beta-lactamases: carbenicillinases

Carbenicillinases are penicillinases able to confer resistance in particular towards carboxy-penicillins such as carbenicillin and ticarcillin and to amino-penicillins such as ampicillin and amoxicillin. Specific motifs belonging to class A beta-lactamases are found in carbenicillinases such as the *bla* active-site (STFK) tetrad at positions 70 to 73 according to the standard numbering scheme of Ambler and the consensual and conserved boxes I to VI (Joris *et al*. 1991). Moreover, box VII consists of the conserved RSG motif which is specific to the carbenicillinases. These bla_{CARB} genes have been broadly dispersed among distantly related bacteria, probably through mobile genetic elements (Petroni *et al*., 2004). Despite the differences in genetic backgrounds, all of the bla_{CARB} genes are contained in cassettes sharing a common array of genetic elements. These elements are located in highly conserved boxes and comprise sequences required for both cassette integration and transcription of bla_{CARB} genes: a unique core site (CS), putative promoters and ribosome-binding sites (RBS) (boxes 1 to 3); a double translation-stop signal (TGATAA), followed by a unique inverse CS (ICS) (box 4), and the 3′ terminus of *attC* and VCRs (box 5). Among the different variants that are known within this enzyme family, CARB-2, CARB-6, CARB-7, CARB-9 have been characterized in *V. cholerae* non/O1-non/O139 (Petroni *et al*., 2004). CARB-17 was identified in *V. parahaemolyticus* (Chiou *et al*., 2015) whereas other carbenicillin-hydrolyzing penicillinases associated to the PSE-type enzymes have been detected in *P. aeruginosa* and *P. mirabilis* genomes (Ito and Hirano, 1997).

Class B beta-lactamases: the metallo beta-lactamases

This class of beta-lactamases is characterized by the ability to hydrolyze carbapenems and by its resistance to the commercially available beta-lactamase inhibitors but susceptibility to inhibition by metal ion chelators. The substrate spectrum is quite broad; in addition to

carbapenems, most of these enzymes hydrolyze cephalosporins and penicillins but lack the ability to hydrolyze aztreonam (monobactam). The mechanism of hydrolysis is dependent on interaction of the beta-lactams with zinc ions in the active site of the enzyme, resulting in the distinctive trait of their inhibition by EDTA, a chelator of Zn^{2+} and other divalent cations (Queenan and Bush, 2007). The class B beta-lactamases are classified into three subclasses B1, B2 and B3 on the basis of sequence alignment. Subclass B1 enzymes share more than 23% identity. These enzymes include the prototypical BcII from *Bacillus cereus* (Hussain *et al*., 1985), CcrA from *Bacteroides fragilis* (Rasmussen *et al*., 1990), BlaB from *Elizabethkingia meningoseptica* (Rossolini *et al*., 1998) and EBR-1 from *Empedobacter brevis*. The acquired IMP-type MBLs found in some clinical isolates of *P. aeruginosa* (Laraki *et al*. 1999), *S.marcescens* (Osano *et al*., 1994), *K. pneumoniae* and *A. baumannii* (Walsh *et al*., 2005), as the VIM-type or SPM-1 enzymes produced by clinical isolates of *P. aeruginosa* (Walsh *et al*., 2005; Toleman *et al*., 2002), belong also to this subclass. The members of subclass B2 show only 11% of identity with subclass B1 members. This subclass includes the enzymes produced by different species of *Aeromonas* (the most studied are CphA produced by *Aeromonas hydrophila* (Massidda *et al*., 1991) and ImiS produced by *Aeromonas veronii* (Walsh *et al*., 1996)) as well as the Sfh-I enzyme produced by *Serratia fonticola* (Saavedra *et al*., 2003). Subclass B3 metallo-beta-lactamases have only nine conserved residues when compared with the other metallo-beta-lactamases. Subclass B3 includes the L1 and GOB-1 metallo-beta-lactamases produced by clinical strains of *Stenotrophomonas maltophilia* (Walsh *et al*., 1994). However, the most common and of clinical interest metallo-beta-lactamase families include the VIM, IMP, GIM, and SIM enzymes, which are located within a variety of integron structures, where they have been incorporated as gene cassettes. Since their initial discoveries, SPM, GIM, and SIM metallo-beta-lactamases have not spread beyond their countries of origin. However, VIM and IMP continue to be detected worldwide, with an overall trend of these two metallo-beta-lactamases moving beyond *P. aeruginosa* and into the *Enterobacteriaceae* (Queenan and Bush, 2007).

Class C beta-lactamases: AmpC cephalosporinases

Class C beta-lactamases are clinically important cephalosporinases encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms (Jacoby, 2009). They are more active on cephalosporins and they can hydrolyze cephamycins such as cefoxitin and cefotetan; oxyiminocephalosporins such as ceftazidime, cefotaxime, and ceftriaxone; and monobactams such as aztreonam but at a rate <1% of that of benzylpenicillin (Jacoby, 2009). They are usually resistant to inhibition by clavulanic acid and active on cephamycins, such as cefoxitin. They have a high affinity for aztreonam in contrast to the class A cephalosporinases (Bush and Jacoby, 2010). In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone and is a problem especially in infections due to *Enterobacter aerogenes* and *Enterobacter cloacae*, where an isolate initially susceptible to these agents may become resistant upon therapy. When produced in large amounts, especially in a host with reduced β-lactam accumulation, these enzymes can provide resistance to carbapenems, especially ertapenem by mutations that reduce influx (outer membrane porin loss) or enhance efflux (efflux pump activation) (Quale *et al*., 2006; Jacoby *et al*., 2004; Bradford *et al*., 1997). Although that, carbapenems can usually be used to treat infections due to AmpC-producing bacteria. Transmissible plasmids have acquired genes for AmpC enzymes, which consequently can now appear in bacteria lacking or poorly expressing a chromosomal bla_{AmpC} gene, such as *E. coli*, *K. pneumoniae* and *P. mirabilis*. Plasmid-mediated enzymes are represent by CMY, ACT, DHA, FOX, MIR, LAT, ACC. The plasmid-determined enzymes are related, sometimes very closely, to chromosomally determined AmpC beta-lactamases (Jacoby, 2009). Like the chromosomally determined AmpC beta-lactamases, the plasmid-mediated enzymes confer resistance to a broad spectrum of beta-lactams including penicillins, oxyimino-beta-cephalosporins, cephamycins, and (variably) aztreonam. Susceptibility to cefepime, cefpirome, and carbapenems is little, if at all, affected.

Class D beta-lactamases

The class D beta-lactamases have been much more elusive and, for the most part, were identifiable only as plasmid-encoded beta-lactamases in Gram-negative bacteria. These early

enzymes were essentially penicillinases, which, unlike the class A beta-lactamases, could hydrolyze and confer resistance to oxacillin as well as penicillins, hence the name oxacillinases and the prefix OXA (Evans and Amies, 2014). The first group of carbapenem-resistant OXA-type beta-lactamases to be identified in *A. baumannii* was the OXA-23 group. The genes for this group of enzymes are frequently plasmid-borne and have been found in many *Acinetobacter* species as well as species belonging to the *Enterobacteriaceae*. The enzymes are able to hydrolyze oxyiminocephalosporins, aminopenicillins, piperacillin, oxacillin, and aztreonam in addition to the carbapenems (Afzal-Shah *et al*., 2001; Paton *et al*., 1993). The second group of OXA-type beta-lactamases from *A. baumannii* to be identified was the OXA-40 group. The founding member of this group, OXA-24 was subsequently renamed OXA-40. The three members of this group are OXA-40, OXA-25 and OXA-26. In general, these enzymes can hydrolyze penicillins but appear to show weak activity against cephalosporins and carbapenems. The largest group of OXA-type beta-lactamases are the OXA-51-like beta-lactamases. These enzymes are intrinsic to *A. baumannii* and are naturally found on the chromosome of this species. Moreover, the huge number of variants, alone, is an indication that these chromosomally encoded enzymes have been under considerable selective pressure from antibiotic use, and these enzymes are not benign and do play a role in resistance (Brown *et al*., 2005; Heritier *et al*., 2005). In addition, these enzymes demonstrate only weak hydrolytic activity towards carbapenems. The first member of the OXA-58 group of enzymes was identified in France in 2003. It was found in a multidrug-resistant *A. baumannii* clinical isolate that also demonstrated carbapenem resistance (Poirel *et al*., 2005b). Analysis of the enzyme kinetics of OXA-58 revealed properties similar to those found in other OXA-type enzymes in *A. baumannii* with weak activity against the carbapenems and penicillins and an ability to hydrolyze cefpirome and cephalothin but not ceftazidime, cefotaxime, or cefepime (Poirel *et al*., 2005b). In 2001, a *K. pneumoniae* isolate was obtained from a patient in Istanbul, Turkey, which was found to be multidrug resistant, including resistance to the carbapenems. In this isolate, a new OXA-type beta-lactamase was identified and named OXA-48 (Poirel *et al*., 2004). This enzyme and its variants are now widespread in *K. pneumoniae* and other *Enterobacteriaceae* and have now been reported in *A. baumannii* as well and they represent one of the most concerning developments in carbapenem resistance in the last decade. Analysis of the enzyme kinetics of OXA-48 showed that the enzyme has a low level of hydrolytic activity against the carbapenems, with much greater activity against imipenem than against meropenem (Poirel *et al*., 2004). Until

the discovery of the OXA-23 beta-lactamase, the OXA enzymes were a relatively minor group of plasmid-encoded beta-lactamases, which were active predominantly against the penicillins. The introduction of the carbapenems and the rise of *A. baumannii* has, for the OXA enzymes, opened "Pandora's box". There has been an explosion of new OXA enzymes, many closely related to each other.

AmpC and OXA-like beta-lactamases in Vibrio and Shewanella genera

Recently, class C beta-lactamases were detected in vibrios. In particular, Li and coworkers (2015) found a $bla_{\text{CMY-2}}$ on a conjugative IncA/C plasmid in a *V. parahaemolyticus* isolate which exhibited resistance to third and fourth generation cephalosporins and obtained from seafood samples purchased from supermarkets and wet markets in Shenzhen, China, in 2013 (Li *et al*., 2015). AmpC was identified also in *V. fischeri* highly resistant to penicillins and susceptible to cephalosporins (Weng *et al*., 2004). Moreover, $bla_{\text{DHA-1}}$ and $bla_{\text{DHA-2}}$ genes, AmpC beta-lactamases, were found in a *V. cholerae* isolated in a 2-year-old child in India resistant to ampicillin, ceftriaxone, cotrimoxazole, and furoxone and sensitive only to ciprofloxacin and tetracycline (Mandal *et al*., 2012). Regarding shewanellae, few data are reported for AmpC beta-lactamases. Otherwise, the literature well supports the presence and the origin of some OXA-like beta-lactamases in *Shewanella* genus. In particular, OXA-55 is reported, as chromosomally encoded gene, to be present in *S. algae* genome. It has 55% amino acid identity with OXA-54 from *Shewanella oneidensis*, 41% with OXA-10, 39% with OXA-23 and -27 from *A. baumannii*, and 33% with the cluster OXA-24, -25, -26, and -40 from *A. baumannii* (Chen *et al*., 2019; Wu *et al*., 2018). OXA-54 is found in *S. oneidensis* sharing 92% of amino acid identity with the plasmid-encoded carbapenem hydrolyzing oxacillinase OXA-48 from *K. pneumoniae* (Poirel *et al*., 2004). *Shewanella xiamenensis* harbors in its chromosome OXA-181, OXA-199, the $bla_{\text{OXA-48}}$-like gene (OXA-416) and OXA-538 beta-lactamases (Yousfi *et al*., 2017; Zong, 2012; Potron *et al*., 2011). Furthermore, Xin and coworkers (2019) describes the presence of $bla_{\text{OXA-58}}$ in *Shewanella* spp. isolated from coastal water in China. OXA-48b, OXA-514, OXA-515, OXA-181, OXA-199 and OXA-252 were also detected in *Shewanella* spp., *S. xiamenensis* and *S. oneidensis* isolated in food producing animals and environmental freshwater in The Netherlands as well as in imported freshwater ornamental fish coming from non European countries (Ceccarelli *et*

al., 2017). OXA-535 is the naturally occurring oxacillinase in *Shewanella bicestrii* (Dabos *et al*., 2018; Jousset *et al*., 2017) whereas Potter and co-authors (2017) reported the genome sequencing of a *S. putrefaciens* carrying $bla_{\text{OXA-436}}$ isolated in the sink handle of a Pakistan hospital room. To conclude *Shewanella* species represent the origins of $bla_{\text{OXA-48}}$ genes which are spreading in *Enterobacteriaceae* (Tacão *et al*., 2018).

The second part of my PhD project has been focused on the beta-lactam resistance profiles of *S. algae* and *Vibrio* spp. strains analysed in this study. A comparison of the amino acid sequences of the beta-lacatmases found in their genomes (OXA-55-like, AmpC and CARB-19) with their respective sequences deposited in Genbank (from the genus *Shewanella*, *Vibrio*, *Acinetobacter* and the *Enterobacteriaceae* family) was performed. Also a phylogenetic analysis of the three enzymes was carried out to investigate the genetic correlation of the marine strains used in this study and to find similarity with the beta-lactamases already known. To the best of my knowledge, this is the first work which tries to highlight the differences that may occur on the beta-lactamase sequences present in MDR marine isolates sampled in different aquaculture centers along the coast of the Adriatic Sea. Furthermore, cloning of AmpC and CARB-19 beta-lactamases in *E. coli* TOP10, susceptible to beta-lactams, was performed in order to evaluate how these beta-lactamases can modify the antimicrobial resistance profile in a member of *Enterobacteriaceae* family if acquired. This experiment was led at the Laboratory of Medical and Molecular Microbiology at the University of Fribourg (Switzerland) under the supervision of Dr. Laurent Poirel and Prof. Patrice Nordmann.

9. MATERIALS AND METHODS

9.1 Phenotypical and molecular identification of the beta-lactamases

The BCT (Pires *et al*., 2013) was performed as biochemical test to detect carbapenemase production whereas resistance to third generation cephalosporins was tested by the strain ability to grow on ChromID ESBL medium (Biomérieux, Marcy l'Étoile, France) at 37°C O/N. Searching for the possible presence of the most important carbapenemases in clinical settings was carried out by multiplex PCR with primers targeting *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, their variants and *bla*$_{OXA-48}$ following protocols reported in literature and showed below (Table 6).

Table 6. List of the primers used for targeting *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, their variants and *bla*$_{OXA-48}$ genes.

Beta-lactamase targeted	**Primer sequence (5'-3')**	**Primer length (bases)**	**Amplicon sizes (bp)**	**References**
***bla*$_{KPC}$ KPC-1 to KPC-5**	KPC_for CATTCAAGGGCTTTCTTGCTGC	22	538	Dallenne *et al*., 2010
	KPC_rev ACGACGGCATAGTCATTTGC	20		
***bla*$_{VIM}$ VIM variants including VIM-1, VIM-2**	VIM_for GATGGTGTTTGGTCGCATA	19	390	Dallenne *et al*., 2010
	VIM_rev CGAATGCGCAGCACCAG	17		
***bla*$_{NDM}$ NDM-1 to NDM-4**	NDM_for CACCTCATGTTTGAATTCGCC	21	984	Kaase *et al*., 2011
	NDM_rev CTCTGTCACATCGAAATCGC	20		

Beta-lactamase targeted	Primer sequence (5'-3')	Primer length (bases)	Amplicon sizes (bp)	References
***bla*$_{IMP}$ IMP variants except IMP-9, IMP-16, IMP-18, IMP-22, IMP-25**	IMP_for TTGACACTCCATTTACDG	18	139	Dallenne *et al*., 2010
	IMP_rev GATYGAGAATTAAGCCACY CT	21		
***bla*$_{OXA-48-like}$**	OXA-48_for GCTTGATCGCCCTCGATT	18	281	Dallenne *et al*., 2010
	OXA-48_rev GATTTGCTCCGTGGCCGAAA	20		

Y = T or C

D = A or G

9.1.1 <u>Protocols for multiplex PCR to detect IMP, VIM, KPC variants and for end-point PCR to detect OXA-48-like and NDM variants</u>

Total DNA was extracted using CTAB and isopropanol precipitation method as described in Annex II. Then, PCR assays were carried out in a Gene Amp PCR System 9700 Thermal cycler (Applied Biosystems, Monza, MB, Italy). Each reaction has been conducted in a final volume of 50 µl, using 2 µl of DNA template, 5 PRIME MasterMix 1X (Quantabio, San Giusto, Milano, Italy), 0.5 µM of VIM and IMP forward and reverse primers and 0.2 µM of KPC forward and reverse primers.

The thermal protocol used was as the following: initial denaturation step at 94°C for 10 min, then 30 cycles with denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec, extension at 72°C for 1 min. The final extension step was at 72°C for 7 min.

For OXA-48-like PCR reaction, 2 µl of DNA template were used in a final volume of 50 µl, 5 PRIME MasterMix 0.9X (Quantabio, San Giusto, Milano, Italy) and 0.4 µM of OXA-48-like forward and reverse primers were used.

The thermal conditions were the following: initial denaturation at 94°C for 10 min, then 30 cycles with denaturation at 94°C for 40 sec, annealing at 57°C for 40 sec and extension at 72°C for 1 min followed by a final extension step at 72°C for 7 min.

Regarding NDM PCR protocol, 2 µl of DNA template were used in a final volume of 50 µl, 5 PRIME MasterMix 1X (Quantabio, San Giusto, Milano, Italy) and 0.4 µM of NDM forward and reverse primers were used.

The thermal conditions used are reported as the following: an initial denaturation step at 94°C for 2 min, then 30 cycles were perfomed with a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min and an extension step at 72°C for 1 min and 15 sec. The final extension step was conducted at 72°C for 7 min.

Amplicons were analyzed on a 1.5% agarose gel for IMP, VIM, KPC and OXA-48-like whereas a 1.2% agarose gel was prepared for NDM variants detection. The gels containing 1X GelRed® (Biotium Inc., Fremont, CA, USA) were analyzed and photographed by the transilluminator GenoSmart2 (VWR, Leicestershire, UK).

9.2 Cloning of $bla_{\text{CARB-19}}$ and bla_{AmpC}

9.2.1 PCR reaction protocol to detect AmpC and CARB-19

The presence of bla_{AmpC} in *V. anguillarum* 28AD and $bla_{\text{CARB-19}}$ in *V. parahaemolyticus* VPE116 was confirmed by end-point PCR using the following primers (Table 7):

Table 7. List of the primers used for targeting *bla*$_{\text{CARB-19}}$ in *V. parahaemolyticus* and *bla*$_{\text{AmpC}}$ in *V. anguillarum.*

Beta-lactamase targeted	**Primer sequence (5'-3')**	**Primer length (bases)**	**Amplicon sizes (bp)**	**References**
***bla*$_{\text{CARB-19}}$ *V. parahaemolyticus* VPE116**	CARB-19_for TGCTTAACATCGCCAAAGTGC	21	975	This study
	CARB-19_rev ACGCATTTGAGAGAGCAGGT	20		
***bla*$_{\text{AmpC}}$ *V. anguillarum* 28AD**	AmpC_for TGATTAGCGTGTCCTATGGTGA	22	1373	This study
	AmpC_rev GTCAGTAAGGCCCCCTGTTT	20		

Primer design was performed using Primer-BLAST (NCBI) and Primer3Plus softwares.

PCR assays were carried out in a Thermal cycler T100 Bio-Rad (Bio-Rad, Hercules, CA, USA). Each reaction was performed in a final volume of 25 µl using 100 ng of DNA template, 0.3 µM of forward and reverse primers, 200 µM of each dNTPs, 2.0 mM of $MgCl_2$ and 1U Taq Polymerase (5U/µl; FirePol, Solis Biodyne, Tartu, Estonia) in 1X Buffer BD (Solis Biodyne, Tartu, Estonia).

The thermal conditions for the detection of CARB-19 and AmpC were 95°C for 4 min, followed by 30 cycles with denaturation at 95°C for 45 sec, annealing at 55°C or 60°C respectively for CARB-19 and AmpC for 30 sec, 72°C for 1 min with a final extension at 72°C for 7 min.

PCR products were then separated on a 1% agarose gel containing 0.5 µg/ml peqGREEN (PeqLAB, Erlangen, Germany) and photographed by Bio-Rad Gel Doc™ XR using Image Lab™ software (Bio-Rad, Hercules, CA, USA).

9.2.2 PCR product purification and cloning

For the cloning step, PCR products were purified using the Zymo DNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instruction reported in Annex II.

