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Surveillance and detection of multidrug resistant bacterial strains and *blaOXA* genes in aquatic environments in Ås and Nordre Follo municipalities

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Biotechnology

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Abstract

The discovery of antibiotics has radically changed the treatment of bacterial infections, making the increasing antibiotic resistance one of the top ten greatest threats to the global health. The main focus of antibiotic resistance research has, until recently, been focused on clinical settings and human and veterinary aspects. It has become more evident that the environment plays a vital role in the evolution, dissemination and prevalence of antibiotic resistant bacteria and genes. Aquatic ecosystems are a known mixing ground for clinical and environmental bacteria and can be a source and reservoir for resistance genes. The transfer of resistance between environmental and clinically important bacteria occurs, and surveillance of the environment is therefore important to better understand this flow of resistance.

The purpose of this thesis was to investigate the occurrence of antibiotic resistant bacteria and genes in aquatic environments in Ås and Nordre Follo municipalities in Norway. Water samples were collected from three different locations was filtered and plated out on Brilliance™ ESBL and CRE chromogenic agar plates selecting for Extended spectrum β-lactamase (ESBL) producing and carbapenem resistant bacteria. Bacterial colonies were isolated, and DNA was extracted, followed by 16S PCR and identification by Sanger sequencing. Multiplex and Singelplex PCR with ESBL-specific primers were utilized to screen for specific ESBL-genes, which were confirmed by sequencing. Selected bacterial strains were chosen for antimicrobial susceptibility testing using five different classes of antibiotics. Illumina MiSeq was utilized for whole genome sequencing (WGS) for three selected bacteria, followed by screening for antibiotic resistance and virulence genes, multidrug efflux pumps and metal resistance genes. Several bacteria genera were detected by selective agar plates and identified by 16S rRNA, including the genera *Pseudomonas*, *Serratia*, *Herbaspirillum*, *Aeromonas*, *Shewanella*, *Chitinophaga* and *Pandoraea*. The susceptibility testing revealed a high amount of resistance to Penicillins and four different Multidrug resistant (MDR) isolates. The bacteria showing the highest amount of resistance was *Pandoraea sp.*, which demonstrated clinical resistance to five out of seven antibiotics tested. The screening for resistance in the three WGS bacteria, *Pseudomonas aeruginosa*, *Chitinophaga silvatica* and *Pandoraea sp.*, revealed several β-lactamases. This included the class D β-lactamases *blaOXA-50*, *blaOXA-158* and two *bla* genes, and the class C β-lactamases *blaPDC-202* and *blaPAO*. Several genes conferring metal resistance and genes for multidrug efflux pumps were also discovered. Together, the results obtained in this work indicate an occurrence of antibiotic resistant bacteria and resistance genes, including several *blaOXA*-variants, in the aquatic environments in Ås and Nordre-Follo municipalities.

Sammendrag

Oppdagelsen av antibiotika har drastisk endret hvordan bakterielle infeksjoner behandles, noe som har ført til at antibiotikaresistens nå blir sett på som en av de ti viktigste truslene mot den globale helsen. Frem til nylig har det meste av forskningen rundt antibiotikaresistens hatt sitt søkelys på det kliniske aspektet, samt menneske og dyrehelse. Det har blitt mer og mer tydelig at miljøet spiller en viktig rolle når det kommer til evolusjon, spredning og forekomst av antibiotikaresistente bakterier og gener. Akvatiske økosystemer er kjente møteplasser for miljøbakterier og klinisk viktige bakterier og er en lagringsplass og kilde til gener som fører til resistens. Overføring av slike gener mellom miljøbakterier og klinisk viktige bakterier forekommer, noe som gjør det viktig å overvåke miljøet for å bedre forstå flyten av resistens.

Hensikten med denne oppgaven har vært å kartlegge forekomsten av antibiotikaresistente bakterier og gener i vannforekomster i Ås og Nordre Follo kommune i Norge. Vannprøver ble innhentet fra tre ulike lokasjoner og ble deretter filtrert og platet ut på Brilliance™ ESBL and CRE agar skåler. Disse kromogene skålene kan skille mellom ulike genus, samt fremme vekst av utvidet spektrum β-laktamase (ESBL) produserende bakterier og Karbapenemresistente bakterier. Totalt ble 27 kolonier isolert, og DNA ble ekstrahert etterfulgt av 16S PCR identifisering ved Sanger sekvensering. Multiplex og Singelplex PCR ble gjennomført med bruken av ESBL-spesifiserte primere for å finne spesifikke resistensgener, etterfulgt av sekvensering for å bekrefte funnene. Antimikrobiell sensitivitet mot fem ulike klasser antibiotika ble testet på et utvalg av isolater. Tre bakterier ble utvalgt for helgenom sekvensering ved bruk av Illumina MISeq, og ble deretter sjekket for tilstedeværelsen av antibiotikaresistente gener, virulens gener, metallresistente gener og multi-medisin efflux pumper. Flere genus ble Identifisert ved bruk av 16S rRNA, inkludert *Pseudomonas*, *Serratia*, *Herbaspirillum*, *Aeromonas*, *Shewanella*, *Chitinophaga* og *Pandoraea*. Sensitivitetstestingen viste en stor mengde med resistens mot Penicillin samt fire multiresistente isolater. Bakterien med mest resistens var *Pandoraea sp.,* som viste resistens mot fem av syv antibiotika. De tre helgenomsekvenserte bakteriene ble identifisert som *Pseudomonas aeruginosa*, *Chitinophaga silvatica* og *Pandoraea sp.* og β-laktamase gener ble funnet hos alle. Det ble funnet fire gener i klasse D β-lakatamse, *blaOXA-50*, *bla OXA-158* og to *bla* gen. I tillegg ble det oppdaget to β-lakatamser i klasse C, *blaPDC-202* og *blaPAO.* Det ble også detektert flere gener som fører til resistens mot ulike metaller samt gener som er viktige i multi-medisin efflux pumper. Resultatene fra dette forsøket indikerer en tilstedeværelse av antibiotikaresistente bakterier og flere *blaOXA*-varianter i de akvatiske miljøene i Ås og Nordre Follo kommune.

Abbreviations

ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant gene
ARM	Antimicrobial resistance
BLAST	Basic Local Alignment Search Tool
BLASTn	BLAST nucleotide
BLASTp	BLAST protein
CARD	The Comprehensive Antibiotic Resistance Database
CF	Cystic fibrosis
CRB	Carbapenem resistant bacteria
CRE	Carbapenem resistant Enterobacteriaceae
DHFR	Dihydrofolate reductase
EARS-Net	European Antimicrobial Resistance Surveillance
ECOFF	Epidemiological cut-off values
EEA	European Economic Area
ESBL	Extended spectrum β-lactamase
EU	European Union
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
HGT	Horizontal Gene Transfer
MDR	Multidrug resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
PCR	Polymerase chain reaction
PubMLST	Public databases for Molecular typing and Microbial genome diversity
rMLST	Ribosomal Multilocus Sequence Typing
VFDB	Virulence Factor Databases
WGS	Whole Genome Sequencing
WHO	World Health Organisation
WWTP	Wastewater Treatment Plant

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2 Introduction

A big turning point in human history was the discovery of antibiotics, which revolutionised how bacterial infections were treated (Liu & Pop, 2009). The appearance of resistant strains has followed the discovery of antibiotics (Davies & Davies, 2010). The high use of these antimicrobial agents can potentially jeopardise their effect of antibiotics (UN (2023).

According to the World Health Organisation (WHO), antimicrobial resistance (AMR) is one of the top ten greatest threats to global health in the 21st century (Hernando-Amado et al., 2019; WHO, 2021a). The estimated number of deaths from AMR in 2019 was 4,95 million, with an estimation of 10 million annually deaths by the year 2050. These estimations emphasise the reality of these significant problems the global community is facing (UN, 2023). Some of these problems include untreatable human infections due to resistant bacteria, the spread of resistance to food through animals and changes in the community composition of environmental bacteria (Singh et al., 2019).

In addition to the emergence of more antibiotic resistant genes (ARG) and antibiotic resistant bacteria (ARB), the development of new antibiotics has declined since the 1990s. There is a reduction in working antibiotics, while the rate of discovery of new antibiotics is decreasing (Pulingam et al., 2022; Singh et al., 2019).

The challenges with AMR must be evaluated in correlation with the triple planetary crisis: climate change, biodiversity loss and pollution and waste. These crisis are all driven by the same factors: unsustainable consumption and production patterns, which are fuelled by population and consumption growth (UN, 2023). The only way to face this big global problem of antibiotic resistance is by knowing the challenges we are facing.

2.1. Antibiotics

Antibiotics are agents that are able to kill or inhibit the growth of bacteria. These agents target major molecular processes and structures in the bacterial cell that are crucial for survival and growth. These include targets in the central dogma, cell membrane and the cell wall (Aiyer et al., 2021, p. 291). The theory and concept of the central dogma was first published by Francis Crick in 1958 (Cobb, 2017). It is the flow of information between DNA, RNA and protein. It includes transcription of DNA to RNA and translation of RNA to proteins (Rinn & Chang, 2012).

Different antibiotics have emerged naturally over time and were produced by microorganisms (Demain & Sanchez, 2009). Since the discovery of antibiotics in the 1920s, the quantity of antibiotics produced has increased, and the cost has decreased, leading to high accessibility. Today, a large amount of antibiotics is produced synthetically. As a defence mechanism, bacteria have naturally developed ways to survive, resulting in antibiotic resistance. High usage of antibiotics leads to a higher prevalence of antibiotic resistant bacteria. This development of resistance across the biosphere is a result of a man-made situation (Davies & Davies, 2010; Demain & Sanchez, 2009; Hernando-Amado et al., 2019). There is a high usage and misuse of antibiotics on a worldwide basis, leading to the spread of these chemical compounds into the environment (Sanseverino et al., 2018).

2.1.1. Antibiotics in the environment

There is a comprehensive understanding that most ecosystems contribute to the emergence, spread and acquisition of AMR. Consequently, the issue of antibiotic resistance cannot be addressed by only examining the problem in healthcare facilities; the ecosystems must also be taken into consideration. Furthermore, environmental bacteria may act as clinically relevant opportunistic agents or spread resistance to other bacteria (Berendonk et al., 2015; Hernando-Amado et al., 2019; Narciso-da-Rocha & Manaia, 2016).

Antibiotic residues can enter the environment through several paths. These include sewage, the manufacturing industry, antibiotic production plants, runoff from livestock manure in agriculture and urban centres. Even though the lifetime of most antibiotics in the environment is considered short, the supply is constant and results in a stable presence (Ben et al., 2019; Singh et al., 2019).

Antibiotic production plants are a massive contributor to antibiotic spillage. There has been a positive correlation between the amount of a specific antibiotic and the presence of the related

ARGs in both macro and micro environments. Today's high production of antibiotics leads to the emergence of ARG and ARB (Singh et al., 2019).

Animal farming can also be a large source of antibiotic spillage. Countries that produce a large amount of meat often use large amounts of antibiotics in the production, as a precaution to prevent future infections. Animal feedlots are also problematic for the development and spread of resistance and are considered a hot spot (Singh et al., 2019).

The current high application of antibiotics results in a high abundance in the environment, which can lead to an enhanced selection pressure on the environmental microbiome (Ben et al., 2019; Done et al., 2015). This can affect bacteria that are usually sensitive to change genetically or by mutation. Thereby leading to an acceleration of the emergence and evolution of ARB and antibiotic resistant genes ARG (Baquero et al., 2008; Ben et al., 2019; Berendonk et al., 2015).

In environments with a high concentration of antibiotics, bacteria with already existing mutations or that mutate may survive and proliferate. For susceptible bacteria, all growth will be inhibited, and all antimicrobial activities will stop, resulting in growth arrest. In an environment with a low concentration of the antibiotics, bacteria will not be totally inhibited. If the concentration is below the inhibitory concentration, cellular response might be triggered. These responses could consist of an alternation of gene expression, induced gene mutation and horizontal gene transfer (HGT), which all could lead to the development of resistance. This will lead to a selection of resistance over time. In this way, both high and low concentrations can potentially lead to a selection pressure for resistance (Ben et al., 2019; Redgrave et al., 2014).

2.1.2. Antibiotic modes of action

There are several types of antibiotics, which are categorised into different classes according to their chemical structure and modes of action. A few examples of antibiotic classes are Cephalosporins, Penicillins, Fluoroquinolones and Carbapenems (Sanseverino et al., 2018). These antimicrobial agents have selective toxicity and can inhibit bacterial growth without harming the human patient (Aiyer et al., 2021, p. 930). Antibiotics do not cause diseases in humans because the target cells have a significantly different biochemical and functional structure than human cells (Madsen, 2016, p. 517).

Different antibiotics use a variety of modes of action, as illustrated in Figure 2.1. Several antibiotics target the central dogma of the bacterial cell, which includes DNA replication, RNA

and protein synthesis, DNA gyrase, topoisomerase IV, RNA polymerase and bacterial ribosomes (Aiyer et al., 2021, p. 931)

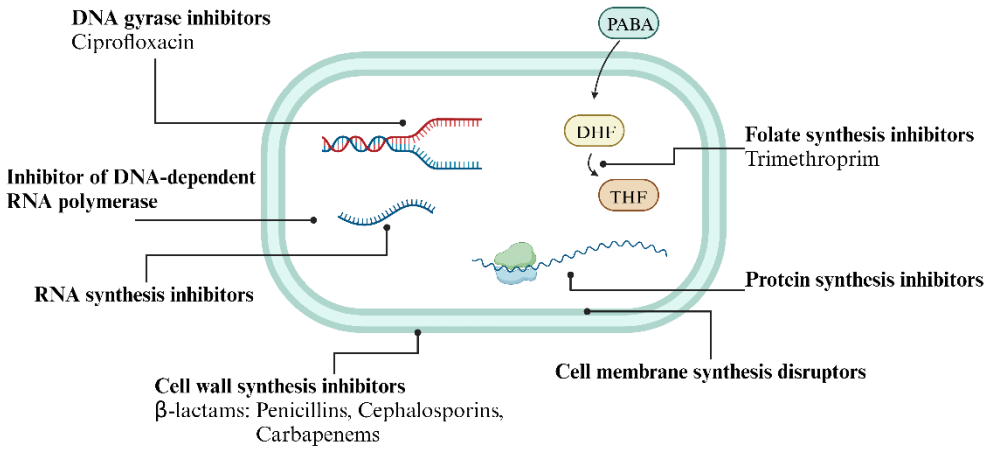


Figure 2.1: Modes of actions of antibiotics. Shows the targets for different antibiotics (Madsen, 2016, p. 517; Sanseverino et al., 2018). Figure created by BioRender.com.

β-lactams

β-lactams are a large group of antibiotics containing Penicillins, Cephalosporins and Carbapenems, among others. Members of this group inhibit cell growth by affecting the cell wall synthesis (Willey et al., 2020, pp. 193-194). A crucial part of the structure is the β-lactam ring. During the synthesis of the bacterial cell wall, new peptidoglycan units are supplemented to the growing cell wall in a process called transpeptidation. In this process, crosslinks are formed between peptidoglycans by the penicillin-binding protein (PBP), which catalyses the transpeptidation reaction. The β-lactam ring of the antibiotic resembles the terminal D-alanine-D-alanine end of the peptidoglycan chains and replaces it in the active site of the PBP protein. This will inhibit the cell wall synthesis and can cause osmotic lysis of the bacterial cell (Aiyer et al., 2021, p. 293; Willey et al., 2020, p. 195). Since the discovery of β-lactams, several new classes have been developed to be able to inhibit more types of bacteria and for inhibition of new specific resistance mechanisms (Bush & Bradford, 2016).

Penicillins is one of the β-lactam antibiotics classes, and the first β-lactam to be used clinically was penicillin G (benzylpenicillin). Today, several types of Penicillin exist, with a variety of effects and uses, including Ampicillin, Penicillin G, Oxacillin, Amoxicillin etc. The different types of Penicillin have different affinities to a variety of bacteria. For instance, Ampicillin has

a higher efficiency against gram negative pathogens compared to Penicillin G (Bush & Bradford, 2016).

Cephalosporins are another class of β-lactam antibiotics. Cefepime and Cefotaxime are two examples of Cephalosporin antibiotics and are known as extended spectrum cephalosporins. These antibiotics have a higher activity against *Enterococci* and *Staphylococci* compared to the earlier cephalosporins. Cefotaxime belongs to the 3rd generation, and Cefepime belongs to the 4th generation of Cephalosporins. From 2004 to 2014, Cephalosporins accounted for more than half of the prescriptions of antibiotics in the USA (Bush & Bradford, 2016).

The group of Carbapenems contain several antibiotics, including Imipenem, Ertapenem and Meropenem. Out of all the β-lactams, carbapenems have the broadest antibacterial spectrum and greatest potential against gram-positive and gram-negative bacteria. This causes Carbapenems to become a "last resort" antibiotic. It is used when patients have severe infections, become very ill or are infected by bacteria resistant to other antibiotics (Papp-Wallace et al., 2011). The structure of carbapenems consists of a β-lactam ring coupled to a carbapenem. This provides broad protection against Extended-Spectrum β-lactamases (ESBL) producing bacteria as well as most β-lactamases, including Metallo- β -lactamase (Codjoe & Donkor, 2017). Carbapenem has been the go-to antibiotic for treating infections with ESBL-producing bacteria. Since the increase in the use of carbapenems, more bacteria resistant to carbapenems have been developed, including CRE – carbapenem resistant *Enterobacteriaceae* (Abban et al., 2023).

Fluoroquinolones

Fluoroquinolones are a group of antibiotics, that includes Ciprofloxacin, among others. Ciprofloxacin is a broad-spectrum antibiotic belonging to the second generation of the fluoroquinolone family and is used for treatment of infections with gram positive and gram-negative bacteria. The principal mechanism of Ciprofloxacin is affecting the DNA supercoiling by binding to DNA gyrase or topoisomerase IV, inhibiting its activity. The binding to DNA gyrase is assumed to prevent the subunit A in the enzyme from releasing the double stranded DNA, affecting the supercoiling. Due to the overuse of Ciprofloxacin, a large amount of bacteria have developed resistance mechanisms against this antibiotic (Shariati et al., 2022).

Trimethoprim

The antibiotic Trimethoprim affects folate synthesis and is a selective inhibitor of bacterial dihydrofolate reductase (DHFR). The synthesis of tetrahydrofolate acid involves six different enzymes, including DHFR. The DHFR reduces dihydrofolate to tetrahydrofolate and is vital in regulating the amount of tetrahydrofolate and other derivates in the cell. Tetrahydrofolate is essential in the production of purines, methionine, and thymidine. Trimethoprim binds to DHFR and thereby inhibits its activity. This leads to an arrest in the synthesis of DNA, RNA and protein, ending cell growth (Hawser et al., 2006). When introduced in the mid-1970s, Trimethoprim showed a wide range of activity against several gram negative and gram-positive bacterial species. Since then, several resistance mechanisms against Trimethoprim have been discovered (Hawser et al., 2006).

2.2. Antibiotic resistance

Antibiotic resistance occurs when bacteria that were earlier susceptible no longer get affected by the drug. These bacteria will no longer be inhibited by the anti-bacterial drugs that earlier were effective (Singh et al., 2019; UN, 2023). Resistance mechanisms may be specific or non-specific. Non-specific resistance can for example be cell membrane permeability. This will affect not only the antibiotics but also other substances trying to cross the cell membrane. Specific resistance could be, for instance, modifying specific substrates or enzymes (Garneau-Tsodikova & Labby, 2016).

The three main categories for antibiotic resistance are adaptive, intrinsic and acquired resistance. The adaptive form of resistance is caused by environmental triggers, which can cause temporary changes in the expression of genes and proteins. This happens for instance in biofilms (Garneau-Tsodikova & Labby, 2016).

When the resistant phenotype is not owed to commonly reported resistance genes and is a typical characteristic for the members of the species, it is considered an intrinsic resistance mechanism (Narciso-da-Rocha & Manaia, 2016). Intrinsic resistance is the natural tendency of bacteria to be resistant against specific classes of antimicrobial agents and happens without the need for mutation or exposure to the drug (Codjoe & Donkor, 2017). These characteristic phenotypes are for instance related to biochemical properties, reduced permeability and efflux systems, and are the result of multiple genes. An example of intrinsic resistance is a naturally low permeability cell wall. This could lead to a lower concentration of antibiotics in the cell and a lower effect of the antibiotic (Garneau-Tsodikova & Labby, 2016; Vaz-Moreira et al.,

2014). These features are not easily transferred by horizontal gene transfer and are not a direct outcome of adaption to antibiotics. Intrinsic resistance is expected to represent an important part of the environmental antibiotic resistome, all the gene that can directly or indirectly confer or contribute to resistance (Perry & Wright, 2013; Vaz-Moreira et al., 2014).

Acquired resistance is an evolutionary response that occurs through HGT, transmission of mobile genetic elements (MGEs), or mutation (Garneau-Tsodikova & Labby, 2016; Martínez et al., 2015). Bacteria with acquired antibiotic resistance will have increased fitness, which entails the ability to survive and reproduce (Vaz-Moreira et al., 2014).

Antibiotic use and selection pressure

Not long after an antibiotic has been approved for clinical use, resistance to the antibiotic appears. The development and spread of antibiotic resistant microbes are caused by selection pressure that derives from prolonged use, persistent underuse, overuse and misuse of antibiotics as well as the release of antibiotics from several sources into the environment (Davies & Davies, 2010; Garneau-Tsodikova & Labby, 2016; Singh et al., 2019). When using antibiotics, the susceptible bacteria will get inhibited, and only resistant strains survive, this creates a selective pressure for resistance. The surviving bacteria, after exposure, would be able to transfer the resistance to other bacteria and communities (Pulingam et al., 2022). Resistance to distinct antibiotics and antimicrobials develops at different rates. This depends on, among other things, the modes of action for each specific antibiotic. Antimicrobials targeting one specific enzyme are more vulnerable to the development of resistance than the ones with several targets (Demain & Sanchez, 2009).

Antibiotic pressure can easily result in resistance against several different antibiotics due to multiple resistance genes often being located in the same plasmids or transposons (Garneau-Tsodikova & Labby, 2016). A Multidrug-resistant (MDR) organism is defined as non-susceptible to a minimum of one agent in three different antimicrobial categories (Magiorakos et al., 2012). Pan-drug resistant is defined as a bacterium with resistance to all antimicrobial categories (Pulingam et al., 2022).

2.2.1. Antibiotic resistance in the environment

Environmental settings play a significant role in the cycling of antibiotic resistance, as discussed by numerous sources (Vaz-Moreira et al., 2014). There is a considerable spillage of both antibiotics and antibiotic bacteria into the environment. This contamination of the environment can lead to interaction between bacteria and can cause a selection pressure. It should be

mentioned that bacteria in these settings often contain intrinsic resistance mechanisms from exposure to naturally occurring antimicrobials in their habitat. These environmental bacteria can serve as reservoirs for resistance genes (Baquero et al., 2008).

Antibiotic resistant hotspots exist in the environment as well as in clinical settings. These areas contain a large number of bacteria in addition to the presence of antibiotic residues. Examples of hot spots are wastewater systems, aquaculture facilities, animal husbandry facilities and pharmaceutical manufacturing effluents (Berendonk et al., 2015). When a substantial quantity of bacteria from various sites are gathered, the chances of transmission of resistance between different bacterial species increase. This could happen among environmental bacteria, as well as between environmental and pathogenic bacteria (Singh et al., 2019).

The microbial habitat of water plays an important part in the evolution and dissemination of ARBs and ARGs, and it is recognised as one of the most significant bacterial habitats on earth. It is well known that aquatic ecosystems are mixing grounds for clinical and environmental bacteria (Perry & Wright, 2013). Aquatic habitats can represent the source of resistance genes and be a reservoir for existing genes, as well as spread these into other ecosystems. Pathogenic and potential pathogenic bacteria are introduced to the aquatic environmens through several sources, including wastewater treatment plants (WWTPs). This can accommodate for the exchange of resistance genes (Baquero et al., 2008; Vaz-Moreira et al., 2014).

2.2.2. β-Lactamases and Carbapenemases

B-lactamases and ESBLs

β-lactamases are a group of enzymes that are able to inactivate β-lactam antibiotics. These enzymes can hydrolyse the β-lactam ring in the antibiotic structure and thereby inhibit their function, and are found in many different bacterial genera. The β-lactamases can be divided into two biochemical divisions, depending on the hydrolysis mechanics. The major group perform hydrolysis by the formation of an acyl-enzyme that contains an active-site serine. The other group facilitate the hydrolytic reaction by an active site with one or two zinc ions, the metallo- β-lactamases. The selective pressure from naturally occurring and from the overuse of drugs containing β-lactams, facilitates the emergence of new β-lactamases (Bush, 2018).

The 1980s was the global arrival of the ESBLs (Bush & Bradford, 2016). ESBL producing bacteria are able to hydrolyse the majority of β-lactam antibiotics, including 3rd generation Cephalosporins, and can be inhibited by β-lactamase inhibitors (Paterson & Bonomo, 2005). Resistance to other antibiotics, including Fluoroquinolones, Sulphonamides and

Aminoglycosides, is often found on the same plasmids that contain the ESBL genes (Abban et al., 2023).

There are two ways of classifying β-lactamases, the Ambler molecular classification or the Jacobi-Medeiros classification. The Ambler classification is based on the molecular structure of the β-lactamase enzymes and is divided into classes A, B, C and D. The enzymes included in class A, C, and D have an active centre using serine. The class B enzymes contains a zinc ion in the active centre. Class A includes TEM, SHV, CTX-M, and class A carbapenemases. Class B includes several carbapenemases like IMP, VIM and NDM, and class D contains the OXA carbapenemase and others (Sawa et al., 2020).

The Jacobi-Medeiros classification is based on the degradation of the substrate and the effect of the inhibition. This classification is divided into three groups. Group 1 contain the β-lactamase that degrades Cephalosporins. Group 2 include the β-lactamase not incorporated in group 1, with a serine active site including TEM, SHV, CTX-M and class A carbapenemases. Group 3 consists of metallo- β-lactamases like carbapenemases IMP, VIM and NDM (Sawa et al., 2020).

Carbapenemases

Carbapenemases are a specific type of β-lactamases which have the capability to hydrolyse Carbapenems. Most carbapenem resistance occurs in gram negative bacteria and the majority of the resistant genes can be transferred between bacteria by plasmids (Codjoe & Donkor, 2017; Papp-Wallace et al., 2011). Much like general resistance, the development of carbapenem resistance is an effect of intrinsic and acquired resistance mechanisms (Codjoe & Donkor, 2017).

Carbapenemases are classified into different classes. All of the members of class A carbapenemases can hydrolase carbapenems. *Klebsiella pneumoniae* carbapenemase (KPC) is the most common enzyme and has caused outbreaks in several countries. The KPC producing bacteria are typically also multidrug resistant to β-lactams (Codjoe & Donkor, 2017). Class B carbapenemases contain several enzymes, including New Delhi metallo-β-lactamase-1 (NDM-1), Imipenem-resistant *Pseudomonas* (IMP)-type of carbapenems and Verona Integron-encoded metallo-β-lactamase (VIM). The hydrolysing carbapenemase (OXA) enzyme is a class D carbapenemases. This enzyme can mutate at a fast rate, providing a high spectrum of activity. There are several different OXA enzymes and OXA-families, and the most usual is OXA-48

(Codjoe & Donkor, 2017). In Norway, two of the most regular carbapenemases are OXA-48 and NDM, with a large increase in occurrence since 2014 (NORM/NORM-VET, 2020)

2.2.3. Resistance mechanisms

Bacteria have several non-ESBL mechanisms to avoid the effects of antibiotics. These include alteration or overproduction of target sight by mutation, regulation of efflux pumps and porins, bypass enzymes, integrons, HGT, and production of β-lactamases and carbapenemases. Combinations of these mechanisms can cause even higher levels of resistance (Aiyer et al., 2021, p. 938; Munita & Arias, 2016; Papp-Wallace et al., 2011; Singh et al., 2019).