Cloning of $bla_{\text{CARB-19}}$ and bla_{AmpC} was carried out using Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Waltham, MA, USA). Zero Blunt® TOPO® PCR Cloning provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of blunt-end PCR products into a plasmid vector. Topoisomerase I from Vaccinia virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products. The plasmid vector (pCR™-Blunt II-TOPO®) is supplied linearized with Vaccinia virus DNA topoisomerase I covalently bound to the 3´ end of each DNA strand (referred to as "TOPO®-activated" vector). The TOPO® Cloning reaction can be transformed into chemically competent cells or electroporated directly into electrocompetent cells. In addition, pCR™-Blunt II-TOPO® allows direct selection of recombinants via disruption of the lethal *E. coli* gene, ccdB. The vector contains the ccdB gene fused to the C-terminus of the LacZα fragment (Figure 14). Ligation of a blunt end PCR product disrupts expression of the lacZα-ccdB gene fusion permitting growth of only positive recombinants upon transformation. Cells that contain nonrecombinant vector are killed upon plating. Therefore, blue/white screening is not required.

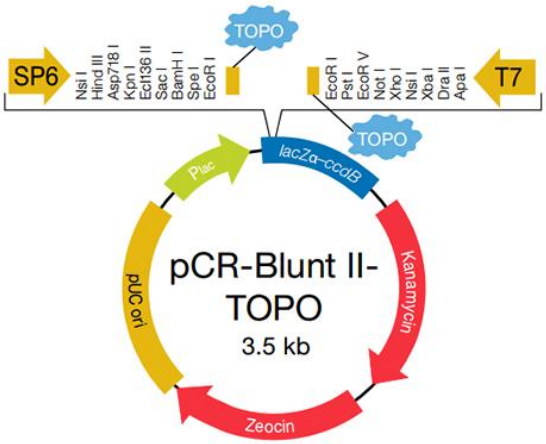

Figure 14. pCR-Blunt II-TOPO vector scheme.

9.2.3 TOPO Cloning reaction

Table 8. TOPO cloning procedure.

Reagent	Volume	This study
Fresh PCR product	0.5–4 µL	4 µL
Salt Solution	1 µL	1 µL
Water	add to a total volume of 5 µL	-
pCR™II-Blunt-TOPO®	1 µL	1 µL
Final Volume	6 µL	6 µL

Mix the reaction (Table 8) gently and incubate for 5 minutes at room temperature (22°C–23°C).

Then, place the reaction on ice and proceed to transform competent *E. coli* TOP10 cells.

9.2.4 Chemical transformation of E. coli TOP10

Competent *E. coli* TOP10 were chosen as recipient cells. They are both chemically competent and susceptible to the most antibiotics used in the further analysis. The protocol adopted is reported in the Annex II.

Antibiotic susceptibility testing was performed using disk diffusion agar method for all the beta-lactams panel (penicillins, cephalosporins, carbapenems and monobactam) both in *V. anguillarum* 28AD WT, *V. parahaemolytcus* VPE116 WT, empty *E. coli* TOP10 and transformed *E. coli* TOP10 expressing $bla_{\text{CARB-19}}$ and $bla_{\text{AmpC,}}$ respectively. The strains were plated on MHA medium at 37°C O/N. Furthermore, MIC for some penicillin, cephalosporin and carbapenem subclass representatives were calculated to confirm results obtained by disk diffusion agar method.

9.3 Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted using the disk diffusion method in Mueller Hinton (MH) agar plates (Bio-Rad, Cressier Switzerland) modifying the EUCAST guidelines as reported in the part I of this thesis. The antibiotics (Bio-Rad, Cressier, Switzerland) used in this study were amoxicillin (25 µg/ml), amoxicillin+clavulanic acid (30 µg/ml), ticarcillin (75 µg/ml), ticarcillin+clavulanic acid (85 µg/ml), piperacillin (75 µg/ml), piperacillin+tazobactam (85 µg/ml), ceftazidime (10 µg/ml), cefepime (30 µg/ml), temocillin (30 µg/ml), cefoxitin (30 µg/ml), cefotaxime (30 µg/ml), cefepime (30 µg/ml), cephalothin (30 µg/ml), aztreonam (30 µg/ml), imipenem (10 µg/ml), ertapenem (10 µg/ml), meropenem (10 µg/ml).

9.4 Whole genome sequencing

Genomic DNA was extracted using CTAB and the isopropanol precipitation method (Annex II). Then, it was sequenced on Illumina NextSeq® 500 platform (Illumina Inc., San Diego, CA, USA) with a paired-end strategy obtaining 2 x 150 bp reads length. Quality control and trimming of produced reads were carried out using FastQC, Scythe v0.991 and Sickle v1.33 softwares, respectively. Genome assembly was performed using SPAdes v3.10.1 assembler. Gene annotation and prediction were carried out using Prodigal v2.6, a tool provided by PROKKA v1.12 software (Seemann, 2014).

9.5 Multiple alignment of the predicted protein sequences involved in AR

The protein sequences involved in beta-lactam resistance (OXA-55, AmpC, CARB-19) and predicted in all the *S. algae* and *Vibrio* spp. genomes were aligned against a collection of representative environmental sequences present in Genbank database (NCBI). Clustal Omega tool (2017) provided by EMBL-EBI was used to find out amino acid substitutions, insertions or deletions and to detect possible sequence changes or similarities at interspecies level between the *Shewanellaceae* and *Vibrionaceae* family members under study. Furthermore, the genetic environment of the mentioned beta-lactamases has been investigated.

9.6 Phylogenetic analysis

A phylogenetic analysis of AmpC and OXA-55 protein sequences was carried out. Multiple alignments and phylogenetic trees were obtained using MEGA version 7.0.16 (Kumar *et al*., 2016) software. Tree methods and statistical models were setted according to the different protein or DNA sequence datasets used. Clinical and environmental reference class D beta-lactamases protein sequences belonging to OXA group and found in *Acinetobacter* spp and *S. algae* strains were retrieved from Genbank database (NCBI). The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method and Poisson model. The robustness of the topology was checked using 1000 bootstrap replications. The same software was used to draw a phylogenetic tree of CARB-19 detected in *V. parahaemolyticus* VPE116 strain and all the CARB variants present in several vibrio genomes found in Genbank database in order to study the evolutionary distances. For this purpose, a NJ method and JTT model were chosen. Regarding AmpC phylogenetic analysis, a database of AmpC protein sequences belonging to *Enterobacteriaceae*, *Shewanellaceae* and *Vibrionaceae* family members was created using sequences stored on Genbank database (NCBI). A NJ phylogenetic tree based on Poisson model was constructed and saved in Newick format by MEGA version 7.0.16. A 1000 bootstrap replication was set up to confirm the robustness of every single node. Subsequently, the tree was graphically elaborated using the online interactive Tree Of Life (iTOL) v4 showing the tree topology (http://itol.embl.de) (iTOL; Letunic and Bork (2016)). The phylogenetic results obtained using protein sequences were confirmed by nucleotide sequence analysis.

9.7 Core-genome alignment analysis of the S. algae genomes

A core-genome multi alignment and analysis were performed using all the core-genomes of the *S. algae* isolates as dataset. For this purpose, Harvest suite v1.1.2 and its tools Parsnp, a fast core-genome multi-aligner, and the Gingr visualization tool were used (Treangen *et al*., 2014). Sample 38LV was used as genome reference for the analysis and the NNI (Net Number of Inconsistencies) statistical model was considered. A Maximum Likelihood (ML) phylogenetic tree was produced and visualized by iTOL v4.

10. RESULTS

10.1 Strain identification

All the marine strains were identified at species level by WGS analysis blasting the predicted 16S rRNA gene. Out of 12 strains under study, 10 were identified as *S. algae* (219VB, 353M, 146bCP, 144bCP, 57CP, 38LV, 83CP, 178CP and 60CP), 1 as *V. anguillarum* (28AD) and 1 as *V. parahaemolyticus* (VPE116) as reported in Table 1.

10.2 Biochemical and molecular detection of beta-lactamases

The Blue Carba test resulted positive for every *S. algae* isolates. Carbapenemase activity was revealed when the test and the negative-control solutions, respectively, were green versus blue as for the *S. algae* strains whereas a doubtful result was obtained for *V. anguillarum* 28AD. The strain VPE116 resulted negative (blue versus blue). Moreover, the growth of all the strains was inhibited on ChromID ESBL medium indicating the susceptibility to third generation cephalosporins and the absence of ESBLs. All the isolates proved negative for multiplex PCR targeting clinical carbapenemases whereas *V. anguillarum* 28AD was positive for bla_{AmpC} (Figure 15) and *V. parahaemolyticus* VPE116 for $bla_{\text{CARB-19}}$ simplex PCRs (Figure 16).

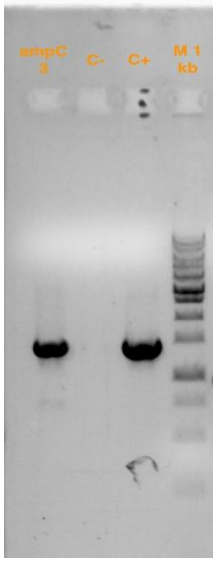

Figure 15. Agarose gel electrophoresis (1%) of AmpC amplicons (1373 bp). Sample 3 represent the results of AmpC cloning in *E.coli* TOP10 whereas the positive control is provided from *V. anguillarum* 28AD.

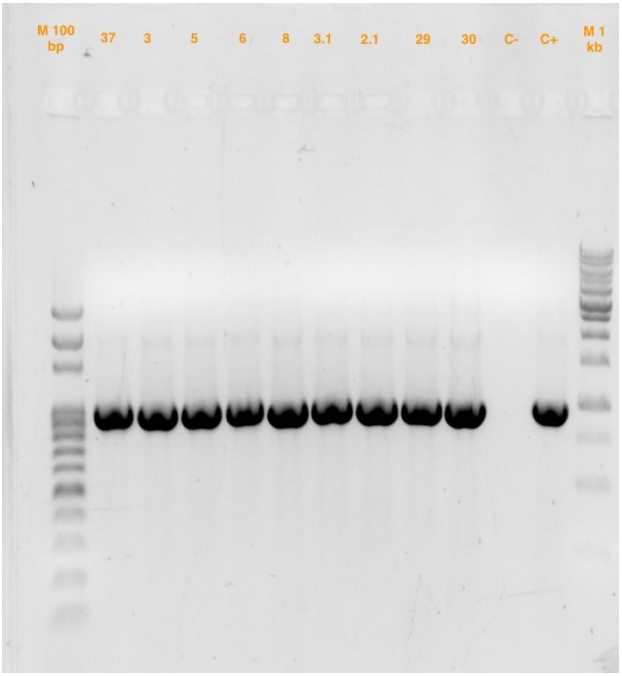

Figure 16. Agarose gel electrophoresis (1%) of CARB-19 amplicons (975 bp). Samples from 37 to 30 represent the results of CARB-19 cloning in *E.coli* TOP10 whereas the positive control is provided from *V. parahaemolyticus* VPE116.

10.3 Antibiotic susceptibility analysis

The antibiotic resistance profiles of *S. algae* and *Vibrio* spp. are shown in Table 1. The results were interpreted modifying the EUCAST guidelines as reported in the first part of this thesis. Most of the strains showed resistance to beta-lactams such as cephalothin, cefoxitin, imipenem and amoxicillin. Moreover, only 57CP and 82CP strains were susceptible to amoxicillin. Resistance to cephalothin, cefoxitin, amoxicillin, ticarcillin, amoxicillin+clavulanic acid, ticarcillin+clavulanic acid and temocillin were reported for 28AD isolate. Conversely, *V. parahaemolyticus* VPE116 was susceptible to beta-lactams, except for amoxicillin and ticarcillin (Table 1).

10.4 Genome features

Genome size, G+C content, total number of proteins and hypothetical proteins are summarized for each strain in Table 2 in the part I of this thesis.

10.5 Analysis of the predicted OXA-55 protein sequences

The class D beta-lactamase OXA-55 protein sequences found in all the *S. algae* isolates were aligned using the Clustal Omega tool (2017) provided by EMBL-EBI and compared to an environmental reference sequence (AAR03105.1) retrieved in Genbank database (NCBI) as reported in Figure 17. BLAST analysis evidenced a high percentage of identity to the amino acid sequences identified as OXA-55 family carbapenem-hydrolyzing enzymes of *S. algae* or *Shewanella* sp. 38A GOM-205m. In particular, 38LV, 82CP, 83CP, 144bCP, 146bCP, 178CP and 219VB shared a 99.65% identity to *S. algae* or *Shewanella* sp. OXA-55 protein sequences deposited in Genbank (WP_082052676.1 for 38LV; WP_025889747.1 for 82CP and 83CP; WP_028779467.1 for 144bCP, 146bCP, 178CP and 219VB). On the contrary, 353M and 60CP reported a 99.31% identity to WP_025889747.1 and WP_082052676.1 OXA-55 amino acid sequence, respectively. Only 57CP registered the lower percentage of identity (98.96%) to WP_117199933.1. On average, the OXA-55-like protein sequences evidenced a 95.5% identity to the reference sequence AAR03105.1. Concerning the primary structure analysis of these enzymes, the 289 amino acid sequences presented several point

mutations that do not involve the catalytic site and in particular the two conserved motifs STFK and KTG found at positions 94 to 97 and 232 to 234, respectively. The other typical motifs of oxacillinases, the YGN and SVV motifs, were also found. Point mutations were present in the 353M (T35A, E167D), 83CP (D39E, H116R, E167D), 57CP (G43E, R187K), 60CP (S76F), 82CP (E167D), 144bCP, 146bCP and 178CP (S269G, Q286L) strains. Most of these mutations have conserved the charge or the polarity of the amino acid side chain but in some cases they changed such as in T35A, C41G, S76F, S269G and Q286L in which the substituted amino acid become not polar whereas in G43E the substitution involved a negative charged amino acid. Moreover, all the strains presented a point mutation in positions 98 and 128 compared to the reference OXA-55 protein sequence with an isoleucine instead of a leucine and an alanine instead of a valine, respectively. Another non polar mutation was located in position 261 with the substitution of a valine present in the reference, 82CP and 83CP sequences and of an isoleucine in all the others. Regarding the genetic environment of this beta-lactamase, it is located in the chromosome and the transcription is mediated by a member of LysR family transcriptional regulator which is located downstream of the gene as confirmed by WGS.

```
                                                35  39;41;43
                                                |   | | |
AAR03105.1   MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVCSEVTAEGWQEVRRWDKLFE  60
82_CP        MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVGSEVTAEGWQEVRRWDKLFE  60
83_CP        MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSEVGSEVTAEGWQEVRRWDKLFE  60
57_CP        MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVSSGVTAEGWQEVRRWDKLFE  60
353_M        MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQAKVSDVGSEVTAEGWQEVRRWDKLFE  60
144B_CP      MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVGSEVTAEGWQEVRRWDKLFE  60
146B_CP      MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVGSEVTAEGWQEVRRWDKLFE  60
178_CP       MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVGSEVTAEGWQEVRRWDKLFE  60
219_VB       MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVGSEVTAEGWQEVRRWDKLFE  60
38_LV        MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVGSEVTAEGWQEVRRWDKLFE  60
60_CP        MNKGLHRKRLSKRLLLPMLLCLLAQQTQAVAAEQTKVSDVGSEVTAEGWQEVRRWDKLFE  60
             *********************************:***:* * *****************

                           76                  98                 116
                           |                   |                  |
AAR03105.1   SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKLPSSLIALETGAVRDETSRFSWD  120
82_CP        SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSHFSWD  120
83_CP        SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
57_CP        SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
353_M        SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
144B_CP      SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
146B_CP      SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
178_CP       SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
219_VB       SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
38_LV        SAGVKGSLLLWDQKRSLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
60_CP        SAGVKGSLLLWDQKRFLGLSNNLSRAAEGFIPASTFKIPSSLIALETGAVRDETSRFSWD  120
             ************** ********************:*****************:****
```

```
                 128                                     167
                  |                                       |
AAR03105.1  GKVREIAVWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
82_CP       GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLDYGNQDIGGQADSF  180
83_CP       GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLDYGNQDIGGQADSF  180
57_CP       GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
353_M       GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLDYGNQDIGGQADSF  180
144B_CP     GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
146B_CP     GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
178_CP      GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
219_VB      GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
38_LV       GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
60_CP       GKVREIAAWNRDQSFRTAMKYSVVPVYQQLAREIGPKVMAAMVRQLEYGNQDIGGQADSF  180
            *******.*************************************:*************
                  187
                   |
AAR03105.1  WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
82_CP       WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
83_CP       WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
57_CP       WLDGQLKITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
353_M       WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
144B_CP     WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
146B_CP     WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
178_CP      WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
219_VB      WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
38_LV       WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
60_CP       WLDGQLRITAFQQVDFLRQLHDNKLPVSERSQRIVKQMMLTEASTDYIIRAKTGYGVRRT  240
            ******:**************************************************
                              261     269             286
                               |       |               |
AAR03105.1  PAIGWWVGWLELDDNTVYFAVNLDLASASQLPLRQQLVKQVLKQEQLLP  289
82_CP       PAIGWWVGWLELDDNTVYFAVNLDLASASQLPLRQQLVKQVLKQEQLLP  289
83_CP       PAIGWWVGWLELDDNTVYFAVNLDLASASQLPLRQQLVKQVLKQEQLLP  289
57_CP       PAIGWWVGWLELDDNTVYFAINLDLASASQLPLRQQLVKQVLKQEQLLP  289
353_M       PAIGWWVGWLELDDNTVYFAINLDLASASQLPLRQQLVKQVLKQEQLLP  289
144B_CP     PAIGWWVGWLELDDNTVYFAINLDLASAGQLPLRQQLVKQVLKQELLLP  289
146B_CP     PAIGWWVGWLELDDNTVYFAINLDLASAGQLPLRQQLVKQVLKQELLLP  289
178_CP      PAIGWWVGWLELDDNTVYFAINLDLASAGQLPLRQQLVKQVLKQELLLP  289
219_VB      PAIGWWVGWLELDDNTVYFAINLDLASASQLPLRQQLVKQVLKQEQLLP  289
38_LV       PAIGWWVGWLELDDNTVYFAINLDLASASQLPLRQQLVKQVLKQEQLLP  289
60_CP       PAIGWWVGWLELDDNTVYFAINLDLASASQLPLRQQLVKQVLKQEQLLP  289
            ********************:*******.**************** ***
```

Figure 17. Comparison among the amino acid sequences of the carbapenem-hydrolyzing class D beta-lactamase OXA-55-like found in the *S. algae* isolates analyzed in this study and that of reference AAR03105.1. Point mutations are highlighted in red whereas numbers indicate their position within the sequence.