One common mode of action of antibiotic resistance is drug target mutation. If mutations occur in the active site of antibiotic binding, the effect of the antibiotic can be compromised. An example is the alternation of the active site in topoisomerase IV or DNA gyrase. Only one nucleotide mutation close to the tyrosine active site can alter the bacterium's affinity to Ciprofloxacin (Hooper & Jacoby, 2015; Shariati et al., 2022). An example of overproduction of target sight by mutation is the regulation of DHFR production. This can result in a very high amount of protein and, thereby reducing the susceptibility to Trimethoprim (Munita & Arias, 2016).

One way for gram negative bacteria to reduce the effect of antibiotics is to reduce the uptake of drugs into the cell. By decreasing the concentration of antibiotics, the effect can be lowered. This can be accomplished by, for instance, changes in the cell membrane porin channels. For hydrophilic antibiotics, including Fluoroquinolones, the main route for entering the cell is porins. This change will alter the membrane permeability, thus preventing the antibiotics from reaching the target (Codjoe & Donkor, 2017; Fernández & Hancock, 2012). One of the most common resistance mechanisms for *P. aeruginosa* against Ciprofloxacin is overexpression of efflux pumps, a type of transport proteins (Shariati et al., 2022). By modulating the active efflux transporter expression, cytoplasmic drug concentrations can be reduced, thus limiting the effect of the antibiotics (Fernández & Hancock, 2012; Shariati et al., 2022).

The use of alternative ways and pathways to bypass the inhibition of the target sight is another way of dodging the influence of antibiotics (Pulingam et al., 2022). An example is the main cause of resistance against Trimethoprim for gram positive bacteria. In this case, MGEs contain Trimethoprim resistant DHFR bypass enzymes that are not affected by the antibiotic causing resistance (Hawser et al., 2006).

Horizontal gene transfer

Horizontal gene transfer consists of transduction, conjugation and transformation (Ben et al., 2019). Transduction is the transfer of resistance genes from bacteriophages to bacteria. Conjugation is the transfer of resistance genes from one bacterium to another and is believed to be the most important way for HGT to spread resistance. This is often conducted by the use of plasmids with resistance genes. Transformation is taking up resistance genes from the environment into the bacteria (Pulingam et al., 2022; Singh et al., 2019). HGT is a very common way of transferring resistance genes from one bacteria to another, both within the population of species and across the species boundaries (Codjoe & Donkor, 2017). Horizontal gene transfer is one of the main reasons why understanding the spread of antibiotic resistance in the environment is challenging (Ben et al., 2019).

Integrons and plasmids

One of the main contributors to the spread of resistance is integrons. Integrons play an active part in the spread of resistance among bacteria, as well as bacterial adaptation and evolution (Deng et al., 2015). They can contain cassettes that contribute to antibiotic resistance and are often carried by mobile genetic elements like transposons or plasmids (Singh et al., 2019).

Plasmids is an common example of a mobile genetic elements. There is a fitness cost for maintaining a plasmid, therefore, its presence must give an advantage, such as resistance to antibiotics or heavy metals. One plasmid often contains ARGs against several antibiotics. A study performed by Gullberg et al. found that the concentration of antibiotics needed for the bacterial population to maintain a multi-resistance plasmid was almost 140-fold lower than the Minimum Inhibitory Concentration (MIC) value for a plasmid-free bacteria (Gullberg et al., 2014). This supports the significance of the antibiotic presence in the environment. These large conjugative plasmids often contain genes that are resistant to heavy metals and biocides. Selection for resistance against heavy metals can, therefore, often lead to a co-selection for resistance against both heavy metals and antibiotics. It has been suggested that both low concentrations of antibiotics and heavy metals in the environment can select for these resistance mechanisms (Gullberg et al., 2014). Other resistance mechanisms like efflux pumps can also confer resistance to both metal and antibiotics (Pal et al., 2017). Plasmid addiction or toxin-antitoxin systems may play a role in the maintenance of plasmid in their host organism. It will release toxins killing cells without the plasmids during replication, thereby ensuring the presence of the plasmid in the next generation even when the plasmid confers no fitness

advantage. In this way, plasmids with resistance genes can remain in the bacteria without selection pressure or the presence of antibiotic residues (Mnif et al., 2013).

2.3. Surveillance of resistance

An important global goal is to reduce the spread of resistance. The risk of transmission of antibiotic resistance to human-associated bacteria is the main concern to world health (Berendonk et al., 2015). The global databases lacks knowledge about the abundance of ARB and ARG in the environment, which is one of the major limitations for the development of the risk assessment (Berendonk et al., 2015).

Surveillance of environments with high bacterial diversity can give a great understanding of the AMR dynamics within the environment. One of these areas includes surface water (Keely et al., 2022). When investigating such an environment, microorganisms with significant health importance are of great interest, including ESBL- producing bacteria (WHO, 2021). According to the World Health Organisation, three sectors are especially important in this surveillance: humans, food systems, and the environment. Surveillance of ARB and ARG in the environment could be used to make a more efficient policy for preventing and combating this worldwide problem (UN, 2023). Examples of some good candidates for target ARGs are *blaTEM*, *blaCTX-M*, *blaVIM*, and *blaNDM* (Berendonk et al., 2015). The abundance of *blaCTX-M* is, among others, proposed to be a good indicator of multi-resistance in the environment (Tacão et al., 2014). During the last decade, the CTX-M type ESBL has taken over from SHV and TEM enzymes and become the most prevalent ESBL across the globe (Mnif et al., 2013).

There are several programs worldwide for monitoring the development of resistance in the clinical setting. The international surveillance in Europe is called the EARS-net, European Antimicrobial Resistance Surveillance. It aims to collect, analyse and report data on AMR from across countries in EU and EEA (ECDE, 2022). In Norway, the NORM surveillance program has been established to register and surveillance antibiotic resistance in human pathogens. The NORM-VET program monitors the antimicrobial resistance in food, feed and animals (VKM et al., 2022). There is still no national surveillance program for environmental resistance Norway today. This could be an interesting addition to better understanding the prevalence of resistance in the environment, and how this can affect the clinical setting. The establishment of a NORM-ECO program has been proposed, with the aim of gaining knowledge about resistance in the environment. It is believed this program may complement the veterinary and clinical data,

and give a broader understanding of the flow of resistance and help in risk assessments (VKM et al., 2022).

There are other measures to be made in addition to surveillance to reduce the development and spread of AMR. One of these is improving the use of antibiotics by decreasing the overuse and better diagnostics to prevent wrong and unnecessary use. Improvement of sanitation at a basic level will also reduce the need for antibiotics. Another measure is improving the removal of antibiotics and resistant bacteria from the wastewater. This can help reduce the opportunities for environmental selection of ARG and ARB, as well as reduce the risk of human exposure (Murray et al., 2021).

2.4. Development of new antibiotics

Great efforts to improve the activity and development of new antibiotics are in progress worldwide. Some of the fields of interest contain modifications of the chemical structure of already existing antibiotics and combinations of different antimicrobial agents. A combination of antimicrobials has been proven to restore bacterial susceptibility in some cases. This creates a synergistic effect that can improve the treatment's effectiveness. There are three different approaches when it comes to combination therapy: inhibition of a target in distinct pathways, using different mechanisms to inhibit the same target, and inhibition of different targets in one pathway (Pulingam et al., 2022**)**.

The use of inhibitors can reduce the effect of β-lactamases. Most inhibitors contain a β-lactam ring and inhibit the activity of β-lactamases by being hydrolysed, reducing their effect on the antibiotic. There are both irreversible and reversible inhibitors. Examples of these inhibitors are Clavulanate, Sulbactam and Tazobactam (Sridhar Rao, 2012). The development of new inhibitors to replace the ones that are no longer operative can also be an effective approach to improve the effect on resistant bacteria (Pulingam et al., 2022**)**.

The development of new antibiotics can potentially be a part of the solution to antibiotic resistance. There are several different approaches, including modification of old antibiotics, exploring uncultivated microorganisms and screening extracted DNA from environmental sources (Demain & Sanchez, 2009). An example is the discovery of Teixobactin, which was identified in 2015 by screening uncultured bacteria (Graham, 2017). Alternatives to antibiotics could also be the narrow-target activity of bacteriocins, peptides produced by bacteria to kill closely related species (Pircalabioru et al., 2021).

2.5. Methodological theory

2.5.1. Phenotypical detection of ESBL and CRE

There are several ways of detecting ESBL producing bacteria. One way is by using Brilliance™ ESBL agar, which is utilised for *in vitro* diagnostics of clinical samples. The agar plate is a chromogenic screening plate that can detect and make a presumptive identification of a range of β-lactamase producing bacteria. This includes *E. coli*, *Klebsiella*, *Serratia*, *Citrobacter* and *Enterobacter*. Brilliance™ ESBL agar can differentiate between different bacteria with the use of two chromogens that specifically target galactosidase and glucuronidase. The breakdown of these chromogens will lead to various colours, which can differentiate species. Antimicrobial agents, including Cefpodoxime, in the agars inhibit non-ESBL bacteria from growing (Oxoid, 2010).

The Brilliance™ CRE agar plates are similar to the ESBL plate but are utilized for screening of carbapenem resistance bacteria. These plates contain a modified Carbapenem and can distinguish between resistant *E.coli* and *Klebsiella/Serratia/Citrobacter* and resistant non-CRE species by a two-chromogen system (Oxoid, 2011).

2.5.2. Polymerase chain reaction - PCR

Polymerase chain reaction (PCR) is a technology designed to amplify a specific nucleic acid fragment. In a PCR reaction, the template DNA is exponentially amplified by a thermo stable polymerase. In addition to polymerase and template DNA, forward and reverse oligonucleotide primers, deoxynucleotide triphosphates (dNTPs) and buffer components are added. The forward and reverse primers are specific for the desired target sight. One PCR reaction starts with an initial denaturation, followed by a cycle containing three main steps, denaturation, annealing and extension. During denaturation, the temperature increases to about 95°C to denature the double-stranded DNA template and any potential secondary structures in the primers. The temperature decreases to about 50-60°C for the annealing step, where the primers hybridise to the target regions on the DNA template. During the extension step, the temperature increases to optimise the conditions for the DNA polymerase activity and extends the primers. The three steps are repeated 20-40 times, depending on the initial DNA concentration and the preferred result. The optimal temperatures during a PCR reaction depend on several factors, including the GC content of the DNA template and primers, the primer's melting point, the length of the wanted target fragment, the DNA polymerase and the DNA concentration (Wages, 2005).

Singleplex VS Multiplex PCR

Normal (*Singleplex)* PCR with one target region has two primers, one forward and one reverse. Multiplex PCR, on the other hand, has several target regions. This reaction works like normal PCR reactions, but there are several primer pairs involved. In this way, it will be possible to screen for several genes in the same reaction (Behind The Bench Staff, 2022). Multiplex PCR can be used as an effective and low-cost method to search for several commonly found β-lactamase genes in one PCR reaction (Dallenne et al., 2010).

Real-time PCR – qPCR

Real-time PCR (qPCR) is a qualitative and quantitative analysis. Similar to a normal PCR reaction, it can quantify the target DNA by using cycles of different temperatures, thermophile polymerase, primers and dNTPs. In qPCR, the product is measured throughout the reaction through the use of fluorescent labels. These labels can be specific or unspecific. The unspecific labels bind to all double-stranded DNA, while the specific type only binds to specific sequences. The concentration of fluorescent can be utilized to calculate the original amount of the template before the PCR reaction. At the end of the PCR cycle, a melting step can be added. In this step, the temperature increases to about 95°C to determine the temperature for the denaturation of the final PCR products. When the PCR product denaturises, it goes from double-stranded to single stranded, causing a drop in measured fluorescence. This will generate a melting curve, which can be utilized to compare the melting temperature of the different PCR products in the reaction. Doble stranded DNA melts at different temperatures, dependent on GC content, structure, length, and chemical formulation of the reaction (Sigma-Aldrich, 2008).

2.5.3. Sanger sequencing and Illumina sequencing (WGS)

Sanger sequencing is a chain-termination DNA sequencing method. The DNA gets amplified by the binding of primers and elongation by DNA-polymerase. This method utilizes both normal dNTPs and dideoxynucleoside triphosphates (ddNTPs) for DNA amplification. The ddNTPs are modified nucleotides that lack a hydroxyl group in the 3’ carbon. When added to a growing DNA strand, they will cause termination of the synthesis of the new DNA. The ddNTPs are also labelled with different fluorescent colours according to their attached base. The result of amplification will be a large amount of DNA fragments with different lengths. Electrophoresis is used to separate the different fragments by size, and a laser beam detects the fluorescent colour of the ddNTP. This produces a chromatogram which can be translated to the nucleotide sequence of the DNA (Willey et al., 2020, pp. 425-426).

Illumina is a Next Generation Sequencing (NGS) method and uses a sequencing by synthesis technique. Similar to the Sanger method, fluorescens is used for the sequencing process. In Illumina sequencing, the amplification of the DNA takes place on a flow cell, creating clusters of double-stranded DNA, each containing identical nucleotide sequences. The double strands are desaturated, leaving single-stranded fragments. Modified nucleotides with a fluorescent tag are incorporate to the complementary single strands. The fluorescent light is captured, and the tag get washed away, and a new modified dNTP is added. This continues, and the detection of the fluorescent light gets translated into a sequence of nucleotides (Willey et al., 2020, pp. 427-430).

2.6. Aim of study

The primary aim of this study was to explore and map the occurrence of resistance, the resistome, in aquatic environments in Ås and Nordre Follo municipalities in Norway. This study is part of an attempt to get a broader understanding of the resistance occurrence and flow and how it relocates from aquatic systems to animals and humans.

3 Method

Water samples were collected from three different locations, filtered, then propagated into two different media. Bacterial colonies were isolated followed by DNA extraction. The extracted DNA was used for 16S rRNA PCR, then sanger sequencing. It was also used for Multiplex and Singleplex PCR, along with whole genome sequencing by Illumina Miseq. Glycerol stocks were made with the isolated bacterial colonies and used for susceptibility testing. An outlie of the complete project is displayed in Figure 3.1.

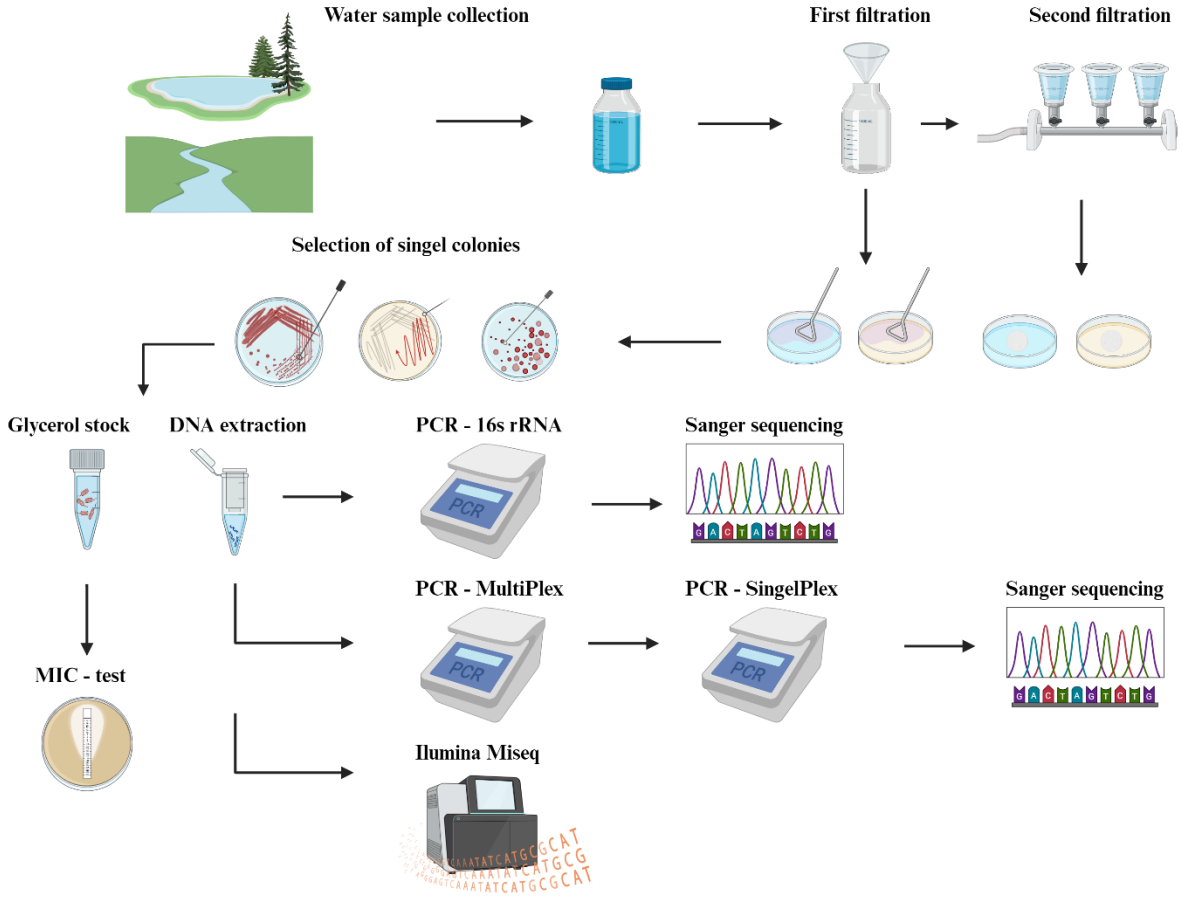


Figure 3.1: Flow chart over the experiment. Shows the different parts of the experiment from water sampling to filtration, selection of single colonies and DNA extraction. The extracted DNA was used for 16S rRNA PCR and Sanger sequencing, Multiplex PCR followed by Singleplex PCR and whole genome sequencing (WGS). Glycerol stocks were made and utilized for susceptibility testing. The figure was created with BioRender.com.

3.1. Water sample collection

Water samples were collected during winter and summer from three different locations in the county of Viken in Norway. There were two collections in the summer, 28. Of August and 11. Of September 2023 in addition to one in the winter 3. Of January 2024. The summer samples where gathered at three different locations W1, W2 and W3, as illustraded in Figure 3.2. Out of the 17 samples collcted during summer, five were from the first sampling, 1C_S_SY, 1E_S_KR, 2E_S_SY, 3E_S_MD and 4E_S_SY. The winter samples were collected at W2 and W3. Collection by Syverdudbekken (W1) was not possible due to snow and ice.

In each location, a total amount of 1 L water was collected in steril Schott Duran glass bottles. After collection, the samples were stored at 4°C for 24 hours. During summer sampling the temprature varied between 15°C and 18°C. It should be noted that there was approximately 50 mm of precipitation the day before the first sampling. This resultet in a high-water flow in the streams, which lead to more turbid water than normal. The temperature was -9°C during winter sampling.

Figure 3.2: Maps of the locations for water sampling. A) Syverdubekken close to Årungen lake. Coordinates for the sampling: 59.6876739°N, 10.7558418°W. B) Kråkstadelva in Kråkstad. Coordinates for the sampling: 59.6827812°N, 10.8893958°W. C) Midsjøvannet close to Ski. Coordinates for the sampling: 59.6827812°N, 10.8893958°W. Pictures retrieved from Norgeskart (Norgeskart.no, 2023).

Locations

The first location for water sampling (W1) was “Syverudbekken”, a small stream in Ås municipality. The samples were collected at the surface in a slower part of the stream, close to its outlet. This area contains a forest with a lot of organic material nearby. During the winter sampling, it was not possible to collect water due to ice and snow.

The second location (W2) was the “Kråkstadelva”, close to Kråkstad railway station in Nordre Follo municipality. This small river flows between fields and forests in cultural landscape. The water samples were collected close to the road and a residential area.

The third location (W3) was the “Midsjøvannet” in Nordre Follo municipality. This small lake is a nature reserve, with a rich wildlife surrounded by farmland (Forskrift om Midtsjøvann naturreservat, 1992). The water samples were collected at a public beach.

3.2. Preparations of water samples

The water samples were coarsely filtered to remove organic substances and debries, while allowing bacteria to pass through. Approximately 400 ml from each sample were filtered through sterile Whatman® filters (589/1 black ribbon ,150 mm diameter) into new steril Schott Duran glass bottles.

After the first filtration, a vacuum-filtration step followed with the Millipore™ Microfil Support Stainless Steel Frit MISP0002 machine (Merck , Darmstadt, Germany). Before use, the machine was cleaned by using the “Flameboy” tool (Integra Bioscineces, Zizers, Switzerland), and the Millipore machine was connected to the sink. EZ-pak® membranefilter with a pore size of 0,45 µm, (Merck KGaA, Darmstadt, Germany) was placed on each stand, before adding Microfil Filtration Funnels (Merck KGaA, Darmstadt, Germany). About 100 ml of the filtered water was added to the plastic funnel, and the valves on the millipore mashine were opend. Water passed through the filter while the existing bacteria were collected on the filter. This was repeated for all of the water samples. The filters were placed on Brilliance™ ESBL plates (Oxoid, Hampshire, United Kingdom) . The filtration step was repeated for all of the samples, and filters were placed on Brilliance™ CRE plates (Oxoid, Hampshire, United kingdom).

In addition to filters, 1 ml of each water sample from the first filtration step were incubated on both CRE and ESBL brilliance plates. The plates were left open to dry before incubation at 37°C. Recommended incubation time was 12-24 hours for CRE plates and 24 hours for ESBL plates (Oxoid, 2010, 2011). The plates were incubated for 48-96 hours, due to slow growth and few visible bacterial colonies after 24 hours.

3.3. Selection of bacterial colonies

Colonies were selected from the CRE and ESBL agar plates and transferred using a sterile inoculation loop. On the CRE and ESBL chromogenic plates different colours indicate different types of bacteria. Using the Oxiod manufacuring's colour quide, bacteria with pink, blue, yellow, green and brown colours were chosen from ESBL plates . From the CRE plates green, blue, red, pink, yellow and brown colour were selected (Oxoid, 2010, 2011). Singel colonies were again transffered and streaked onto new plates followed by incubation at 37°C, for 24 hours. This step was repeated 2-3 times to ensure no contamination of other bacteria and growth of one single bacterial strains. Once the bacterial strains were isolated, DNA was extracted and glycerol stocks were made for further analysis.

Glycerol stocks

Glycerol stocks were made by adding 1 ml of 17% glycerol solution to a cryotube. By the use of an inoculation loop, bacteria were collected directly from ESBL or CRE plates, and transferred to the glycerol solution. The samples were homogenised by pipetting the solution up and down several times. The cryotubes were stored at -80°C until further use.

3.4. DNA extraction

The DNeasy® PowerFood® kit (Quiagen, Hilden, Germany) was utilized for DNA extraction. The extraction was conducted according to the manufacturer's protocol with some exceptions (Qiagen, 2017).

Bacteria was collected directly from Brilliance ESBL and CRE petri dishes with an inoculation loop, and transferred to a 2 ml collection tube containing 1 ml Ringers solution. The mixture was homogenised by vortexing and pipetting up and down. The collection tube was centrifuged at maximum speed for one minute (23 238 x g). Supernatant was removed, and the DNA extraction continued at step 3 of the manufacturer's protocol. During the elution step, 50 µl EB elution buffer was used instead of 100 µl, and the samples were incubated at room temperature for 2 minutes before centrifugation.

The DNA extraction was followed by measuring of DNA quantity and quality by NanoDrop 2000 (Thermo Fisher scientific, Waltham, USA). The blanking solution was the EB elution buffer from DNeasy® PowerFood® DNA extortion kit. A total of 2 µl sample was loaded onto the machine for measuring. Extracted DNA was stored at -20°C until futher analysis.

3.5. Sanger sequencing

3.5.1. 16S amplification

Sanger sequencing was conducted to identify the isolated bacteria, by using the 16S rRNA region. 16S Polymerase chain reaction (PCR) was conducted to amplify the 16S region in the bacterial genome prior to Sanger sequencing. The Q5 Hot start High-Fidelity 2X MasterMix polymerase and protocol and was utilized (New England Biolabs, Ipswich, USA). The concentration and amount of added regents for the master mix was as according to the manufacturers suggestions and are described in Table 3.2. The universal forward and reverse primers 1F and 5R were utilized to amplify the 16S rRNA gene sequence, and is presented in Table 3.1. A total of 25 µl liquid was loaded into each PCR tube, contaning 24 µl master mix and 1 µl DNA. The PCR reaction was conducted on a 1000™ Thermal Cycler (Bio-Rad Laboratories Inc, California, USA) and the PCR program was followed as described in Table 3.3.

Table 3.1: Primer pair for 16S rRNA amplification. Shows the name of the primers, primer sequence and length of amplified product.

Primer	Primer-sequence (5'-3')	Length of product
Forward (1F)	GAGTTTGATCCTGGCTCAG	1505 bp
Reverse (5R)	GGTTACCTTGTTACGACTT	

Table 3.2: Reagents for PCR master mix for Q5 Hot start polymerase. Shows the final concentration and amount per reaction for each reagent.

Reagent	25 µl R X N	Final concentration
10 µM forward primer	1,25 µl	0,5 µM
10 µM reverse primer	1,25 µl	0,5 µM
DNA Template *	1 µl	< 1 ng
Q5 Hot start High-Fidelity 2x MasterMix	12,5 µl	1 x
Nuclease free water	9 µl	

Table 3.3: Program for the PCR-reaction of 16S amplification. Shows the temperature and duration of the different steps during the PCR reaction.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1
Denaturation	98 °C	10 seconds	
Annealing	55 °C	30 seconds	32
Extension	72 °C	42 seconds	
Final extension	72 °C	2 minutes	1
Hold	4°C	Infinite	

3.5.2. Agarose Gel electrophoresis

PCR reaction was followed by gel electrophoresis of the PCR product. This was conducted to ensure PCR product with the right length before Sanger sequencing.

A 1,5 % agarose gel was prepared by adding 3,15 g SeaKem® LE Agarose (Lonza Rockland Inc, Rockland, USA) to 120 ml 1X TAE buffer. The mixture was heated in the microwave until all agarose was dissolved, and cooled to 60°C. For each 10 µl TAE buffer, 0,5 µl SYBER® Safe DNA stain was added (Edvotek, Washington, USA). The gel mixture was transferred to a gel trey to solidify. The samples were prepared for application, by adding 2 µl PCR product, 2 µl Gel loading Dye Purple (6X), no SDS (New England Biolabs, Ipswich, USA) and 7 µl nuclease free water in a container. A total amount of 10 µl was applied in each well, in addition to one well with a 100 bp DNA Ladder (New England Biolabs, Ipswich, USA), diluted 1:1 with 1 X TAE buffer and a negative control. The negative control contained 2 µl nuclease free water instead of PCR product. The gel electrophoresis was performed at approximately 90V for 45 ml gels, and 120 V for 210 ml gel. Depending on the wanted separation and length of PCR product, the gel electrophoresis were performed for about 45 min to 1 hour and 30 minutes. Gels were visualised by the use of UV light with Molecular Imager® Gel Doc ™ XR Image system (Life science, Bio-Ras Laboratories Inc.).

3.5.3. PCR clean-up and preparation for sequencing

For PCR cleanup of the 16S PCR product, the GeneElute ™ PCR Clean-Up kit (Sigma-Aldrich, Spruce Street, USA) was utilized. The protocol was followed as described by the manufacturer. A total of 23 µl PCR-product and 115 µl of Bindings solution was used for each sample. This protocol was performed on the 16S PCR product before sanger sequencing.

Before the preparation of the samples for sequencing, Nanodrop was conducted to measure the concentration of DNA in the cleaned PCR products. For each sample, two 1,5 ml safe-lock tubes were prepared and marked with barcodes. In each container, 5 µl primer and 5 µl PCR product was added. The concentration of the primer was 5 µM and the concentration of the cleaned PCR product depended on the length of the PCR product. The 16S fragment were about 1505 bp long, thereby a concentration of 10 ng/µl PCR product was added. Reagents were diluted with nuclease free water to obtain the right concentration. For each sample, one tube with forward and one tube with reverse primer were sent to Eurofins in Germany for Sanger sequencing.

3.5.4. Processing of results from Sanger sequencing

BioEdit *Sequence Alignment Editor version 7.7.1* was utilized for analysing the Sanger sequencing results. The sequences were cleaned up by Eurofins, by removing parts of the beginning and ending of the sequence. The quality of the sequences was evaluated by visual inspection of the electropherograms for each sequence provided by Eurofins. The forward and reverse sequence for all samples were imported into the BioEdit program. Using the Accessory Application tool and CAP contig assembly program, a consensus sequence for each sample were assembled.

The consensus sequences were inserted into the Nucleotide Basic Logical Alignment Search Tool (BLASTn®) from the National Center for Biotechnology Information (NCBI). The sequences were compared to sequences in the Nucleotide collection (nr/nt) database.

3.6. Detection of resistance genes

3.6.1. Multiplex PCR

PCR with five different Multiplex primer solution mixes were conducted to screen for specific ESBL-genes. Multiplex PCR assay is an effective and low-cost method for screening and monitoring spread and emergence of β -lactamases. These Multiplex mixes contain two to three different primer pairs each, as described in Table 3.4 and Table 3.5 (Dallenne et al., 2010; Finton et al., 2020). The primer sequences are displayed in Appendix v.1.

Table 3.4: Overview of the different primers in the primer mixes. Shows the different primer pairs in each Multiplex mix.

Multiplex 1	**Multiplex 2**	**Multiplex 3**	**Multiplex 4**	**Multiplex 5 - *control***
blaCTX-M gr. 2	*blaCTX-M gr. 9*	*blaNDM*	*blaCMY*	*rpoB*
blaOXA	*blaCTX-M gr. 1*	*blaVIM*	*blaOXA-48*	*16S rRNA*
blaSHV	*blaTEM*	*blaKPC*	*blaIMP*	

Table 3.5: Overview of primers in Multiplex mixes. Shows the different primers, products and the reference.

Multiplex	**Primer pair**	**Reference**	**Comment**
Multiplex 1	*blaCTX-M gr. 2*	Dallenne et al., 2010	Class A ESBL
	blaOXA	Dallenne et al., 2010	blaOXA carbapenemase
	blaSHV	Dallenne et al., 2010	SHV ESBL, including SHV-1
Multiplex 2	*blaCTX-M gr. 9*	Dallenne et al., 2010	Class A ESBL
	blaCTX-M gr. 1	Dallenne et al., 2010	Class A ESBL
	blaTEM	Dallenne et al., 2010	ESBL, variants including both TEM-1 and TEM-2
Multiplex 3	*blaNDM*	Finton et al., 2020	New Delhi Metallo-β-Lactamase, carbapenemase
	blaVIM	Finton et al., 2020	Verpna Integron-Mediated Metallo-β-Lactamase, Carbapenemase
	blaKPC	Finton et al., 2020	*Klebsiella pneumoniae* carbapenemas (KPC)
Multiplex 4	*blaCMY*	Finton et al., 2020	Resistance to 3rd-generation cephalosporins
	blaOXA-48	Dallenne et al., 2010	OXA-48 Carbapenemase
	blaIMP	Finton et al., 2020	Metallo-β-Lactamase, confers resistance til many B-lactams
Multiplex 5 *control*	*rpoB*	Universal primers	Positive for Enterobacteriaceae DNA
	16S rRNA	Universal primers	16S RNA

The Quick-Start protocol from QIAGEN® Multiplex PCR kit (QIAGEN, Hilden, Germany) was followed. Half of the recommended amount of each reagent was used, with a total volume of 25 µl, as described in Table 3.6. The optional reagent "Q-solution" was not utilized. The PCR setup was as described in the manufacturer protocol and Table 3.7. The PCR reaction was conducted on a 1000™ Thermal Cycler (Bio-Rad Laboratories Inc, California, USA).

Table 3.6: Reagents for Multiplex PCR master mix. Shows the amount of each reagent per reaction. The proportion of DNA compared to the rest of the master mix was as suggested by Dallenne et al (Dallenne et al., 2010).

Reagents	25 µl R X N	Final concentration
2x QIAGEN Multiplex PCR Master Mix	12,5 µl	1x
Primer mix	2,5 µl	0,2 µM for each primer
DNA Template	1 µl	≤ 1µg DNA/reaction
Nuclease free water	9 µl	

Table 3.7: PCR program for Multiplex PCR. Shows the temperature and duration of the different steps during the PCR reaction.

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	15 minutes	1
Denaturation	94 °C	30 seconds	
Annealing	60 °C	1,5 minutes	35
Extension	72 °C	1,5 minutes	
Final extension	72 °C	10 minutes	1
Hold	4°C	Infiniti	

The positive control was an in-house strain of *Klebsiella pneumoniae*. This strain was only used for positive control for some of the genes of interest, as the genome only contained the *blaSHV*, *blaTEM*, *blaCTX-M gr.1* and *blaOXA* genes. These genes were confirmed by whole genome sequencing. The negative controls contained 1 µl nuclease free water instead of DNA.

Multiplex PCR was followed by agarose gel electrophoresis. This was conducted as described in chapter 3.5.2 "Agarose gel electrophoresis". Bands on the gel were compared to the 100 bp ladder to estimate the length of the PCR products. Bacterial strains with bands with a length close to the desired product sizes, were selected for Singleplex PCR.

3.6.2. Singleplex PCR

For amplification of specific areas by the use of one primer pair, the Q5 Hot Start High-Fidelity 2x MasterMix polymerase (New England Biolabs, Ipswich, USA) was used. The setup for reagents and the PCR reaction was the same as described in 3.5.1 "16S amplification", and Table 3.2 and Table 3.3.

Gel electrophoresis was conducted to visualise the product from Singleplex PCR. A 1,5% agarose gel was prepared, and gel electrophoresis was executed as described in section 3.5.2. The bands were compared to the 100 bp ladder to estimate the length of the PCR products.

Bands estimated to contain PCR product with the right length, were cut out of the gel. This was executed by increasing the amount of PCR product loaded onto the gel, to ensure a high enough DNA concentration for Sanger sequencing. For the *blaOXA-48, blaTEM* and *blaVIM* PCR product from the summer samples, 23 µl PCR product and 2 µl Gel Loading dye Purple (6X), no SDS (New England Biolabs, Ipswich, USA) with added 200 µl sucrose, was loaded onto the gel. Due to unspecific bands and smear with the use of larger volumes, the amount of PCR product was modified to 5 µl for the remaining samples, and the Gel Loading dye Purple was used without the added sucrose.

3.6.3. Clean-up of gel pieces and sequencing

After Gel electrophoresis, bands of interest were excised from the gel by visualising with UV light and cutting using a sterile scalpel. The NucleSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co, Düren, Germany) was used for gel clean-up. The protocol was followed as described in the protocol guides section 5.2, "DNA extraction from agarose gels", with some exceptions. In the first step, a total amount of 500 µl Buffer NTI was added to the tube, regardless of the mass of the gel piece. In the fourth step, "Dry silica membrane", the samples were centrifuged for 3 minutes instead of 1 minute. This was executed to ensure the proper drying of the membrane. In step 5 "Elute DNA", 15 µl Buffer NE was applied to the NucleoSpin® Gel and PCR Clean-up Column, and the column was incubated for 5 minutes before centrifugation at 11,000 x g. The elution step was repeated by applying the flow through to the column and centrifuge again at same speed to ensure optimal elution.

Before the preparation of the samples for sequencing, Nanodrop was conducted to measure the concentration of DNA in the cleaned PCR products.

In the samples containing *blaOXA-48, blaTEM* and *blaVIM*, the amount of cleaned PCR product sent to sequencing was calculated using the nanodrop values. The desired length of the PCR products varied from 281 bp to 800 bp, as a result, the concentration of the PCR products was determined to be 5 ng/µl. This concentration was obtained by diluting the PCR products with nuclease free water. For the remaining PCR products, a total amount of 8 µl sample and 2 µl primer was sent to sequencing. This was to ensure high enough concentration of the PCR product for good sequencing results. For each sample, one tube containing forward primer and PCR product were sent to sequencing by Eurofins, except for *blaOXA-48*. For these samples, two tubes per sample were sent, one with forward primer and PCR product, and one with reverse primer and PCR product.

3.6.4. Analysing sequencing results from detection of resistance genes

The consensus sequences of the sequencing results for the *blaOXA-48* were assembled with BioEdit in the same way as the 16S PCR samples, as described in section 3.5.4. The sequences were then inserted into the BLASTn tool from NCBI. For the remaining samples with only forward primer, the sequences were inserted directly into the BLASTn, without assembling a consensus sequence. The same Nucleotide collection (nr/nt) database was used for the BLASTn searches.

3.7. PCR troubleshooting and optimization

Prior studies using the same Multiplex mixes, showed only specific band on gels after PCR (Dallenne et al., 2010). Due to a large amount of unspecific bands in both Multiplex and Singleplex PCR products, several possible explanations were explored. The factores that were investigated included annealing temperature, concentration and age of primers, quality of DNA and type of polymerases.

Different annealing temperatures were tested to find an optimal temperature for the PCR reactions. For the Multiplex PCRs, firstly 60°C and 62°C were tested as suggested by Dallenne et al. (Dallenne et al., 2010). For the Singleplex PCRs, the optimal temperatures for the specific primers were calculator by the use of a Tm calculator from New England BioLabs (https://tmcalculator.neb.com/#!/main). The Q5 Hot start High-Fidelity 2X MasterMix polymerase (New England Biolabs, Ipswich, USA) was selected and primer sequence and concentration were inserted into the Tm calculator. The blaCTX-M gr.1 primers were selected for the testing of optimization, and the calculated optimal annealing temperatures was 66°C. Three different annealing temperatures were tested; 55°C, 63°C ,and 66°C.

When calculating the optimal annealing temperature, the Tm calculator (New England Biolabs) suggested a different concentration of primers. This was tested by repeating the Singleplex PCR reaction with blaCTX-M gr.9 primers, using half the concentration (0,25 µM).

The primers used for both the Multiplex PCR and the Singleplex PCRs were from last years students. The working solutions for these primers had been used several times, and had been exposed for repeated thawing followed by freezing. New primers were acquired and tested out and compared to the prior primers. The same polymerase, PCR set-up and program as described in 3.5.1 was utilized.

After DNA extraction, the quality was tested by Nanodrop. This showed an acceptable concentration an purity of the DNA. After preparing for several PCR reactions through this thesis, the DNA samples had been exposed to thawing followed by freezing several times. To test if this had degraded the DNA in the samples, DNA extraction was repeated. The bacteria were retrieved from the glycerol stocks, cultured on plates followed by DNA extraction. Multiplex PCR was conducted to look after a significant difference in the PCR results. This experiment was conducted by Eirunn Øvregaard Søyland.

Different polymerases were tested, the first beeing the Hemo KlenTaq® (New England Biolabs, Ipswich, USA). This polymerase was designed to tolerate temperatures up to 68°C, which was closer to the calculated Tm for the annealing temperature for blaCTX-M gr. 1 and blaCTX-M gr.9. Hemo KlenTaq® was tested on the same samples as Q5, at 63°C. The PCR program and master mix components are described in Appendix iii.1 and Appendix iii.2. The second polymerase tested was the iProof™ High Fidelity DNA Polymerase (Bio-Rad Laboratories Inc, Lithuania), a polymerase adapted to higher temratures. This polymerase was tested on samples with the blaCTX-M gr.1 primers with an annealing temperature at 63°C and 66°C, as described in Appendix iv.1 and Appendix iv.2.

3.8. MIC – Antibiotic susceptibility testing

Minimum inhibitory concentration testing was conducted to test selected bacterial species for resistance to different antibiotics. From the summer samples, 6 bacterial strains were chosen for testing. Isolated from ESBL plates, *Serratia fonticola* (1E_S_KR), *Pseudomonas aeruginosa* (5E_S_SY) (8E_S_MD) and *Shewanella sp.* (3C_S_KR), *Chitinophaga sp.* (4C_S_KR) and *Aeromonas sp.* (5C_S_KR) from CRE plates were selected. From the winter samples, *Pseudomonas indoloxydans* (13E_S_MD) and *Pseudomonas siliginis* (14E_S_SY) isolated from ESBL plates, and *Herbaspirillum huttiense* (7C_W_MD), *Pandoraea sp.*

(8C_W_KR) and *Cohnella sp.* (10C_W_MD) isolate from CRE plates were chosen. These selections were based on results in BLASTn from the 16S sequencing and results from Multiplex and Singleplex PCR.

The MIC protocol was established based on guidelines for disk diffusion methods for antimicrobial susceptibility testing proposed by EUCAST (EUCAST, 2023). The bacterial species of interest were re-plated on new ESBL and CRE plates using glycerol stocks and a sterile inoculation loop. After 24 hours incubation at 37°C, solutions of bacteria were prepared. Sterile inoculation loops were used to transfer bacteria directly from ESBL or CRE plates into a tube containing 10 ml ringer solution. The solutions were homogenised by pipetting up and down and vortexing. A concentration of 1-2 x 10^8 CFU/ml was obtained by using a counting chamber (Marienfeld superior, Lauda-Königshofen, Germany). Laborlux K Microscope (Lecia Microsystems, Wetzlar, Germany) was utilized to count the number of cells in the B square in the counting chamber. A magnification of 400X was used, and five different B squares were counted, and the mean was calculated to find the CFU in the solution. The suspensions were spread evenly on a Müller-Hinton (MH) agar plate by the use of a sterile cotton swab. The swab was dipped into the cell suspension, and streaked evenly across the plate, the plate was rotated 90 degrease and the swab was streaked over the plate again, as explained in Figure 3.3. This was repeated to ensure a confluent lawn of growth. In the case of *Cohnella sp.* (10C_W_MD), there were not sufficient growth on the Müller-Hinton agar plate, and PCA agar was utilized.

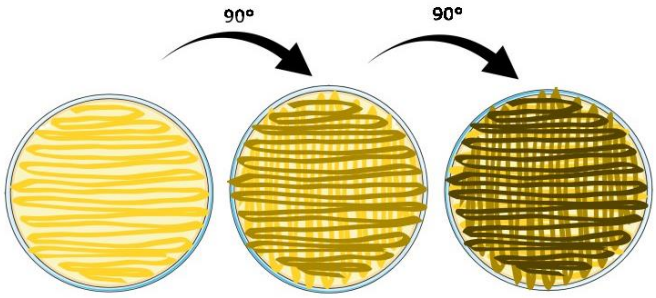


Figure 3.3: MIC- inoculation. Shows how the cell suspensions were streaked onto the MH-plates to secure a confluent lawn of growth. Figure made by "Inkscape. Draw freely." version 1.3.

The plates were left to dry in a fume hood for 5-10 minutes before MIC-test strips (Liofilchem, Roseto delgi Anruzzi, Italy) were applied in the middle of each petri dish using a sterile tweezer. Each of the 11 selected strains were tested for 7 different antibiotics, as described in Table 3.8. The plates were incubated at 37°C for 24 hours, and the minimum inhibition values were observed and recorded.

Table 3.8: The classes of antibiotics and concentrations used. Overview over the different antibiotics and classes of antibiotics used during the MIC-testing. Shows the different concentrations on the MIC-strips for each antibiotic strips.

Class of antibiotics	Antibiotic	Concentrations (µg/mL)
Penicillin	Ampicillin (AMP)	0,016 - 256
	Penicillin G (P)	0,016 - 256
Carbapenem	Meropenem (MRP)	0,002 - 32
Cephalosporin	Cefepime (FEP)	0,002 - 32
	Cefotaxime (CTX)	0,016 - 256
Fluoroquinolone	Ciprofloxacin (CIP)	0,002 - 32
Trimethoprim	Trimethoprim (TM)	0,002 - 32

3.9. Whole genome sequencing (WGS)

Three bacterial strains were selected for whole genome sequencing (WGS), *Chitinophaga sp.* (4C_S_KR), *Pandoraea sp.* (8C_W_KR) and *P. aeruginosa* (5E_S_SY). *Chitinophaga sp.* was collected from the Kråkstadelva during the second summer sampling. *Pandoraea sp.* was retrieved at the same location, sampled during the winter. *P. aeruginosa* was isolated from water samples from Syverudbekken from the second summer sampling. The selection of bacterial strains for WGS was based on the results from Multiplex and Singleplex PCR, the BLASTn results from 16S sequencing and the MIC results.

3.9.1. Preparations for whole genome sequencing

Prior to whole genome sequencing, the selected strains were retrieved from the glycerol stocks, and replated onto CRE and ESBL plates followed by DNA extraction. The DNA extraction was performed as described in section 3.4.

Nanodrop and Qubit were conducted to ensure a high enough DNA quality and concentration. Nanodrop was performed as described in section 3.4. The Qubit™ 1X dsDNA HS Assay Kit (Invitrogen™ Life Technologies Corporation, Eugene, USA) was utilized as according to the manufactures protocol. For each sample, 197 µl working solution and 3µl DNA was mixed and incubated for 2 minutes at room temperature. The samples were measured using a Qubit® 2.0 Fluorometer (Thermo Fisher scientific, Waltham, USA). A total amunt of 30 µl of high qualty DNA from each isolate was sendt to Invitrogen in England for Illumina Miseq.

3.9.2. Processing results from whole genome sequencing

The reads from the WGS Illumina Miseq were oppoaded to the Galaxy platform (https://usegalaxy.eu/). Forward and reverse reads were assembbled to "contigs", using the "Shovill Faster SPAdes assemblly for Illumina reads" tool (Seemann, 2017), and the "Trim reads" option was selected using the "Trimmomatic" program. The contigs-files were scanned in the ABRicate program (Seemann, 2016) against three different databases, "The Comprehensive Antibiotic Resistance Database" (CARD), "Bacterial Antimicrobial Resistance Reference Gene Database" (NCBI) and "The Virulence Factor Database" (VFDB). This program scans the contigs to detect antibiotic resistance or virulence genes. The "PROKKA" tool was used for the annotation of possible genes in the assembly data (Cuccuru et al., 2014; Seemann, 2014). The gbk genebank results from PROKKA were screened for genes contaning "Lactamases", "resistance" or "virulence ". The protein sequence of the enzymes of interest weere inserted into BLASTp and searched through NCBI. The selected database was "Non-redundant protein sequence (nr)" and the chosen organism was "bacteria (taxid:2)".

To confirm the 16S rRNA results from the Sanger sequencing, the contig sequences were submitted to the Ribosomal Multilocus sequence typing (rMLST) tool by Public databases for molecular typing and microbiaal genome diversity (PubMLST) (Jolley et al., 2018). This was preformed for the three samples that were whole genome sequenced.

The contig files were further analyzed by the use of several tools from "Center for Genomic Epidemiology" (https://www.genomicepidemiology.org/). "ResFinder" was utilized to search for resistance genes and point mutations that can cause resistance for all three species. The "PathogenFinder" tool was used on all three bacteria to predict the possibility of pathogenicity towards human hosts. A "MGE" toll was also utilized for 5E_S_Sy and 8C_W_KR to search for mobile genetic elements with relation to virulence factors for antimicrobial species. Some of the tools were only utilized on 5E_S_SY *P. aeruginosa,* including "PAst", "MLST" and "KmerResistance". The "PAst" tool was used to find the serotype for the *P. aeruginosa*. The "MLST" tool classified the species by comparing it to 7 different housekeeping genes. The "KmerResistance" looked for acquired resistance genes by using Kmers.

3.10. Real-time PCR

Real-time PCR (qPCR) was conducted to look for differences in the three *blaOXA-48* products. The three samples tested were 3C_S_KR *Shewanella sp*., 4C_S_KR *Chitinophaga sp*. and 1E_S_KR *S. fonticola*. To test the qPCR with different primers, three reactions were performed with the same qPCR program, described in Table 3.11 and Table 3.12. The PowerUpTM SYBRTM Green Master Mix (Thermo Fisher scientific, Waltham, USA) was used for all reactions. One of the three reactions used the Multiplex 4 primer mix and DNA as the template. The amount of each reagent is described in Table 3.10. The other two reactions used the forward and reverse blaOXA-48 primers. One used the extracted DNA as template while the other utilized cleaned Singleplex PCR product from the PCR reaction with blaOXA-48 primers as template. The reagents for the qPCR reactions using the blaOXA-48 primers are described in Table 3.9. The qPCR machine utilized was the LightCycler®480 II from Roche (Roche diagnostics, Basel, Switzerland).

Table 3.9: Reagents for qPCR Singleplex master mix. Shows the different reagents and their respective volumes at three different total volumes.

Reagents	X 1	X 8	X 3
PowerUp^{TM} polymerase	5 µl	40 µl	15 µl
Primer bla_{OXA-48} R	0,5 µl	4 µl	1,5 µl
Primer bla_{OXA-48} F	0,5 µl	4 µl	1,5 µl
DNA/Cleaned PCR product	1 µl	8 µl	6 µl
Water	3 µl	24 µl	6 µl
Total	*10* µl	*80* µl	*30* µl

Table 3.10: Reagents for qPCR Multiplex master mix. Shows the "Table 2 Standard cycling mode (primer $T_m \geq 60°C$)" setup from the manufacturers protocol.

Reagents	X 1	X 8	X 3
PowerUp^{TM} polymerase	5 µl	40 µl	15 µl
Primer (Multi 4)	1 µl	8 µl	3 µl
Primer F	0,5 µl	4 µl	1,5 µl
DNA	1 µl	8 µl	6 µl
Water	3 µl	24 µl	6 µl
Total	*10* µl	*80* µl	*30* µl

Table 3.11: Program for the qPCR-reaction. Shows the temperature and duration of the different steps during the PCR reaction.

Step	Temperature	Time	Cycles
UDG activation	50 °C	2 minutes	1
Activation	95 °C	2 minutes	1
Denaturation	95 °C	15 seconds	40
Annealing/Extend	60 °C	1 minut	

Table 3.12: Overview of dissociation curve conditions for qPCR. Shows the "Table 4 Dissociation curve conditions (melt curve stages)" table from the manufacturers protocol.

Step	Ramp rate	Temperature	Time
1	1,6°C/Second	95 °C	15 seconds
2	1,6°C/Second	60 °C	1 minute
3[1]	0,15°C/Second	95 °C	15 seconds

4 Results

4.1. Phenotypical detection

Isolation of bacterial strains was conducted on water samples from three different locations, Syverudbekken, Kråkstadelva and Midsjøvannet. A total number of 27 different colonies were selected from the chromogenic Oxoid Brilliance™ CRE and ESBL plates, 13 and 14 colonies, respectively. The colonies were selected based on the colour of the agar plates as well as their ability to grow independently. DNA was extracted and sequenced from all the isolated samples, and the sequencing results are displayed in Table 4.1. All 27 isolates are presented in the table, including origin for each sample, the season of sampling, the chromogenic agar plate used, and the top BLASTn results from the 16S Sanger sequencing.

Table 4.1: Results from the isolation of bacterial colonies and 16S Sanger sequencing. The table shows the origin of each sample, the location for water sampling, the season the samples were collected, and from which agar plate the colony was isolated. These three variables are also included in the bacterial ID. The colours in the table represent the colour of the samples when isolated on the chromogenic agar plates. The species collum presented in the table have the highest scored or most occurring results when using BLASTn in NCBI. The sequence similarity for the BLASTn is also presented.

W1: Syverudbekken (SY), W2: Kråkstadelva (KR), W3: Midsjøvannet (MD)

CRE/ ESBL	Summer/ Winter	Bacterial ID	Location	Colour	Species	Sequence Similarity
CRE	Summer	1C_S_SY	W1	Green	*Stenotrophomonas sp.*	99,65%
		2C_S_SY	W1	Blue	*Raoultella ornithinolytica*	99,58%
		3C_S_KR*	W2	Yellow	*Shewanella sp.*	99,79%
		4C_S_KR *	W2	Green	*Chitinophaga sp.*	98,16%
		5C_S_KR *	W2	Blue	*Aeromonas sp.*	99,86%
		6C_S_SY	W1	Blue	*Pseudomonas boreopolis*	99,37%
	Winter	7C_W_MD*	W3	Red	*Herbaspirillum huttiense*	99,79%
		8C_W_KR*	W2	Yellow	*Pandoraea sp.*	99,79%
		9C_W_KR	W2	Yellow	*Pandoraea pulmonicola*	99,86%
		10C_W_MD*	W3	Blue	*Cohnella sp.*	99,03%
		11C_W_MD	W3	Pink	*Herbaspirillum frisingense*	99,79%
		12C_W_MD	W3	Pink	*Herbaspirillum huttiense*	99,65%
		13C_W_MD	W3	Red	*Herbaspirillum frisingense*	99,65%
ESBL	Summer	1E_S_KR*	W2	Blue-Green	*Serratia fonticola*	99,86 %
		2E_S_SY	W1	Blue-Green	*Serratia fonticola*	99,79%
		3E_S_MD	W3	Pink	*Bordetella sp / Kerstersia sp..*	99,37%
		4E_S_SY**	W1	Green	*Bordetella sp. / Kerstersia sp.*	-
		5E_S_SY*	W1	Brown	*Pseudomonas aeruginosa*	99,72%
		6E_S_KR	W2	Blue-Green	*Serratia fonticola*	99,93%
		7E_S_SY	W1	Brown	*Pseudomonas aeruginosa*	99,72%
		8E_S_MD*	W3	Brown	*Pseudomonas aeruginosa*	99,93%
		9E_S_MD	W3	Blue-Green	*Serratia fonticola*	99,93%
		10E_S_SY	W1	Pink	*Bordetella sp./ Kerstersia sp.*	99,44%
		11E_S_KR	W2	Pink	*Bordetella sp./ Kerstersia gyiorum*	98,1%
	Winter	12E_W_KR	W2	Yellow	*Pseudomonas siliginis*	99,79%
		13E_W_KR*	W2	Yellow	*Pseudomonas indoloxydans*	99,79%
		14E_W_MD*	W3	Yellow	*Pseudomonas siliginis*	99,86%

*Selected for MIC- antimicrobial susceptibility testing.