10.6 Analysis of the predicted AmpC protein sequences

Class C beta-lactamases were predicted in *S. algae* and *V. anguillarum* 28AD genomes. These beta-lactamases are able to confer resistance to cephalosporins and, with minor

efficacy, to amino- and carboxypenicillins. BLAST analysis revealed identity to *S. algae* or *Shewanella* spp. class C beta-lactamases for the AmpC found in our *S. algae* strains. In particular, 38LV, 82CP and 219VB shared a 100% identity to WP_101059234.1 protein sequence whereas 57CP and 60CP reported a 99.48% identity to WP_096142539.1. The strains 144bCP, 146bCP and 178CP were more similar to OHY57436.1 protein sequence registering an identity percentage of 98.96%. The 83CP isolate showed a 98.18% identity to WP_082052691.1 whereas 353M evidenced a 98.45% identity to WP_123115713.1. On the contrary, *V. anguillarum* 28AD strain showed an identity percentage of 99.74% to class C beta-lactamase *Vibrio* sp. V12 P9A6T4 (WP_094166136.1). A 10 *S. algae* AmpC protein sequence dataset was created as reported before and the sequences were aligned to all the predicted *S. algae* AmpC protein sequences (384-386 amino acids) under analysis to find some substitutions (Figure 18). In particular, most of the point mutations were found in the 83CP strain (M11V, V17A, I32M, S99C, V220M, K379R). Moreover, 144bCP, 146bCP, 178CP and 353M created a first group of strains presenting the same substitutions in the same positions (D/N26A, S108N, K115R, V119L) whereas a second group was made of 219VB, 38LV and 82CP presenting the point mutations G41D, E/R80Q, S103N, G154E, A245G. A third group was created by the 57CP and 60CP isolates containing the substitution from an alanine to a valine in position 101.

```
                                 11 14 17       26    32       41
                                 |  |  |        |     |        |
WP_093983723.1    MHGDRMINRVTKLLAMVLGFSVHFAMAETPDANIQSITDEVDQQAGMLMEKYHIPGMAIA 60
83_CP             -----MINRVTKLLAVVLGFSAHFAMAETPNANIQSMTDEVDQQAGMLMEKYHIPGMAIA 55
WP_037474920.1    MHGDRMINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIA 60
WP_082050130.1    MHGDRMINRVTKLLAVVLGFSVHFAMAETPNANIQSITDEVDQQAGMLMEKYHIPGMAIA 60
57_CP             -----MINRVTKLLAMVLEFSVHFAMAETPDANIQSITDEVDQQADMLMEKYHIPGMAIA 55
60_CP             -----MINRVTKLLAMVLGFSVHFAMAETPDANIQSITDEVDQQADMLMEKYHIPGMAIA 55
WP_096142539.1    MHGDRMINRVTKLLAMVLGFSVHFAMAETPDANIQSITDEVDQQADMLMEKYHIPGMAIA 60
PBQ25716.1        MHGDRMINRVTKLLAMVLGFSVHFAMAETPDANIQSITDEVDQQADMLMEKYHIPGMAIA 60
WP_082052691.1    MHGDRMINRVTKLLAMVLGFSVHFAMAETPNANIQSITDEVDQQAGMLMEKYHIPGMAIA 60
144B_CP           -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIA 55
146B_CP           -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIA 55
178_CP            -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIA 55
353_M             -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIA 55
WP_082813108.1    ---------------MVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIA 45
219_VB            -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQADMLMEKYHIPGMAIA 55
38_LV             -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQADMLMEKYHIPGMAIA 55
82_CP             -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQADMLMEKYHIPGMAIA 55
WP_101059234.1    -----MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQADMLMEKYHIPGMAIA 55
WP_037467572.1    MHGDRMINRVTKLLAMVLGFSVHFAMAETPNANIQSITDEVDQQAGMLMEKYHIPGMAIA 60
WP_083390194.1    MHGDRMINRVTKLLTMVLGFSVHFAMAETPNANIQSITDEVDQQAGMLMEKYHIPGMAIA 60
                                 :** **.******** *****:********.**************
```

```
                                    75   80                99;101;103;106;108;115
                                    |    |                 | | |  | |      |
WP_093983723.1   ISIGGEQHFYHYGMADVNAGIKVSEHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAK 120
83_CP            ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGCYAQSLGTFNLEDNAAK 115
WP_037474920.1   ISIGGEQHFYHYGMADVNADIKVSRHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAK 120
WP_082050130.1   ISIGGEQHFYHYGMADVNASIKVSEHTLFELGSISKTFTAILGSYAQSLGAFSLEDNAAK 120
57_CP            ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYVQSLGAFSLEDNAAK 115
60_CP            ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYVQSLGAFSLEDNAAK 115
WP_096142539.1   ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYVQSLGAFSLEDNAAK 120
PBQ25716.1       ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYVQSLGAFSLEDNAAK 120
WP_082052691.1   ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGCYAQSLGTFNLEDNAAK 120
144B_CP          ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAAR 115
146B_CP          ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAAR 115
178_CP           ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAAR 115
353_M            ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAAR 115
WP_082813108.1   ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAAR 105
219_VB           ISIGGEQHFYHYGMADVNAGIKVSQHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAK 115
38_LV            ISIGGEQHFYHYGMADVNAGIKVSQHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAK 115
82_CP            ISIGGEQHFYHYGMADVNAGIKVSQHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAK 115
WP_101059234.1   ISIGGEQHFYHYGMADVNAGIKVSQHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAK 115
WP_037467572.1   ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAK 120
WP_083390194.1   ISIGGEQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAAR 120
                 ******************.****.******************.*.*.**:*.******:
                    119                                154
                    |                                  |
WP_093983723.1   YVKLWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTQRLYS 180
83_CP            YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 175
WP_037474920.1   YVKVWEGSPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 180
WP_082050130.1   YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 180
57_CP            YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 175
60_CP            YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 175
WP_096142539.1   YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 180
PBQ25716.1       YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 180
WP_082052691.1   YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 180
144B_CP          YVKLWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 175
146B_CP          YVKLWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 175
178_CP           YVKLWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 175
353_M            YVKLWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 175
WP_082813108.1   YVKLWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 165
219_VB           YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDEMLAYYKAWQPEFAAGTHRLYS 175
38_LV            YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDEMLAYYKAWQPEFAAGTHRLYS 175
82_CP            YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDEMLAYYKAWQPEFAAGTHRLYS 175
WP_101059234.1   YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDEMLAYYKAWQPEFAAGTHRLYS 175
WP_037467572.1   YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 180
WP_083390194.1   YVKVWEGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYS 180
                 ***:***:****************************** ****************:****
                                                       220
                                                       |
WP_093983723.1   NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
83_CP            NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRMAQYAFGYNAKNEPVR 235
WP_037474920.1   NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
WP_082050130.1   NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
57_CP            NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
60_CP            NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
WP_096142539.1   NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
PBQ25716.1       NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
WP_082052691.1   NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
144B_CP          NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
146B_CP          NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
178_CP           NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
353_M            NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
WP_082813108.1   NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 225
219_VB           NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
38_LV            NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
```

```
82_CP             NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
WP_101059234.1    NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 235
WP_037467572.1    NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
WP_083390194.1    NPSIGLYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVR 240
                  ****************************************:***************
                           245     253
                           |       |
WP_093983723.1    VSTGMLDAEAYGVKSTSVDMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 300
83_CP             VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
WP_037474920.1    VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQVAAAIKETHKGYYDTASFTQALGW 300
WP_082050130.1    VSPGMLDAEAYGVKSTSVDMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 300
57_CP             VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
60_CP             VSPGMLDAEAYGVKSTSVDMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
WP_096142539.1    VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 300
PBQ25716.1        VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 300
WP_082052691.1    VSPGMLDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 300
144B_CP           VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
146B_CP           VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
178_CP            VSPGMLDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
353_M             VSPGMLDAEAYGVKSTSVDMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
WP_082813108.1    VSPGMLDAEAYGVKSTSVDMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 285
219_VB            VSPGMLDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
38_LV             VSPGMLDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
82_CP             VSPGMLDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
WP_101059234.1    VSPGMLDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 295
WP_037467572.1    VSPGMLDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 300
WP_083390194.1    VSPGMLDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGW 300
                  ** ******.*******.******************:************************

WP_093983723.1    EIYECPLTLSKLLEGNSREIASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
83_CP             EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
WP_037474920.1    EIYEYPLTLSKLLEGNSREMILQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
WP_082050130.1    EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
57_CP             EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
60_CP             EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
WP_096142539.1    EIYEYPLTLSKLLEGNSREIASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
PBQ25716.1        EIYEYPLTLSKLLEGNSREIASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
WP_082052691.1    EIYEYPLTLSQLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
144B_CP           EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
146B_CP           EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
178_CP            EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
353_M             EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
WP_082813108.1    EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 345
219_VB            EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
38_LV             EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
82_CP             EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
WP_101059234.1    EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 355
WP_037467572.1    EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
WP_083390194.1    EIYEYPLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPA 360
                  **** *****:********:  ************************************
                                      379
                                      |
WP_093983723.1    KKLAIVMLANKSYPISARVEAAYKILAAAQK  391
83_CP             KKLAIVMLANKSYPISARVEAAYRILAAA--  384
WP_037474920.1    KKLAIVMLANKSYPISARVEAAYKILAAAQE  391
WP_082050130.1    KKLAIVMLANKNYPISARVEAAYKILAAAQE  391
57_CP             KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
60_CP             KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
WP_096142539.1    KKLAIVMLANKSYPISARVEAAYKILAAAQE  391
PBQ25716.1        KKLAIVMLANKSYPISARVEAAYKILAAAQE  391
WP_082052691.1    KKLAIVMLANKSYPISARVEAAYKILAAAQE  391
144B_CP           KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
146B_CP           KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
178_CP            KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
```

```
353_M              KKLAIVMLANKSYPISARVEAAYRILAAAQE  386
WP_082813108.1     KKLAIVMLANKSYPISARVEAAYRILAAAQE  376
219_VB             KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
38_LV              KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
82_CP              KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
WP_101059234.1     KKLAIVMLANKSYPISARVEAAYKILAAAQE  386
WP_037467572.1     KKLAIVMLANKSYPISARVEAAYKILAAAQE  391
WP_083390194.1     KKLAIVMLANKSYPISARVEAAYKILAAAQE  391
                   ***********.***********:*****
```

Figure 18. Multiple alignment of the *S. algae* AmpC sequences compared to a dataset of AmpC reference sequences retrieved in Genbank database. Point mutations are reported in red whereas the numbers indicate the positions in which they are found within the protein sequence.

The 385 amino acid AmpC sequence detected in *V. anguillarum* 28AD was aligned to a small dataset made of 12 AmpC protein sequences found in other environmental *V. anguillarum* or *Vibrio* spp. strains deposited in Genbank database and used for further phylogenetic analysis (Figure 19). Multiple alignment was performed using Clustal Omega tool (2017) provided by EMBL-EBI and denoted very few mutations. In particular, point mutations did not affect the active site and the main motifs FELGS and SKTF which are involved in the beta-lactam hydrolytic activity.

```
                         10
                         |
AmpC_28_AD         MCVRRLTINAIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
OXX51557.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
WP_094166136.1     MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
OXX41694.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
OXX63112.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
OXX69469.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
WP_094133832.1     MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
ASW82655.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
AQM18085.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
WP_029388315.1     MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
ASO30330.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
OQQ11210.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
AQM14661.1         MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
WP_081245207.1     MCVRRLTINTIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEGKT  60
                   *********:**************************************************
                                                96
                                                |
AmpC_28_AD         YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLTSYAQVKGQLSLTDTAEQHMPELKG 120
OXX51557.1         YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLTSYAQVKGQLSLTDTAEQHMPELKG 120
WP_094166136.1     YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLTSYAQVKGQLSLTDTAEQHMPELKG 120
OXX41694.1         YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
OXX63112.1         YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
OXX69469.1         YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
WP_094133832.1     YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
ASW82655.1         YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
AQM18085.1         YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
WP_029388315.1     YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
```

```
ASO30330.1        YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
OQQ11210.1        YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
AQM14661.1        YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
WP_081245207.1    YFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLASYAQVKGQLSLTDTAEQHMPELKG 120
                  ************************************:************************

                                                     161
                                                     |
AmpC_28_AD        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
OXX51557.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
WP_094166136.1    STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
OXX41694.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWNEVYPSNTKRVYSNPSIGLF 180
OXX63112.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWNEVYPSNTKRVYSNPSIGLF 180
OXX69469.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWNEVYPSNTKRVYSNPSIGLF 180
WP_094133832.1    STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWNEVYPSNTKRVYSNPSIGLF 180
ASW82655.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
AQM18085.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
WP_029388315.1    STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
ASO30330.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
OQQ11210.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
AQM14661.1        STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
WP_081245207.1    STIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIGLF 180
                  ****************************************:*******************

AmpC_28_AD        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
OXX51557.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
WP_094166136.1    GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
OXX41694.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
OXX63112.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
OXX69469.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
WP_094133832.1    GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
ASW82655.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
AQM18085.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
WP_029388315.1    GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
ASO30330.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
OQQ11210.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
AQM14661.1        GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
WP_081245207.1    GYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGVLD 240
                  ************************************************************

AmpC_28_AD        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
OXX51557.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
WP_094166136.1    AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
OXX41694.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
OXX63112.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
OXX69469.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
WP_094133832.1    AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
ASW82655.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
AQM18085.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
WP_029388315.1    AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
ASO30330.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
OQQ11210.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
AQM14661.1        AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
WP_081245207.1    AEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPYPS 300
                  ************************************************************

                                                     342
                                                     |
AmpC_28_AD        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
OXX51557.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
WP_094166136.1    QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
OXX41694.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
OXX63112.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
OXX69469.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
WP_094133832.1    QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
```

```
ASW82655.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTSGFGAYVVYIPSKESGIVI 360
AQM18085.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTSGFGAYVVYIPSKESGIVI 360
WP_029388315.1    QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTSGFGAYVVYIPSKESGIVI 360
ASO30330.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
OQQ11210.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
AQM14661.1        QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
WP_081245207.1    QLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGIVI 360
                  ****************************************.******************

AmpC_28_AD        LANKNYPNQERVKAAFRILQAGLEQ   385
OXX51557.1        LANKNYPNQERVKAAFRILQAGLEQ   385
WP_094166136.1    LANKNYPNQERVKAAFRILQAGLEQ   385
OXX41694.1        LANKNYPNQERVKAAFRILQAGLEQ   385
OXX63112.1        LANKNYPNQERVKAAFRILQAGLEQ   385
OXX69469.1        LANKNYPNQERVKAAFRILQAGLEQ   385
WP_094133832.1    LANKNYPNQERVKAAFRILQAGLEQ   385
ASW82655.1        LANKNYPNQERVKAAFRILQAGLEQ   385
AQM18085.1        LANKNYPNQERVKAAFRILQAGLEQ   385
WP_029388315.1    LANKNYPNQERVKAAFRILQAGLEQ   385
ASO30330.1        LANKNYPNQERVKAAFRILQAGLEQ   385
OQQ11210.1        LANKNYPNQERVKAAFRILQAGLEQ   385
                  *************************
```

Figure 19. Multiple alignment of the *V. anguillarum* 28 AD AmpC sequences compared to a dataset of *V. anguillarum* and *Vibrio* spp. reference AmpC sequences retrieved in Genbank database. Point mutations are reported in red whereas the numbers indicate the positions in which they are found within the protein sequence.

A multiple alignment of all the predicted AmpC protein sequences found in *V. anguillarum* 28AD and *S. algae* isolates was performed as reported in Figure 20. The amino acid similarity among *S. algae* and *V. anguillarum* 28AD AmpC was 47.3%. Although some amino acid blocks are conserved, suggesting a role in the beta-lactamase activity (catalytic site) and protein folding, a discrete variability is described between the AmpC protein sequences originated from the two genera under study.

```
AmpC_28_AD    MCVR--RLTINAIALAMLFAPNVRAAGVGEKLKNEVDKQAQALMQNYHIPGMAFGVVVEG  58
83_CP         MINRVTKLLAVVLGFSAHFAMAETPNANIQSMTDEVDQQAGMLMEKYHIPGMAIAISIGG  60
219_VB        MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQADMLMEKYHIPGMAIAISIGG  60
38_LV         MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQADMLMEKYHIPGMAIAISIGG  60
82_CP         MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQADMLMEKYHIPGMAIAISIGG  60
144B_CP       MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIAISIGG  60
146B_CP       MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIAISIGG  60
178_CP        MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIAISIGG  60
353_M         MINRVTKLLAMVLGFSVHFAMAETPAANIQSITDEVDQQAGMLMEKYHIPGMAIAISIGG  60
57_CP         MINRVTKLLAMVLEFSVHFAMAETPDANIQSITDEVDQQADMLMEKYHIPGMAIAISIGG  60
60_CP         MINRVTKLLAMVLGFSVHFAMAETPDANIQSITDEVDQQADMLMEKYHIPGMAIAISIGG  60
              *  *  :*   .: ::  **      .  :.:.:***:**  **::********:.: : *

AmpC_28_AD    KTYFYNYGVADTAQNQPVSEATIFELGSVSKTFAATLTSYAQVKGQLSLTDTAEQHMPEL 118
83_CP         EQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGCYAQSLGTFNLEDNAAKYVKVW 120
219_VB        EQHFYHYGMADVNAGIKVSQHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAKYVKVW 120
38_LV         EQHFYHYGMADVNAGIKVSQHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAKYVKVW 120
```

```
82_CP          EQHFYHYGMADVNAGIKVSQHTLFELGSISKTFTAILGSYAQNLGAFSLEDNAAKYVKVW 120
144B_CP        EQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAARYVKLW 120
146B_CP        EQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAARYVKLW 120
178_CP         EQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAARYVKLW 120
353_M          EQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYAQSLGAFNLEDNAARYVKLW 120
57_CP          EQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYVQSLGAFSLEDNAAKYVKVW 120
60_CP          EQHFYHYGMADVNAGIKVSRHTLFELGSISKTFTAILGSYVQSLGAFSLEDNAAKYVKVW 120
               : :**:**:**.  .  **. *:*****:****:* * .*.*  * :.* *.* :::

AmpC_28_AD     KGSTIGARTLQELGTYIAGGLPLQFPDTVKNNQDMVQYYRNWDEVYPSNTKRVYSNPSIG 178
83_CP          EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYSNPSIG 180
219_VB         EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDEMLAYYKAWQPEFAAGTHRLYSNPSIG 180
38_LV          EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDEMLAYYKAWQPEFAAGTHRLYSNPSIG 180
82_CP          EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDEMLAYYKAWQPEFAAGTHRLYSNPSIG 180
144B_CP        EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYSNPSIG 180
146B_CP        EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYSNPSIG 180
178_CP         EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYSNPSIG 180
353_M          EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYSNPSIG 180
57_CP          EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYSNPSIG 180
60_CP          EGTPIGNTKLINLATYTAGGLPLQFPNEVTTNDGMLAYYKAWQPEFAAGTHRLYSNPSIG 180
               :*: **  .* :*.** *********: *..*: *: **: *:  : :.*:*:*******

AmpC_28_AD     LFGYIAALSLKEDYVSAMEKNVFPALSMSNTYIHVPDNKMKEYAFGYNANGEAVRVNPGV 238
83_CP          LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRMAQYAFGYNAKNEPVRVSPGM 240
219_VB         LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
38_LV          LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
82_CP          LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
144B_CP        LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
146B_CP        LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
178_CP         LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
353_M          LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
57_CP          LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
60_CP          LYGYLAALSMKREFSELMEQTVLPQLAMNNTFVKVPDNRVAQYAFGYNAKNEPVRVSPGM 240
               *:**:****:*.:: . **:.*:* *:*.**:::****:: :*******:.* ***.**:

AmpC_28_AD     LDAEAYGVKSTSSDMVQYIKANLGLAVVATDMQQALVNNRTGFYQSSTFMQGLAWEMYPY 298
83_CP          LDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
219_VB         LDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
38_LV          LDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
82_CP          LDAEGYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
144B_CP        LDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
146B_CP        LDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
178_CP         LDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
353_M          LDAEAYGVKSTSVDMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
57_CP          LDAEAYGVKSTSADMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
60_CP          LDAEAYGVKSTSVDMLHYLEANMGLKPLSPQIAAAIKETHKGYYDTASFTQALGWEIYEY 300
               ****.******* **::*::**:**  :: ::  *: :.:.*:*:::::* *.*.**:* *

AmpC_28_AD     PSQLSALLEGNSTDVIIKPQTIQINTPPTPPTNGVWVNKTGSTGGFGAYVVYIPSKESGI 358
83_CP          PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
219_VB         PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
38_LV          PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
82_CP          PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
144B_CP        PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
146B_CP        PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
178_CP         PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
353_M          PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
57_CP          PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
60_CP          PLTLSKLLEGNSREMASQPTKVKASDDIVEKTGEVWINKTGSTGGFGAYVAFIPAKKLAI 360
               *  ** ****** ::  :* .:: .   .  *. **:**************.:**:*: .*

AmpC_28_AD     VILANKNYPNQERVKAAFRILQAGLEQ  385
83_CP          VMLANKSYPISARVEAAYRILAAA---  384
219_VB         VMLANKSYPISARVEAAYKILAAAQE-  386
```

```
38_LV          VMLANKSYPISARVEAAYKILAAAQE-  386
82_CP          VMLANKSYPISARVEAAYKILAAAQE-  386
144B_CP        VMLANKSYPISARVEAAYKILAAAQE-  386
146B_CP        VMLANKSYPISARVEAAYKILAAAQE-  386
178_CP         VMLANKSYPISARVEAAYKILAAAQE-  386
353_M          VMLANKSYPISARVEAAYRILAAAQE-  386
57_CP          VMLANKSYPISARVEAAYKILAAAQE-  386
60_CP          VMLANKSYPISARVEAAYKILAAAQE-  386
               *:****.** . **:**::** *.
```

Figure 20. Multiple alignment of all the predicted AmpC protein sequences found in *V. anguillarum* 28AD and *S. algae* strains analyzed in this study. Domains involved in catalytic site and tertiary structure of the enzyme are highlighted in yellow whereas in red are reported the point mutations.

This chromosome-encoded beta-lactamase has resulted to be located near *cat* (chloramphenicol acetyltransferase), HTH-type transcriptional regulator DmlR, *czcA* (cobalt-zinc-cadmium resistance protein), *OprM* (Outer membrane protein OprM) and *pqiAB* (paraquat-inducible protein AB) genes for our *S. algae* isolates. Its induction is mediated by beta-lactams and regulated by AmpD, AmpE, AmpR, a LysR-type regulator and AmpG. Concerning the bla_{AmpC} of *V. anguillarum* 28AD, it is regulated by the same system described above but the *cat* gene is not reported in this case. Finally, the presence of AmpC and class C beta-lactamases in *V. anguillarum* genomes retrieved in Genbank has been investigated. These beta-lactamases were found in 12 out of 72 (16.7%) complete genomes, complete chromosomes, scaffolds and contigs analyzed. In addition, some other *V. anguillarum* isolates (10/72; 13.9%) harbor in their genomes a metallo beta-lactamase not found in our strains.

10.7 Analysis of the predicted CARB-19 nucleotide and amino acid sequences

A 283 amino acid sequence predicted as CARB-19 was detected in *V. parahaemolyticus* VPE116 genome. This class A beta-lactamase confers resistance to penicillins, in particular to amino- and carboxypenicillins. BLAST analysis revealed a 100% identity to carbenicillin-hydrolyzing class A beta-lactamase CARB-19 found in *V. parahaemolyticus* (WP_063857870.1). The nucleotide sequences alignment revealed the presence of several point mutations (Figure 21).

```
NG_048721.1    ATGAAAAAGTTATTCCTGTTGGCTGGGCTGATGGTTTGCTCAACTGTTAGTTACGCCTCC  60
CARB-19        ATGAAAAAGTTATTCCTGTTGGCTGGGCTGATGGTTTGCTCAACTGTTAGTTACGCCTCC  60
               ************************************************************

NG_048721.1    AAATTAAACGAAGACATATCCCTCATCGAGAAACAAACATCTGGGCGAATTGGAGTGTCA  120
CARB-19        AAATTAAACGAAGACATATCCCTCATCGAGAAACAAACATCTGGGCGAATTGGAGTGTCA  120
               ************************************************************

NG_048721.1    GTCTGGGATACACAAACGGACGAGCGTTGGGATTATCGCGGAGACGAACGTTTCCCATTA  180
CARB-19        GTCTGGGATACACAAACGGACGAGCGTTGGGATTATCGCGGAGACGAACGTTTCCCATTA  180
               ************************************************************
                                                        225
                                                        |
NG_048721.1    ATGAGCACATTCAAAACGTTAGCGTGTGCCACCATGCTAAGCGATATGGACAGCGGCAAA  240
CARB-19        ATGAGCACATTCAAAACGTTAGCGTGTGCCACCATGCTAAGCGACATGGACAGCGGCAAA  240
               ****************************************** ***************
                             255
                             |
NG_048721.1    CTCAACAAAAATGCTACAGCGAAAATCGATGAACGCAATATTGTGGTTTGGTCTCCGGTG  300
CARB-19        CTCAACAAAAATGCCACAGCGAAAATCGATGAACGCAATATTGTGGTTTGGTCTCCGGTG  300
               ************** ********************************************
                                                      348
                                                      |
NG_048721.1    ATGGATAAACTGGCTGGACAAAGCACACGTATCGAACACGCTTGTGAGGCCGCCATGTTG  360
CARB-19        ATGGATAAACTGGCTGGACAAAGCACACGTATCGAACACGCTTGTGAAGCCGCCATGTTG  360
               ********************************************* ************

NG_048721.1    ATGAGCGACAACACCGCCGCGAACTTAGTGCTAAATGAAATTGGTGGTCCTAAAGCGGTC  420
CARB-19        ATGAGCGACAACACCGCCGCGAACTTAGTGCTAAATGAAATTGGTGGTCCTAAAGCGGTC  420
               ************************************************************
                 423         435                                     474
                 |           |                                       |
NG_048721.1    ACGCTGTTTTTGCGATCTATTGGCGACAAAGCAACGCGACTTGACCGATTGGAACCCCGT  480
CARB-19        ACACTGTTTTTGCGCTCTATTGGCGACAAAGCAACGCGACTTGACCGATTGGAGCCCCGT  480
               ** *********** ************************************* ******
                                    504
                                    |
NG_048721.1    TTGAATGAAGCAAAACCGGGCGATAAGCGAGACACCACAACGCCTAACGCCATGGTAAAC  540
CARB-19        TTGAATGAAGCAAAACCGGGCGACAAGCGAGACACCACAACGCCTAACGCCATGGTAAAC  540
               ********************** ***********************************
                       549
                       |
NG_048721.1    ACCCTACATACCTTGATGGAAGATAACGCCCTATCTTACGAGTCACGCACACAGCTGAAA  600
CARB-19        ACCCTACACACCTTGATGGAAGATAACGCCCTATCTTACGAGTCACGCACACAGCTGAAA  600
               ******** ***************************************************
                             615               633         645   651
                             |                 |           |     |
NG_048721.1    ATCTGGATGCAAGATAACAAAGTATCGGATTCTCTCATGCGCTCCGTTCTACCAAAAGGC  660
CARB-19        ATCTGGATGCAAGACAACAAAGTATCGGATTCGCTCATGCGCTCTGTTCTGCCAAAAGGC  660
               ************** ***************** *********** ***** *********

NG_048721.1    TGGTCGATTGCAGACCGCTCTGGCGCAGGTAACTACGGTTCACGCGGCATTAGCGCGATG  720
CARB-19        TGGTCGATTGCAGACCGCTCTGGCGCAGGTAACTACGGTTCACGCGGCATTAGCGCGATG  720
               ************************************************************

NG_048721.1    ATCTGGAAAGACAACTACAAGCCGGTTTACATCAGTATTTACGTCACAGACACCGACCTT  780
CARB-19        ATCTGGAAAGACAACTACAAGCCGGTTTACATCAGTATTTACGTCACAGACACCGACCTT  780
               ************************************************************

NG_048721.1    TCGCTTCAAGCTCGCGATCAACTGATCGCGCAAATCAGCCAACTGATTTTAGAGCACTAC  840
CARB-19        TCGCTTCAAGCTCGCGATCAACTGATCGCGCAAATCAGCCAACTGATTTTAGAGCACTAC  840
               ************************************************************
```

```
NG_048721.1     AAAGAAAGTTAG  852
CARB-19         AAAGAAAGTTAA  852
                ***********
```

Figure 21. Nucleotide sequence comparisons of *bla*$_{\text{CARB-19}}$ gene found in *V. parahaemolyticus* VPE 116 and the corresponding reference sequence (NG-048721.1) present in Genbank database. Silent mutations are indicated in red.

They are described along the gene sequence but they do not change the primary structure of the carbenicillinase (Figure 22).

```
WP_063857870.1  MKKLFLLAGLMVCSTVSYASKLNEDISLIEKQTSGRIGVSVWDTQTDERWDYRGDERFPL  60
CARB-19         MKKLFLLAGLMVCSTVSYASKLNEDISLIEKQTSGRIGVSVWDTQTDERWDYRGDERFPL  60
                ************************************************************

WP_063857870.1  MSTFKTLACATMLSDMDSGKLNKNATAKIDERNIVVWSPVMDKLAGQSTRIEHACEAAML  120
CARB-19         MSTFKTLACATMLSDMDSGKLNKNATAKIDERNIVVWSPVMDKLAGQSTRIEHACEAAML  120
                ************************************************************

WP_063857870.1  MSDNTAANLVLNEIGGPKAVTLFLRSIGDKATRLDRLEPRLNEAKPGDKRDTTTPNAMVN  180
CARB-19         MSDNTAANLVLNEIGGPKAVTLFLRSIGDKATRLDRLEPRLNEAKPGDKRDTTTPNAMVN  180
                ************************************************************

WP_063857870.1  TLHTLMEDNALSYESRTQLKIWMQDNKVSDSLMRSVLPKGWSIADRSGAGNYGSRGISAM  240
CARB-19         TLHTLMEDNALSYESRTQLKIWMQDNKVSDSLMRSVLPKGWSIADRSGAGNYGSRGISAM  240
                ************************************************************

WP_063857870.1  IWKDNYKPVYISIYVTDTDLSLQARDQLIAQISQLILEHYKES    283
CARB-19         IWKDNYKPVYISIYVTDTDLSLQARDQLIAQISQLILEHYKES    283
                ******************************************
```

Figure 22. Multiple alignment of the predicted CARB-19 protein sequence found in *V. parahaemolyticus* VPE 116 genome evidencing the perfect match with the corresponding reference sequence retrieved in Genbank (NCBI) database.

Moreover, the *bla*$_{\text{CARB-19}}$ gene, found in our strain, is located near *cusAB* genes which are involved in silver, copper and fosfomycin resistance. The presence of class A beta-lactamases and in particular of carbenicillinases is reported in *V. parahaemolyticus*. However, other CARB variants are described in the sequenced genomes present in Genbank (CARB-18, CARB-20, CARB-22 and CARB-30), in addition to other serine beta-lactamases and metallo beta-lactamases.

10.8 Phylogenetic analysis of the clinical and environmental class D beta-lactamase protein sequence variants

A NJ phylogenetic tree based on the clinical and environmental 69 protein sequences of *Acinetobacter* spp. and *Shewanella* spp. derived OXA variants retrieved in Genbank database (NCBI) was constructed (Figure 23).

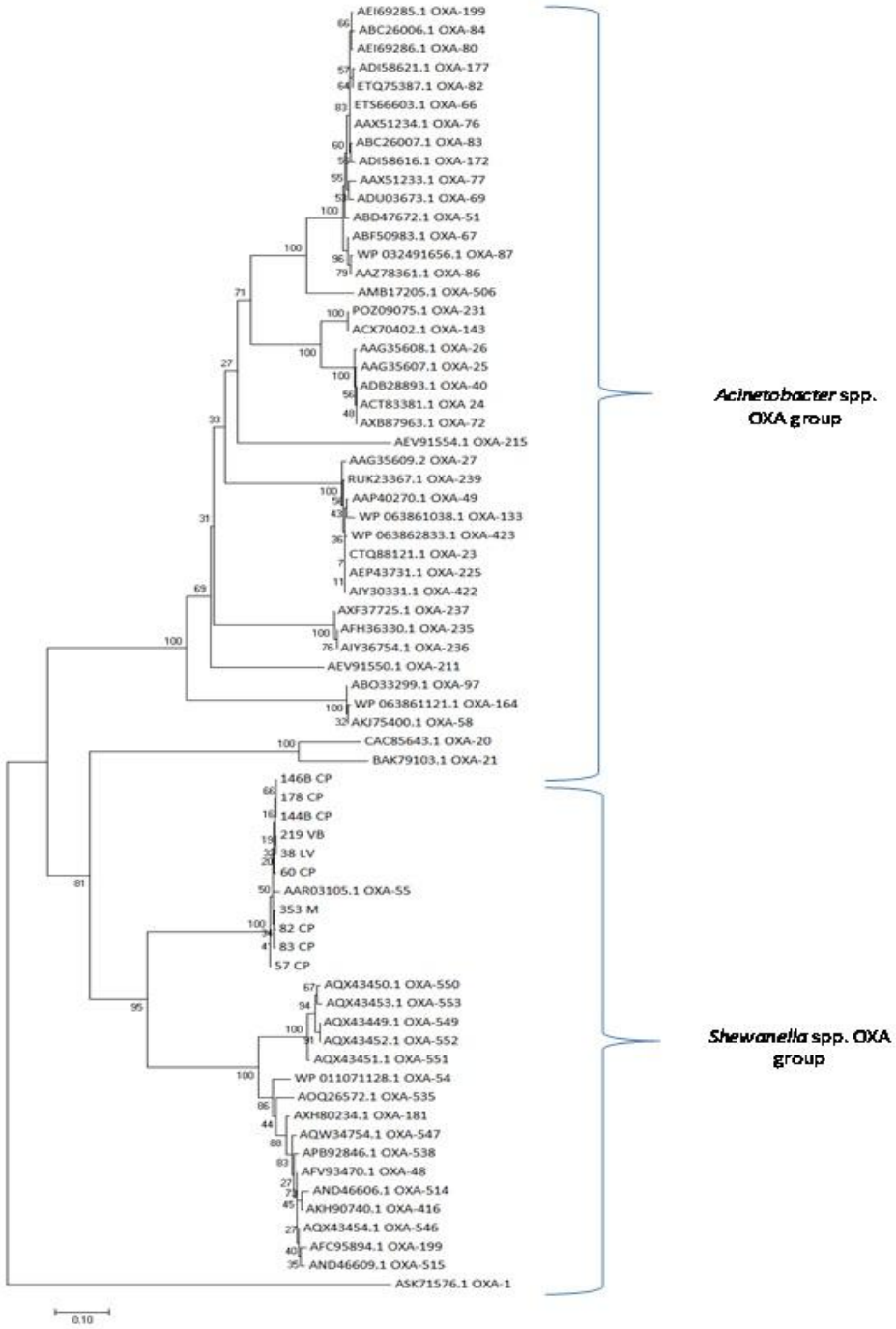

Figure 23. Neighbor-Joining phylogenetic tree based on the OXA-55-like amino acid sequences found in *S. algae* strains under study. A comparison of the known OXA variants present in Genbank database and detected in *Acinetobacter* and *Shewanella* genera was performed. Robustness of the analysis was checked using a 1000 bootstrap replication test whereas the distances were computed using the Poisson correction method.

The phylogenetic tree was inferred from the OXA-55 protein sequences of *S. algae* genomes obtained in this study. Comparative analyses of the OXA groups allowed to separate them in two main groups of variants. The first one includes the clinical and environmental variants present in the *Acinetobacter* spp. genomes whereas the second one can locate the environmental variants mainly present in the genomes of *Shewanella* genus. Within these two groups, OXA variants can generate several clusters based on the similarity of the protein sequences. Interestingly, in the *Acinetobacter* spp. OXA group, OXA-20 and OXA-21 constitute a subgroup that are more related to *Shewanella* spp. OXA variants respect to the *Acinetobacter* variants. These results are confirmed by the phylogenetic analysis performed using the nucleotide sequences as well (Figure 24).

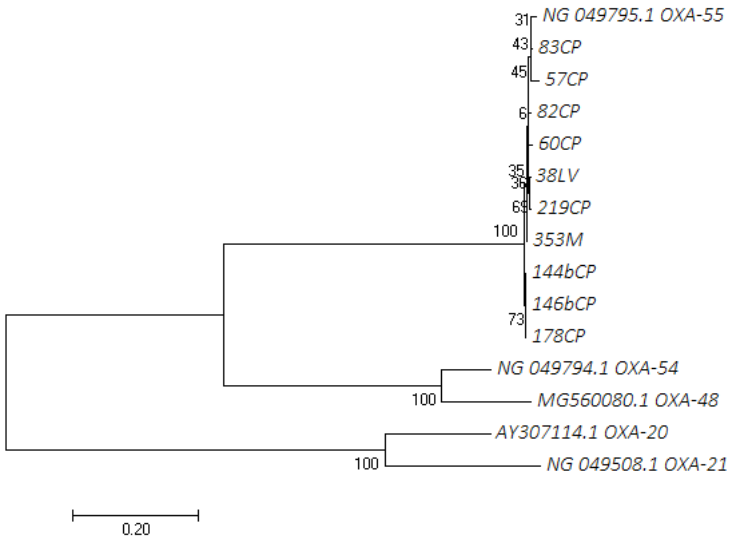

Figure 24. Phylogenetic analysis involving 15 class D beta-lactamase (OXA) nucleotide sequences. The evolutionary history was inferred using the Neighbor-Joining method using 1000 bootstrap replicates while distances were computed using the Kimura 2-parameter method.

Regarding the *Shewanella* spp. OXA group, it harbors 4 clusters. The first one locates the OXA-55 cluster in which the OXA-55 predicted protein sequences of the *S. algae* analyzed in this study are included. The intragroup heterogeneity suggests that the single amino acid

substitutions present in the protein sequences of the isolates are able to differentiate them in OXA-55-like if compared to OXA-55 reference used in the analysis.

10.9 Phylogenetic analysis of the clinical and environmental class C beta-lactamase protein sequence variants

A 165 amino acid sequences NJ tree was constructed using the predicted AmpC protein sequences found in the *Shewanella* spp. and *V. anguillarum* 28AD genomes in order to study the level of amino acid relatedness and divergence among AmpC isolated in members of the same genus and in clinical strains belonging to *Enterobacteriaceae* family. Figure 25 shows the tree topology obtained. The graphical view of the tree evidenced many clusters belonging to several groups. *S. algae* and *V. anguillarum* 28AD are included in a group which is composed of 3 clusters: other *Vibrio* species, *S. algae*/*Shewanella* spp. and *V. anguillarum*/*Vibrio* spp. clusters.

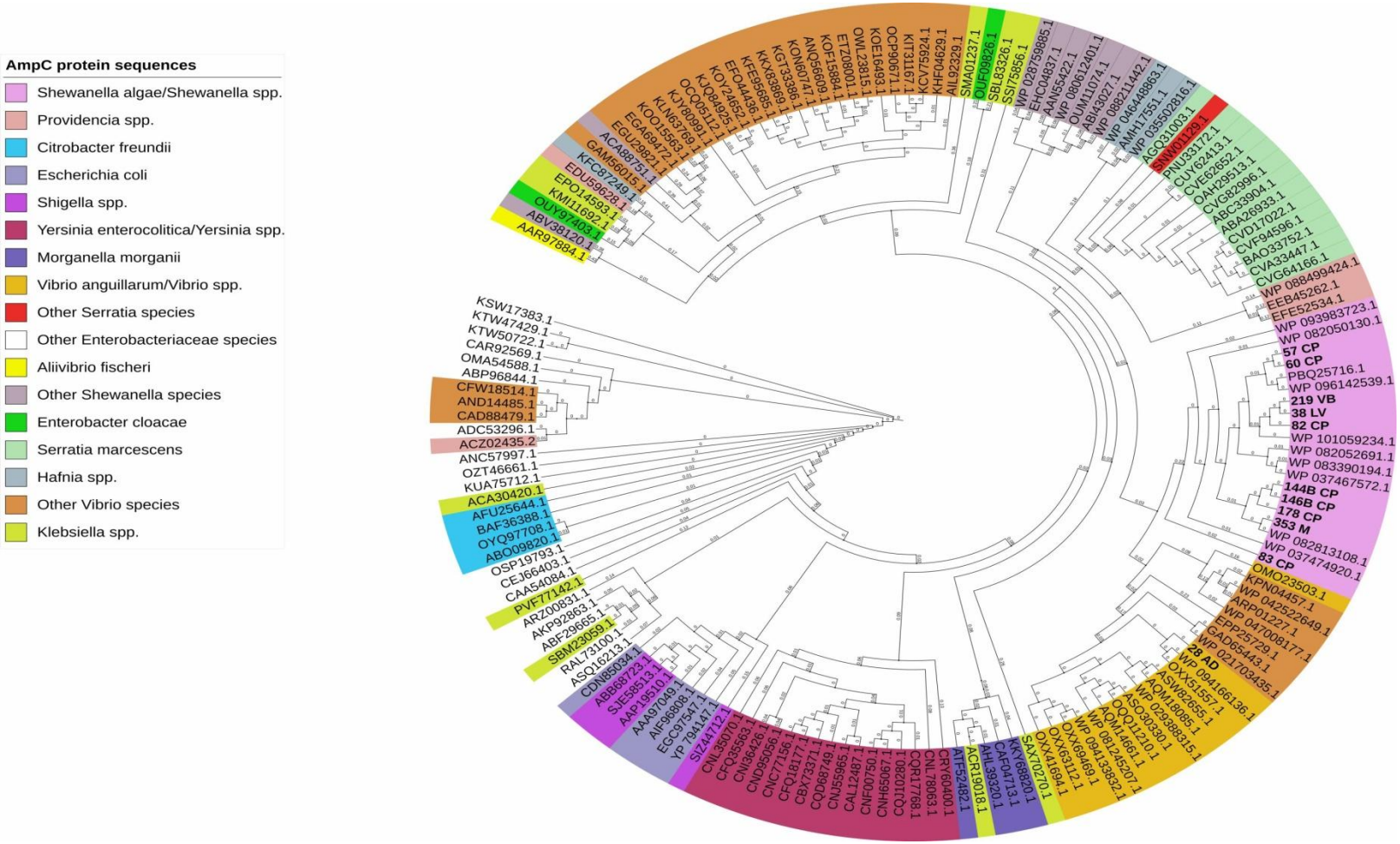

Figure 25. Phylogenetic analysis, based on Neighbor-Joining method, of the predicted AmpC protein sequences found in *V. anguillarum* 28 AD, *Shewanella algae* genomes and AmpC protein sequences present in *Enterobacteriaceae* family members. Here, the tree topology is reported. The analysis involves 165 amino acid sequences and is conducted in MEGA7. The legend associates colours to the different species or genera considered. The evolutionary distances between AmpC are reported on each branch.