** Low sequence quality

There were seven bacterial isolates with a high sequence similarity to species in the *Pseudomonas* genus. This included 6C_S_SY, 5E_S_SY, 7E_S_SY, 8E_S_MD, 12E_W_KR, 13E_W_KR and 14_W_MD, with a sequence similarity of ≥ 99,37%. All the *Pseudomonas* species isolated from ESBL plates had a yellow or brown colour, these colours were not described by the manufacturer colour guide. One *Pseudomonas* sample was isolated from a CRE plate, 6C_S_SY, and displayed a blue colour. Out of the seven *Pseudomonas* samples, three were identified as *Pseudomonas aeruginosa.* Four different *Serratia fonticola* species were detected, all with a blue-green colour as indicated by the ESBL chromogenic guide. These included 1E_S_KR, 2E_S_SY, 6E_S_KR and 9E_S_MD. For some of the isolates from the ESBL plates, there were not possible to distinguish between two different genera, the *Bordetella* and *Kerstersia* genus. These isolates, 3E_S_MD, 4E_S_SY, 10E_S_SY and 11E_S_KR, all demonstrated a pink colour on the agar plates with the exception of 4E_S_SY. The 4E_S_SY isolate had a green colour and demonstrated a low-quality result from the 16S rRNA Sanger sequencing.

From the CRE plates, the four *Herbaspirillum* species detected, 7C_W_MD,11C_W_MD, 12C_W_MD and 13_W_MD all showed a pink colour. The isolates identified as *Pandoraea* demonstrated a yellow colour, with 8C_W_KR and 9C_W_KR. In addition to the previously mentioned genus, six others were identified from isolates form the CRE plates, including *Stenotrophomonas, Raoultella, Shewanella, Chitinophaga, Aeromonas* and *Cohnella,* 1C_S_SY, 2C_S_Y, 3C_S_KR, 4C_S_KR, 5C_S_KR, 10C_W_MD respectively. They demonstrated a green (*Stenotrophomonas* and *Chitinophaga*), blue (*Raoultella, Aeromonas* and *Chonella*) and yellow *(Shewanella)* colours, none of which were defined by the CRE chromogenic guide. All had a high sequence similarity of ≥ 99,03 %.

4.2. PCR optimization

Due to poor results from the visualization of the PCR product on agarose gel, several factors affecting the PCR were tested. This included temperature, primer concentration, age of primers and quality of DNA.

Temperature

Firstly, two different annealing temperatures were tested for Multiplex 2 PCR, 60°C and 62°C, as displayed in Figure 4.1.

A) Multiplex 2: 60°C *B) Multiplex 2: 62°C*

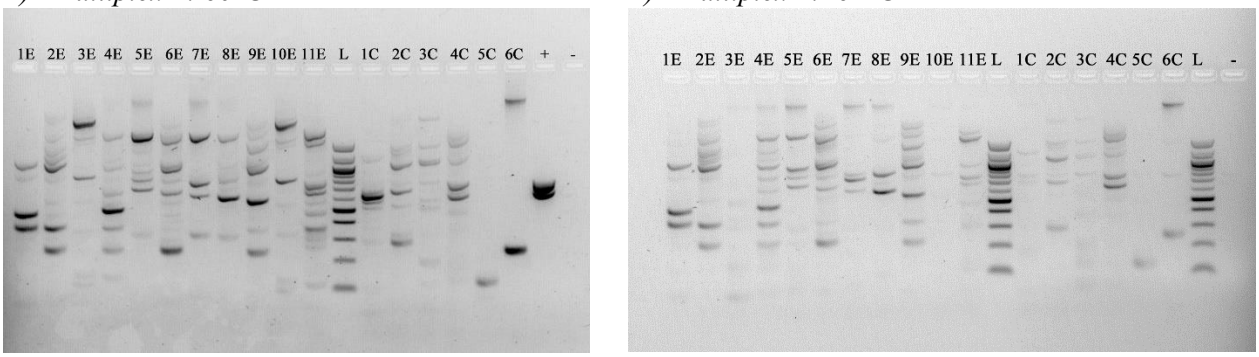


Figure 4.1: PCR optimization with different annealing temperatures – Multiplex 2. A) Shows Multiplex 2 PCR results on gel electrophoresis. The annealing temperature was 60°C. B) Shows the Multiplex 2 PCR reaction results with an annealing temperature of 62°C.

Figure 4.1 shows the difference between two PCR reactions containing the same reagents and using the same PCR program with an exception in annealing temperature. Agarose gels for both temperatures revealed several different PCR products for each sample. When comparing the two gels, there were no significant difference in the number of unspecific bands.

Other temperatures were tried out to find the optimal annealing temperature for Singleplex PCR reactions. The three annealing temperatures 55°C, 63°C and 66°C were tested, and are visualized in Figure 4.2.

A) Singleplex CTX-M gr.1: 55°C *B) Singleplex CTX-M gr.1: 63°C* *C) Singleplex CTX-M gr.1: 66°C*

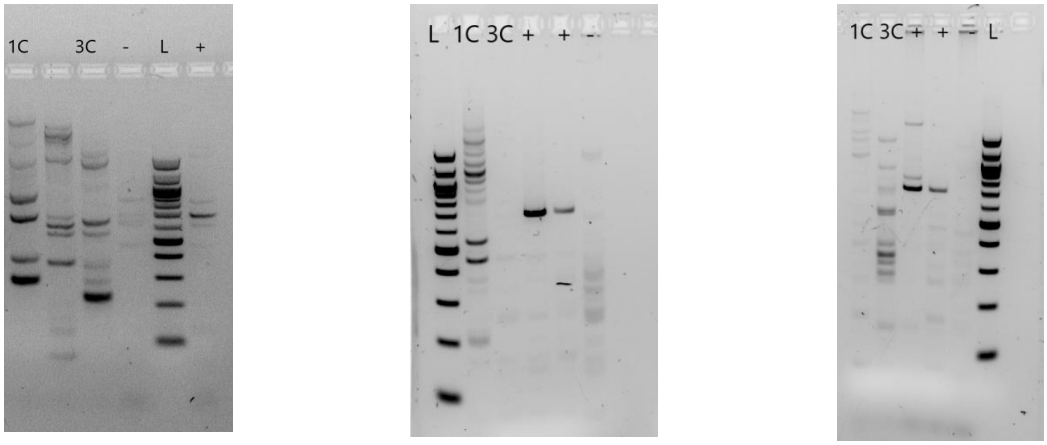


Figure 4.2: PCR optimization with different annealing temperatures - Singleplex. A) Shows the Singleplex PCR results on gel electrophoresis with the CTX-M gr.1 primers. The annealing temperature was 55°C. B) Shows Singleplex PCR products with CTX-M gr.1 primers at 63°C. C) Shows Singleplex PCR results with CTX-M gr.1 primers at 66°C.

Figure 4.2 illustrates the PCR products from three PCR reactions with different annealing temperatures. All three reactions used the blaCTX-M gr.1 primer pair. To illustrate a possible difference, two samples were chosen for the PCR reactions, 1C_S_SY and 3C_S_KR. Figure

4.2 A) had an annealing temperature of 55°C, B) 63°C and C) 66°C. It should be noted that the negative control in all three gels had some vague and low saturated bands, which questions the credibility of these results.

Sample 1C_S_SY had a large number of unspecific bands for all three temperatures. Figure A) demonstrated a clear visible band with the same length as the positive control for 1C_S_SY, while the higher temperature lacked a band with this length. In Figure C), the saturation of the bands was fainter for 1C_S_SY compared to the two other temperatures. For sample 3C_S_KR, both PCR products from 55°C and 66°C had an abundance of unspecific bands in addition to a well saturated band with the same length as the positive control.

Primers

In addition to temperature, two different concentrations of primers were investigated. The same PCR reactions were conducted with a difference in primer concentration, one reaction with 0,5 µM and the other 0,25 µM. For this experiment, the blaCTX-M gr.9 primer pairs were utilized. Different ages of the primers were tested by conducting two identical PCR reactions, one with old and one with new primers. After visualisation by gel electrophoresis, there was no visual difference in the amount of unspecific PCR products for neither primer concentration nor age.

DNA quality

The matter of DNA quality was examined by executing a new DNA extraction for an assortment of bacteria. The chosen bacteria were retrieved from glycerol stock and grown on selective media, followed by DNA extraction and PCR. The PCR product was visualised on gel electrophoresis and compared with previous gels and showed no reduction in the number of unspecific bands.

Polymerase

Three different polymerases were tested, including Q5 Hot start High-Fidelity 2X MasterMix Polymerase (New England Biolabs, Ipswich, USA), Hemo KlenTaq® (New England Biolabs, Ipswich, USA) and iProof™ High Fidelity DNA Polymerase (Bio-Rad Laboratories Inc, Lithuania). The Hemo KlenTaq® polymerase was tested using the blaCTX-M gr.1 and blaCTX-M gr.9 primer pairs. The PCR product was visualized on gels and showed few visible bands and a large amount of smear, but demonstrated clear and visible ladders. The results from the iProof™ High Fidelity DNA polymerases show no bands and no ladder.

4.3. PCR identification of resistance genes

Multiplex PCR was followed by visualization of products with agarose gel. A total of five different Multiplex PCRs were conducted, each containing two or three primer pairs, as explained in Table 3.4. All the gels from Multiplex PCR are presented in Appendix ii.2. After visual confirmation by agarose gels, samples with a band in the desired length were chosen for Singleplex PCR. The Singleplex PCR products were visualised on agarose gels, as seen in Appendix ii.3. Visible bands of the desired length were excised from the gel, cleaned and sent to Sanger sequencing. A summary of all sequenced bands is presented in Table 4.2.

Table 4.2: Bands chosen for Sanger sequencing. Shows which bands were excised from the gel, cleaned, and sent to sequencing. A total of 26 bands were chosen, using six different primers: blaCTX-M gr.9, blaCTX-M gr.1, blaTEM, blaVIM, blaKPC and blaOXA-48. Six of the bands were confirmed as resistance genes after BLASTn search in NCBI (+), the remaining samples were not identified as resistance genes (-). Some of the samples had insufficient sequencing quality (-*).

Bacterial ID	Multiplex 2			Multiplex 3		Multiplex 4
	CTX-M gr.9	CTX-M gr.1	TEM	VIM	KPC	OXA-48
1E_S_KR			+			+
2E_S_SY	-*	-	+			
3E_S_MD			-			
4E_S_SY		-				
5E_S_SY			-			
6E_S_KR			-			
9E_S_MD			+			
12E_W_KR		-				
13E_W_KR	-			-		
14E_W_MD	-*	-*				
2C_S_SY		-			-*	
3C_S_KR				-*		+
4C_S_KR		-*				+
7C_W_MD	-					
8C_W_KR	-					
11C_W_MD	-					
12C_W_MD	-					
13C_W_MD	-					

* Low-quality sequencing

After visual inspection of the PCR product on agarose gels, 26 bands were sent to sanger sequencing. All of the sequenced bands from blaCTX-M gr.9, blaCTX-M gr.1, blaVIM and blaKPC showed no resemblance to their respective gene products when searched in NCBI. A total of six of the sequenced bands revealed low sequencing quality.

From the blaTEM PCR products, six bands were chosen for sequencing. Out of these six, three were revealed to have a high sequence similarity to BLASTn results containing a TEM-type enzyme. These three belonged to 1E_S_KR, 2E_S_SY and 9E_S_MD, all identified as *S. fonticola* by 16S sequencing. All the bands chosen for sequencing from blaOXA-48 PCR revealed a high similarity to sequences containing *blaOXA-48* genes. These three sequences belonging to 1E_S_KR, 3C_S_KR and 4C_S_KR. The BLASTn results for the six samples with high similarity to TEM and OXA-48 enzymes are presented in Table 4.3.

Table 4.3: BLASTn results for blaOXA-48 and blaTEM PCR products. Shows the top results after BLASTn in NCBI with the consensus sequence for blaOXA-48 and blaTEM sequences.

Genes	Bacterial ID	Species	Sequence Similarity	BLASTn Results
blaOXA-48	1E_S_KR	*S. fonticola*	99,65 %	*Citrobacter freundii* strain CF2 plasmid pCF2_OXA48, complete sequence
	3C_S_KR	*Shewanella sp.*	99,29 %	*Citrobacter freundii* strain CF2 plasmid pCF2_OXA48, complete sequence
	4C_S_KR	*Chitinophaga sp.*	99.29 %	*Citrobacter freundii* strain CF2 plasmid pCF2_OXA48, complete sequence
blaTEM	1E_S_KR	*S. fonticola*	99,48 %	*Shigella sonnei blaTEM-1* gene for extended-spectrum beta-lactamase, partial cds, strain: *S. sonnei*-w9
	2E_S_SY	*S. fonticola*	96,88 %	*Acinetobacter baumannii* 12KPTEM *blaTEM-1* gene for class A extended-spectrum beta-lactamase, partial seq
	9E_S_MD	*S. fonticola*	99,87 %	*Escherichia coli strain* SKGH_24 beta-lactamase (*blaTEM*) gene, partial cds

There were six consensus sequences that contained a high sequence identity to resistance genes in the NCBI database. 1E_S_KR, 3C_S_KR and 4C_S_KR were collected in the Kråkstadelva (W2) and had a sequence similarity of $\geq$ 99,29 % to *blaOXA-48* genes. These genes were located at a pCF2 plasmid from *Citerobacter freundii* strain CF2.

The other three samples had a high sequence similarity to TEM-containing sequences. For 1E_S_KR and 2E_S_SY, the genes were specifically described as *blaTEM-1* in the NCBI database, while the results for 9E_S_MD was as a *blaTEM* gene. The BLASTn results showed a sequence similarity of 99,48 %, 96,88% and 99,87%, respectively. The three strains were collected at different locations, 1E_S_KR was collected in the Kråkstadelva (W2), 2E_S_SY was from Syverudbekken (W1) and 9E_S_MD from Midsjøvannet (W3). The matched sequences in the database belong to *Shigella sonnei, Acinetobacter baumannii* and *Escherichia coli*.

4.3.1. Real-Time PCR - OXA-48

In order to further examine the blaOXA-48 results, three real-time PCR reactions were conducted. The real-time PCR melting curve results with the blaOXA-48 primers and Singleplex PCR product templates is visualised in Figure 4.3

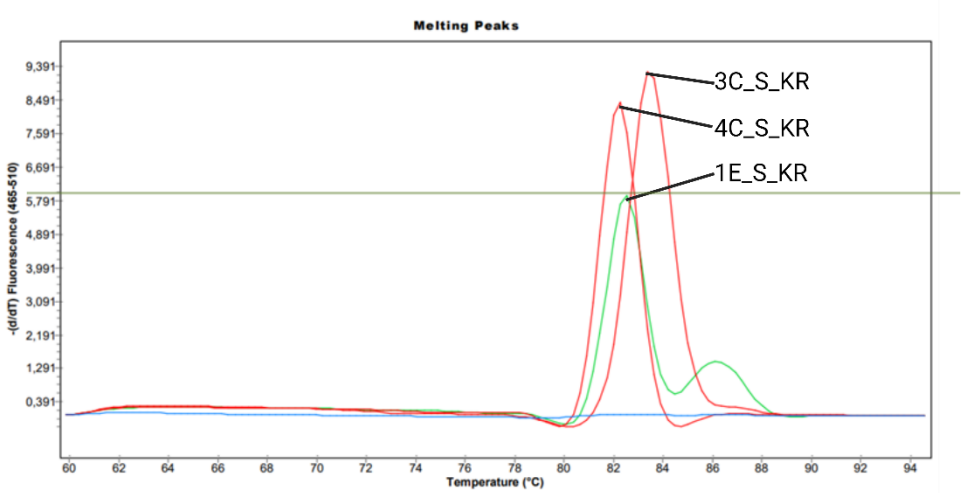


Figure 4.3: Real-time PCR results for blaOXA-48. Shows the melting temperature (x) and fluorescence (y) of the three samples containing the *blaOXA-48* gene sequence, *S. fonticola* (1E_S_KR), *Shewanella sp.* (3C_S_KR) and *Chitinophaga sp*. (4C_S_KR). This real-time PCR reaction used the blaOXA-48 primers and the Singleplex bloaOXA-48 PCR products as template.

The product from the real-time PCR shows a slight difference in melting temperatures between the three samples. The melting temperature for 4C_S_KR *Chitinophaga sp.* was approximately 82°C, for 1E_S_KR *S. fonticola,* the temperature was closer to 83°C. The third sample, 3C_S_KR *Shewanella sp.* had a melting temperature closer to 84°C. The y-axis shows that 3C_S_KR had the highest fluorescence concentration out of the three.

4.4. Antimicrobial susceptibility testing

The susceptibility to seven different antibiotics were tested for an assortment of bacteria. The chosen bacteria were 1E_S_KR *S. fonticola*, 5E_S_SY *P. aeruginosa*, 8E_S_MD *P. aeruginosa*, 13E_W_KR *Pseudomonas indoloxydans*, 14E_W_MD *Pseudomonas siliginis,* 3C_S_KR *Shewanella sp.*, 4C_S_KR *Chitinophaga sp.*, 5C_S_KR *Aeromonas sp.*, 7C_W_MD *Herbaspirillum huttiense*, 8C_W_KR *Pandoraea sp.* and 10C_W_MD *Cohnella sp.*. Susceptibility was tested by applying Minimum Inhibitory Concentration (MIC) test strips on to MH-agar plates with a lawn of growth of the selected bacteria. Due to insufficient growth, PCA agar was used for the 10_W_MD *Cohnella sp.* isolate. The MIC test was performed twice for each strain, and the mean MIC values were calculated and are displayed in Table 4.4. The MIC and Epidemiological cut-off values (ECOFF) used for the determination of resistance are retrieved from EUCAST and are presented in Table 4.5.

Table 4.4: Antimicrobial susceptibility testing. Shows the mean MIC values (µl/mL) for the 11 tested bacterial isolates with seven different antibiotics.

Antibiotics	1E_ S_KR	5E_ S_SY	8E_ S_KR	13E_ W_KR	14E_ W_MD	3C_ S_KR	4C_ S_KR	5C_ S_KR	7C_ W_MD	8C_ W_KR	10C_ W_MD
Ampicillin	≥256	≥256	≥256	≥256	≥256	2,5	≥256	192*	8	≥256	2
Cefepime	0,048	0,5	0,75	0,12	3	0,023	≥32	0,142	0,032	3,5	0,625
Cefotaxime	1	12	16	2	48	0,035	72	0,38	0,12	3	0,25
Ciprofloxacin	0,0175	0,19	0,079	0,01	0,055	1,5	2,25	1,75	0,315	1	0,22
Meropenem	0,04	0,16	0,064	0,023	0,75	0,38	1,88	0,02	0,19	≥32	1,25
Penicillin G	≥256	≥256	≥256	≥256	≥256	8	72	≥256	3	≥256	1,25
Trimethoprim	0,38	≥32	≥32	≥32	≥32	≥32	0,69	16,75*	1,125	≥32	≥32

* No inhibition was registered during the second repetition of the susceptibility testing.
Red numbers: values higher than ECOFF or MIC values retrieved from EUCAST.

Table 4.5: MIC- values and ECOFF values. Shows the MIC and ECOFF values used to determine resistance. All values are retrieved from EUCAST and are explained in Appendix vii.2. All the numbers in the table are MIC-values, with the exception of the values for 5E_S_SY and 8E_S_KR, which demonstrates ECOFF values.

Antibiotics	1E_ S_KR	5E_ S_SY	8E_ S_KR	13E_ W_KR	14E_ W_MD	3C_ S_KR	4C_ S_KR	5C_ S_KR	7C_ W_MD	8C_ W_KR	10C_ W_MD
Ampicillin	S ≤ 8 > R	-	-	-	-	8	8	8	8	8	0,5
Cefepime	S ≤ 1, 4 > R	16*	16*	S≤0,001 8 > R	S≤0,001 8 > R	NA	NA	S ≤ 1 4 > R	NA	NA	NA
Cefotaxime	S ≤ 1, 2 > R	64*	64*	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5
Ciprofloxacin	S ≤ 0,25 0,5 > R	0,5*	0,5*	S≤ 0,001 0,5<R	S≤ 0,001 0,5<R	0,25	0,25	S ≤0,25 0,5 > R	0,25	0,25	0,5
Meropenem	S ≤ 2 8 > R	2*	2*	S≤ 2 2<R	S≤ 2 2<R	2	2	2	2	2	2
Penicillin G	-	-	-	-	-	0,5	0,5	0,5	0,5	0,5	0,25
Trimethoprim	S ≤ 4 > R	-	-	-	-	NA	NA	NA	NA	NA	NA

*ECOFF values.
NA: no values were found.

As displayed in Table 4.4, the strain demonstrating the highest amount of resistance was 8C_W_KR *Pandoraea sp.* This strain had MIC values higher than the values established for gram negative bacteria by EUCAST for five antibiotics. These included Ampicillin, Cefotaxime, Ciprofloxacin, Meropenem and Penicillin G, and thereby defining *Pandoraea sp.* as clinically multidrug resistant. The MIC value for Cefepime was also high, with a value of 3,5 µg/mL. In addition, no inhibition was detected for Trimethoprim.

The two *P. aeruginosa* strains tested had both higher MIC values for Ampicillin, Penicillin G and Trimethoprim than the registered ECOFF values, classifying them as epidemiological resistant. The other *Pseudomonas* strains were clinically resistant against Ampicillin, Cefotaxime, Penicillin G and Trimethoprim, with higher registered MIC values than the provided breakpoints.

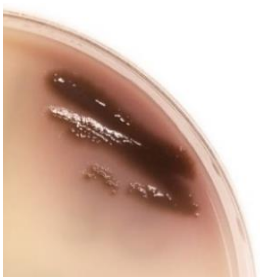
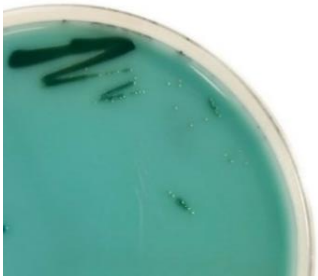
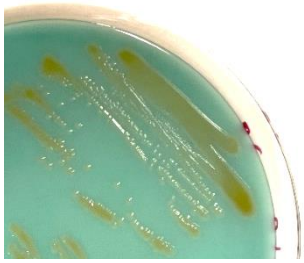
The three strains 3C_S_KR *Shewanella sp,* 4C_S_KR *Chitinophaga sp*. and 5C_S_KR *Aeromonas sp*. were less susceptible to Ampicillin, Ciprofloxacin and Penicillin G, with MIC values higher than proposed values by ECUAST. The three samples 1E_S_KR *S. fonticola*, 10C_W_MD *Cohnella sp*. and 10_W_MD *H. huttiense* were resistant to Penicillin G in addition to Ampicillin (1E_S_KR and 7C_W_MD) and Ciprofloxacin (7C_W_MD).

4.5. Whole Genome Sequencing

4.5.1. Identification of species, serotype and sequence type

Three bacterial strains were chosen for Illumina MiSeq whole genome sequencing to further investigate their resistance and virulence genes. Galaxy was used to assemble the reads to contigs, and an assembly quality assessment was performed using the "Quast" tool. The quality assembly assessments are presented in Appendix viii.1. The genus and species were identified by using the MLST tool by PubMLST. Sample 4C_S_KR was identified as *Chitinophaga silvatica* (100%), 5E_S_SY was defined as *Pseudomonas aeruginosa* (100%), while 8C_W_KR was identified as *Pandoraea sp.* with 84% support for *Pandoraea nosoerga,* as shown in Table 4.6.

Table 4.6: Pictures of the three WGS bacteria. Shows the bacterial ID, species determination by 16S sequencing and WGS, and the colour of the colonies. The table also shows the sequence type and serotype for the *P. aeruginosa* isolate.

	Isolates		
Bacterial ID	5E_S_SY	4C_S_KR	8C_W_KR
16S rRNA	*P. aeruginosa* (99,72 %)	*Chitinophaga sp.* (98,16 %)	*Pandoraea sp*. (99,79%)
WGS	*P. aeruginosa* (100 %)	*Chitinophaga silvatica* (100 %)	*Pandoraea sp.* (84 %)
Sequence type	ST 3436	-	-
Serotype	O5	-	-
Colour	Brown	Green	Yellow
Picture			

The serotype for 5E_S_SY *P. aeruginosa* was determined to be the O5 serotype group, having a value of 98 %. The O5 serotype group includes serotypes O5, O18 and O20. The sequence type was ST3436, with 100% similarity to all seven housekeeping genes tested. It was not possible to use the "PAst" and "MLST" tools for the *C. silvatica* and *Pandoraea sp.* isolates. The *P. aeruginosa* isolate was predicted to be a human pathogen at a probability level of 0,711 by "PathogenFinder". *Pandoraea sp.* was predicted to have a human pathogen probability of 0,383.

The "MGE" tool in Galaxy was utilized to search for mobile genetic elements in the samples. No mobile elements were found in 4C_S_KR *C. silvatica*. In 5E_S_SY *P. aeruginosa*, four mobile elements were detected, three IS-elements and one ICE-element. The ISPa6 IS-element detected contained a *fosA* gene conferring resistance to Fosfomycin. One IS-element was found in 8C_W_KR *Pandoraea sp.*, with no connection to a resistance gene. All the MGEs results are presented in Appendix x.1.

4.5.2. Detection of resistance genes

Several tools were utilized to screen for resistance genes, including the NCBI and CARD databases, the "ResFinder" tool and searching the annotated genes from PROKKA. The genes conferring antibiotic resistance annotated by PROKKA were searched with the BLASTp tool using the "non-redundant protein sequence (nr)" database. An assortment of the discovered resistance genes are presented in Table 4.7. All the resistance genes are presented in Appendix xi.1 and Appendix xi.2.

Table 4.7: Detected resistance genes. Shows the resistance genes identified by ResFinder, NCBI and CARD and annotated by PROKKA. The annotated genes were searched through BLASTp and the "non-redundant protein sequence (nr)" database. Alignment of the β-lactamase genes are presented in Appendix ix.1, Appendix ix.2 and Appendix ix.3.

Resistance genes	Database	Identity (%)	Query coverage (%)	Accession number	Comment
4C_S_KR *C. silvatica*					
bla	nr*	67,92	100	WP_167016720.1	Class D β-lactamases
bla	nr*	89,89	100	WP_245950823.1	Class D β-lactamases
5E_S_SY *P. aeruginosa*					
aph(3')-IIb	NCBI	98,51	100	NG_047424.1	Aminoglycoside O-phosphotransferase, Kanamycin resistance
blaPDC-202	NCBI	99,83	100	NG_054987.1	Class C β-lactamase, Cephalosporin resistance
blaPAO	ResFinder	97,57	-	AY083592	Class C β -lactamase
blaOXA-50	NCBI**	98,73	100	NG_049777.1	Class D β-lactamase, OXA-50
fosA	NCBI**	98,04	100	NG_047883.1	Fosfomycin resistance
catB7	NCBI	97.65	100.00	NG_047614.1	Chloramphenicol O-acetyltransferase, Chloramphenicol resistance
8C_W_KR *Pandoraea sp.*					
ceoB	CARD	81,78	99,25	U97042:1263-4347	CeoAB-OpcM efflux pump
blaOXA-158	NCBI**	99,30	100	NG_049457.1	Class D β -lactamase OXA-158-like, OXA-62 family, Resistance to Amoxicillin, Ampicillin, Imipenem, Meropenem and Piperacillin
ampC	nr*	99,27	100	WP_287496540.1	PNC family class C β-lactamase

*The gene was annotated by PROKKA and found in NCBI using BLASTp and the "non-redundant protein sequence (nr)" database.

** Genes found in both NCBI and CARD, displaying the NCBI search results.

All three sequence bacteria contained at least two β-Lactamases, as illustrated by Table 4.7. No resistance genes were detected in 4C_S_KR *Pandoraea sp.* by using the NCBI or CARD databases. However, PROKKA annotated two *bla* genes. These genes showed a 67,92% and 89,89% sequence identity to class D β-Lactamases. According to PROKKA, these were *blaOXA-2* and *blaOXA-10* genes respectively. In 8C_W_KR *C. silvatica*, the same *blaOXA-158*-like gene was detected by NCBI, CARD and ResFinder with a sequence identity of 99,30% in NCBI. This gene belonged to Amblers class D β-Lactamases and the OXA-62 family, and according to ResFinder, it can confer resistance to Amoxicillin, Ampicillin, Imipenem, Meropenem and Piperacillin. PROKKA also detected an *ampC* gene in *C. silvatica*. It belongs to the PNC family of the class C β-Lactamases and had a sequence identity of 99,27%. Three β-Lactamases were detected in 5E_S_SY *P. aeruginosa*, two of which belonged to class C β-Lactamases and one to class D. The class D gene, *blaOXA-50,* was detected by the NCBI and CARD databases and had a sequence identity of 98,73% in NCBI. The two class C β-Lactamase genes were *blaPDC-202* and *blaPAO,* found by NCBI and ResFinder, respectively. They had a sequence similarity of 99,83% and 97,57 %.

In addition to β-Lactamases, one other resistance gene was discovered in 8C_W_KR *C. silvatica*, *ceoB*. The *ceoB* had a sequence identity of 81,78% and belongs to the CeoAB-OpxM efflux pump system. For 5E_S_SY *P. aeruginosa*, an additional three genes were detected by NCBI, *aph(3')-IIb*, *fosA* and *catB7*. The *aph(3')-IIb* gene is an aminoglycoside O-phosphotransferase and confers resistance to Kanamycin. The *fosA* provides resistance to Fosfomycin and *catB7* is a Chloramphenicol O-acetyltransferase providing Chloramphenicol resistance.

4.5.3. Virulence genes

Genes related to virulence factors were detected by searching the VFDB database and the annotated genes by PROKKA. A selection of virulence factors for 5E_S_SY *P. aeruginosa* and 8C_W_KR *C. silvatica* are presented in Table 4.8. No virulence genes were detected by VFDB for 4C_S_KR *Pandoraea sp.* Alle virulence genes detected for 5E_S_SY and 8C_W_KR are presented in the Appendix.

Table 4.8: Detected virulence genes. Shows the detected genes in VFDB for 5E_S_SY *P. aeruginosa* and 8C_W_KR *C. silvatica*.

Virulence genes	Database	Identity (%)	Query coverage (%)	Accession number	Comment
5E_S_SY *P. aeruginosa*					
alg44	VFDB	99,74	100	NP_252232	Alginate
alg8		99,60	100	NP_252231	
algA		99.38	100	NP_252241	
algB		98.81	100	NP_254170	
algC		100.00	10	NP_254009	
algD		99.08	100	NP_252230	
algE		99.19	100	NP_252234	
algF		99.08	100	NP_252240	
algG		99.20	100	NP_252235	
algI		99.42	100	NP_252238	
algJ		99.75	100	NP_252239	
algK		99.23	100	NP_252233	
algL		99.09	100	NP_252237	
algP/algR3		96,76	98,87	NP_253940	
algQ		98.76	100	NP_253942	
algR		99.47	100	NP_253948	
algU		99.83	100	NP_249453	
algW		99.14	100	NP_253136	
algX		99.30	100	NP_252236	
algZ		99.54	100	NP_253949	
lasA	VFDB	97.61	100	NP_250562	Protease precursor
lasB		98.73	100	NP_252413	
lasI		98.84	100	NP_250123	
plcH	VFDB	99.22	100	NP_249535	Hemolytic phospholipase
plcN	nr*	100	100	WCV80199.1	C precursor
toxA	*Not detected*				Exotoxin A synthesis
toxR	nr*	100	100	WP_003120800.1	Transcriptional regulator
aprA	VFDB	99,31	100	NP_249940	Alkaline metalloproteinase precursor
exoS	VFDB	91.25	100	NP_252530	Type III secretion system
exoT		98.11	100	NP_248734	effector/regulator
exoU	*Not detected*				
exoY	*Not detected*				
exlA	*Not detected*				
Exotoxin A (PEA)	*Not detected*				
lip1	VFDB	99.57	100	NP_248770	Lipoprotein
lipA	*Not detected*				
loxA	*Not detected*				
Leukocidin	*Not detected*				
Pyocyanin (PCN)	*Not detected*				
8C_W_KR *Pandoraea sp.*					
cheB	VFDB	99,74	100	YP_109897	Chemotaxis-specific methylesterase, Flagella
cheW	VFDB	9960	100	YP_109901	Chemotaxis protein, Flagella

*The gene was annotated by PROKKA and found in NCBI using BLASTp and the "non-redundant protein sequence (nr)" database.

The VFDB database found two genes related to virulence in 8C_W_KR *C. silvatica*, *cheB* and *cheW*. They both are involved in the chemotaxis of the bacterium. In 5E_S_SY *P. aeruginosa*, a large amount of virulence genes were detected. Many different *alg* genes were discovered, which are genes affecting different parts of the production and function of alginate, an acetylated polymer. Different precursors were detected, including protease precursors, *las* genes, and alkaline metalloproteinase precursor, *aprA*. Two genes connected to type III secretion systems were discovered by VFDB, *exoS* and *exoT*.

4.5.4. Metal resistance genes

Genes conferring metal resistance were detected by searching through the annotated genes from PROKKA for the three whole genome sequence bacteria. The discovered metal resistance genes are presented in Table 4.9.

Table 4.9: Discovered metal resistance. Shows different genes conferring metal resistance, which were annotated by PROKKA in 5E_S_SY, 4C_S_KR and 8C_W_KR.

Gene	Product
5E_S_SY *P. aeruginosa*	
ars	Arsenic resistance
arsB	Arsenical pump membrane protein
arsB	Arsenical pump membrane protein
cntO	Metal-pseudopaline receptor
copA	Copper resistance protein
czc	Cobalt-zinc-cadmium resistance protein
czcD	Metal cation efflux system protein
feiF	Ferrous-iron efflux pump
fosB	Metallothiol transferase
ftsH	ATP-dependent zinc metalloprotease
impA	Immunomodulating metalloprotease
merR1	Mercuric resistance
mntH	Divalent metal cation transporter
mntP	Putative manganese efflux pump
pmbA	Metalloprotease
tldD	Metalloprotease
ycaL	Metalloprotease
ycfH	putative metal-dependent hydrolase
yciC	Putative metal chaperone
yjjV	putative metal-dependent hydrolase
yycJ	Putative metallo-hydrolase
4C_S_KR *C. silvatica*	
arsR	Arsenical resistance operon repressor
cnrB	Nickel and cobalt resistance protein
cntO	Metal-pseudopaline receptor
czc	Cobalt-zinc-cadmium resistance protein
ftsH	ATP-dependent zinc metalloprotease
mntH	Divalent metal cation transporter
pmbA	Metalloprotease
tldD	Metalloprotease
yciC	Putative metal chaperone
yfiT	Putative metal-dependent hydrolase
yycJ	Putative metallo-hydrolase
8C_W_KR *Pandoraea sp.*	
acr3	Arsenical-resistance protein
atm1	ATM1-type heavy metal exporter
cntO	Metal-pseudopaline receptor
czc	Cobalt-zinc-cadmium resistance protein
ftsH	ATP-dependent zinc metalloprotease
mntH	Divalent metal cation transporter
pmbA	Metalloprotease
tldD	Metalloprotease
ycfH	putative metal-dependent hydrolase
yciC	Putative metal chaperone
yjjV	putative metal-dependent hydrolase
yycJ	Putative metallo-hydrolase

Several genes conferring resistance to metals were discovered, as described in Table 4.9. All three bacterium contain the *cnc* gene, which can confer resistance to cobalt, zinc and cadmium. In 5E_S_SY *P. aeruginosa* and 4C_S_KR *Pandoraea sp.*, an arsenical resistance gene was discovered, *ars*. A *copA* and a *mer* gene were also discovered in 5E_S_SY and can confer copper resistance and arsenic resistance, respectively. In addition, the 4C_S_KR contained a *cnr* gene, conferring nickel and cobalt resistance.

4.5.5. Multidrug Efflux Pumps

Genes related to multidrug efflux pumps and other multidrug resistance transporters were found by screening the annotated genes from PROKKA. The genes found in 5E_S_SY *P. aeruginosa*, 4C_S_KR *Pandoraea sp.* and 8C_W_KR *C. silvatica* are presented in Table 4.10.

Table 4.10: Discovered efflux pumps. Shows different genes related to different Multidrug efflux pumps and different transporters annotated by PROKKA in 5E_S_SY, 4C_S_KR and 8C_W_KR.

Gene	Product
5E_S_SY – *P. aeruginosa*	
acr	Multidrug efflux
bmrA	Multidrug resistance ABC transporter
emr	Multidrug export protein
mdt	Multidrug resistance protein
mex	Multidrug efflux system
oqxB4	Multidrug efflux RND transporter
stp	Multidrug resistance protein
4C_S_KR – *Chitinophaga silvatica*	
emrB	Multidrug export protein
imrA	Multidrug resistance ABC transporter
mdt	Multidrug resistance protein
mex	Multidrug efflux system
stp	Multidrug resistance protein
ybh	Multidrug ABC transporter
yhel	Multidrug resistance ABC transporter
8C_W_KR – *Pandoraea sp.*	
acrB	Multidrug efflux pump
bmr3	Multidrug resistance protein
emr	Multidrug export protein
mdf	Multidrug transporter
mdt	RND-type drug exporter, Multidrug resistance protein
mep	Multidrug export protein
mex	Multidrug efflux system
norM	Multidrug resistance protein, Multidrug efflux pump
opXB27	Multidrug efflux RND transporter
ybhR	Putative Multidrug ABC transporter

All three bacteria contained several multidrug efflux system genes, including *mdt* and *mex* genes. Several other multidrug efflux systems, Multidrug RND transporters and ABC transporters were detected in all samples as demonstrated in Table 4.10.

5 Discussion

5.1. Phenotypical detection

This experiment used Brilliance™ ESBL and CRE plates to screen for antibiotic resistant bacteria in water samples collected in Nordre Follo and Ås municipalities. A total of 27 different bacterial strains were isolated and selected.

The CRE plates were designed for rapid and easy identification of Carbapenem-resistant *Enterobacteriaceae* (CRE) colonies. They facilitate a more targeted treatment and enable early detection, thereby contributing to avoiding the spread of pathogens and resistance genes. The plates contain a modified Carbapenem and chromogenic chemicals to differentiate between *E. coli*, in a pink colour, and *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC) with a blue colour (Oxoid, 2011). The KESC-group are common pathogenic bacteria in hospitals (Gupta et al., 2021; Hansen et al., 2004; Liu et al., 2018; Zhou et al., 2017). None of the colonies isolated from CRE plates in this study were designed for these plates, being environmental bacteria. It is therefore difficult to draw any conclusions about the genera of the isolates based only on colour. This identification of genera by colour will probably work better in clinical settings with fewer environmental bacteria.

The ESBL plates can detect Extended Spectrum β-lactamase producing bacteria and can differentiate between Enterobacteriaceae and non-*Enterobacteriaceae*. The plates contain Cefpodoxime and other antibacterial agents to select for only ESBL producers. It was also developed for and by clinical samples, similar to the CRE plates. It contains two chromogens, helping differentiate between *E.coli* with blue and pink colours and bacteria in the KESC group with a green colour. Non-*Enterobacteriaceae* are supposed to be displayed as colourless colonies (Oxoid, 2010).

In this study, all species in the *Bordetella* or *Kerstersia* genus demonstrated a pink or green colour, which should have been colourless according to the colour guide. The samples in the *Serratia* genus had a blue-green colour, as expected, and are also more common in hospital settings. The *Pseudomonas* species had a brown or yellow colour. In a study by Huang et al. this colour was also observed for *Pseudomonas* species. Huang et al. explored the sensitivity of the ESBL plates regarding colour determination and ESBL-producing bacteria. They found a high sensitivity to both, but tested only clinically important strains (Huang et al., 2010). Even

though these selective chromogenic plates can detect resistance phenotypes, these results imply a tendency for higher colour specificity in clinical samples compared to environmental samples.

The ESBL plates, are effective in identifying bacteria with resistance mechanisms that makes them able to grow on the media. However, it is challenging to be certain that the observed resistance to the antibiotics is caused by ESBL producing bacteria and not other resistance mechanisms. A genotypic investigation is necessary to determine which mechanisms are responsible for the observed resistance.

5.2. 16S rRNA identification

The Sanger sequencing of the 16S PCR amplifications, revealed the presence of several bacterial genera in the aquatic environments tested. The most occurring genus was *Pseudomonas,* with seven different isolates. Several bacterial isolates belonging to the *S. fonticola* species were also discovered. Other genera discovered were *Bordetella/Kerstersia, Herbaspirillum, Pandoraea, Stenotrophomonas, Raoultella, Shewanella, Chitinophaga, Aeromonas* and *Cohnella*. The WGS indicated 16S sequencing to be an accurate estimation of bacterial genus or species. However, the use of 16S had difficulties differentiating between the *Bordetella* and *Kerstersia* genera. These discoveries demonstrated a presence of phenotypic antibiotic resistance in a wide variety of genera.

5.3. Genotypical detection

5.3.1. The *blaOXA-48*-like and *blaTEM* resistance genes

During the PCR screening, six resistance genes were detected and confirmed with Sanger sequencing. Three of the detected genes had a high sequence similarity to a *blaTEM* gene, all belonging to *S. fonticola* isolates. The TEM β-lactamases became widespread during the 1990s and the beginning of the 2000s and is still a commonly occurring allele in bacterial populations (Dallenne et al., 2010; Mroczkowska & Barlow, 2008). The *blaTEM* gene is suggested to be a good candidate for an indication gene for resistance in the environment (Berendonk et al., 2015).

The three remaining genes were assumed to be *blaOXA-48* and were revealed as *blaOXA-48-like* genes by Sanger sequencing. The OXA enzymes have a high mutation rate and harbours a high spectrum of activity (Codjoe & Donkor, 2017). One of the bacteria harbouring this gene was the 4C_S_KR *C. silvatica*, the two others were *Shewanella sp.* and *S. fonticola*. Interestingly, Tanner et al. found a *Shewanella* species isolated from drinking water contain a *blaOXA-48* type carbapenemas (Tanner et al., 2019). When screening for resistance genes in *C.*

silvatica, two *blaOXA* genes were annotated by PROKKA, one probably being the *blaOXA* gene detected by PCR screening. The two genes were defined as *blaOXA-2* and *blaOXA-10* by PROKKA. All six genes were detected in bacteria isolated from Kråkstadelva (W2). This indicates the presence of both class A and class D β -lactamases in Kråkstadelva in several different bacterial genera.

The three OXA sequences were also tested by real-time PCR to look for differences in melting temperatures. This revealed a slight difference in melting temperature, which shows that it is possible to differentiate between the sequences using real-time PCR. Nevertheless, the sequences amplified by the blaOXA-48 primers are only a part of the gene sequence and thus not represent the entirety of the sequence.

Another point addressing the specificity of the primers is the resistance genes detected. Huang et al. demonstrated that out of the ESBL producing bacteria, the CTX-M types are the most occurring and most prevalent ESBL's across the globe (Cantón & Coque, 2006; Huang et al., 2010; Mnif et al., 2013). In this study, no *blaCTX-M* genes were discovered, but the absence of detection does not necessarily signify that they were not present. There is a possibility that the genes were present in the isolated bacteria, but the primers failed to detect them in the PCR reaction. These results demonstrate the specificity of the primers used on environmental isolates. They were only specific enough to identify six genes rightfully, by the use of the blaOXA-48 and blaTEM primers.

5.3.2. Troubleshooting for PCR reactions

Quite a few bands with the expected size were observed through the PCR screening, in addition to a large number of unspecific bands. The low number of discovered resistance genes was odd in light of the low susceptibility shown in the MIC testing. The majority of the isolates were non-susceptible to at least one antibiotic. There can be several explanations for the low occurrence of resistance genes, including other resistance mechanisms or the production of other β-lactamases then tested for. Another reason may be faults in the PCR setup.

Different factors that could affect the PCR results were tested to optimize the PCR protocol. Several different annealing temperatures were assessed to look for differences in the number of unspecific bands. New primers were tested due to previous applications by former students, which led to continuous thawing and freezing. Two different primer concentrations were also examined, owing to a suggestion by the Tm calculator from New England Biolabs. By visual

inspection of the PCR results on agarose gels, there was no indication of a difference in the number of unspecific bands for any of the factors tested.

In addition, three different polymerases were utilized to search for an improvement in the PCR product. The results from using iProof™High Fidelity DNA Polymerase and Hemo KlenTaq® Polymerase showed no improvement from the Q5 Hot Start High-Fidelity polymerase outcomes. The 5Q Hot Start Polymerase was therefore used for the remainder of the experiment. Ideally, the different polymerases should have been tested several times, but this was not executed due to limited time and resources. The last factor tested was the DNA quality, where DNA extraction was repeated with no improvement in results. After exploring several possible reasons for the high number of unspecific bands, no conclusion could be reached but indicated that another factor probably contributed to these results.

5.3.3. Suggestion for optimization of PCR reactions

There are several challenges when using PCR-based methods to screen for specific genes, such as sequence conservation. If the target sequence has changed too substantially, the primers will not be able to amplify this sequence. Another problem is false positives by nonspecific amplification (Grenni, 2022). Most approaches for detecting resistance genes and resistant bacteria are based on comprehensive studies of clinically significant bacteria (VKM et al., 2022). This makes these methods less ideal when studying environmental bacteria, as demonstrated in this study.

The Multiplex mixes used in this experiment were designed and assembled by Dallenne et al. and Finton et al. Several well-known β-lactamase-producing Enterobacteriaceae strains were used as references, the majority isolated from hospitalized patients (Dallenne et al., 2010; Finton et al., 2020). The designed primers would thereby be adapted for use in clinical samples. In this study, there were a large amount of unspecific genes when using both Singleplex and Multiplex primers. Interestingly, there were no unspecific bands when testing the PCR setup with the positive control (*K. pneumoniae*). The *K. pneumoniae* is a known clinical bacterium and has been researched thoroughly (Wyres et al., 2020). The low abundance of unspecific bands indicates that the primers lack of specificity to environmental samples may be the reason for the large number of unspecific bands. It should be mentioned that as a commonly found bacteria in clinical settings, *P. aeruginosa* isolates, should have less unspecific bands than other environmental bacteria, but no such parallel was observed (Thi et al., 2020).

For further research, it would be favourable to design new primers based on environmental bacteria. This could increase the specificity and potentially reduce the number of unspecific PCR products, making it easier for further research.

5.4. Whole genome sequencing

From the isolated bacteria, three were chosen for whole genome sequencing. These three were the 4C_S_KR, 5E_S_SY and 8C_W_KR isolates, and were selected based on the 16S sequencing results, the Multi and Singleplex PCRs and the susceptibility testing.

The 4C_S_KR isolate was identified to belong to the *Chitinophaga* genus by 16S sequencing. This genus has mainly been identified in environmental settings but have in some rare cases caused infection in humans (Crémet et al., 2009; Tran et al., 2020). The main reason for selecting this bacterium for WGS was the discovery of a *blaOXA-48-like* gene by Singleplex PCR screening. The 5E_S_SY isolate was defined as *P. aeruginosa* by 16S sequencing. This species is known to be an opportunistic pathogenic bacterium and is known to contain several antibiotic resistance mechanisms (Asfeldt et al., 2023; Breidenstein et al., 2011). In addition to being a species of interest, the susceptibility testing showed a low or non susceptibility to several antibiotics. The 8C_W_KR isolate was identified as *Pandoraea sp.* by 16S sequencing. The isolate belongs to a genus which was relatively recently discovered, and is a common environmental bacterium (Coenye et al., 2000). However, this bacterium demonstrated the highest resistance of all the tested isolates and was therefore interesting to investigate further.

5.4.1. *Chitinophaga silvatica*

The 4C_S_KR sample was determined to be a species in the *Chitinophaga* genus with a 98,16% sequence identity by 16S rRNA. Through WGS, the genus was confirmed, and the species was established as *Chitinophaga silvatica* with 100% support.

The first description of the *Chitinophaga* genus was by Sangkhobol and Skerman in 1981. Their description was based on several gram-negative rod-shaped bacterial isolates from soil and freshwater, with the ability to hydrolyse chitin (Sangkhobol & Skerman, 1981; Tran et al., 2020). The majority of the discovered species in this genus are environmental and have been found in soil, roots, sludge, fresh water and plant rhizosphere (Tran et al., 2020). The *C. silvatica* species was first discovered and defined by Yao et al. in 2021. The bacteria was isolated from a soil sample from a forest in the Guangdong province of China (Yao et al., 2021).

Only two cases have shown human bacteremia caused by a species in the *Chittinophaga* genus. The first discovery was in 2009 in France, with *Chitinophaga terrae*. This bacterium was

isolated from a blood sample from a severely immunosuppressed patient and was shown to be Multidrug resistant, including resistance to several β-lactamsase and Fluoroquinolones. The *C. terrae* species is usually an environmental bacteria, but in this case, it acted like an opportunistic pathogen (Crémet et al., 2009). The second discovery was in Vietnam in 2020 with the novel species *Chitinophaga vietnamensis*, isolated from human clinical samples (Tran et al., 2020). This illustrates that strains in the *Chitinophaga* genus have been detected to cause infection in humans.

Virulence genes

When screening for virulence factors, no genes were found in the VFDB database. However, when using the PROKKA tool for annotating genes, one virulence gene was found, *virF* (90,10%). This gene codes for a *araC* family transcription regulator. The *virF* gene is an activator and is essential for the virulence expression in *Shigella* and activates the Type III Secretion System (T3SS) which promotes invasion of the human intestinal mucosa (Trirocco et al., 2023). When screening the annotated genes, no genes coding for T3SS were found in *C. silvatica*.

Resistance genes

From the PCR screening with the blaOXA-48 primers, a band with the same length as the *blaOXA-48* gene was detected and sent to sequencing, revealing it to be a *blaOXA-48-like* gene. When screening for resistance genes in NCBI, CARD and ResFinder, no genes were found. However, searching through the annotated genes from PROKKA revealed two *bla* genes belonging to Class D β-lactamases. These genes were described as *blaOXA-2* and *blaOXA-10* genes by PROKKA, one of them probably being the *blaOXA-48-like* gene detected by PCR.

Susceptibility to antibiotics

The 4C_S_KR specimen was isolated on a CRE plate, revealing its ability to grow in the presence of carbapenems. This resistance could be caused by the two Class D β-lactamases located in the bacteria. Even though only two β-lactamases were discovered, this isolate was observed to be non susceptible to Ampicillin, Cefepime, Cefotaxime, Ciprofloxacin, and Penicillin G. Following the MIC values for gram negative bacteria proposed by EUCAST, the *C. silvatica* specimen could be considered Multidrug resistant.

5.4.2. *Pandoraea sp.*

Sanger sequencing of the 16S rRNA region determined 8C_W_KR to be a species in the *Pandoraea* genus which was later confirmed by WGS. This sample could not be identified to a species level, with the closest similarity being the *Pandoraea nosoerga* species with an 84% support.

The *Pandoraea* genus was proposed by Coenye et al. based on bacterial isolates from environmental samples and from patients with Cystic fibrosis. This genus consists of rod-shaped gram negative, which can act as opportunistic pathogens and are well known to be a problem for patients with Cystic fibrosis (Coenye et al., 2000; Peyclit et al., 2021). Cystic fibrosis (CF) is a chronic disease caused by faults in genes regulating the transmembrane channels. This disease can affect several organs, especially the lungs and can trigger inflammatory responses (Costello et al., 2011; Kruis et al., 2023; Rafeeq & Murad, 2017). The leading cause of fatality for patients with CF is lung infections by pathogen and opportunistic pathogen bacterial strains like *P. aeruginosa, Staphylococcus aureus* and *Pandoraea sp.* (Pither et al., 2021). Cases with *Pandoraea sp.* infections in patients without apparent immunodeficiency have been observed, revealing a possibility for the spread of infection between CF patients and non-CF patients (Kruis et al., 2023). Bacteria in the *Pandoraea* genus can contain several antimicrobial resistance and biodegradation genes at the same time, thereby being able to persist for a long time in a hospital or clinical setting (Kruis et al., 2023).

Virulence genes

When screening for virulence genes, two genes were found using the VFDB database. These genes were *cheB* (80,28%) and *cheW* (87,12%), both involved in bacterial chemotaxis, and are not considered important virulence factors (Xu et al., 2021).

Resistance genes

The screening for resistance genes revealed one gene with a 99,30% identity similarity to *blaOXA-158*. The same *blaOXA-158* gene was discovered in a study by Schneider and Bauernfeind. Their study had problems differentiating between *Pandoraea* isolates at a species level and proposed using OXA-variants to distinguish between the species. It has also been suggested that species in the *Pandoraea* genus may function as natural reservoirs for carbapenem-hydrolysing oxacillinases and are proposed to be the forerunners for some of the acquired β-Lactamases (Schneider & Bauernfeind, 2015). Interestingly, a *blaOXA-158* gene has been discovered in *P. nosoerga* isolate, causing severe infection in CF patients (Peyclit et al.,

2021). In addition to the *blaOXA-158* gene, an *ampC* gene was discovered. This gene belongs to the PNC family in class C β -Lactamases.

Susceptibility to antibiotics

The MIC testing showed a low susceptibility to several antibiotics. Only two of the tested antibiotics, Cefepime and Trimethoprim, had lower observed MIC values than MIC values proposed by EUCAST, which defined this isolate as a multidrug resistant bacterium. Kokcha et al. also states that species in the *Pandoraea* genus could be considered multidrug resistant pathogenic bacteria when it comes to infections in CF patients (Kokcha et al., 2013). The presence of class D β-lactamases was discovered in an outbreak of *Pandoraea spp*. in non-CF patients in Germany. In this study, the isolated strains were shown to be resistant to many antibiotic agents, including Penicillins, Fluoroquinolones, Cephalosporins and Meropenem (Kruis et al., 2023). According to ResFiner, the observed *blaOXA-158* gene has been found to confer resistance to several antibiotics, including Ampicillin and Meropenem, as confirmed in thus study by the susceptibility testing. The antibiotic treatment of infections by species in the *Pandoraea* genus has been shown to be challenging due to a broad spectre of resistance (Kruis et al., 2023; Schneider & Bauernfeind, 2015). Resistance to several antibiotics have also been demonstrated for the 4C_S_KR isolate.

5.4.3. *Pseudomonas aeruginosa*

The 5E_S_SY isolate was identified as *P. aeruginosa* by 16S rRNA Sanger sequencing and whole genome sequencing (100% support). The strain type was ST3436, and the serotype was group O5, which includes serotypes O5, O18 and O20.