Homogeneity of the AmpC protein sequences is detected within the *S. marcescens*, *S. algae*/*Shewanella* spp. and *V. anguillarum*/*Vibrio* spp clusters indicating little divergence among members belonging to these species. Although the results evidenced a great amino acid sequence heterogeneity within the same genus, a great homogeneity is observed at intraspecies level, in particular among the *S. algae*, *V. anguillarum*, *V. parahaemolyticus* and *S. marcescens* clusters. Of interest is the presence of the same groups evidenced in the multiple alignment analysis according to the mutations found in the protein sequence. Observing the *S. algae* and *Shewanella* spp. cluster we can find a first group formed by 353M, 178CP, 146bCP and 144bCP with the last two strains more closely related than the others. In particular, it is important to evidence that the 353M strain, sampled in the open sea, is related to the AmpC protein sequences of the *S. algae* isolated in the inlet water described previously but more similar to WP_082813108.1. Then, a second group is represented by 219VB, 38LV and 82CP in which the first two strains are more related than the 82CP strain and their reference WP_101059234.1. Besides, a third one was made of 57CP and 60CP which are distinct from PBQ25716.1 and WP_096142539.1 located in the same group. Finally, the fourth one is given by 83CP which represents the strain with the high number of mutations and less related not only to the other AmpC protein sequences of our *S. algae* strains but also to the other protein sequences used as references. Also, the phylogenetic analysis using the nucleotide sequences of *S. algae* and *V. anguillarum* 28AD confirmed these results (Figure 26). Moreover, the phylogenetic analysis showed how the AmpC of *V. anguillarum*/*Vibrio* spp. and *S. algae*/*Shewanella* spp. are more closely related than the other clusters belonging to *Enterobacteriaceae* members. In fact, among them *Hafnia*, *Serratia*, *Enterobacter*, *Klebsiella*, *Morganella* and *Providencia* genera seem to be less divergent compared to the others. In addition, they are related to the "other *Shewanella* species" cluster.

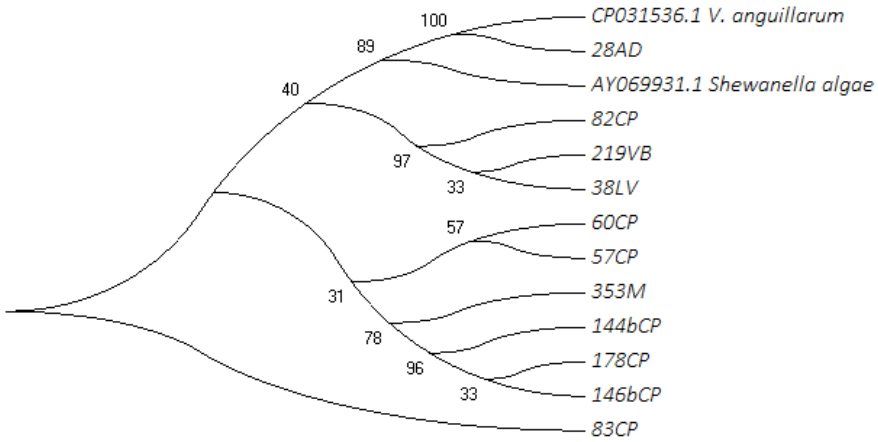

Figure 26. Phylogenetic analysis of the class C beta-lactamase (bla_{AmpC}) nucleotide sequences found in the chromosomes of *S. algae* and *V. anguillarum* 28AD. The evolutionary analysis was conducted using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were calculated using the Kimura 2-parameter method.

10.10 Phylogenetic analysis of the CARB-19 amino acid sequence and its variants detected in Vibrio genus

Evolutionary analysis of the CARB-19 protein sequence predicted in *V. parahaemolyticus* VPE116 strain and its variants was inferred using the NJ method (Figure 27). A total of 36 amino acid sequences, found in *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae* and retrieved in Genbank database, were analyzed. The analysis reported the presence of 5 clusters. The first one is represented by all the CARB variants found in *V. parahaemolyticus*, in which CARB-19 isolated in *V. parahaemolyticus* VPE116 is located. The relatedness is high (100%) to its reference amino acid sequence (WP_063857870.1) compared to the other sequences present in the same cluster.

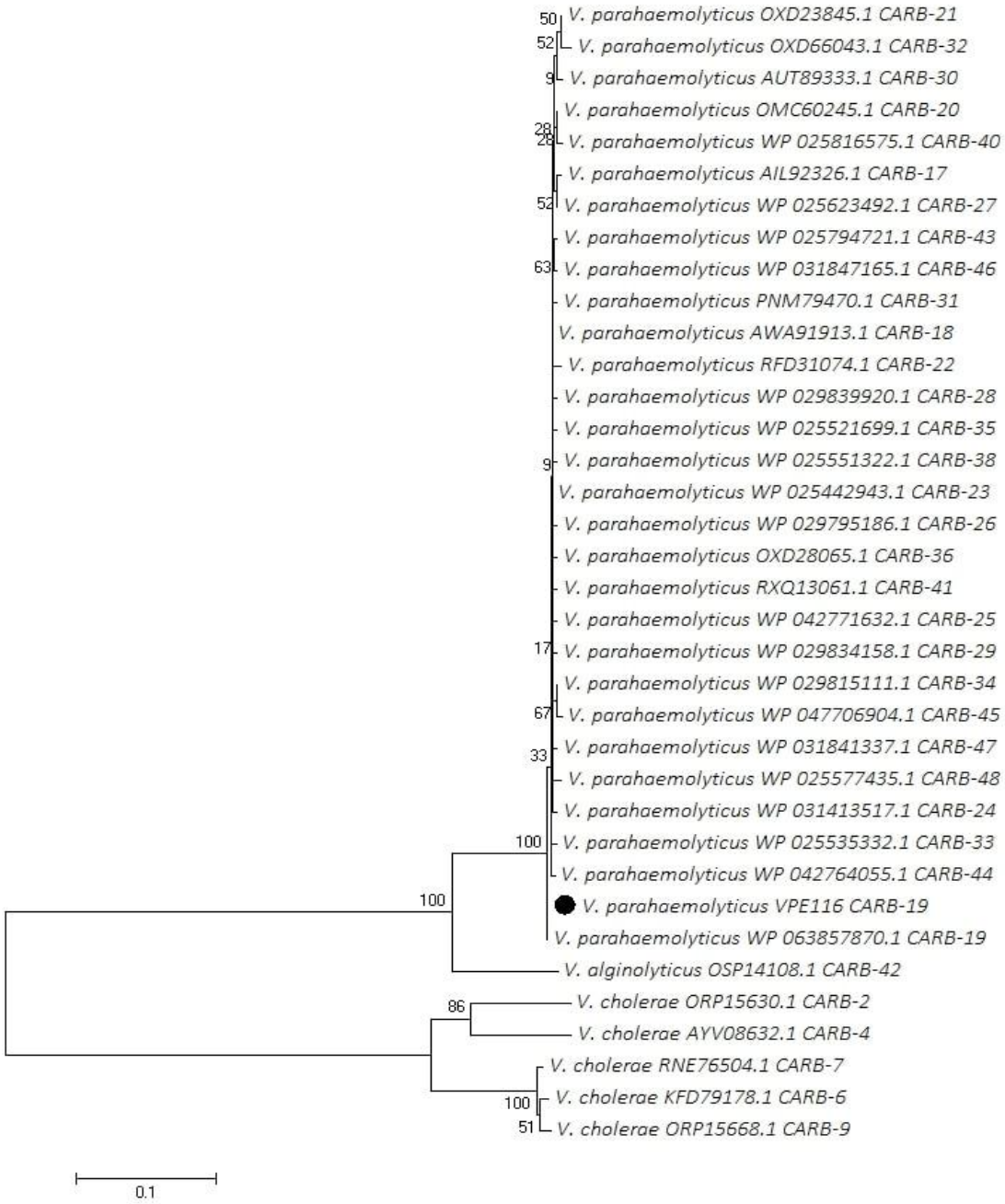

Figure 27. Evolutionary analysis of CARB and its variant found in *Vibrio* spp. genomes. It was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated CARB sequences clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The distances were computed using the JTT method and are in the units of the number of amino acid substitutions per site. The analysis involved 36 amino acid sequences and was conducted in MEGA7.

10.11 Core-genome alignment

The core-genome alignment of all the *S. algae* genomes under study evidenced an identity of 89% among the 10 strains considered. Of interest is the confirmation of some groups already found with the AmpC protein sequence multiple alignment and with the phylogenetic analysis (Figure 28). In particular, the group comprising 144bCP, 146bCP, 178CP and 353M is maintained suggesting the possibility for 144bCP, 146bCP and 178CP to be the same clone circulating in the inlet water sampled in June 2011 in a single aquaculture center. In addition, the 353M strain sampled in the open sea is confirmed to be more related to 144bCP, 146bCP and 178CP rather than to the other isolates. Another cluster is made of the 219VB, 82CP, 38LV and 60CP strains isolated in three different fish farms. The 82CP strain is more related to the 38LV isolate sampled in the Varano lake, a fish farm located well distant in the Southern Adriatic Sea than to 60CP, a strain obtained in the same aquaculture center or to strain 219VB, sampled in another northern fish farm. Finally, both 83CP and 57CP are separated from the other clusters evidencing more genetic divergence compared to the other strains although sampled in the same center, in the same source, inlet water, and in the same date.

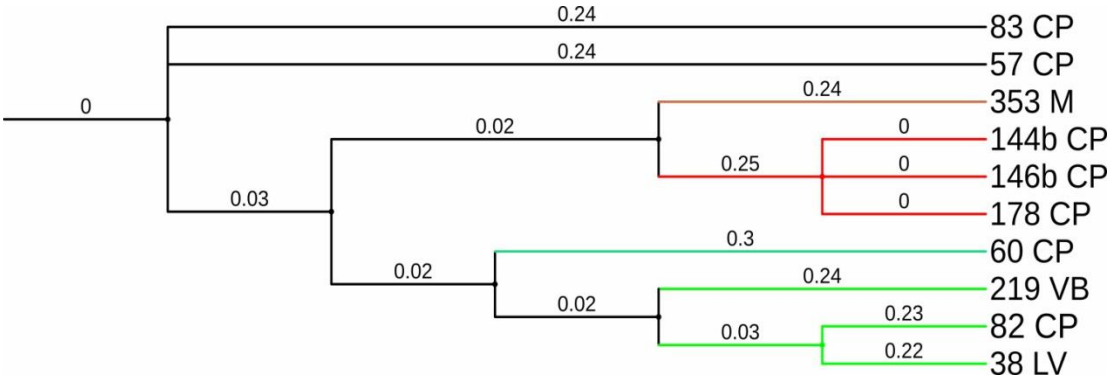

Figure 28. Multiple alignments of the 10 *S. algae* core-genomes using 38LV as reference and Harvest suite v1.1.2. A Maximum Likelihood tree was performed using the NNI model.

10.12 AST of the cloned bla_{AmpC} and $bla_{CARB-19}$

The antimicrobial susceptibility testing showed the resistance to amoxicillin and ticarcillin in *V. parahaemolyticus* VPE116 whose susceptibility was restored by the adjunct of a beta-

lactamase inhibitor (Figure 29, left). Conversely, *V. anguillarum* 28AD revealed resistance to more classes of beta-lactams included amoxicillin, ticarcillin, cephalothin, cefoxitin and temocillin. In general, susceptibility to beta-lactams is lower than *V. parahaemolyticus* VPE116, in particular for carbapenems. Moreover, the presence of clavulanic acid did not restore the susceptibility to amoxicillin and ticarcillin (Figure 29, right) as previously seen in *V. parahaemolyticus*.

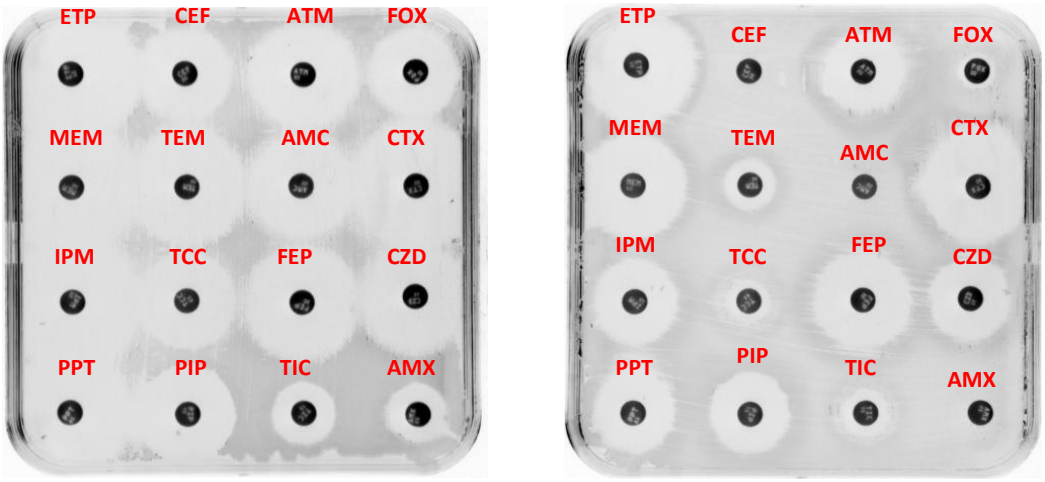

Figure 29. Antibiograms for the beta-lactam panel of *V. parahaemolyticus* VPE116 (left) and *V. anguillarum* 28AD (right).

ETP, ertapenem; CEF, cephalothin; ATM, aztreonam; FOX, cefoxitin; MEM, meropenem; TEM, temocillin; AMC, amoxicillin+clavulanate; CTX, cefotaxime; IPM, imipenem; TCC, ticarcillin+clavulanate; FEP, cefepime; CZD, ceftazidime; PPT, piperacillin+tazobactam; PIP, piperacillin; TIC, ticarcillin; AMX, amoxicillin.

Regarding the clones of bla_{AmpC} and $bla_{\text{CARB-19}}$ in *E. coli* TOP10, the Kirby-Bauer test has showed different resistance profiles as reported below (Figure 28). Of interest is the complete susceptibility of the whole panel of beta-lactams in *V. anguillarum* 28AD (left) except for cephalothin and amoxicillin. Concerning *V. parahaemolyticus* VPE116 the resistance to amino- and carboxypenicillins is confirmed. In addition, reduced activity of clavulanic acid is reported as well.

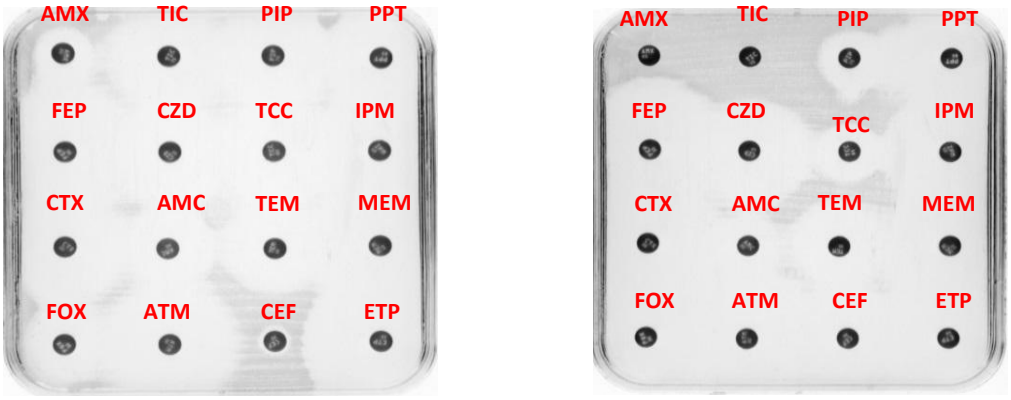

Figure 30. Antibiograms of *E. coli* TOP10 expressing bla_{AmpC} (left) and $bla_{CARB-19}$ (right).

ETP, ertapenem; CEF, cephalothin; ATM, aztreonam; FOX, cefoxitin; MEM, meropenem; TEM, temocillin; AMC, amoxicillin+clavulanate; CTX, cefotaxime; IPM, imipenem; TCC, ticarcillin+clavulanate; FEP, cefepime; CZD, ceftazidime; PPT, piperacillin+tazobactam; PIP, piperacillin; TIC, ticarcillin; AMX, amoxicillin.

Furthermore, MIC values for some amino- and carboxypenicillins, ureidopenicillins, cephalosporins (first and third generations) and carbapenems were calculated for *V. parahaemolycus* VPE116 WT, *V. anguillarum* 28AD WT, the transformed *E.coli* TOP10 and the empty *E. coli* TOP10 as reported in Table 9, below. The results showed that in pCARB-19 resistance to piperacillin is increased in *E. coli* expression system whereas resistance to amoxicillin and ticarcillin is confirmed. Regarding pAmpC, decreasing MIC values were obtained compared to *V. anguillarum* 28AD WT (see Table 9 and Figures 29-30) in particular against amino- and carboxypenicillins and ureidopenicillins whereas resistance to first generation cephalosporin is confirmed also in *E. coli*.

Table 9. MICs for some beta-lactam subclass representatives of *V. parahaemolycus* VPE116 WT, *V. anguillarum* 28AD WT, pCARB-19 and pAmpC *E. coli* TOP10.

	M.I.C. µg/ml				
	***V. parahaemolyticus* VPE116 ($bla_{CARB-19}$)**	***E. coli* TOP10 (pCARB-19)**	***V. anguillarum* 28AD (bla_{AmpC})**	***E. coli* TOP10 (pAmpC)**	***E. coli* TOP10**
Ampicillin	128	>256	128	8	2
Amoxicillin	256	>256	256	16	2
Ticarcillin	128	>256	64	4	2
Piperacillin	16	256	8	2	0.5
Cephalothin	16	16	>256	256	4
Cefotaxime	0.125	0.06	0.25	0.25	0.06
Ceftriaxone	0.25	0.03	1	0.25	0.06
Imipenem	0.125	0.5	4	1	1
Ertapenem	<0.015	<0.015	0.125	0.06	<0.015

11. DISCUSSION

The rise of community-acquired infections caused by antibiotic resistant bacteria has addressed the interest to the presence of antibiotic resistant (AR) genes in natural environments also including seawaters. It has been well documented a dramatic increase of bacterial resistance to beta-lactams, the most used drugs both in human and veterinary medicine (Bush and Bradford, 2016; Poole, 2004). Considering this situation, few data are reported in Italy describing the current prevalence of AR emerging and opportunistic bacteria such as shewanellae and vibrios present in seawaters and causing a number of human infections. Most of them focus on resistance to most common used beta-lactams in veterinary medicine such as penicillins and cephalosporins (Ottaviani *et al*., 2013) or to other classes of antibiotics such as tetracycline, sulfonamides and trimethoprim (Labella *et al*., 2013). A recent study on *S. algae* infections in Italy is reported by Torri and colleagues (2018) in which susceptibilities to third-generation cephalosporins and gentamycin were described in addition to a good activity showed by amikacin, carbapenems and piperacillin/tazobactam.

The current study aims at investigate the incidence of carbapenemase- and beta-lactamase-producer strains among those isolated in aquaculture centers located in the coastal areas of the Adriatic Sea in Italy (Labella *et al*., 2013). For this purpose, NGS technology was considered as it has permitted to decipher the less common beta-lactamases and their genetic surroundings rarely detected in human pathogens but that can have a role in maintaining an ARGs reservoir in the aquatic environment. First of all, *in vitro* tests were used to rapidly detect ESBL and carbapenemase producers within the non-*Enterobacteriaceae* aquatic microflora. The test has revealed able to detect the presence of carbapenem hydrolyzing class D beta-lactamases (CHDLs) in all the *S. algae* strains. Subsequently, these results were confirmed by genome sequencing with all the strains showing an enzyme encoding gene belonging to the OXA-55 group and an AmpC encoding gene. A doubtful result for the BCT test was obtained for *V. anguillarum* 28AD with the impossibility to interpret it. As a carbapenemase-negative control we used *V. parahaemolyticus* VPE116, an environmental strain which showed to be susceptible to imipenem and cefotaxime but resistant to cephalothin.

To better understand the molecular characteristics of class A, C and D beta-lactamases circulating in *S. algae*, *V. parahaemolyticus* and *V. anguillarum* strains isolated in our area, we performed multiple alignments and phylogenetic analysis. This has permitted to compare the results not only at intra and interspecies level within the same genus but also in reference to members of the *Enterobacteriaceae* family.