P. aeruginosa is a well-known environmental bacteria commonly found in places containing soil and water as well as in the intestinal flora of humans (Folkehelseinstituttet, 2010). This bacterium has high metabolic flexibility and can adapt and survive in a broad spectrum of different environmental conditions (Abban et al., 2023). It is known to harbour a high amount antibiotic resistance, which is a result of several mechanisms working at once (Breidenstein et al., 2011). In a study by Nasrin et al., more than 400 *P. aeruginosa* strains were tested, and 26,7% were defined as MDR (Nasrin et al., 2022). *P. aeruginosa* has a naturally low permeability of the outer membrane, which causes a high intrinsic resistance to several antibiotics. In addition to the intrinsic mechanism, it also easily gains resistance genes through HGT (Breidenstein et al., 2011). This can for example be through integrons and MEGs, such as IS-elements (Botelho et al., 2019; Chen et al., 2009; Evans & Segal, 2007). This species is

commonly found to be resistant to antibiotics, including Fluoroquinolones and β-lactams (Abban et al., 2023; Dewi et al., 2021)

This bacterium rarely leads to infection in patients with a functional immune system but is opportunistic and can potentially cause severe infection both within and outside the hospital. *P. aeruginosa* was responsible for 7,1% of all healthcare-associated infections in the United States in 2017. It is also well-known for causing infections in CF patients (Thi et al., 2020). On the priority list of pathogens proposed by The World Health Organisation in 2017, one of the critical bacteria needing urgent attention is the carbapenem resistant *P. aeruginosa* (Abban et al., 2023)

Virulence genes

Many genes connected to virulence were discovered in the VFDB database for the *P. aeruginosa* isolate. These genes included *alg, las, apr* and *exo* genes. Many CF patients gets infected by *P. aeruginosa* strains early in life, which can later transition into mucoid variants. This transition increases the alginate production along with the patient's morbidity and mortality (Pritt et al., 2007). The *alg* genes are involved in alginate production, which in mucoid strains protects the bacterium, helps in biofilm maturation, and can decrease the flow of antibiotics through the biofilm (Thi et al., 2020). Some of the genes included in the *las* system regulates genes encoding important virulence factors, thereby being and important contributor for the pathogenicity of *P. aeruginosa (Kiratisin et al., 2002).* The *lasA* gene have been shown to modulate the invasion of *P. aeruginosa,* and mutations in this region can reduce the the levels of toxin produced and reduce invasion (Cowell et al., 2003). Genes involved in secretion systems are also important virulence factors and can cause decreased susceptibility to antibiotics (Irum et al., 2021).

Resistance genes

Several genes conferring resistance were discovered for *P. aeruginosa.* Three different β-lactamases were detected, *blaOXA-50* (98,73 %), *blaPDC202* (99,83 %), and *blaPAO* (97,57 %). Both *blaOXA-50* and *blaPDC (ampC)* are considered to be naturally occurring in *P. aeruginosa* (Girlich et al., 2004). The *blaOXA-50* gene belongs to the class D β-lactamases and was first identified in *P. aeruginosa.* It is considered to have a weak hydrolysing activity by itself, but combined with other resistance mechanisms, it can lead to carbapenem resistance (Girlich et al., 2004; Petrova et al., 2019). When first discovered, it was proven to confer resistance to ampicillin, Ticarcillin, Meropenem and Moxalactam (Girlich et al., 2004). It is

common to detect *blaOXA-50* genes in *P. aeruginosa* but has been suggested to not be intrinsic (Petrova et al., 2019). The *blaPAO* and *blaPDC202* genes belongs to class C β-lactamases. PDC stands for *Pseudomonas*-derived Cephalosporinases, and overproduction of these genes is linked to virulence and resistance to Cephalosporins (Philippon et al., 2022). According to ResFinder, the *blaPAO* gene can confer resistance to several antibiotics, including Cefotaxime, Cefepime, Ampicillin, and Amoxicillin.

The other resistance genes detected in addition to β-Lactamases were *aph(3')-Ilb, fosA* and *catB7.* These genes confer resistance to Aminoglycosides, Fosfomycin and Chloramphenicol, respectively (Josino et al., 2021; Wang & Liu, 2004; Zeng & Jin, 2003). Interestingly, a *fosA* gene was located in a detected IS element, ISPa6. As commonly known, IS elements, like other MGEs, can be transferred between different bacteria and are known to harbour antibiotic resistance genes (Khedkar et al., 2022). This indicates that this *P. aeruginosa* bacteria has a great potential to transfer this gene across the species barrier. This IS element was also found in a studies by Irum et al. (Irum et al., 2021). The resistance genes discovered in the 5E_S_SY *P. aeruginosa* isolate have commonly been found in several clinically isolated *P. aeruginosa* samples (Irum et al., 2021; Subedi et al., 2018).

Susceptibility to antibiotics

According to Poole et al., *P. aeruginosa* is considered to be naturally resistant to many antibiotics, including first- and second generation Cephalosporins, Cefotaxime, Quinolones and Trimethoprim (Poole, 2011). When testing for susceptibility, there was no inhibition of growth by Ampicillin, Penicillin G or Trimethoprim. According to the ECOFF values proposed by EUCAST, this isolated *P. aeruginosa* is epidemiological resistant against these three antibiotics. The resistance exhibited could be caused by the resistance genes discovered, other resistance mechanisms, or a combination.

5.4.4. Efflux pumps and metal resistance

As mentioned earlier, an increase in the activity of efflux pumps is a known resistance mechanism (Bush & Bradford, 2016). Upregulation of the expression by regulating genes or by mutation can reduce the concentration of antibiotics in the cell, thus preventing the effect of the drugs (Shariati et al., 2022; Singh et al., 2019). Multidrug efflux pumps are an important intrinsic resistance mechanism in bacteria such as *P. aeruginosa* (Breidenstein et al., 2011). Several Multidrug efflux pumps were discovered in all three sequenced bacteria. In *P. aeruginosa, bmrA, emr, mex* and *stp* were detected. Genes including *emr, imr, mdt,*

mex and *yhel* were discovered in *C. silvatica* while *acr, bmr, emr, mdt, mep* and *mex* in *Pandoraea sp.* In addition to removing antibiotics from the cell, some efflux pumps can confer resistance to both metals and antibiotics (Pal et al., 2017).

Resistance to heavy metals can lead to co-selections and increased antibiotic resistance in the environment. Several connections have been observed between metal resistance and increased antibiotic resistance (Chen et al., 2015). In an environment with a high heavy metal concentration, there will be a selection of metal resistant bacteria. If these bacteria already contain antibiotic resistance genes, the ARG in this environment will increase. Another possible explanation to this correlation is that the occurrence of metals may induce sensitive antibiotic resistance mechanisms and lead to resistance. Some metal transporters in the bacteria can also become sensitive to antibiotics and transport both metals and antibiotics. A study by Berg et al. showed an increase in cobber resistance and an indirect selection for antibiotic resistance in soil environments with a higher cobber concentration (Berg et al., 2005). Interestingly, genes conferring resistance to cobber (*cop*) and mercuric *(mer)* were found in the 5E_S_KR isolate. Other resistance genes detected were arsenic resistance (*ars*) and cobalt-zinc-cadmium resistance*(aza),* which have been spotted in all three isolates. The presence of metal resistance and multidrug efflux pump genes in the three sequenced bacteria reveals a high potential for non-ESBL-production resistance mechanisms.

5.5. Susceptibility testing and resistance definitions

Defining bacteria as susceptible, intermediate, or resistant can be challenging, especially concerning environmental bacteria. Clinical standards, like the MIC values proposed by EUCAST, are insufficient when assessing antibiotic susceptibility for environmental bacteria. The clinical breakpoints are based on parameters only relevant to therapeutic use (Berendonk et al., 2015). The epidemiological cut-off values (ECOFF) separates the populations with resistance mechanisms from the wild-type, which are susceptible. These values are based on wild-type distribution and phenotypical traits (Berendonk et al., 2015; Kahlmeter & Turnidge, 2022). The use of ECOFF values can be valuable in environmental surveillance and provide proof of emerging acquired resistance mechanisms. It should be mentioned, however, that these ECOFF estimations are largely based on databases containing a high majority of isolates with a clinical origin. It can be debated if these values reflect the accurate relationship between the wild type and non-wild type in the environment (Berendonk et al., 2015).

For the purpose of this study, ECOFF values were used for the *P. aeruginosa* samples, while MIC values were used for the remaining bacteria. This was due to a lack of ECOFF values for the other discovered species.

The susceptibility testing revealed that all bacteria, except 7C_W_MD, were resistant to at least two antibiotics. These include the two penicillin antibiotics tested, Penicillin G and Ampicillin. Resistance to penicillins is normal among several bacteria, like *P. aeruginosa*, *Staphylococcus aureus* and *Enterobacteriaceae* (Botelho et al., 2019; Lobanovska & Pilla, 2017). More concerningly was the discovery of an isolate that displayed resistance to the carbapenem Meropenem. This isolate was *Pandoraea sp.,* and it was the bacteria with the lowest amount of inhibition by antibiotics of all the tested strains. In this strain, one class D β-lactamase was discovered, which may be the reason for the lack of inhibition by Meropenem. This bacterium was isolated from the Kråkstadelva, where also two other class D β-lactamase were detected. Carbapenems are the go-to antibiotics when dealing with ESBL-producing bacteria and are considered a "last resort" drug (Abban et al., 2023). This makes the increase in Carbapenem resistance even more challenging and makes discoveries of carbapenem resistance more noteworthy (Papp-Wallace et al., 2011).

When using MIC values proposed by EUCAST, four of the strains were defined as multi-resistant. These were two *Pseudomonas sp.* isolates 13E_W_KR and 14E_W_MD, 4C_S_KR *C. silvatica* and 8C_W_KR *Pandoraea sp.* These findings show a large amount of resistance in the aquatic environments tested.

5.6. Further work and challenges

Surveillance is the first step in comprehending the challenges the global society is facing, especially understanding the connection between the environment, animal and human health (Singh et al., 2019). To better estimate the flow of ARB and ARG between the environment and clinically relevant bacteria, more information is needed on the existing antibiotic resistance in the environment. This includes both characterisation and quantification (Berendonk et al., 2015)

There are several aspects that would be interesting to investigate further. One interesting angle is the occurrence of antibiotic residues in the water tested. Studies have shown that antibiotic resistance can develop and increase with the level of antibiotics through genetic drift and natural selection (Peterson & Kaur, 2018; Sanz-García et al., 2023). The concentration of antibiotics could therefore give an indication of the development of resistance in these aquatic ecosystems. However, calculating the concentration of antibiotic residues could be proven difficult in

environments like rivers and small streams due to the constant movement of water. A potential calculation would furthermore only represent a momentary image and temporal trends of the antibiotic frequency.

Another interesting factor to investigate is the seasonal differences. A study by Calero-Caceres et al. found a significant difference in the abundance of ARGs in different seasons in samples from rivers. The greatest quantity of ARGs was found in the winter samples, with the exception of the *blaTEM* gene (Calero-Cáceres et al., 2017). This has also been observed by former master's students of Bjørn-Arne Lindstedt and the study by Finton et al. (Finton et al., 2020). The three *blaTEM* and three *blaOXA-48-like* genes discovered in this study were all found in bacteria isolated from the summer collections. This contradicts the findings by Calero-Caceres et al., however, possibly due to nonspecific primers, only a few genes were detected through the PCR screenings. This implies these six genes may not be the only ARG present, and it is thereby not possible to draw any conclusion by looking at the discovered ARG in this thesis. More extensive research and comparison between summer and winter samples must be conducted to draw further conclusions.

A challenge when mapping resistance in environmental bacteria is the use of culture-dependent studies. A large number of bacteria are not detected with culture-based methods, resulting in a substantial part of the environmental community not being explored. An option to avoid this problem is using DNA based methods, by testing the metagenome of environmental samples with methods like quantitative PCR (Berendonk et al., 2015). This can help reveal resistance genes and mechanisms in the environment. It will however be difficult to know the phenotype of the bacterium only by investigating the presence of the resistance genes (Kahlmeter & Turnidge, 2022).

Another challenge is the databases used. When searching the CARD, NCBI and VFDB databases, few genes were detected for the *Pandoraea sp.* and *C. silvatica* samples. These isolates are environmental bacteria, and little is known about them compared to other species, like *P. aeruginosa*. Therefore, the absence of resistance genes in these searches does not necessarily mean they are not present in the bacterial genome. A proof of this is the search of resistance genes for *C. silavtica.* There was a lack of detected genes in the NCBI and CARD databases, while two class D β-lactamases were discovered when screening through the annotations by PROKKA. An improved database is needed to better assess the ARGs in the environment (Ben et al., 2019).

5.7. Concluding remarks

The purpose of this study was to investigate the occurrence of resistance in aquatic environments in Norway. Through phenotypic and genotypic detection, several ESBL-producing bacteria were detected. By PCR screening three *OXA-48-like* genes were discovered in different bacteria isolated from the Kråkstadelva in Nordre Follo municipality. Several β-lactamases were also found in the three isolates chosen for whole genome sequencing. There were two class D genes in *C. silvatica,* and a *blaOXA-158* and *ampC* gene in *Pandoraea sp.*, both isolated from Kråkstadelva. A *P. aeruginosa* ST3436 O5 isolated was discovered in the Syverudbekken in Ås municipality. It contained several virulence and resistance genes, including the ESBL genes *blaPDC-202, blaPAO* and *blaOXA-50.* In addition to ESBL-genes, several multidrug efflux pumps and metal resistance genes were discovered in all three bacteria, revealing a high potential for non-ESBL-production resistance mechanisms. Sensibility testing revealed resistance to several antibiotic classes in the tested bacteria. All but one were resistant to both Ampicillin and Penicillin G. and resistance to Cefotaxime, Ciprofloxacin, Trimethoprim and Meropenem were detected. Even though the discovered bacteria were primarily environmental and likely non-pathogen, their resistance and resistance genes can still pose a threat to animal and human health. The aquatic ecosystems are naturally meeting grounds for a large assortment of bacteria, where transfer of resistance gene from environmental to clinically important bacteria may occur. More research and surveillance are needed to get a broader understanding of the antibiotic resistance situation, and how environmental bacteria can affect the animal and human health.

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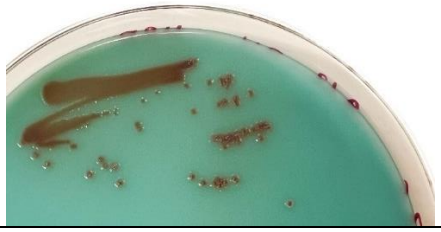
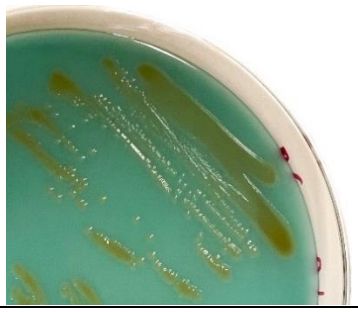
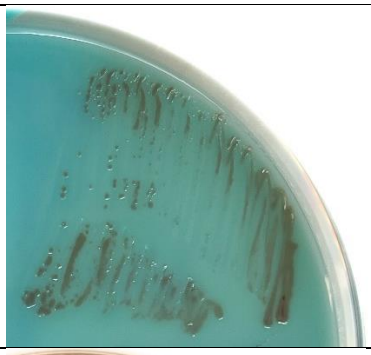
Appendix

i. Picture of isolated bacteria

Appendix i.1: Picture of isolated bacteria on CRE and ESBL plates. Shows an assortment of the isolated bacteria. Pictures of the remaining bacterial isolates can be provided by request.

Bacterial ID	Species	Colour	Picture
1C_S_SY	*Stenotrophomonas sp.*	Green	
2C_S_SY	*Raoultella ornithinolytica*	Blue	
3C_S_KR	*Shewanella sp.*	Yellow	
4C_S_KR	*Chitinophaga sp.*	Green	

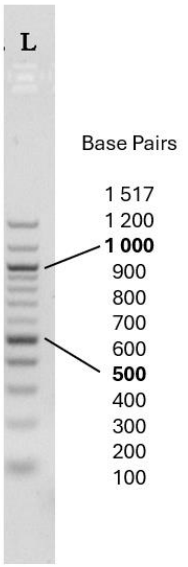
5C_S_KR	*Aeromonas sp.*	Blue	
6C_S_SY	*Pseudomonas boreopolis*	Blue	
7C_W_MD	*Herbaspirillum huttiense*	Red	
8C_W_KR	*Pandoraea sp.*	Yellow	
9C_W_KR	*Pandoraea pulmonicola*	Yellow	
10_W_MD	*Cohnella sp.*	Blue	

13C_W_MD	*Herbaspirillum frisingense*	Red	
1E_S_KR	*S. fonticola*	Blue-Green	
3E_S_MD	*Bordetella sp / Kerstersia sp..*	Pink	
4E_S_SY	*Bordetella sp. / Kerstersia sp.*	Green	
5E_S_SY	*P. aeruginosa*	Brown	

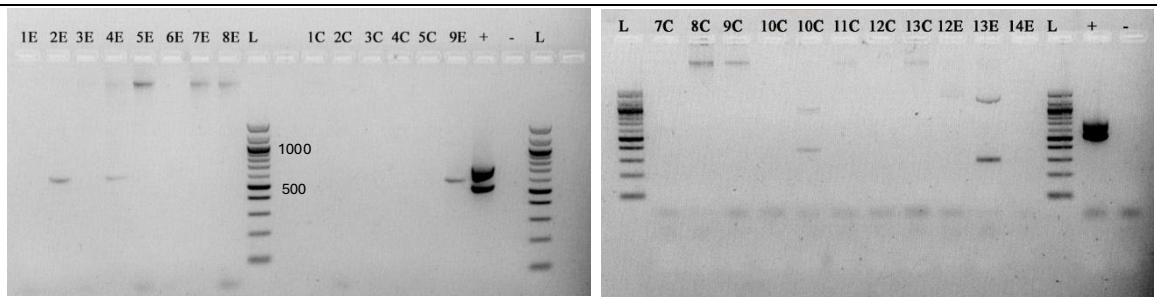
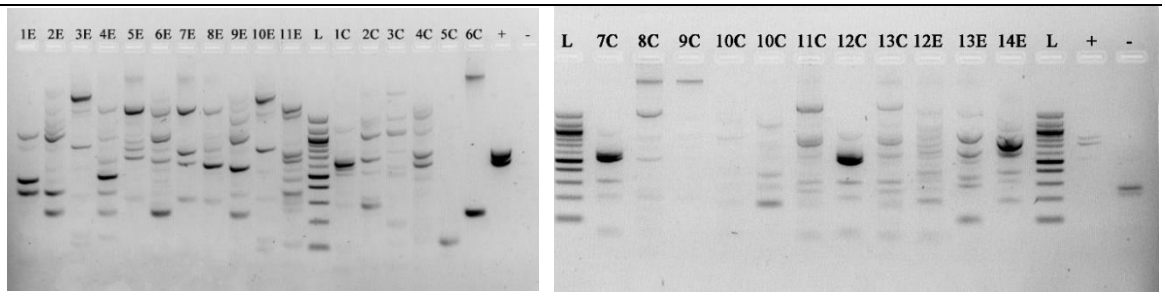
8E_S_MD	*P. aeruginosa*	Brown	
13E_W_KR	*Pseudomonas indoloxydans*	Yellow	
14E_W_MD	*Pseudomonas siliginis*	Yellow	

ii. Agarose gel electrophoresis - pictures

Appendix ii.1: The ladder used for all gel electrophoresis. The ladder is the 100 bp DNA Ladder from New England Biolabs (New England Biolabs, Ipswich, US).

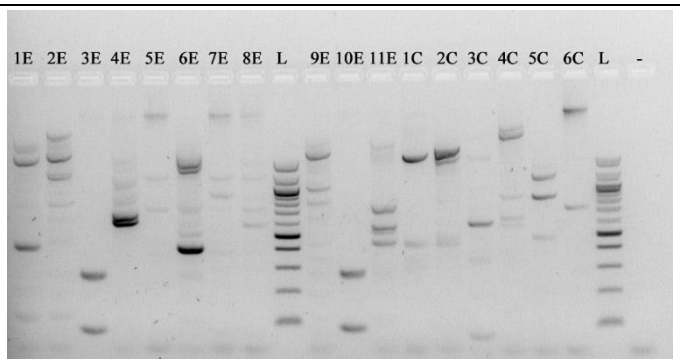
Ladder – Base Pairs


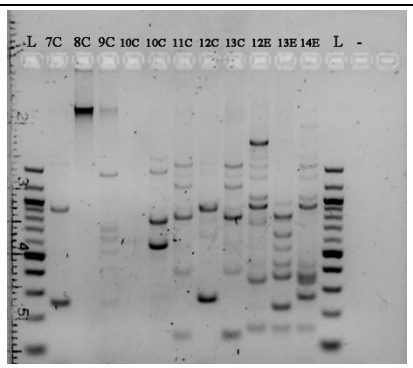
Appendix ii.2: The results from Multiplex PCR. Shows all the gel electrophoresis of the Multiplexes from 1 to 5.

Multiplex	Agarose gels
Multiplex 1	
Multiplex 2	

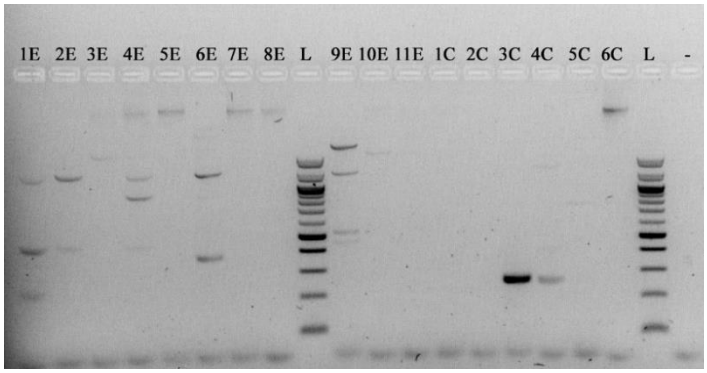
Appendix

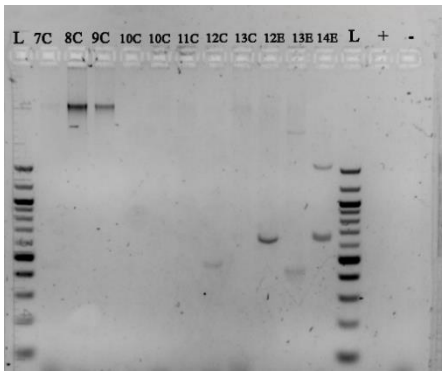
Multiplex 3



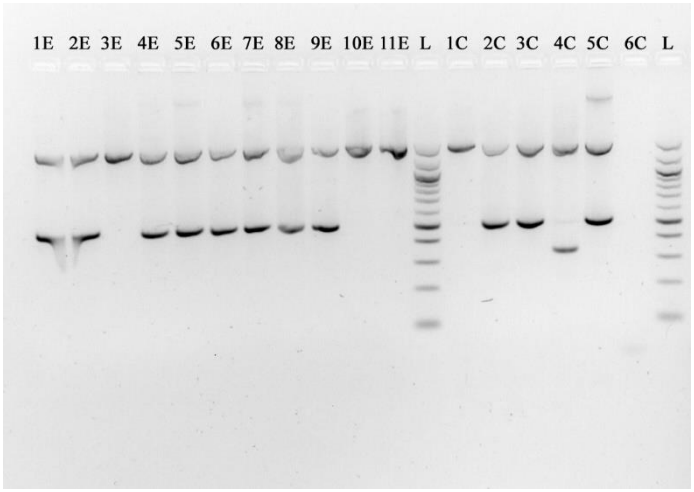


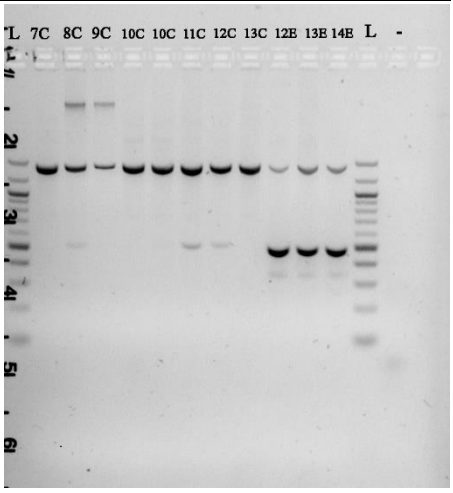
Multiplex 4



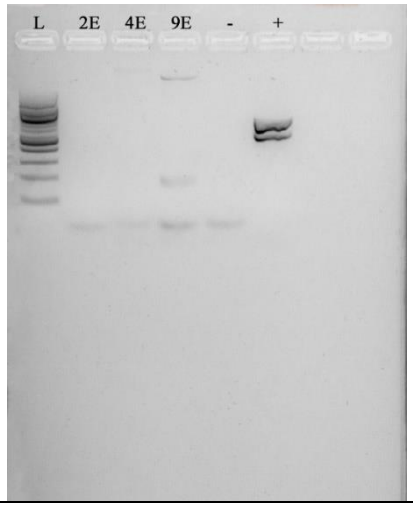
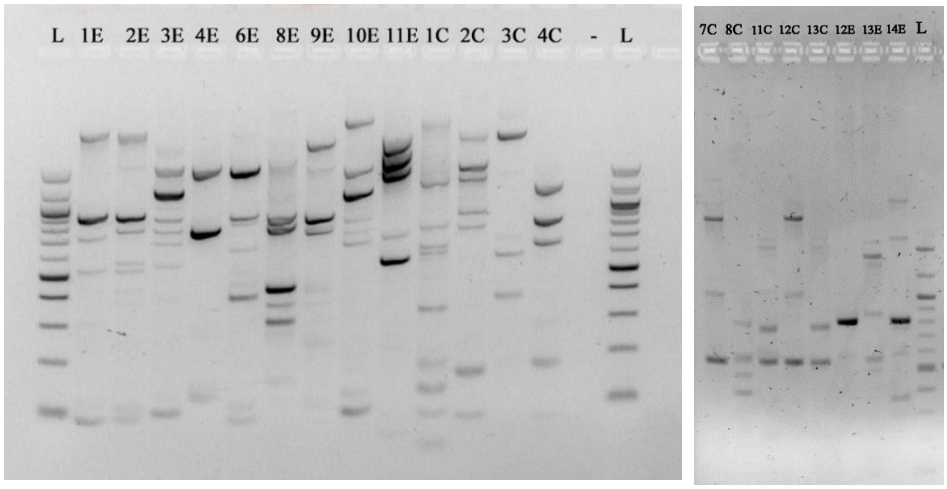
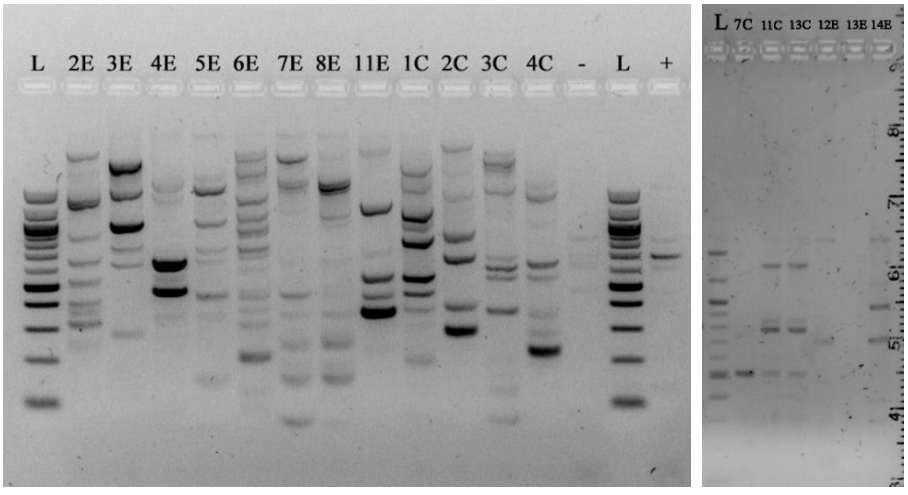


Multiplex 5

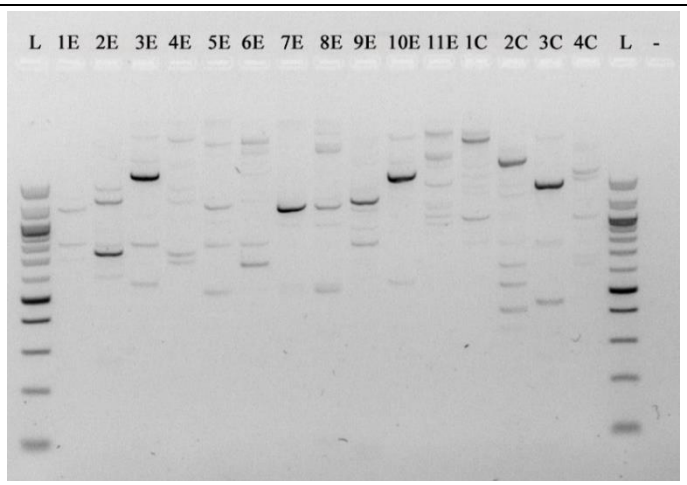


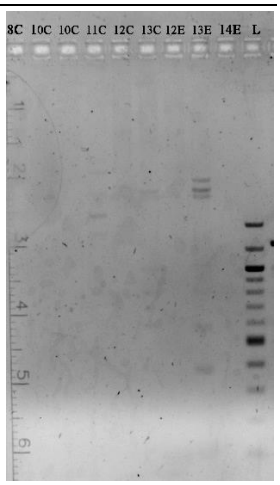


Appendix ii.3: Results from Singelplex PCR. Shows all of the PCR results from Singelplex PCR on agarose gels.