The multiple alignment of the class D beta-lactamases found in all the analyzed *Shewanella* strains evidenced that although all the protein sequences are located in the OXA-55 cluster, a little divergence is reported within the same cluster indicating the presence of slightly different OXA-55-like enzymes in our area if compared among them and to the reference sequence (AAR03105.1). Their average 95.5% identity to the OXA-55 reference sequence confirms they belong to an OXA-55-like beta-lactamase family as it is evidenced also in the phylogenetic analysis. The analysis confirms the high phylogenetic relatedness to the cluster harboring OXA variants found in other *Shewanella* species including OXA-48 and OXA-48-like. Moreover, we evaluated the divergence rate respect to clinical and environmental *Acinetobacter* spp. OXA variants as it is the bacterial species of medical interest in which carbapenem resistance is mainly due to CHDLs (Evans and Amies, 2014). Of interest is the similarity between the *Shewanella* spp. OXA group and OXA-20, a chromosome encoded CHDL and OXA-21, an OXA-2-like enzyme, detected in *Acinetobacter* spp. respect to other OXA variants found in *Acinetobacter* spp. that is statistically well supported and less related to the other *Acinetobacter* enzymes.

Regarding class C beta-lactamases, AmpC was found in all the *S. algae* and in *V. anguillarum* 28AD strains. AmpC found in the analyzed *S. algae* strains showed different mutation patterns with higher variability and molecular heterogeneity if compared to other AmpC belonging to the *S. algae* and *Shewanella* spp. isolates retrieved in Genbank database. The results obtained has allowed to subdivide the analyzed strains in 4 groups on the basis of their AmpC mutation patterns. Interestingly, 146bCP, 144bCP and 178CP shared the same mutations and are all isolated in the same aquaculture center in the Veneto region. On the contrary, the second group formed by 219VB, 82CP and 38LV were found in different farms also distant each other considering that the LV center is located in the Southern Italy. The third group (57CP and 60CP) includes isolates sampled in the inlet water in the same center and in the same date. The strain 83CP, originating from the outlet water and isolated in the same day, is instead separated from the other strains on the basis of the AmpC sequence. The core-genome multiple alignment analysis and the phylogenetic analysis performed using

AmpC nucleotide sequences confirmed totally the cluster composed by 144bCP, 146bCP, 178CP and 353M evidencing the genetic relatedness of the aquaculture isolates to a clone circulating in the open sea and containing the same mutations. Moreover, the cluster including 219VB, 38LV and 82CP is also related but with a lower DNA similarity level to the 60CP isolate as revealed also for AmpC. Of interest is the similarity of the 38LV strain isolated in the Varano lake forming a cluster with the strains collected in the northern aquaculture centers and suggesting the circulation of *S. algae* clones in the Adriatic Sea carrying the same OXA and AmpC mutations. Furthermore, 83CP and 57CP, isolated in the same fish farm and in the same date are related neither to any cluster nor among them.

Regarding the 28AD isolate, it is more related to WP_094166136.1, a class C beta-lactamase of *Vibrio* sp. OXX51557.1, although being in the group comprising *V. anguillarum* and *Vibrio* spp. class C beta-lactamases. To conclude, the presence of AmpC and class C beta-lactamases in some of the whole sequence genomes, chromosomes, scaffolds or contigs of *V. anguillarum*, present in Genbank, can suggest a recent acquisition of these enzymes and consequently of the cephalosporin resistance.

Surely, a core-genome multiple alignment can better describe the relatedness rate between the *S. algae* strains isolated in the inlet and outlet water of aquaculture. By the way, it is interesting to find correspondences with some clusters formed taking into account only the AmpC protein or nucleotide sequences, in particular those comprising the open sea strain (353M) and the Varano lake strain (38LV). According to these data, the AmpC mutations reflect the different clones of the *S. algae* found in the inlet and outlet water of aquaculture centers in different sampling dates and sites along the Adriatic coast. Moreover, these results evidenced the molecular heterogeneity present not only at intraspecies level but also between the genera *Shewanella* and *Vibrio* even though they are phylogenetically related to other *Enterobacteriaceae* members.

Cloning of bla_{AmpC} and $bla_{\text{CARB-19}}$ in *E. coli* TOP10 has been performed to study the behaviour of these genes if transferred in an *Enterobacteriaceae* family member. For pCARB-19, both the antibiograms and MIC values showed the involvement of similar mechanisms of expression resulting in the resistance to all penicillins included piperacillin. Regarding pAmpC, it is possible to appreciate a different expression level of this beta-lactamase in *V. anguillarum* 28AD and in *E. coli* TOP10. In fact, in the *V. anguillarum* 28AD WT strain, resistance to cephalosporins in particular to first generation cephalosporin such as cephalothin, penicillins in general and to carbapenems (imipenem) is showed. Conversely,

once the beta-lactamase is cloned, only resistance to cephalothin is detected, with a significant decrease of resistance to penicillins and carbapenems showing a different expression level in *E. coli* rather than in *V. anguillarum*. Probably, the higher expression level of the chromosomal-encoded AmpC in *V. anguillarum* 28AD is due to the presence of the system AmpD, AmpE, AmpG and AmpR which can exacerbate and regulate the expression of this gene leading to resistance also to other beta-lactams such as penicillins and carbapenems.

In conclusion, bla_{AmpC} and $bla_{\text{CARB-19}}$ seem to have different expression levels depending on the host microorganism. The results here presented have showed how this difference is more evident in bla_{AmpC} than in $bla_{\text{CARB-19}}$ indicating a better activity in *V. anguillarum* 28AD, its original host. Conversely, $bla_{\text{CARB-19}}$ has seemed to be more adaptable to other host bacteria genomes belonging to *Enterobacteriaceae* family expressing similar activity if compared to its original host, *V. parahaemolyticus* VPE116.

12. CONCLUSIONS

The class A, C and D beta-lactamases found in the chromosomes of our MDR marine isolates has confirmed their essential role as ARGs reservoir in the Adriatic Sea. Interestingly, the mutation analysis either at protein level or at nucleotide level has evidenced how they were sampling site-related indicating the dissemination of different clones along the Adriatic Sea water. Although most of the drug resistances that have been recovered were intrinsic, it is noticed that their potential transfer in a MGE can facilitate their spread horizontally to the microbiota present in the environment included pathogenic bacterial species.

In this study, the beta-lactamases analyzed were all chromosomal-encoded and their mobilization, in particular of bla_{AmpC} and $bla_{\text{CARB-19}}$ into a plasmid in an *Enterobacteriaceae* family member has showed how their expression level can vary if transferred to another host cell. The different resistance profiles obtained if compared to the two wild type vibrios has highlighted possible different mechanisms of expression involved, in particular for bla_{AmpC}.
The contribution of these analyses is essential to understand how the aquatic environment can provide ARGs able to interact and to integrate into the chromosome or MGEs of other pathogenic bacterial species.
Furthermore, the study of their mutation profiles can help us to investigate the mutation rate in these enzymes and if they can evolve in other potential variants that would become later of clinical interest and a public health concern.
To conclude, this is the first report about the specific beta-lactamases involved in the beta-lactam resistance in strains isolated in Italian aquaculture centers. A survey of these enzymes in this field has been essential to monitor the contribution of aquaculture in arising to AMR in veterinary medicine and in the environment.

13. CONCLUDING REMARKS AND PERSONAL CONSIDERATIONS

The results obtained from the present investigation has allowed to decipher the resistome, mobilome and virulome of some emerging pathogens providing new insights facilitated by the use of NGS technology. The detailed analysis of the resistance genes, in particular of enzymes and efflux pumps as well as of mobilomes has been of crucial importance to understand the players that are involved in the maintenance of this ARG reservoir. We also found out that bacteriophages can have a role in the spread of these genes included virulence genes in the species analyzed. Moreover, thanks to NGS technology the genetic background of some beta-lactamases has been investigated evidencing that they are mostly associated to heavy metal resistance genes that can stimulate their expression following the exposure in the aquatic environment.

Although NGS can provide only the presence or the absence of a specific ARG, nothing is reported about its expression, so sometimes genotype and phenotype can differ hypothesizing other mechanisms of regulation or expression involved. Another big issue is the presence of a large number of hypothetical proteins that will remain unsolved until a similar protein member of the same family will be annotated in Genbank. It is, in fact, difficult to discover new resistance genes if the databases used are not well curated. In my opinion, NGS needs to be supported from updated databases that can give more information at least at protein family level in order to hypothetisize its function. This is the case of fosfomycin resistance present in all our *S. algae* strains which has been detected phenotypically but not genotypically, so supposing only the presence of the mechanisms involved.

These data, although providing evidence of the presence of ARGs in the Adriatic Sea water and sediment, have noted that most of the genes are responsible for intrinsic resistance in these strains and some of them are also species specific (OXA-55, CARB-19, qnrA3). Aquatic microbiota can be a reservoir of new possible variants or determinants of specific resistance genes but the nosocomial settings are the perfect environments where spreading, acquiring more mutations and more resistance genes is easier in particular due to the direct exposure to antibiotics. To conclude, this thesis is the result of several collaborations

occurred within different departments and laboratories of the University of Verona and University of Fribourg (Switzerland), including bioinformaticians, microbiologists and biochemists in order to have a multidisciplinary approach to the question.

At the end of this PhD project, I can express my personal satisfaction about the results obtained. Furthermore, I had the opportunity to travel a lot, to attend national and international congresses, workshops, meetings, to meet researchers who could help me in discussing and improving my results and to collaborate with researchers of very known research level.

14. ACKNOWLEDGEMENTS

I really want to thank my academic tutors Prof. *Maria Lleo* and Prof. *Giovanni Malerba* for all the support and the help during these three years of my PhD.

Prof. *Patrice Nordmann* and Dr. *Laurent Poirel* for giving me the chance to attend their laboratory at the Faculty of Medicine and Science at Fribourg University.

Laura Veschetti for helping me in performing some of my bioinformatic analysis and discussing the results.

Dr. *Carolina Conter* and Dr. *Alessandra Astegno* for supporting me in some of my experiments.

15. BIBLIOGRAPHY

Aberkane S., Compain F., Barraud O., Ouédraogo A.S., Bouzinbi N., Vittecoq M., Jean-Pierre H., Decré D., Godreuil S. (2015) Non-O1/non-O139 *Vibrio cholerae* avian isolate from France cocarrying the bla_{VIM-1} and bla_{VIM-4} genes. Antimicrob Agents Chemother 59:6594–96.

Afzal-Shah M., Woodford N., Livermore D.M. (2001) Characterization of OXA-25, OXA-26, and OXA-27, molecular class D beta-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. Antimicrob Agents Chemother. 45(2):583-8.

Ahmed H.A., El Bayomi R.M., Hussein M.A., Khedr M.H.E., Abo Remela E.M., El-Ashram, A.M.M. (2018) Molecular characterization, antibiotic resistance pattern and biofilm formation of *Vibrio parahaemolyticus* and *V. cholerae* isolated from crustaceans and humans. Int J Food Microbiol 274:31-7.

Aldred K.J., Kerns R.J., Osheroff N. (2014) Mechanism of quinolone action and resistance. Biochemistry. 53(10):1565–74.

Anes J., McCusker M.P., Fanning S., Martins M. (2015) The ins and outs of RND efflux pumps in *Escherichia coli*. Front Microbiol 10;6:587.

Arndt D., Grant J.R., Marcu A., Sajed T., Pon A., Liang Y. Wishart D.S. (2016) PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res 44(W1):W16-21.

Ashbolt N.J., Amezquita A., Backhaus T. Borriello P, Brandt K.K., Collignon P., Coors A., Finley R., Gaze W.H., Heberer T., Lawrence J.R., Larsson D.G., McEwen S.A., Ryan J.J., Schönfeld J., Silley P., Snape J.R., Van den Eede C., Topp E. (2013) Human Health Risk Assessment (HHRA) for Environmental Development and Transfer of Antibiotic Resistance. Environ Health Perspect 121:993–1001.

Bado I., Papa-Ezdra R., Cordeiro N., Outeda M., Caiata L., García-Fulgueiras V., Seija V., Vignoli R. (2018) Detection of qnrVC6, within a new genetic context, in an NDM-1-producing *Citrobacter freundii* clinical isolate from Uruguay. J Glob Antimicrob Resist 14:95-98.

Baker-Austin C., Oliver J.D., Alam M., Ali A., Waldor M.K., Qadri F., Martinez-Urtaza J. (2018) *Vibrio* spp. infections. Nat Rev Dis Primers.4(1):8.

Balcázar J.L., Subirats J., Borrego C.M. (2015) The role of biofilms as environmental reservoirs of antibiotic resistance. Front Microbiol 6:1216.

Balcázar J.L. (2014) Bacteriophages as vehicles for antibiotic resistance genes in the environment. PLoS Pathog. 10(7):e1004219.

Bankevich A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin V.M., Nikolenko S.I., Pham S., Prjibelski A.D., Pyshkin A.V., Sirotkin A.V., Vyahhi N., Tesler G., Alekseyev M.A., Pevzner P.A. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19(5):455-77.

Baruah F.K., Grover, R.K. (2014) Case report and literature review of carbapenem resistant *Shewanella putrefaciens* isolated from ascitic fluid. J Clin Diagn Res 8:DD01-02.

Bengtsson-Palme J., Kristiansson E., Larsson D.G.J. (2018) Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Ecology 42:68-80.

Bier N., Schwartz K., Guerra B., Strauch E. (2015) Survey on antimicrobial resistance patterns in *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 in Germany reveals carbapenemase-producing *Vibrio cholerae* in coastal waters. Front Microbiol 6:1179.

Björklund H.V., Råbergh C.M.J., Bylund G. (1991) Residues of oxolinic acid and oxytetracycline in fish and sediments from fish farms. Aquaculture 97:85–96.

Blaser M.J. (2014) The microbiome revolution. J Clin Invest 124:4162–65.

Boccard F., Smokvina T., Pernodet J.L., Friedmann A., Guérineau M. (1989) Structural analysis of loci involved in pSAM2 site-specific integration in *Streptomyces*. Plasmid. 21(1):59-70.

Bondarczuk K., Markowicz A., Piotrowska-Seget Z. (2015) The urgent need for risk assessment on the antibiotic resistance spread via sewage sludge land application. Environ Int 87:49–55.

Bonomo R.A. (2017) Beta-Lactamases: A Focus on Current Challenges. Cold Spring Harb Perspect Med 7:a025239.

Bradford P.A., Urban C., Mariano N., Projan S.J., Rahal J.J., Bush K. (1997) Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC beta-lactamase, and the foss of an outer membrane protein. Antimicrob Agents Chemother. 41(3):563-9.

Brown-Jaque M., Calero-Cáceres W., Muniesa M. (2015) Transfer of antibiotic-resistance genes via phage-related mobile elements. Plasmid 79:1–7.

Brown S., Young H.K., Amyes S.G. (2005) Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. Clin Microbiol Infect. 11(1):15-23.

Brüssow H. (2005) Phage therapy: the *Escherichia coli* experience. Microbiology. 151(Pt 7):2133-40.

Buchan B.W., Ledeboer N.A. (2014) Emerging Technologies for the Clinical Microbiology Laboratory. Clinical Microbiology Reviews 27(4):783-822.

Burrus V., Marrero J., Waldor M.K. (2006) The current ICE age: biology and evolution of SXT-related integrating conjugative elements. Plasmid. 55(3):173-83.

Bush K, Bradford PA. (2016) β-Lactams and β-Lactamase inhibitors: an overview. Cold Spring Harb Perspect Med 6:a025247.

Bush K. (2018) Past and Present Perspectives on β-Lactamases. Antimicrob Agents Chemother. 24;62(10).

Bush K. (2013) Proliferation and significance of clinically relevant beta-lactamases. Ann. N.Y. Acad. Sci. 1277:84–90.

Bush K., Jacoby G.A. (2010) Updated functional classification of beta-lactamases. Antimicrob Agents Chemother. 54:969–976.

Cabello F.C., Godfrey H.P., Buschmann A.H., Dölz H.J. (2016) Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. Lancet Infect Dis 16: e127–33.

Cabello F.C., Godfrey H.P., Tomova A., Ivanova L., Dölz H., Millanao A., Buschmann A.H. (2013) Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environ Microbiol 15:1917–42.

Cabezón E., Ripoll-Rozada J., Peña A., De la Cruz F., Arechaga I. (2015) Towards an integrated model of bacterial conjugation. FEMS Microbiol Rev. ;39(1):81-95.

Calero-Cáceres W., Melgarejo A., Colomer-Lluch M., Stoll C., Lucena F., Jofre J., Muniesa M. (2014) Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions. Environ Sci Technol. 48(13):7602-11.

Cambray G., Guerout A.M., Mazel D. (2010) Integrons. Annu Rev Genet. 44:141-66.

Cantas L., Midtlyng P.J., Sørum H. (2012) Impact of antibiotic treatments on the expression of the R plasmid tragenes and on the host innate immune activity during pRAS1 bearing *Aeromonas hydrophila* infection in zebrafish (*Danio rerio*). BMC Microbiol 12:37.

Carraro N., Poulin D., Burrus V. (2015) Replication and Active Partition of Integrative and Conjugative Elements (ICEs) of the SXT/R391 Family: The Line between ICEs and Conjugative Plasmids Is Getting Thinner. PLoS Genet. 11(6):e1005298.

Carraro N., Burrus V. (2014) Biology of Three ICE Families: SXT/R391, ICEBs1, and ICESt1/ICESt3. Microbiol Spectr. 2(6).

Cassini A., Högberg L.D., Plachouras D. Quattrocchi A., Hoxha A., Simonsen G.S., Colomb-Cotinat M., Kretzschmar M.E., Devleesschauwer B., Cecchini M., Ouakrim D.A., Oliveira T.C., Struelens M.J., Suetens C., Monnet D.L.; Burden of AMR Collaborative Group. (2019) Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis 19:56–66.

Ceccarelli D., Van Essen-Zandbergen A., Veldman K.T., Tafro N., Haenen O., Mevius D.J. (2017) Chromosome-based bla_{OXA-48}-like variants in *Shewanella* species isolates from food-producing animals, fish, and the aquatic environment. Antimicrob Agents Chemother 61:(2).e01013-16.

Ceccarelli D., Alam M., Huq A., Colwell R.R. (2016) Reduced Susceptibility to Extended-Spectrum β-Lactams in *Vibrio cholerae* Isolated in Bangladesh. Front Public Health. 18;4:231

Chancey S.T., Zähner D., Stephens D.S. (2012) Acquired inducible antimicrobial resistance in Gram-positive bacteria. Future Microbiol. 2012 Aug;7(8):959-78.

Chen Y.J., Tung K.C., Hong Y.K., Chen S.Y., Huang Y.T., Liu P.Y. (2019) Genome Sequence of Colistin-Resistant Bacteremic *Shewanella algae* Carrying the Beta-Lactamase Gene bla_{OXA-55}. Can J Infect Dis Med Microbiol. 2019:3840563.

Chen D.Q., Yang L., Luo Y.T., Mao M.J., Lin Y.P., Wu A.W. (2013) Prevalence and characterization of quinolone resistance in *Laribacter hongkongensis* from grass carp and Chinese tiger frog. J Med Microbiol 62:1559–64.

Chiou J., Li R., Chen S. (2015) CARB-17 family of β-lactamases mediates intrinsic resistance to penicillins in *Vibrio parahaemolyticus*. Antimicrob Agents Chemother 59:3593-5.

Chowdhury G., Pazhani G.P., Sarkar A., Rajendran K., Mukhopadhyay A.K., Bhattacharya M.K. Ghosh A., Ramamurthy T. (2016) Carbapenem resistance in clonally distinct clinical strains of *Vibrio fluvialis* isolated from diarrheal samples. Emerg Infect Dis 22:1754-61.

Cimmino T., Olaitan A.O., Rolain, J.M. (2016) Whole genome sequence to decipher the resistome of *Shewanella algae*, a multidrug-resistant bacterium responsible for pneumonia, Marseille, France. Expert Rev Anti Infect Ther 14:269-75.

Clarke B., Hiltz M., Musgrave H., Forward K.R. (2003) Cephamycin resistance in clinical isolates and laboratory-derived strains of *Escherichia coli*, Nova Scotia, Canada. Emerg Infect Dis. 9(10):1254-9.

Collis C.M., Kim M.J., Partridge S.R., Stokes H.W., Hall R.M. (2002) Characterization of the class 3 integron and the site-specific recombination system it determines. J Bacteriol. 2002 Jun;184(11):3017-26.

Correia S., Poeta P., Hébraud M., Capelo J.L., Igrejas G. (2017) Mechanisms of quinolone action and resistance: where do we stand? J Med Microb 66:551-9.

Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origins. Official Journal of the European Communities. 18.08.1990. No L 224/1.

Crofts T.S., Gasparrini A.J., Dantas G. (2017) Next-generation approaches to understand and combat the antibiotic resistome. Nat Rev Microbiol. 15(7):422–34.