Gene	Agarose gels
Multiplex 1	
blaOXA	
Multiplex 2	
blaCTX-M gr. 9	
blaCTX-M gr. 1	

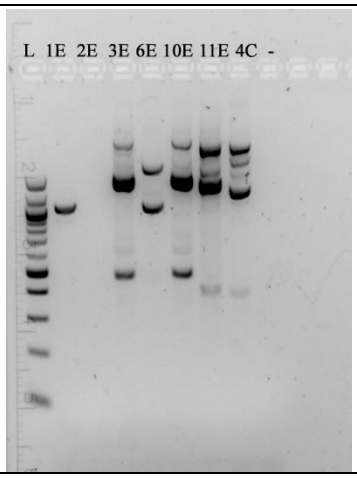
blaTEM

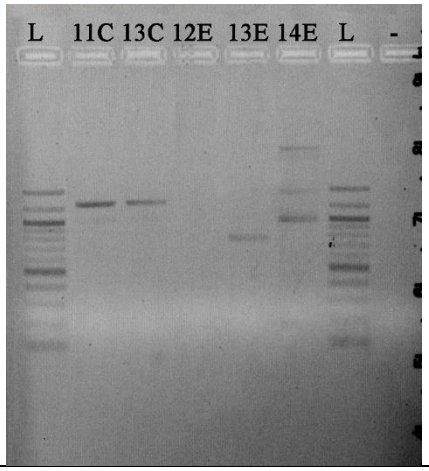




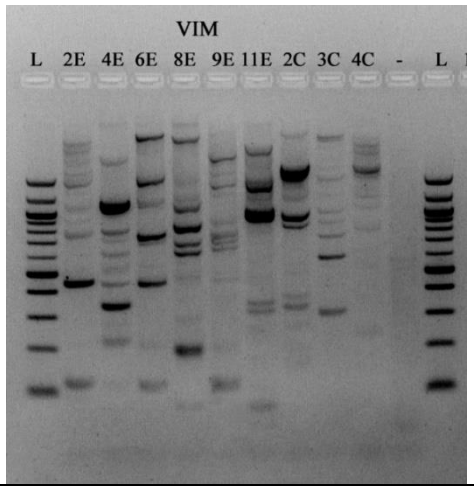
Multiplex 3

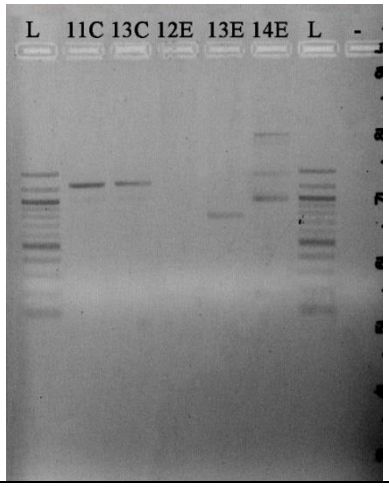
blaNDM



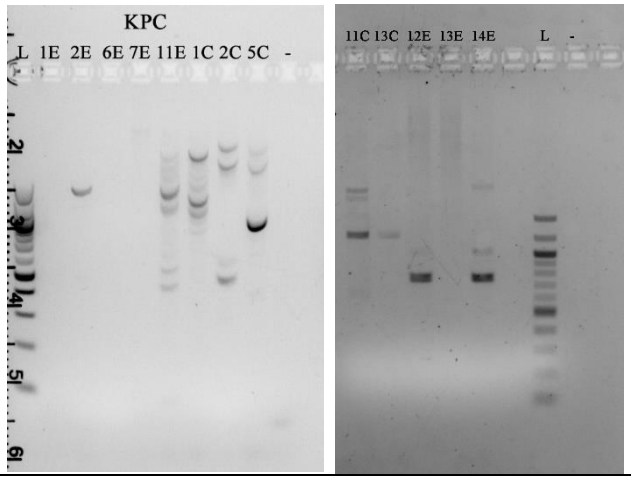
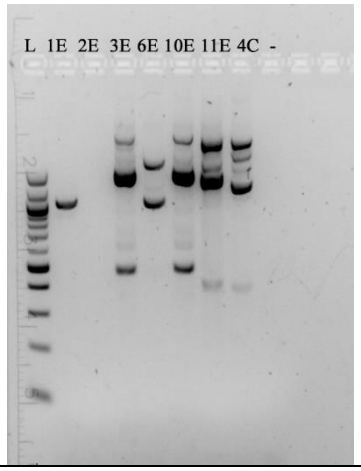
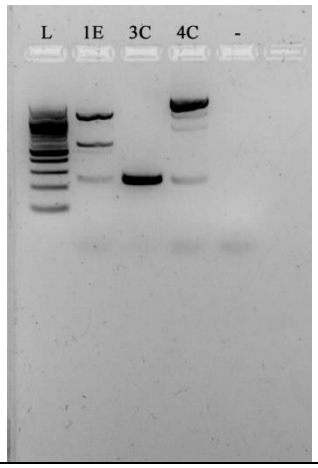


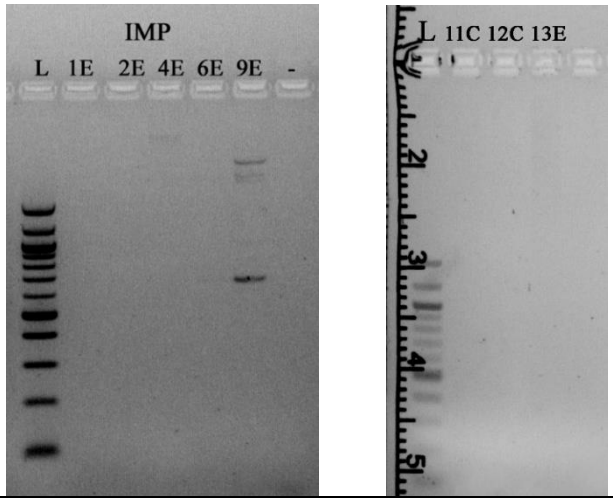
blaVIM





Appendix

blaKPC	
Multiplex 4	
blaCMY	
blaOXA-48	

blaIMP	

iii. PCR program for Hemo KlenTaq® Polymerase

Appendix iii.1: Reagents for the PCR master mix for Hemo KlenTaq® polymerase. Shows the amount of each reagent per reaction for the Hemo KlenTaw® polymerase.

Reagent	25R µl X N	Final concentration
5X Hemo KlenTaq Reaction Buffer	5 µl	1 X
10 mM dNTPs	0,5 µl	0,2 mM
10 µM Forward Primer	0,75 µl	0,3 µM
10 µM Reverse Primer	0,75 µl	0,3 µM
Hemo KlenTaq MasterMix	2 µl	
Nuclease free water	15 µl	
DNA template	1 µl	

Appendix iii.2: Program for the PCR reaction with the Hemo KlenTaq® polymerase. Shows the temperature and duration of the different steps during the PCR reaction. Two different annealing temperatures were tested, 63 °C and 66 °C.

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	3 minutes	1
Denaturation	95 °C	30 seconds	
Annealing	63/66 °C*	1,5 minutes	30
Extension	68 °C	1,5 minutes	
Final extension	68 °C	10 minutes	1
Hold	4°C	Infiniti	

*Both 63°C and 66°C annealing temperatures were tested.

iv. PCR program for iProof™ High Fidelity DNA Polymerase

Appendix iv.1: Reagents for the PCR master mix for iProof™ High-Fidelity DNA polymerase. Shows the amount of each reagent per reaction for the iProofTM High-Fidelity DNA polymerase.

Reagent	25R µl X N	Final concentration
5X iProof HF Buffer	5 µl	1 X
dNTPs mix	0,5 µl	200 µM
10 µM Forward Primer	1,25 µl	0,5 µM
10 µM Reverse Primer	1,25 µl	0,5 µM
iProof DNA polymerase	0,25µl	
Nuclease free water	15,75 µl	
DNA template	1 µl	

Appendix iv.2: Program for the PCR reaction with the iProof™ High-Fidelity DNA polymerase. Shows the temperature and duration of the different steps during the PCR reaction. Two different annealing temperatures were tested, 63 °C and 66 °C.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1
Denaturation	98 °C	10 seconds	
Annealing	63/66 °C*	30 seconds	30
Extension	72 °C	30 seconds	
Final extension	72 °C	10 minutes	1
Hold	4°C	Infiniti	

*Both 63°C and 66°C annealing temperature were tested.

v. Primers

Appendix v.1: Overview of the primers used to determine resistance genes. Shows the name of the primers, the length of the desired product and the primer sequences (forward (F) and reverse (R)).

Name	Product length	Sequence (5'-3')
blaCMY	188 bp	F- GCATCTCCCAGCCTAATCCC R- TTCTCCGGGACAACTTGACG
blaCTX-M gr. 1	688 bp	F- TTAGGAARTGTGCCGCTGYA R- CGATATCGTTGGTGGTRCCAT
blaCTX-M gr. 2	404 bp	F- CGTTAACGGCACGATGAC R- CGATATCGTTGGTGGTTCCAT
blaCTX-M gr. 9	561 bp	F- TCAAGCCTGCCGATCTGGT R- TGATTCTCGCCGCTGAAG
blaIMP	393 bp	F- ACAGGGGGAATAGAGTGGCT R- AGCCTGTTCCCATGTACGTT
blaKPC	460 bp	F- TCCGTTACGGCAAAAATGCG R- GCATAGTCATTTGCCGTGCC
blaNDM	157 bp	F- TGGCCCGCTCAAGGTATTTT R- GTAGTGCTCAGTGTCGGCAT
BlaOXA	564 bp	F- GGCACCAGATTCAACTTTCAAG R- GACCCCAAGTTTCCTGTAAGTG
blaOXA-48	281 bp	F- GCTTGATCGCCCTCGATT R- GATTTGCTCCGTGGCCGAAA
blaSHV	713 bp	F- AGCCGCTTGAGCAAATTAAAC R- ATCCCGCAGATAAATCACCAC
blaTEM	800 bp	F- CATTTCCGTGTCGCCCTTATTC R- CGTTCATCCATAGTTGCCTGAC
blaVIM	564 bp	F- ATAGAGCTCAGTGTGTCGGCAT R- TTATTGGTCTATTTGACCGCGT

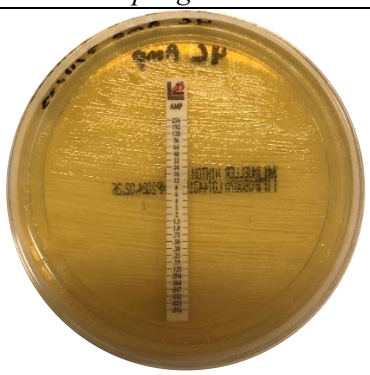
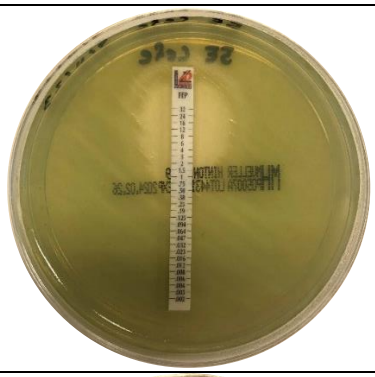
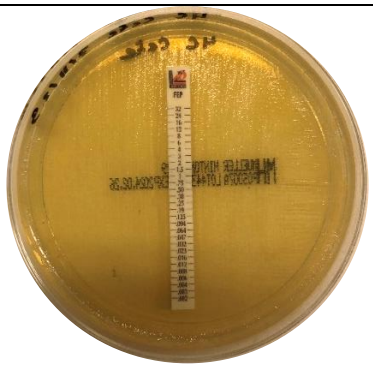
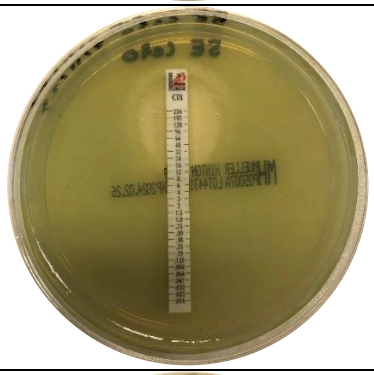
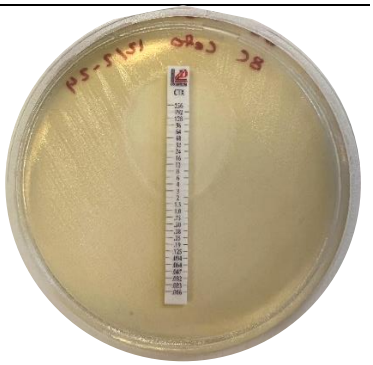
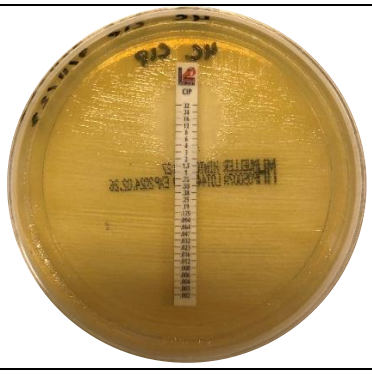
vi. Nanodrop - Quantification and quality

Appendix vi.1: Nanodrop values for DNA extraction. Shows the 260/280, 260/230 and the concentration (ng/µl) for the DNA aster DNA extraction.

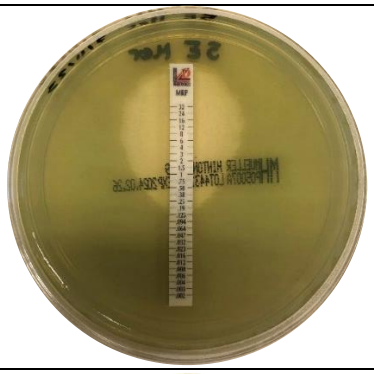
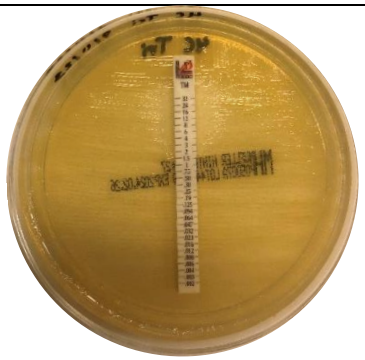
Bacterial ID	Nanodrop		
	260/280	**260/230**	**Concentration (ng/µl)**
1C_S_SY	8,51	0,37	23,3
2C_S_SY	8,19	0,73	23,7
3C_S_KR	2,9	0,8	49,5
4C_S_KR	2,02	1,17	203
5C_S_KR	0,34	-0,17	-3,9
6C_S_SY	2,03	1,86	346,1
7C_W_MD	1,95	1,49	87,6
8C_W_KR	1,95	2	269,2
9C_W_KR	1,95	2	269,2
10C_W_MD	2,36	0,47	10,2
11C_W_MD	1,95	1,79	151,9
12C_W_MD	2,08	0,26	55
13C_W_MD	1,94	1,56	319
1E_S_KR	3,69	0,76	37,1
2E_S_SY	2,6	1,18	63,5
3E_S_MD	1,82	0,98	98
4E_S_SY	1,89	1,4	224,5
5E_S_SY	2,13	1,33	179
6E_S_KR	2,14	1,51	144,8
7E_S_SY	2,13	1,28	187,1
8E_S_MD	2,23	1,07	122,9
9E_S_MD	2,67	0,13	65,9
10E_S_SY	3,02	0,62	52,2
11E_S_KR	2,64	1,45	68,4
12E_W_KR	1,93	2,11	485,9
13E_W_KR	1,93	1,95	515,7
14E_W_MD	1,94	1,29	127,7

vii. Antimicrobial Susceptibility testing

Appendix vii.1: Antimicrobial susceptibility testing. Shows three of the eleven testes bacterial strains after 24-hour incubation from the first repetition of the antimicrobial susceptibility testing. Pictures of the remaining bacterial isolates can be provided by request.

Antibiotics	**Bacteria**		
	5E_S_SY *Pseudomonas aeruginosa*	4C_S_KR *Chitinophaga silvatica*	8C_W_KR *Pandoraea sp.*
Ampicillin			
Cefepime			
Cefotaxime			
Ciprofloxacin			

Appendix

Meropenem			
Penicillin G			
Trimethoprim			

Appendix vii.2: Explanation of MIC and ECOFF values. The Minimum inhibitory concentration (MIC) values and Epidemiological cut-off value (ECOFF) are retrieved from ECUAST web page (1: https://mic.eucast.org/search/?search%5Bmethod%5D=mic&search%5Bantibiotic%5D=-1&search%5Bspecies%5D=-1&search%5Bdisk_content%5D=-1&search%5Blimit%5D=50), EUCAST Antimicrobial Susceptibility Testing breakpoint table (2:https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_13.1_Breakpoint_Tables.pdf) and EUCAST guidance on breakpoints MIC values for Gram positive and Gram negative bacteria aerobic bacteria (3 : When_there_are_no_breakpoints_2024-02-29.pdf (eucast.org)).

According to the manual from EUCAST (3), a "dash" instead of numerical values means "the microbe can me reported resistant without further testing".

Green: MIC values for Enterobacteriaceae family (2)
Yellow: ECOFF values for P. aeruginosa (1)
Orange: MIC values for Pseudomonas spp. (2)
Purple: MIC values for Gram negative bacteria (3)
Blue: MIC values for Gram positive bacteria (3)
Pink: MIC values for Aeromonas spp. (2)
Blank: No values were found.

Antibiotics	1E_ S_KR	5E_ S_SY	8E_ S_KR	13E_ W_KR	14E_ W_MD	3C_ S_KR	4C_ S_KR	5C_ S_KR	7C_ W_MD	8C_ W_KR	10C_ W_MD
Ampicillin	S ≤ 8 > R	-	-	-	-	8	8	8	8	8	0,5
Cefepime	S ≤ 1 4 > R	16*	16*	S≤0,001 8 > R	S≤0,001 8 > R			S ≤ 1 4 > R			
Cefotaxime	S ≤ 1, 2 > R	64*	64*	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5
Ciprofloxacin	S ≤ 0,25 0,5 > R	0,5*	0,5*	S≤ 0,001 0,5<R	S≤ 0,001 0,5<R	0,25	0,25	S ≤0,25 0,5 > R	0,25	0,25	0,5
Meropenem	S ≤ 2 8 > R	2*	2*	S≤ 2 2<R	S≤ 2 2<R	2	2	2	2	2	2
Penicillin G	-	-	-	-	-	0,5	0,5	0,5	0,5	0,5	0,25
Trimethoprim	S ≤ 4 > R	-	-	-	-						

viii. Genome assembly quality assessment

A) 4C_S_KR – *C. sylvatica*

	Shovill_on_data_5_and_data_6__Contigs
# contigs (>= 0 bp)	103
# contigs (>= 1000 bp)	43
Total length (>= 0 bp)	6464688
Total length (>= 1000 bp)	6448942
# contigs	52
Largest contig	680870
Total length	6454949
GC (%)	49.36
N50	277872
N90	85146
auN	320786.7
L50	8
L90	22
# N's per 100 kbp	0.00

B) 5E_S_SY – *P. aeruginosa*

	Shovill_on_data_1_and_data_3__Contigs
# contigs (>= 0 bp)	140
# contigs (>= 1000 bp)	59
Total length (>= 0 bp)	6554258
Total length (>= 1000 bp)	6534883
# contigs	68
Largest contig	541885
Total length	6541129
GC (%)	66.29
N50	239732
N90	67376
auN	259949.6
L50	9
L90	29
# N's per 100 kbp	0.00

C) 8C_W_KR – *Pandoraea sp*

	Shovill_on_data_8_and_data_7__Contigs
# contigs (>= 0 bp)	126
# contigs (>= 1000 bp)	41
Total length (>= 0 bp)	4874684
Total length (>= 1000 bp)	4856059
# contigs	50
Largest contig	1217022
Total length	4862061
GC (%)	66.04
N50	504121
N90	78281
auN	622526.0
L50	3
L90	12
# N's per 100 kbp	0.00

Appendix viii.1: Genome assembly quality assessment. Shows the Genome assembly assessment by the use of the "Quast" tool in Galaxy (https://usegalaxy.eu/) (Gurevich et al., 2013; Mikheenko et al., 2018; Mikheenko et al., 2016). All statistics are based on contigs of size >= 500 bp, unless otherwise noted.
A) Quality assessment of the genome assembly by the "Shovill" tool for 4C_S_KR *C. sylvatica.*
B) Quality assessment of the genome assembly by the "Shovill" tool for 5E_S_SY *P. aeruginosa.*
C) Quality assessment of the genome assembly by the "Shovill" tool for 8C_W_KR *Pandoraea sp.*

ix. Sequence alignments of β-lactamase genes

A)

Score	Expect	Method	Identities	Positives	Gaps
394 bits(1013)	6e-136	Compositional matrix adjust.	180/265(68%)	212/265(80%)	1/265(0%)

```
Query  1    MRIIFLAAVLLLLCCPRAGAQDPAAFFRDCGVTGSTTIYDYNNRKWHYTDSADAQQASLP  60
            ++IIF   VLL +C   A   D    F+DC VTGSTT+YDY  ++W +TDS DA +ASLP
Sbjct  12   IKIIF-CIVLLFICSSAAAQHDYQQLFKDCHVTGSTTVYDYKQQRWIFTDSTDANKASLP  70

Query  61   ASTFKIINLLVALQAGVISSVHDTVRWPGSTDTVKYGYRPDIYHDMPVDEAFRLSAGWVF  120
            ASTFKIINLL+AL+ G I + +DTVRWPGSTDT KYGYRPDIYHDM V EAF++SAGW F
Sbjct  71   ASTFKIINLLIALETGTIKTENDTVRWPGSTDTTKYGYRPDIYHDMSVKEAFQVSAGWAF  130

Query  121  VELAKKIGRDKYRYYLRACNYGNGRLDEADADFWNFGAFGISPQNQVEFLLKVYREKLPF  180
            VELAK+IGR+KYRYYL+ CNYGN +LDE DADFWNFGAFGI+P NQ+ FL  VY +K+PF
Sbjct  131  VELAKRIGREKYRYYLKKCNYGNNQLDEKDADFWNFGAFGITPVNQIGFLKNVYEDKVPF  190

Query  181  AAKYIRTLKEVMITETTPAYTIHSKTGWTRPDGYDLGWWTGYVESGKNIYFFATRIWKPG  240
            + + IR LK+VMITE TP YTIHSKTGWTRPDGYD+GWWTGYVE+  N+YFFATRIWK
Sbjct  191  SKRNIRILKDVMITEKTPQYTIHSKTGWTRPDGYDMGWWTGYVETTDNVYFFATRIWKER  250

Query  241  STENPGFSRCRQQITKEILKQLGAL  265
               N  FS+CRQQITK ILK + A+
Sbjct  251  KIVNDNFSKCRQQITKSILKDIQAI  275
```

B)

Score	Expect	Method	Identities	Positives	Gaps
502 bits(1293)	1e-178	Compositional matrix adjust.	240/267(90%)	253/267(94%)	1/267(0%)

```
Query  1    MKQLGWVLAAVMISLAACAPNNVTEEKGWEKYFSQYKVEGTFMLFNNSQGIFKVYNLDRA  60
            MKQLGWVLA VMISL ACAPNNV EEKGWEKYFSQYKVEGTFMLFNNSQG+FKVYNLDRA
Sbjct  2    MKQLGWVLAVVMISLTACAPNNVKEEKGWEKYFSQYKVEGTFMLFNNSQGVFKVYNLDRA  61

Query  61   KERFLPASTFKIFNSLVGLQTGAIKDTGMVIPWDGVPREEP-WNKDLSMQEAFKVSAVPY  119
            KERFLPASTFKIFNSLVGL TG IKDTGMVIPWDGV RE P WN+DLSMQ+AFK+SAVPY
Sbjct  62   KERFLPASTFKIFNSLVGLTTGVIKDTGMVIPWDGVTREVPGWNQDLSMQQAFKLSAVPY  121

Query  120  YQEVARRIGKQTMQLWLDSVKYGNMKISRIDTFWLDNSLQISPDEELGFVKKLYFDQLPF  179
            YQEVARRIGK  MQ+WLDSVKYGNMKISRIDTFWLDN+LQISPDEELGFVKKLYFDQLPF
Sbjct  122  YQEVARRIGKDQMQMWLDSVKYGNMKISRIDTFWLDNTLQISPDEELGFVKKLYFDQLPF  181

Query  180  SKTAMKAVRDVMLMEKTPKYELRYKTGWGVTGKKSIGWIVGWIEENRHPSFFVLNFESED  239
            SKTAMK+VRDVM+MEKTPKYEL YKTGWGV GKK+I W+VG+IEENRHPSFFVLNFE+ED
Sbjct  182  SKTAMKSVRDVMVMEKTPKYELSYKTGWGVVGKKNIAWVVGYIEENRHPSFFVLNFETED  241

Query  240  PKLDLVKARMDILRGILTDAGYFKGEM  266
            PKLDL+KARMDILR ILTDAGYFKGEM
Sbjct  242  PKLDLIKARMDILRNILTDAGYFKGEM  268
```

Appendix ix.1: Sequence alignments of β-lactamase genes detected in 4C_S_KR *C. sylvatica.* Sequence alignments for genes found in 4C_S_KR by PROKKA and the top result by BLASTp in NCBI.
A) Shows the alignment of a discovered *blaOXA-2* gene and Class D β-lactamases gene from NCBI (WP_167016720.1)
B) Shows the alignment of a discovered *blaOXA-10* gene and Class D β-lactamases gene from NCBI (WP_245950823.1)

Score	Expect	Method	Identities	Positives	Gaps
800 bits(2066)	0.0	Compositional matrix adjust.	395/397(99%)	396/397(99%)	1/397(0%)

```
Query  1    MRDTGFPCLCGIAASTLLFAATSAIAGEAPADRLKTLVDAAVQPVMKANDIPGLAVAISL  60
            MRDTGFPCLCGIAASTLLFAATSAIAGEAPADRLKTLVDAAVQPVMKANDIPGLAVAISL
Sbjct  1    MRDTGFPCLCGIAASTLLFAATSAIAGEAPADRLKTLVDAAVQPVMKANDIPGLAVAISL  60

Query  61   KGEPHYFSYGLASKEDGRRVTPETLFEIGSVSKTFTATLAGYALAQDKMRLDDRASLHWP  120
            KGEPHYFSYGLASKEDGRRVTPETLFEIGSVSKTFTATLAGYALAQDKMRLDDRASLHWP
Sbjct  61   KGEPHYFSYGLASKEDGRRVTPETLFEIGSVSKTFTATLAGYALAQDKMRLDDRASLHWP  120

Query  121  ALQGSRFDGISLLDLATYTAGGLPLQFPDSVQKDQAQIR-YYRQWQPTYTPGSQRLYSNP  179
            ALQGSRFDGISL+DLATYTAGGLPLQFPDSVQKDQAQIR YYRQWQPTYTPGSQRLYSNP
Sbjct  121  ALQGSRFDGISLIDLATYTAGGLPLQFPDSVQKDQAQIRDYYRQWQPTYTPGSQRLYSNP  180

Query  180  SIGLFGYLAARSLGQPFERIMEQQLFPALGLEQTHLDVPEAALAQYAQGYGKDDRPLRVG  239
            SIGLFGYLAARSLGQPFERIMEQQLFPALGLEQTHLDVPEAALAQYAQGYGKDDRPLRVG
Sbjct  181  SIGLFGYLAARSLGQPFERIMEQQLFPALGLEQTHLDVPEAALAQYAQGYGKDDRPLRVG  240

Query  240  PGPLDAEGYGVKTSAADLLRFVDANLHPERLDKPWAQALDATHRGYYKVGDMTQGLGWEA  299
            PGPLDAEGYGVKTSAADLLRFVDANLHPERLDKPWAQALDATHRGYYKVGDMTQGLGWEA
Sbjct  241  PGPLDAEGYGVKTSAADLLRFVDANLHPERLDKPWAQALDATHRGYYKVGDMTQGLGWEA  300

Query  300  YDWPISLKRLQAGNSTPMALQPHRIARLPAPQALEGQRLLNKTGSTNGFGAYVAFIPGRD  359
            YDWPISLKRLQAGNSTPMALQPHRIARLPAPQALEGQRLLNKTGSTNGFGAYVAFIPGRD
Sbjct  301  YDWPISLKRLQAGNSTPMALQPHRIARLPAPQALEGQRLLNKTGSTNGFGAYVAFIPGRD  360

Query  360  LGLVILANRNYPNAERVKIAYAILSGLEQQAKVPLKR  396
            LGLVILANRNYPNAERVKIAYAILSGLEQQAKVPLKR
Sbjct  361  LGLVILANRNYPNAERVKIAYAILSGLEQQAKVPLKR  397
```

Appendix ix.2: Sequence alignments of β-lactamase genes detected in 5E_S_SY K *P. aeruginosa.* Sequence alignments for a *blaPDC-202* gene found in 5E_S_SY by PROKKA and the top result by BLASTp in NCBI.