Dabos L., Jousset A.B., Bonnin R.A., Fortineau N., Zavala A., Retailleau P., Iorga B.I., Naas T. (2018) Genetic and Biochemical Characterization of OXA-535, a Distantly Related OXA-48-Like β-Lactamase. Antimicrob Agents Chemother. 24;62(10).

Dallenne C., Da Costa A., Decré D., Favier C., Arlet G. (2010) Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in *Enterobacteriaceae*. J. Antimicrob Chemother 65:490-5.

Dark M.J. (2013) Whole-genome sequencing in bacteriology: state of the art. Infect. Drug Resist. 6, 115–123.

Delavat F., Miyazaki R., Carraro N., Pradervand N., Van der Meer J.R. (2017) The hidden life of integrative and conjugative elements. FEMS Microbiol Rev. 2017 Jul 1;41(4):512-537.

Deurenberg R.H., Bathoorn E., Chlebowicz M.A., Couto N., Ferdous M., Cobos S.G., Kooistra-Smid A.M.D., Raangs E.C., Rosema S., Veloo A.C.M., Zhou K., Friedrich A.W., Rossen J.W.A. (2017) Application of next generation sequencing in clinical microbiology and infection prevention. Journal of Biotechnology 243:16-24.

Diana J.S., Egna H.S., Chopin T., Peterson M.S., Cao L., Pomeroy R., Verdegem M., Slack W.T., Bondad-Reantaso M.G., Cabello F. (2013) Responsible aquaculture in 2050: valuing local conditions and human innovations will be key to success. Bioscience 63:255–62.

Di D.Y.W., Shin H., Han D., Unno T., Hur H.G. (2019) High genetic diversity of *Vibrio parahaemolyticus* isolated from tidal water and mud of southern coast of South Korea. FEMS Microbiol Ecol 95:fiz022.

Domingues S., Harms K., Fricke W.F., Johnsen P.J., Da Silva G.J., Nielsen K.M. (2012) Natural transformation facilitates transfer of transposons, integrons and gene cassettes between bacterial species. PLoS Pathog 8:e1002837.

Dönhöfer A., Franckenberg S., Wickles S., Berninghausen O., Beckmann R., Wilson D.N. (2012) Structural basis for TetM-mediated tetracycline resistance. Proc Natl Acad Sci USA. 16; 109(42):16900–5.

Drawz S.M., Bonomo R.A. (2010) Three decades of b-lactamase inhibitors. Clin Microbiol Rev 23:160– 201.

Elbashir S., Parveen S., Schwarz J., Rippen T., Jahncke M., DePaola A. (2018) Seafood pathogens and information on antimicrobial resistance: A review. Food Microbiol. 70:85-93.

Ellington M.J., Ekelund O., Aarestrup F.M., Canton R., Doumith M., Giske C., Grundman H., Hasman H., Holden M.T.G., Hopkins K.L., Iredell J., Kahlmeter G., Köser C.U., A. MacGowan A., Mevius D., Mulvey M., Naas T., Peto T., Rolain J.-M., Samuelsen Ø., Woodford N. (2017) The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clinical Microbiology and Infection 23:2-22.

Escudero J.A., Loot C., Nivina A., Mazel D. (2015) The integron: adaptation on demand. Microbiol Spectr 3:MDNA3-0019-2014.

Evans B.A., Amies S.G. (2014) OXA β-lactamases. Clin Microbiol Rev 27:241-63.

Fancello L., Desnues C., Raoult D., Rolain J.M. (2011) Bacteriophages and diffusion of genes encoding antimicrobial resistance in cystic fibrosis sputum microbiota. J Antimicrob Chemother. 66(11):2448-54.

Fang Y., Wang Y., Li Z., Liu Z., Li X., Diao B., Kan B., Wang D. (2018) Distribution and genetic characteristics of SXT/R391 Integrative Conjugative Elements in *Shewanella* spp. from China. Front Microbiol 11;9:920.

FAO. Antimicrobial Resistance in the environment. Summary Report of an FAO Meeting of Experts FAO Antimicrobial Resistance Working Group, 2018.

Ferri M., Ranucci E., Romagnoli P., Giaccone V. (2017) Antimicrobial resistance: A global emerging threat to public health systems. Crit Rev Food Sci Nutr. 57(13):2857-76.

Finley R.L., Collignon P., Larsson D.G., McEwen S.A., Li X.Z., Gaze W.H., Reid-Smith R., Timinouni M., Graham D.W., Topp E. (2013) The scourge of antibiotic resistance: the important role of the environment. Clin Infect Dis 57:704–10.

Fisher J.F., Mobashery S. (2014) The sentinel role of peptidoglycan recycling in the beta-lactam resistance of the Gram negative *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Bioorg Chem 56:41– 48.

Fishovitz J., Taghizadeh N., Fisher J.F., Chang M., Mobashery S. (2015) The Tipper–Strominger hypothesis and triggering of allostery in penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus* (MRSA). J Am Chem Soc137:6500– 6505.

Frans I., Michiels C.W., Bossier P., Willems K.A., Lievens B., Rediers H. (2011) *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. J Fish Dis. 34(9):643-61.

Gayet S., Chollet R., Molle G., Pagès J.M., Chevalier J. (2003) Modification of outer membrane protein profile and evidence suggesting an active drug pump in *Enterobacter aerogenes* clinical strains. Antimicrob Agents Chemother. 47(5):1555-9.

Gazin M., Paasch F., Goossens H., Malhotra-Kumar S., MOSAR WP2 and SATURN WP1 Study Teams. (2012) Current trends in culture-based and molecular detection of extended-spectrum-β-lactamase-harboring and carbapenem-resistant *Enterobacteriaceae*. J Clin Microbiol. 50(4):1140-6.

Grossman T.H. (2016) Tetracycline antibiotics and resistance. Cold Spring Harb Perspect Med 6(4):a025387.

Gupta S.K., Padmanabhan B.R., Diene S.M., Lopez-Rojas R., Kempf M., Landraud L., Rolain JM. (2014) ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother 58(1):212-20.

Hektoen H., Berge J.A., Hormazabal V., Yndestad M. (1995) Persistence of antibacterial agents in marine sediments. Aquaculture 133:175–84.

Héritier C., Poirel L., Fournier P.E., Claverie J.M., Raoult D., Nordmann P. (2005) Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. Antimicrob Agents Chemother. 49(10):4174-9.

Héritier C., Poirel L., Nordmann P. (2004) Genetic and biochemical characterization of a chromosome-encoded carbapenem-hydrolyzing Ambler class D beta-lactamase from *Shewanella algae*. Antimicrob Agents Chemother 48(5):1670-5.

Heuer O.E., Kruse H., Grave K., Collignon P., Karunasagar I., Angulo F.J. (2009) Human health consequences o fuse of antimicrobial agents in aquaculture. Clin Infect Dis. 49(8):1248-53.

Hickman A.B., Dyda F. (2015) Mechanisms of DNA transposition. Microbiol Spectr 3:MDNA3-0034-2014.

Holmes A.H., Moore L.S., Sundsfjord A., Steinbakk M,. Regmi S., Karkey A., Guerin P.J., Piddock L.J. (2016) Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 387(10014):176-87.

Holt H.M., Søgaard P., Gahrn-Hansen B. (1997) Ear infections with *Shewanella alga*: a bacteriologic, clinical and epidemiologic study of 67 cases. Clin Microbiol Infect 3:329-34.

Huang Y.H., Tang Y.Y., Cheng J.F., Wu Z.Y., Mao Y.C., Liu P.Y. (2018) Genome analysis of multidrug-resistant *Shewanella algae* isolated from human soft tissue sample. Front Pharmacol 9:419.

Hussain M., Carlino A., Madonna M.J., Lampen J.O. (1985) Cloning and sequencing of the metallothioprotein beta-lactamase II gene of *Bacillus cereus* 569/H in *Escherichia coli*. J Bacteriol. 164(1):223-9.

Ito Y., Hirano T. (1997) Carbenicillin-hydrolysing penicillinase mediated by a plasmid of *Proteus mirabilis* and its relationship to the PSE-type enzymes of *Pseudomonas aeruginosa*. J Appl Microbiol. 83(2):175-80.

Jacob-Kokura S., Chan C.Y., Kaplan L. (2014) Bacteremia and empyema caused by *Shewanella algae* in a trauma patient. Ann Pharmacother 48:128-36.

Jacobs-Slifka, K.M., Newton, A.E., Mahon, B.E. (2017) *Vibrio alginolyticus* infections in the USA, 1988-2012. Epidemiol Infect 145:1491-99.

Jacoby G.A. (2009) AmpC beta-lactamases. Clin Microbiol Rev. 22(1):161-82.

Jacoby G.A, Mills D.M, Chow N. (2004) Role of beta-lactamases and porins in resistance to ertapenem and other beta-lactams in *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 48(8):3203-6.

Janda J.M., Abbott S.L. (2014) The genus *Shewanella*: from the briny depths below to human pathogen. Crit Rev Microbiol 40:293-312.

Jia B., Raphenya A.R., Alcock B., Waglechner N., Guo P., Tsang K.K., Lago B.A., Dave B.M., Pereira S., Sharma A.N., Doshi S., Courtot M., Lo R., Williams L.E., Frye J.G., Elsayegh T., Sardar D., Westman E.L., Pawlowski A.C., Johnson T.A., Brinkman F.S., Wright G.D., McArthur A.G. (2017) CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 45(D1):D566-D573.

Joris B., Ledent P., Dideberg O., Fonzé E., Lamotte-Brasseur J., Kelly J.A., Ghuysen J.M., Frère J.M. (1991) Comparison of the sequences of class A beta-lactamases and of the secondary structure elements of penicillin-recognizing proteins. Antimicrob Agents Chemother. 35(11):2294-301.

Jousset A.B., Dabos L., Bonnin R.A., Girlich D., Potron A., Cabanel N., Dortet L., Glaser P., Naas T. (2017) CTX-M-15-producing *Shewanella* species clinical isolate expressing OXA-535, a chromosome-encoded OXA-48 variant, putative progenitor of the plasmid-encoded OXA-436. Antimicrob Agents Chemother 62:e01879.

Kaase M., Nordmann P., Wichelhaus T.A., Gatermann S.G., Bonnin R.A., Poirel L. (2011) NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt. J Antimicrob Chemother. 66(6):1260-2.

Khameneh B., Diab R., Ghazvini K., Fazly Bazzaz B.S. (2016) Breakthroughs in bacterial resistance mechanisms and the potential ways to combat them. Microb Pathog. 95:32-42.

Khashe S., Janda J.M. (1998) Biochemical and pathogenic properties of *Shewanella alga* and *Shewanella putrefaciens*. J Clin Microbiol 36(3):783-7.

Kim D.M., Kang C.I., Lee C.S., Kim H.B., Kim E.C., Kim N.J., Oh M.D., Choe K.W. (2006) Treatment failure due to emergence of resistance to carbapenem during therapy for *Shewanella algae* bacteremia. J Clin Microbiol 44:1172–74.

Kleinheinz, K.A., Joensen, K.G., Larsen, M.V. (2014) Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and *E. coli* virulence genes in bacteriophage and prophage nucleotide sequences. Bacteriophage 4(1):e27943.

Köser C.U., Ellington M.J., Peacock S.J. (2014) Whole-genome sequencing to control antimicrobial resistance. Trends Genet. 30(9):401-7.

Kumar S., Stecher G., Tamura K. (2016) MEGA 7: Molecular Evoltutionary Genetics Analysis version 7 for bigger datasets. Mol Biol Evol. Jul;33(7):1870-4.

Kwon D.H., Dore M.P., Kim J.J., Kato M., Lee M., Wu J.Y., Graham D.Y. (2003) High-level beta-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. Antimicrob Agents Chemother. 47(7):2169-78.

Labella A., Gennari M., Ghidini V., Trento I., Manfrin A., Borrego J.J., Lleo M.M. (2013) High incidence of antibiotic multi-resistant bacteria in coastal areas dedicated to fish farming. Mar Pollut Bull 70:197-203.

Laganà P., Caruso G., Minutoli E., Zaccone R., Santi D. (2011) Susceptibility to antibiotics of *Vibrio* spp. and *Photobacterium damsela* spp. *piscicida* strains isolated from Italian aquaculture farms. New Microbiol 34(1):53-63.

Laraki N., Franceschini N., Rossolini G.M., Santucci P., Meunier C., de Pauw E., Amicosante G., Frère J.M., Galleni M. (1999) Biochemical characterization of the *Pseudomonas aeruginosa* 101/1477 metallo-beta-lactamase IMP-1 produced by *Escherichia coli*. Antimicrob Agents Chemother. 43(4):902-6.

Lascols C., Podglajen I., Verdet C., Gauthier V., Gutmann L., Soussy C.J., Collatz E., Cambau E. (2008) A plasmid-borne *Shewanella algae* gene, qnrA3, and its possible transfer *in vivo* between *Kluyvera ascorbata* and *Klebsiella pneumonia*. J Bacteriol 190:5217–23.

Ledingham K. (2019) Antibiotic resistance: using a cultural contexts of health approach to address a global health challenge. WHO, Cultural Contexts of Health and Well-being, Policy brief no. 2.

Lee L.H., Ab Mutalib N.S., Law J.W., Wong S.H., Letchumanan V. (2018) Discovery on antibiotic resistance patterns of *Vibrio parahaemolyticus* in Selangor reveals carbapenemase producing *Vibrio parahaemolyticus* in marine and freshwater fish. Front Microbiol 9:2513.

Lemaire O.N., Méjean V., Iobbi-Nivol C. (2020) The *Shewanella* genus: ubiquitous organisms sustaining and preserving aquatic ecosystem. FEMS Microbiol Rev. Jan 10;fuz031.

Letunic I, Bork P. (2016) Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other tree. Nucleic Acids Res 44(W1):W242-5.

Li R., Lin D., Chen K., Wong M.H., Chen S. (2015) First detection of AmpC β-lactamase *bla*(CMY-2) on a conjugative IncA/C plasmid in a *Vibrio parahaemolyticus* isolate of food origin. Antimicrob Agents Chemother. 59(7):4106-11.

Li W., Atkinson G.C., Thakor N.S., Allas U., Lu C.C., Chan K.Y., Tenson T., Schulten K., Wilson K.S., Hauryliuk V., Frank J. (2013) Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). Nat Commun. 4:1477.

Liu J., Yang L., Chen D., Peters B.M., Li L., Li B., Xu Z., Shirtliff M.E. (2018) Complete sequence of pBM413, a novel multidrug resistance megaplasmid carrying *qnrVC6* and $bla_{\text{IMP-45}}$ from *Pseudomonas aeruginosa*. Int J Antimicrob Agents 51(1):145-50.

Liu M., Li X., Xie Y., Bi D., Sun J., Li J., Tai C., Deng Z., Ou H.Y. (2019) ICEberg 2.0: an updated database of bacterial integrative and conjugative elements. Nucleic Acids Res 47(D1):D660-D665.

Lloyd N.A., Nazaret S., Barkay T. (2018) Whole genome sequences to assess the link between antibiotic and metal resistance in three coastal marine bacteria isolated from the mummichog gastrointestinal tract. Marine Pollution Bulletin 135:514-20.

Love D.C., Rodman S., Neff R.A., Nachman K.E. (2011) Veterinary drug residues in seafood inspected by the European Union, United States, Canada, and Japan from 2000 to 2009. Environ Sci Technol 45:7232–40.

Mahillon J., Chandler M. (1998) Insertion sequences. Microbiol Mol Biol Rev. 62(3):725-74. Review.

Mandal J., Sangeetha V., Ganesan V., Parveen M., Preethi V., Harish B.N., Srinivasan S., Parija S.C. (2012) Third-generation cephalosporin-resistant *Vibrio cholerae*, India. Emerg Infect Dis. 18(8):1326-8.

Mangat C.S., Boyd D., Janecko N., Martz S.L., Desruisseau A., Carpenter M., Reid-Smith R.J., Mulvey M.R. (2016) Characterization of VCC-1, a novel Ambler class A carbapenemase from *Vibrio cholerae* isolated from imported retail shrimp sold in Canada. Antimicrob Agents Chemother 60:1819–25.

Marshall B.M., Levy S.B. (2011) Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev. 24(4):718-33.

Martinez J.L. (2008) Antibiotics and antibiotic resistance genes in natural environments. Science 321:365–7.

Martinez-Martinez L., Pascual A., Jacoby G.A. (1998) Quinolone resistance from a transferable plasmid. Lancet. 351:797–9.

Massidda O., Rossolini G.M., Satta G. (1991) The *Aeromonas hydrophila* cphA gene: molecular heterogeneity among class B metallo-beta-lactamases. J Bacteriol. 173(15):4611-7.

Matamp N., Bhat S.G. (2019) Phage Endolysins as Potential Antimicrobials against Multidrug Resistant *Vibrio alginolyticus* and *Vibrio parahaemolyticus*: Current Status of Research and Challenges Ahead. Microorganisms. 2019 Mar 18;7(3).

Minot S., Sinha R., Chen J., Li H., Keilbaugh S.A., Wu G.D., Lewis J.D., Bushman F.D. (2011) The human gut virome: inter-individual variation and dynamic response to diet. Genome Res. (10):1616-25.

Miranda, C.D., Kehrenberg, C., Ulep, C., Schwarz, S., Roberts, M.C. (2003) Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. Antimicrob Agents Chemother. 47(3):883-8.

Miranda C.D., Zemelman R. (2001) Antibiotic resistant bacteria in fish from the Concepción Bay, Chile. Mar Pollut Bull. 42(11):1096-102.

Modi S.R., Lee H.H., Spina C.S., Collins J.J. (2013) Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. Nature. 499(7457):219-22.

Mohr M., Köstler J., Salzberger B., Hanses F. (2016) Polymicrobial soft tissue infection including *Shewanella putrefaciens*. Infection 44:563-4.

Motro Y., Moran-Gilad J. (2017) Next-generation sequencing applications in clinical bacteriology. Biomolecular detection and quantification (14):1-6.

Moura A., Soares M., Pereira C., Leitão N., Henriques I., Correia A. (2009) INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. Bioinformatics 25(8):1096-8.

Munita J.M., Arias C.A. (2016) Mechanisms of antibiotic resistance. Microbiol Spectr. 4(2). doi:10.1128/microbiolspec.VMBF-0016-2015.

Muñoz-Gallego I., Chaves F., Orellana M.A. (2016) Epidemiological and clinical characteristics of *Shewanella* spp. infections in a tertiary hospital in Madrid. Infect Dis (Lond) 48(10):760-2.

Navarrete P., Mardones P., Opazo R., Espejo R., Romero J. (2008) Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon. J Aquat Anim Health 20:177–83.

Nikaido H. (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 67(4):593–656.

Nikaido H. (2000) Crossing the envelope: how cephalosporins reach their targets. Clin Microbiol Infect. 3:22-6.

Nordmann P., Boulanger A.E., Poirel L. (2012) NDM-4 metallo-β-lactamase with increased carbapenemase activity from *Escherichia coli*. Antimicrob Agents Chemother 56:2184–86.

OIE. 2018. Report of the meeting of the OIE ad hoc group on antimicrobial resistance. Paris, France.

Okamoto K., Gotoh N., Nishino T. (2002) Extrusion of penem antibiotics by multicomponent efflux systems MexAB-OprM, MexCD-OprJ, and MexXY-OprM of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 46(8):2696-9.

Osano E., Arakawa Y., Wacharotayankun R., Ohta M., Horii T., Ito H., Yoshimura F., Kato N. (1994) Molecular characterization of an enterobacterial metallo beta-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob Agents Chemother. 38(1):71-8.

Ottaviani D., Bacchiocchi I., Masini L., Leoni F., Carraturo A., Giammarioli M., Sbaraglia G. (2001) Antimicrobial susceptibility of potentially pathogenic halophilic vibrios isolated from seafood. International Journal of Antimicrobial Agents 18:135 – 40.

Ottaviani D., Leoni F., Talevi G., Masini L., Santarelli S., Rocchegiani E., Susini F., Montagna C., Monno R., D'Annibale L., Manso E., Oliva M., Pazzani C. (2013) Extensive investigation of antimicrobial resistance in *Vibrio parahaemolyticus* from shellfish and clinical sources, Italy. Letters to the Editor / International Journal of Antimicrobial Agents 42:187– 93.

Overbeek R., Olson R., Pusch G.D., Olsen G.J., Davis J.J., Disz T., Edwards R.A., Gerdes S., Parrello B., Shukla M., Vonstein V., Wattam A.R., Xia F., Stevens R. (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Research 42:D206–D214.

Pal C., Bengtsson-Palme J., Kristiansson E., Larsson D.G.J. (2016) The structure and diversity of human, animal and environmental resistomes. Microbiome 4:54.

Pal C., Bengtsson-Palme J., Kristiansson E., Larsson D.G.J. (2015) Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16:964.

Parsley L.C., Consuegra E.J., Thomas S.J., Bhavsar J., Land A.M., Bhuiyan N.N., Mazher M.A., Waters R.J., Wommack K.E., Harper W.F. Jr., Liles M.R. (2010) Census of the viral metagenome within an activated sludge microbial assemblage. Appl Environ Microbiol. 76(8):2673-7.

Partridge S.R., Kwong S.M., Firth N., Jensen S.O. (2018) Mobile genetic elements associated with antimicrobial resistance. Clin Microbiol Rev 31:e00088-17.

Paton R., Miles R.S., Hood J., Amyes S.G., Miles R.S., Amyes S.G. (1993) ARI 1: beta-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*. Int J Antimicrob Agents. 2(2):81-7.

Petersen A., Andersen J.S., Kaewmak T., Somsiri T., Dalsgaard A. (2002) Impact of integrated fish farming on antimicrobial resistance in a pond environment. Appl Environ Microbiol. 68(12):6036-42.

Petroni A., Melano R.G., Saka H.A., Garutti A., Mange L., Pasterán F., Rapoport M., Miranda M., Faccone D., Rossi A., Hoffman P.S., Galas M.F. (2004) CARB-9, a carbenicillinase encoded in the VCR region of *Vibrio cholerae* non-O1, non-O139 belongs to a family of cassette-encoded beta-lactamases. Antimicrob Agents Chemother 48:4042-6.

Pires J., Novais, A., Peixe, L. (2013) Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. J Clin Microbiol 51:4281-3.

Pirnay J.P., De Vos D., Mossialos D., Vanderkelen A., Cornelis P., Zizi M. (2002) Analysis of the *Pseudomonas aeruginosa* oprD gene from clinical and environmental isolates. Environ Microbiol. 4(12):872-82.

Poirel L., Cattoir V., Nordmann P. (2012a) Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies. Front Microbiol 2;3-24.

Poirel L., Bonnin R.A., Nordmann P. (2012b) Genetic support and diversity of acquired extended-spectrum β-lactamases in Gram-negative rods. Infect Genet Evol. 12(5):883-93.

Poirel L., Rodriguez-Martinez J.M., Mammeri H. Liard A., Nordmann P. (2005a) Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob Agents Chemother 49:3523–25.

Poirel L., Marqué S., Héritier C., Segonds C., Chabanon G., Nordmann P. (2005b) OXA-58, a novel class D {beta}-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 49(1):202-8.

Poirel L., Héritier C., Nordmann, P. (2004) Chromosome-encoded Ambler class D-lactamase of *Shewanella oneidensis* as a progenitor of carbapenem-hydrolyzing oxacillinase. Antimicrob Agents Chemother 48:348–51.

Poole K. (2007) Efflux pumps as antimicrobial resistance mechanisms. Ann Med 39(3):162-76.

Poole K. (2005) Efflux-mediated antimicrobial resistance. J Antimicrob Chemother. 56(1):20–51.

Poole K (2004) Resistance to beta-lactam antibiotics. Cell Mol Life Sci. 61(17):2200-23.

Potron A., Poirel L., Nordmann P. (2011) Origin of OXA-181, an emerging carbapenem-hydrolyzing oxacillinase, as a chromosomal gene in *Shewanella xiamenensis*. Antimicrob Agents Chemother 55:4405-7.

Potter R.F., D'Souza A.W., Wallace M.A., Shupe A., Patel S., Gul D., Kwon J.H., Andleeb S., Burnham C.-A.D., Dantas G. (2017) Draft genome sequence of the $bla_{\text{OXA-436}}$- and $bla_{\text{NDM-1}}$-harboring *Shewanella putrefaciens* SA70 isolate. Genome Announc 5:e00644-17.

Pruden A., Larsson D.G.J., Amezquita A., Collignon P., Brandt K.K., Graham D.W., Lazorchak J.M., Suzuki S., Silley P., Snape J.R., Topp E., Zhang T., Zhu Y.G. (2013) Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ Health Perspect. 121:878–85.

Quale J., Bratu S., Gupta J., Landman D. (2006) Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. Antimicrob Agents Chemother. 50(5):1633-41.

Quale J., Bratu S., Landman D., Heddurshetti R. (2003) Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. Clin Infect Dis. 37(2):214-20.

Queenan A.M., Bush K. (2007) Carbapenemases: the versatile beta-lactamases. Clin Microbiol Rev. 20(3):440-58.

Quintiliani R. Jr., Courvalin P. (1996) Characterization of Tn1547, a composite transposon flanked by the IS16 and IS256-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. Gene. 12;172(1):1-8.

Quirós P., Colomer-Lluch M., Martínez-Castillo A., Miró E., Argente M., Jofre J., Navarro F., Muniesa M. (2014) Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. Antimicrob Agents Chemother. 58(1):606-9.

Rahman T., Yarnall B., Doyle D.A. (2017) Efflux drug transporters at the forefront of antimicrobial resistance. Eur Biophys J. 2017 Oct;46(7):647-653.

Ramírez M.S., Bello H., González Rocha G., Márquez C., Centrón D. (2010a) Tn7::In2-8 dispersion in multidrug resistant isolates of *Acinetobacter baumannii* from Chile. Rev Argent Microbiol. 42(2):138-40.

Ramírez M.S., Merkier A.K., Almuzara M., Vay C., Centron D. (2010b) Reservoir of Antimicrobial Resistance Determinants Associated with Horizontal Gene Transfer in Clinical Isolates of the Genus *Shewanella*. Antimicrob Agents and Chemiother 54:4516–7.

Rasmussen B.A., Gluzman Y., Tally F.P. (1990) Cloning and sequencing of the class B beta-lactamase gene (ccrA) from Bacteroides fragilis TAL3636. Antimicrob Agents Chemother. 34(8):1590-2.

Rhoads D.D. (2018) Lowering the barriers to routine whole-genome sequencing of bacteria in the clinical microbiology laboratory. J Clin Microbiol 56:e00813-18.

Rodríguez-Martínez J.M., Cano M.E., Velasco C., Martínez-Martínez L., Pascual A. (2011) Plasmid-mediated quinolone resistance: an update. J Infect Chemother. 17(2):149–82.

Romero J., Ringo E., Merrifield D.E. (2014) The gut microbiota of fish. In: Merrifield D.E., Ringo E., eds. Aquaculture nutrition: gut health, probiotics and prebiotics. Hoboken, NJ: Wiley-Blackwell, 75–100.

Rosenblatt-Farrell N. (2009) The landscape of antibiotic resistance. Environ Health Perspect. 117:A244-50.

Rossen J.W.A., Friedrich A.W., Moran-Gilad J., on behalf of the ESCMID Study Group for Genomic and Molecular Diagnostics (ESGMD) (2018) Practical issues in implementing whole-genome-sequencing in routine diagnostic microbiology. Clinical Microbiology and Infection 24:355-60.

Rossolini G.M., Franceschini N., Riccio M.L., Mercuri P.S., Perilli M., Galleni M., Frere J.M., Amicosante G. (1998) Characterization and sequence of the *Chryseobacterium* (*Flavobacterium*) *meningosepticum* carbapenemase: a new molecular class B beta-lactamase showing a broad substrate profile. Biochem J. 332 (Pt 1):145-52.

Rozov R., Brown Kav A., Bogumil D., Shterzer N., Halperin E., Mizrahi I., Shamir R. (2017) Recycler: an algorithm for detecting plasmids from de novo assembly graphs. Bioinformatics 33(4):475-82.

Saavedra M.J., Peixe L., Sousa J.C., Henriques I., Alves A., Correia A. (2003) Sfh-I, a subclass B2 metallo-beta-lactamase from a *Serratia fonticola* environmental isolate. Antimicrob Agents Chemother. 47(7):2330-3.

Samuelsen O.B., Torsvik V., Ervik A. (1992) Long-range changes in oxytetracycline concentration and bacterial resistance toward oxytetracycline in a fish farm sediment after medication. Sci Total Environ 114:25–36.

Santos L., Ramos F. (2018) Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. Int J Antimicrob Agents 52:135-43.

Satomi M. (2014) The family *Shewanellaceae*. Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds). The prokaryotes, 4th edn. Springer, Heidelberg, New York, Dordrecht, London.

Sboner A., Mu X.J., Greenbaum D., Auerbach R.K., Gerstein M.B. (2011) The real cost of sequencing: higher than you think! Genome Biol. 12, 125.

Scarano C., Piras F., Virdis S., Ziino G., Nuvoloni R., Dalmasso A., De Santis E.P.L., Spanu C. (2018) Antibiotic resistance of *Aeromonas* ssp. strains isolated from *Sparus aurata* reared in Italian mariculture farms. Int J Food Microbiol. 2018 Nov 2;284:91-97.

Scarano C., Spanu C., Ziino G., Pedonese F., Dalmasso A., Spanu V., Virdis S., De Santis E.P. (2014) Antibiotic resistance of *Vibrio* species isolated from *Sparus aurata* reared in Italian mariculture. New Microbiol 37(3):329-37.

Schürch A.C., Van Schaik W. (2017) Challenges and opportunities for whole-genome sequencing–based surveillance of antibiotic resistance. Ann. N.Y. Acad. Sci. 1388:108–20.

Seemann T. (2014) Prokka: rapid prokaryotic genome annotation. Bioinformatics 30(14):2068-9.

Seiler C., Berendonk T.U. (2012) Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front Microbiol 3:399.

Shintani M., Sanchez Z.K., Kimbara K. (2015) Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. Front Microbiol. 2015 Mar 31;6:242.

Shuman S. (1994) Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. J Biol Chem. 269(51):32678-84.

Shuman S. (1991) Site-specific interaction of vaccinia virus topoisomerase I with duplex DNA. Minimal DNA substrate for strand cleavage in vitro. J Biol Chem. 1991 266(17):11372-9.

Siguier P., Perochon J., Lestrade L., Mahillon J., Chandler M. (2006) ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 34 (Database issue):D32-6.

Srinivasiah S., Bhavsar J., Thapar K., Liles M., Schoenfeld T., Wommack K.E. (2008) Phages across the biosphere: contrasts of viruses in soil and aquatic environments. Res Microbiol 159:349–57.

Stürenburg E., Sobottka I., Mack D., Laufs R. (2002) Cloning and sequencing of *Enterobacter aerogenes* OmpC-type osmoporin linked to carbapenem resistance. Int J Med Microbiol. 291(8):649-54.

Tacão M., Araújo S., Vendas M., Alves A., Henriques I. (2018) *Shewanella* species as the origin of $bla_{\text{OXA-48}}$ genes: insights into gene diversity, associated phenotypes and possible transfer mechanisms. Int J Antimicrob Agents. 51(3):340-348.

Tagini F., Greub G. (2017) Bacterial genome sequencing in clinical microbiology: a pathogen-oriented review. Eur J Clin Microbiol Infect Dis 36:2007–20.

Telke A.A., Rolain J.M. (2015) Functional genomics to discover antibiotic resistance genes: The paradigm of resistance to colistin mediated by ethanolamine phosphotransferase in *Shewanella algae* MARS 14. Int J Antimicrob Agents 46:648-52.

Thomas C.M. (2000). Paradigms of plasmid organization. Mol Microbiol 37:485– 491.

Toleman M.A., Simm A.M., Murphy T.A., Gales A.C., Biedenbach D.J., Jones R.N., Walsh T.R. (2002) Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. J Antimicrob Chemother. 50(5):673-9.

Topp E., Larsson D.G.J., Miller D.N., Van den Eede C., Virta M.P.J. (2018) Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. FEMS Microbiol Ecol 1;94(3).

Torri A, Bertini S, Schiavone P, Congestrì F, Matteucci M, Sparacino M, Testa G, Pedna MF, Sambri V. (2018) *Shewanella algae* infection in Italy: report of 3 years' evaluation along the coast of the northern Adriatic Sea. New Microbes New Infect 23:39–43.

Treangen T.J., Ondov B.D., Koren S., Philippy A.M. (2014) The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 15:524.

Xin R., Zhang K., Wu N., Zhang Y., Niu Z. (2019) The pollution level of the blaOXA-58 carbapenemase gene in coastal water and its host bacteriacharacteristics. Environ. Pollut. Jan;244:66-71.

Walsh T.R., Toleman M.A., Poirel L., Nordmann P. (2005) Metallo-beta-lactamases: the quiet before the storm? Clin Microbiol Rev. 18(2):306-25.

Walsh T.R., Gamblin S., Emery D.C., MacGowan A.P., Bennett P.M. (1996) Enzyme kinetics and biochemical analysis of ImiS, the metallo-beta-lactamase from *Aeromonas sobria* 163a. J Antimicrob Chemother. 37(3):423-31.

Walsh T.R., Hall L., Assinder S.J., Nichols W.W., Cartwright S.J., MacGowan A.P., Bennett P.M. (1994) Sequence analysis of the L1 metallo-beta-lactamase from *Xanthomonas maltophilia*. Biochim Biophys Acta. 1218(2):199-201.

Watts J.E.M., Schreier H.J., Lanska L., Hale M.S. (2017) The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Mar Drugs 15:158.

Weindorf H., Schmidt H., Martin H.H. (1998) Contribution of overproduced chromosomal beta-lactamase and defective outer membrane porins to resistance to extended-spectrum beta-lactam antibiotics in *Serratia marcescens*. J Antimicrob Chemother. 41(2):189-95.

Weng S.F., Chao Y.F., Lin J.W. (2004) Identification and characteristic analysis of the ampC gene encoding beta-lactamase from *Vibrio fischeri*. Biochem Biophys Res Commun. 314(3):838-43.

Wozniak A.F., Waldor M.K. (2010) Integrative and conjugative elements: mosaic mobile genetic elements inabling dynamic lateral gene flow. Nature Review Microbiol 8: 552-63.

Wright G.D. (2010) Antibiotic resistance in the environment: a link to the clinic? Curr Opin Microbiol 13:589–94.

Wu Z.Y., Ho S.P., Cheng J.F., Tung K.C., Hong Y.K., Chen S.Y., Menzer A., Chen Y.J., Huang Y.T., Liu P.Y. (2018) Whole-genome characterization of *Shewanella algae* strain SYT3 isolated from seawater reveals insight into hemolysis. Future Microbiol. 13:1709-1717.

Yousfi K., Bekal S., Usongo V., Touati A. (2017) Current trends of human infections and antibiotic resistance of the genus *Shewanella*. Eur J Clin Microbiol Infect Dis 36:1353–62.

Zerbino D.R., Birney E. (2008) Velvet: Algorithms for *de novo* short read assembly using de Bruijn graphs. Cold Spring Harbor Laboratory Press 18:821-9.

Zhang G., Sun K., Ai G., Li J., Tang N., Song Y., Wang C., Feng J. (2019) A novel family of intrinsic chloramphenicol acetyltransferase CATC in *Vibrio parahaemolyticus*: Naturally occurring variants reveal diverse resistance levels against chloramphenicol. Int J Antimicrob Agents. 2019 Jul;54(1):75-79.

Zong Z. (2012) Discovery of *bla*(OXA-199), a chromosome-based *bla*(OXA-48)-like variant, in *Shewanella xiamenensis*. PLoS One. 7(10):e48280.

16. ANNEX I

Culture Media

TCBS

TCBS (Oxoid Ltd, Basingstoke, Hampshire, England) agar is a medium used for the selective isolation and cultivation of vibrios. It allows the identification of *Vibrio* spp., including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* from food, animal feeding stuffs and environmental samples in the area of food production and food handling. Sodium citrate serves to maintain an alkaline pH and along with sodium thiosulfate and ox bile are selective agents, inhibiting Gram-positive organisms and suppressing coliforms. Sodium thiosulfate serves also as a sulfur source and, in combination with ferric citrate, detects hydrogen sulfide production. Sodium chloride maintains the osmotic balance of the medium and stimulates vibrios growth. Sucrose is the fermentable carbohydrate whereas bromothymol blue and thymol blue are pH indicators.

TSB

TSB (Oxoid Ltd, Basingstoke, Hampshire, England), is a general purpose liquid enrichment medium used for the enrichment and cultivation of aerobic microorganisms that are not excessively fastidious. It supports the growth of a wide variety of microorganisms, especially common aerobic and facultatively anaerobic bacteria. The supplementation with agar forms TSA.

ChromID ESBL medium

ChromID® ESBL medium (Biomérieux, Marcy l'Étoile, France) is a selective chromogenic medium for the screening of Extended Spectrum ß-Lactamase producing enterobacteria in chronic carrier patients or patients at risk. ChromID® ESBL agar consists of a rich nutritive base including a variety of peptones. It contains a mixture of antibiotics, including cefpodoxime, enabling the selective growth of ESBL-producing enterobacteria; two chromogenic substrates and one natural substrate enabling the direct identification of the most frequently encountered ESBL-producing enterobacteria.

- *Escherichia coli*: spontaneous coloration (pink to burgundy) of ß-glucuronidase-producing strains (ß-GUR).

- *Klebsiella, Enterobacter, Serratia, Citrobacter* (KESC): spontaneous green, brownish-green or blue colouration of strains expressing a ß-glucosidase (ß-GLU).

- *Proteeae* (*Proteus*, *Providencia*, *Morganella*): spontaneous dark brown to light brown colouration of strains expressing a deaminase.

Mueller Hinton Agar

Mueller-Hinton Agar (Bio-Rad, Cressier, Switzerland) is used for Antimicrobial Susceptibility Testing (AST). It has become the standard medium for the Kirby-Bauer method, and is specified by the Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Moreover, it has a few properties that make it excellent for antibiotic use. First of all, it is a nonselective, nondifferential medium. This means that almost all organisms plated on it will grow. Additionally, it contains starch. Starch is known to absorb toxins released from bacteria, so that they cannot interfere with the antibiotics. Second, it is a loose agar. This allows for better diffusion of the antibiotics than most other plates. A better diffusion leads to a truer zone of inhibition.

17. ANNEX II

Genomic DNA extraction from bacterial cells (CTAB method)

- Dissolve bacterial colonies in 1 ml TE (pH 8.0) 1X buffer and centrifuge the cells at 14000 rpm for 2 minutes.
- Discard the surnatant and resuspend the pellet in 567 µl of TE 1X buffer.
- Add 30 µl of SDS 10% and 3 µl of proteinase K (20 mg/ml). Mix and incubate at 37°C for 1 hour.
- Add 100 µl of NaCl 5M and 80 µl of CTAB/NaCl solution. Mix and incubate at 65°C for 10 minutes.
- Add 780 µl of SEVAG (24:1 chloroform: isoamyl alcohol), mix and centrifuge at 14000 rpm for 5 minutes.
- Transfer the surnatant in a new eppendorf and add an equal volume (~ 500 µl) of phenol/chloroform/isoamyl alcohol.
- Centrifuge at 14000 rpm for 5 minutes.
- Transfer the surnatant in a new eppendorf and add 300 µl of isopropanol. Invert the samples twice.
- Incubate the samples in -20°C for 1 hour and 30 minutes.
- Centrifuge at 14000 rpm for 2 minutes and keep the pellet.
- Wash the DNA with 900 µl of ethanol 70% and centrifuge at 14000 rpm for 5 minutes.
- Carefully remove all the surnatant and dry the pellet in a thermal block at 37°C.
- Resuspend the pellet in 100 µl of water or TE buffer.

PCR product purification and cloning

For the cloning step, PCR products were purified using the Zymo DNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instruction.

The protocol is reported below:

1. In a 1.5 ml microcentrifuge tube, add 2-7 volumes of DNA Binding Buffer to each volume of DNA sample. Mix briefly by vortexing. For PCR fragments I used a volume ratio 5:1, DNA Binding Buffer : Sample.
2. Transfer mixture to a provided Zymo-Spin™ Column in a Collection Tube.
3. Centrifuge at 15.000 x g for 30 seconds. Discard the flow-through.
4. Add 200 µl DNA Wash Buffer to the column. Centrifuge for 30 seconds. Repeat the wash step.

Add ≥ 6 µl DNA Elution Buffer or water directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.

1. Add 2 µL of the TOPO® Cloning reaction into a vial of chemically competent *E. coli* TOP10 and mix gently. Do not mix by pipetting up and down.
2. Incubate on ice for 5–30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes on ice.
5. Add 250 µL of room temperature S.O.B. medium.
6. Cap the tube tightly and incubate at 37°C for 1 hour.
7. Spread 100 µL from each transformation on a prewarmed LB plate containing 30 µg/ml of kanamycin and incubate overnight at 37°C.
8. An efficient TOPO® Cloning reaction will produce several hundred colonies. Pick at least 10 colonies for analysis.