A)

Score	Expect	Method	Identities	Positives	Gaps
835 bits(2157)	0.0	Compositional matrix adjust.	407/410(99%)	409/410(99%)	0/410(0%)

```
Query  1    MSKPRPTAFTTLAALAAATAIVWMGLSTRSHAAQPQPENDPRLAEVRSLVDQTVEPLMAR  60
            MSKPRPTAFTTLAALAAATAIVWMGLSTRSHAAQPQPENDPRLAEVRSLVDQTVEPLMAR
Sbjct  1    MSKPRPTAFTTLAALAAATAIVWMGLSTRSHAAQPQPENDPRLAEVRSLVDQTVEPLMAR  60

Query  61   QQIPGMAVGVAFDGKSYVFDYGVADKADNRPVTPDTLFEIGSVSKTFTATLATYAQGAGA  120
            QQIPGMAVGVAFDGKSYVFDYGVADKADNRPVTPDTLFEIGSVSKTFTATLATYAQGAGA
Sbjct  61   QQIPGMAVGVAFDGKSYVFDYGVADKADNRPVTPDTLFEIGSVSKTFTATLATYAQGAGA  120

Query  121  LSLRDKTSRFIPEVAGTPFGNISLVNLATHTTGGMPLQVPDDVTTDEQLLQYLEAWKPAQ  180
            LSLRDKTSRFIPEVAGTPFGNISLVNLATHTTGGMPLQVPDDVTTDEQLLQYLEAWKPAQ
Sbjct  121  LSLRDKTSRFIPEVAGTPFGNISLVNLATHTTGGMPLQVPDDVTTDEQLLQYLEAWKPAQ  180

Query  181  PAGTVRTYSNVSIGMLGRIVARAMRGDFATLMTQHVFRPLELGHTYIRVPADQMQHYAWG  240
            PAGTVRTYSNVSIGMLGRIVARAMRGDFATLMTQHVFRPLELGHTY+RVPADQMQHYAWG
Sbjct  181  PAGTVRTYSNVSIGMLGRIVARAMRGDFATLMTQHVFRPLELGHTYLRVPADQMQHYAWG  240

Query  241  YGKEGNPVRVSPGLLEAEAYGVKTTAGDLLRFVNANLGKPVFDHRLRHAIYAARTGYFFD  300
            YGK+GNPVRVSPGLLEAEAYGVKTTAGDLLRFVNANLGKPVFDHRLRHAIYAARTGYFFD
Sbjct  241  YGKDGNPVRVSPGLLEAEAYGVKTTAGDLLRFVNANLGKPVFDHRLRHAIYAARTGYFFD  300

Query  301  KPMTQDLIWEQYPYPVSVETLLEGNSAKMAYEPTPVREFSPPMAPTPVAWVNKTGSTNGF  360
            KPMTQDLIWEQYPYPVSVETLLEGNSAKMAYEPTPVREFSPPMAPTPVAWVNKTGSTNGF
Sbjct  301  KPMTQDLIWEQYPYPVSVETLLEGNSAKMAYEPTPVREFSPPMAPTPVAWVNKTGSTNGF  360

Query  361  GAYVAFVPYRQMGIVMLANKNYPIDERVRAAHRILTVLDGMPRTPVVPQP  410
            GAYVAFVP RQMGIVMLANKNYPIDERVRAAHRILTVLDGMPRTPVVPQP
Sbjct  361  GAYVAFVPSRQMGIVMLANKNYPIDERVRAAHRILTVLDGMPRTPVVPQP  410
```

B)

Score	Expect	Method	Identities	Positives	Gaps
578 bits(1491)	0.0	Compositional matrix adjust.	282/283(99%)	282/283(99%)	0/283(0%)

```
Query  1    MKKTLSRWRRGALALRFLGALASPVVFAMPGHAAEPAHSSAVRIAERADWGKYFADEGVK  60
            MKKTLSRWRRGALALR LGALASPVVFAMPGHAAEPAHSSAVRIAERADWGKYFADEGVK
Sbjct  1    MKKTLSRWRRGALALRLLGALASPVVFAMPGHAAEPAHSSAVRIAERADWGKYFADEGVK  60

Query  61   GTVIVLDGRTQTYQAYDAARAERRMSPASTYKIFNSLLALESGALDNEREIIPWDGKPRR  120
            GTVIVLDGRTQTYQAYDAARAERRMSPASTYKIFNSLLALESGALDNEREIIPWDGKPRR
Sbjct  61   GTVIVLDGRTQTYQAYDAARAERRMSPASTYKIFNSLLALESGALDNEREIIPWDGKPRR  120

Query  121  VKAWNAALDLRNAFRVSCLPCYQVVSHKIPRQYAQAKLNEAGYGNRTIGRAAHAYWIDDS  180
            VKAWNAALDLRNAFRVSCLPCYQVVSHKIPRQYAQAKLNEAGYGNRTIGRAAHAYWIDDS
Sbjct  121  VKAWNAALDLRNAFRVSCLPCYQVVSHKIPRQYAQAKLNEAGYGNRTIGRAAHAYWIDDS  180

Query  181  LQISAREQVDFLQRLATGTLPFSARSQDIVRNISIVEANVDYVLHGKTGWFTEKKPDIGW  240
            LQISAREQVDFLQRLATGTLPFSARSQDIVRNISIVEANVDYVLHGKTGWFTEKKPDIGW
Sbjct  181  LQISAREQVDFLQRLATGTLPFSARSQDIVRNISIVEANVDYVLHGKTGWFTEKKPDIGW  240

Query  241  WVGWLERDGNLTMIALNIDIQTDADAPKRARIVRNVLKDLKLI  283
            WVGWLERDGNLTMIALNIDIQTDADAPKRARIVRNVLKDLKLI
Sbjct  241  WVGWLERDGNLTMIALNIDIQTDADAPKRARIVRNVLKDLKLI  283
```

Appendix ix.3: Sequence alignments of β-lactamase genes detected in 8C_W_KR *Pandoraea sp.*. Sequence alignments for genes found in 8C_W_KR by PROKKA and the top result by BLASTp in NCBI.
A) Shows the alignment of a discovered *ampC* gene and Class D β-lactamases gene from NCBI (WP_287496540.*1*)
B) Shows the alignment of a discovered *blaOXA-158* gene and Class D β-lactamases gene from NCBI (NG_049457.1)

x. Mobile genetic elements

Appendix x.1: Mobile genetic elements (MGE) found in the sequence bacteria. Shows the results from search of Mobile Genetic Elements using the "MGE" tool by Center for Genomic Epidemiology. Shows the detected Insertion sequences (IS) and Integrative Conjugative Elements (ICE). Includes potential genes located in the MGEs, sequence identity, alignment coverage and accession number for MGEs and genes.

MGE	Identity MGE (%)	Coverage MEG (%)	Accession MGE	Gene	Identity gene (%)	Coverage gene (%)	Accession number	Phenotype gene
5E_S_SY: *Pseudomonas aeruginosa*								
ISPa6	96,49	98,27	U16784	*fosA*	98,04	100	ACWU01000146	Fosfomycin resistance
ISPa86	93,2	99,1	MF344569	-	-	-	-	-
ICE(Tn4371)6041	98,44	99,86	AAKW01000024	-	-	-	-	-
ISPa32	98,86	98,95	NC_002516	-	-	-	-	-
8C_W_KR								
IS407	97,17	99,6	M82980	-	-	-	-	-

xi. Resistance genes

Appendix xi.1: Resistance genes detected by CARD and NCBI. Shows all the resistance genes detected by the databases NCBI and CARD for 5E_S_SY *P. aeruginosa* and 8C_W_KR *Pandoraea sp.* No genes were detected by these databases for 4C_S_KR *C. silvatica.*

Resistance genes	Database	Identity (%)	Query coverage (%)	Accession number	Comment
5E_S_SY *P. aeruginosa*					
bcr-1	CARD	99.09	100.00	CP012901.1:5980365-5979156	Transmembrane protein, confer bicyclomycin resistance
APH(3')-IIb	CARD	97.52	100.00	X90856:387-1194	Aminoglycoside phosphotransferase
PDC-10	CARD	97.57	100.00	FJ666073:0-1194	Extended-spectrum beta-lactamase, resistance to carbapenem, cephalosporin and monobactam
mexX	CARD	97.63	100.00	AB015853:145-1315	MexXY-OprM multidrug efflux complex
mexY	CARD	97.93	99.81	AB015853:1330-4471	
MexC	CARD	98.45	100.00	U57969:294-1458	MexCD-OprJ multidrug
MexD	CARD	97.67	99.97	U57969:1485-4617	
OprJ	CARD	97.99	100.00	U57969:4622-6062	
mexK	CARD	97.82	100.00	AE004091.2:4119265-4116187	MexJK multidrug efflux protein
mexJ	CARD	99.37	100.00	AE004091.2:4120373-4119269	

mexL	CARD	98.28	100.00	AE004091.2:4120468-4121107	
ArmR	CARD	98.15	100.00	AE004091.2:4165880-4165718	Antirepressor, upregulate MexAB-OprM
OXA-50	CARD	98.73	100.00	AY306130:0-789	Beta-lactamase, confer decreased susceptibility to ampicillin, ticarcillin, moxalactam and meropenem
CpxR	CARD	99.70	100.00	LT673656.1:1885022-1884344	Activation of expression of RND efflux pump MexAB-OprM
TriA	CARD	99.13	100.00	AE004091.2:177306-178458	Efflux pump TriABC-OpmH
TriB	CARD	99.16	100.00	AE004091.2:178454-179525	
TriC	CARD	98.89	100.00	AE004091.2:179521-182569	
fosA	CARD	97.79	100.00	AE004091.2:1221690-1222098	Fosfomycin resistance
opmE	CARD	98.10	100.00	AB219524.1:4334-5810	Multidrug efflux pump MexPQ-OpmE
mexQ	CARD	98.96	100.00	AB219524.1:1176-4338	
mexP	CARD	98.70	100.00	AB219524.1:22-1180	
arnA	CARD	99.20	100.00	AE004091.2:3982020-3984009	Modifies lipid A with 4-amino-4-deoxy-L-arabinose (Ara4N)
MexE	CARD	98.72	100.00	AE004091.2:2808742-2809987	MexEF-OprN multidrug efflux complex
MexF	CARD	99.22	100.00	AE004091.2:2810008-2813197	
OprN	CARD	98.52	100.00	AE004091.2:2813193-2814612	
OpmB	CARD	97.87	100.00	AE004091.2:2847779-2846282	MuxABC-OpmB efflux pumps
MuxC	CARD	99.16	100.00	AE004091.2:2850886-2847775	
MuxB	CARD	99.01	100.00	AE004091.2:2854014-2850882	
MuxA	CARD	98.67	100.00	AE004091.2:2855291-2854010	
soxR	CARD	99.15	100.00	AE004091.2:2503895-2503424	Transcriptional activator
basS	CARD	99.09	100.00	JQ340365:0-1434	Histidine protein kinase sensor Lipid A modification gene
catB7	CARD	97.65	100.00	NC_002516.2:780101-779462	chromosome-encoded cat gene
mexW	CARD	99.18	100.00	NC_002516.2:4904646-4907703	Efflux complex MexVW-OprM
mexV	CARD	98.94	100.00	AE004091.2:4903465-4904596	
aph(3')-IIb	NCBI	98.51	100.00	NG_047424.1	Aminoglycoside O-phosphotransferase APH(3')-

					IIb, confer kanamycin resistance
blaPDC-202	NCBI	99.83	100.00	NG_054987.1	Class C beta-lactamase PDC-202
blaOXA-50	NCBI	98.73	100.00	NG_049777.1	Oxacillin-hydrolyzing class D beta-lactamase OXA-50
fosA-354827590	NCBI	98.04	100.00	NG_047883.1	Fosfomycin resistance glutathione transferase
catB7	NCBI	97.65	100.00	NG_047614.1	type B-4 chloramphenicol O-acetyltransferase CatB7, Chloramphenicol resistance
8C_W_KR *Pandoraea sp.*					
ceoB	CARD	81.78	99.25	U97042:1263-4347	CeoAB-OpcM efflux pump
OXA-158	CARD	99.30	100.00	KP771986.1:1028-1880	OXA-158 is a beta-lactamase, resistance to cephalosporin and penam
blaOXA-158	NCBI	99.30	100.00	NG_049457.1	OXA-62 family carbapenem-hydrolyzing class D beta-lactamase OXA-158

Appendix xi.2: Detected resistance genes by PROKKA for 4C_S_KR. Shows the detected resistance genes for 4C_S_KR *C. silvatica* by PROKKA. Alle detected genes were searched in BLASTp by NCBI.

Resistance genes	**Database**	**Identity (%)**	**Query coverage (%)**	**Accession number**	**Comment**
4C_S_KR *C. silvatica*					
bla	nr*	67,92	100	WP_167016720.1	Class D beta-lactamase
bla	nr*	89,99	100	WP_245950823.1	Class D beta-lactamase
bcr	nr*	81.60	100	WP_255492060.1	Multidrug effflux MFS transporter
ble	nr*	75.42	100	WP_111600268.1	VOC family protein
drrA	nr*	78.60	100	WP_291941056.1	ATP-binding cassette domain-containing protein
emrA	nr*	89.66	100	WP_245950924.1	HlyD family secretion protein
fsr	nr*	88.70	98	WP_073084226.1	MFS transporter
mdtA	nr*	90.38	100	WP_332002400.1	Efflux RND transporter periplasmic adaptor subunit
mdtB	nr*	90.31	100	WP_291942020.1	Efflux RND transporter
mdtC	nr*	94.34	100	WP_111591022.1	permease subunit
mdtE	nr*	85.29	100	WP_291948072.1	Efflux RND transporter periplasmic adaptor subunit
mdtG	nr*	91.04	99	WP_211117778.1	MFS transporter
mdtK	nr*	91.13	99	WP_291951515.1	MATE family efflux transporter
Mecl	nr*	86.55	99	WP_291944399.1	BlaI/MecI/CopY family transcriptional regulator
mexA	nr*	73.99	97	WP_169227130.1	Efflux RND transporter periplasmic adaptor subunit
stp	nr*	75.88	99	WP_291907478.1	MFS transporter
tetA	nr*	84.07	100	WP_218376083.1	TCR/Tet family MFS transporter

xii. Virulence genes

Appendix xii.1: Virulence genes detected by VFDB. Shows all the resistance genes detected by the VFDB databases for 5E_S_SY *P. aeruginosa* and 8C_W_KR *Pandoraea sp*. No genes were detected for 4C_S_KR *C. silvatica.*

Virulence genes	Database	Identity (%)	Query coverage (%)	Accession number	Comment
8C_W_KR *C. silvatica*					
cheB	VFDB	80.28	88.50	YP_109897	Chemotaxis-specific methylesterase [Flagella (VF0430)] [Burkholderia pseudomallei K96243]
cheW	VFDB	83.26	87.12	YP_109901	Chemotaxis protein CheW [Flagella (VF0430)] [Burkholderia pseudomallei K96243]
5E_S_SY *P. aeruginosa*					
alg44	VFDB	99.74	100.00	NP_252232	Alginate biosynthesis protein Alg8 [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
alg8	VFDB	99.60	100.00	NP_252231	Alginate-c5-mannuronan-epimerase AlgG [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algA	VFDB	99.38	100.00	NP_252241	Phosphomannose isomerase / guanosine 5'-diphospho-D-mannose pyrophosphorylase [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algB	VFDB	98.81	100.00	NP_254170	Two-component response regulator AlgB [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algC	VFDB	100.00	100.00	NP_254009	Phosphomannomutase AlgC [Alginate biosynthesis (CVF522)] [Pseudomonas aeruginosa PAO1]
algD	VFDB	99.08	100.00	NP_252230	(algD) GDP-mannose 6-dehydrogenase AlgD [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algE	VFDB	99.19	100.00	NP_252234	Alginate biosynthetic protein AlgK precursor [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algF	VFDB	99.08	100.00	NP_252240	Alginate o-acetyltransferase AlgF [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algG	VFDB	99.20	100.00	NP_252235	Outer membrane protein AlgE [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algI	VFDB	99.42	100.00	NP_252238	Alginate o-acetyltransferase AlgI [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algJ	VFDB	99.75	100.00	NP_252239	Alginate o-acetyltransferase AlgJ [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algK	VFDB	99.23	100.00	NP_252233	Alginate biosynthesis protein Alg44 [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algL	VFDB	99.09	100.00	NP_252237	Poly(beta-d-mannuronate) lyase precursor AlgL [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algP/algR3	VFDB	96.13	98.87	NP_253940	Alginate regulatory protein AlgP [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algQ	VFDB	98.76	100.00	NP_253942	Alginate regulatory protein AlgQ [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algR	VFDB	99.47	100.00	NP_253948	Alginate biosynthesis regulatory protein AlgR [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algU	VFDB	99.83	100.00	NP_249453	Alginate biosynthesis protein AlgZ/FimS [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algW	VFDB	99.14	100.00	NP_253136	AlgW protein [Alginate regulation (CVF523)] [Pseudomonas aeruginosa PAO1]

algX	VFDB	99.30	100.00	NP_252236	Alginate biosynthesis protein AlgX [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
algZ	VFDB	99.54	100.00	NP_253949	Sigma factor AlgU [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
aprA	VFDB	99.31	100.00	NP_249940	Alkaline metalloproteinase precursor [Alkaline protease (VF0090)] [Pseudomonas aeruginosa PAO1]
chpA	VFDB	98.12	99.72	NP_249104	Still frameshift probable component of chemotactic signal transduction system [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
chpB	VFDB	99.22	100.00	NP_249105	Probable methylesterase [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
chpC	VFDB	98.82	100.00	NP_249106	Probable chemotaxis protein [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
chpD	VFDB	98.74	100.00	NP_249107	Probable transcriptional regulator [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
chpE	VFDB	97.55	100.00	NP_249108	Probable chemotaxis protein [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
clpV1	VFDB	98.93	100.00	NP_248780	Type VI secretion system AAA+ family ATPase [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
dotU1	VFDB	98.89	100.00	NP_248768	Type VI secretion system protein DotU [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
exoS	VFDB	91.25	100.00	NP_252530	Type III secretion system effector ExoS ADP ribosyltransferase activity and GTPase-activating protein activity [ExoS (VF0096)] [Pseudomonas aeruginosa PAO1]
exoT	VFDB	98.11	100.00	NP_248734	Type III secretion system effector ExoT ADP ribosyltransferase activity and GTPase-activating protein activity [ExoT (VF0097)] [Pseudomonas aeruginosa PAO1]
exsA	VFDB	99.40	100.00	NP_250404	Type III secretion system regulatory protein ExsA [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
exsB	VFDB	99.28	100.00	NP_250403	(exsB) type III secretion system piolitin ExsB [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
exsC	VFDB	98.63	100.00	NP_250401	Type III secretion system regulatory protein ExsC [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
exsD	VFDB	98.80	100.00	NP_250405	Type III secretion system regulatory protein ExsD [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
exsE	VFDB	97.97	100.00	NP_250402	Type III secretion system regulatory protein ExsE [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
fha1	VFDB	99.06	99.60	NP_248771	Type VI secretion system forkhead-associated protein Fha1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
fimT	VFDB	97.25	100.00	NP_253239	Type 4 fimbrial biogenesis protein FimT [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
fimU	VFDB	99.41	100.00	NP_253240	Type 4 fimbrial biogenesis protein FimU [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
fimV	VFDB	98.22	100.00	NP_251805	Putative Type IV pili related protein [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
fleN	VFDB	99.53	100.00	NP_250145	Flagellar synthesis regulator FleN [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fleQ	VFDB	99.05	100.00	NP_249788	Transcriptional regulator FleQ [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]

fleR	VFDB	98.11	100.00	NP_249790	Two-component response regulator [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fleS	VFDB	99.26	100.00	NP_249789	Two-component sensor [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
flgA	VFDB	99.00	100.00	NP_252040	Flagellar basal body P-ring biosynthesis protein FlgA [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
flgB	VFDB	99.75	100.00	NP_249768	Flagellar basal body rod protein FlgB [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
flgC	VFDB	99.55	100.00	NP_249769	Flagellar basal-body rod protein FlgC [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgD	VFDB	100.00	100.00	NP_249770	Flagellar basal-body rod modification protein FlgD [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgE	VFDB	99.71	100.00	NP_249771	Flagellar hook protein FlgE [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgF	VFDB	100.00	100.00	NP_249772	Flagellar basal-body rod protein FlgF [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgG	VFDB	99.75	100.00	NP_249773	(flgG) flagellar basal-body rod protein FlgG [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgH	VFDB	99.42	100.00	NP_249774	Flagellar L-ring protein precursor FlgH [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgI	VFDB	98.83	100.00	NP_249775	Flagellar P-ring protein precursor FlgI [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgJ	VFDB	97.09	100.00	NP_249776	Flagellar rod assembly protein/muramidase FlgJ [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgK	VFDB	85.74	99.71	NP_249777	Flagellar hook-associated protein 1 FlgK [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flgM	VFDB	99.07	100.00	NP_252041	Negative regulator of flagellin synthesis [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
flgN	VFDB	98.30	100.00	NP_252042	Flagella synthesis protein FlgN [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
flhA	VFDB	98.96	100.00	NP_250143	Flagellar biosynthesis protein FlhA [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flhB	VFDB	98.68	100.00	NP_250140	Flagellar biosynthetic protein FlhB [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
flhF	VFDB	99.22	100.00	NP_250144	Flagellar biosynthesis protein FlhF [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliA	VFDB	99.33	100.00	NP_250146	Flagellar biosynthesis sigma factor FliA [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
fliE	VFDB	98.48	100.00	NP_249791	Flagellar hook-basal body complex protein FliE [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliF	VFDB	99.11	100.00	NP_249792	Flagellar M-ring protein FliF [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliG	VFDB	99.41	100.00	NP_249793	Flagellar motor switch protein G [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]

fliH	VFDB	97.89	100.00	NP_249794	Flagellar assembly protein H [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliI	VFDB	98.89	100.00	NP_249795	Flagellum-specific ATP synthase FliI [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliJ	VFDB	99.10	100.00	NP_249796	Flagellar protein FliJ [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliK	VFDB	98.21	100.00	NP_250132	Flagellar hook-length control protein FliK [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
fliL	VFDB	98.85	100.00	NP_250133	Flagellar basal body protein FliL [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
fliM	VFDB	99.59	100.00	NP_250134	Flagellar motor switch protein FliM [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliN	VFDB	99.37	100.00	NP_250135	Flagellar motor switch protein FliN [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliO	VFDB	98.90	100.00	NP_250136	Flagellar protein FliO [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliP	VFDB	98.96	100.00	NP_250137	Flagellar biosynthetic protein FliP [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliQ	VFDB	99.63	100.00	NP_250138	Flagellar biosynthetic protein FliQ [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fliR	VFDB	98.96	99.36	NP_250139	Flagellar biosynthetic protein FliR [Flagella (VF0273)] [Pseudomonas aeruginosa PAO1]
fptA	VFDB	98.61	100.00	NP_252911	Fe(III)-pyochelin receptor precursor [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
fpvA	VFDB	99.55	100.00	NP_251088	Ferripyoverdine receptor FpvA [Pyoverdine (VF0094)] [Pseudomonas aeruginosa PAO1]
hcp1	VFDB	99.39	100.00	NP_248775	Type VI secretion system substrate Hcp1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiA1	VFDB	98.07	100.00	NP_248772	Type VI secretion system hcp secretion island protein HsiA1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiB1/vipA	VFDB	99.42	100.00	NP_248773	Type VI secretion system tubule-forming protein VipA [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiC1/vipB	VFDB	99.60	100.00	NP_248774	Type VI secretion system tubule-forming protein VipB [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiE1	VFDB	99.17	100.00	NP_248776	Type VI secretion system hcp secretion island protein HsiE1 interacting with HsiB1 to form a novel subcomplex of the T6SS [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiF1	VFDB	98.63	100.00	NP_248777	Type VI secretion system hcp secretion island protein HsiF1 a gp25-like protein but not exhibit lysozyme activity [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiG1	VFDB	98.87	100.00	NP_248778	Type VI secretion system hcp secretion island protein HsiG1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiH1	VFDB	98.85	100.00	NP_248779	Type VI secretion system hcp secretion island protein HsiH1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
hsiJ1	VFDB	99.03	100.00	NP_248769	Type VI secretion system hcp secretion island protein HsiJ1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
icmF1/tssM1	VFDB	99.30	100.00	NP_248767	Type VI secretion system protein IcmF1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]

lasA	VFDB	97.61	100.00	NP_250562	LasA protease precursor [LasA (VF0088)] [Pseudomonas aeruginosa PAO1]
lasB	VFDB	98.73	100.00	NP_252413	Elastase LasB [LasB (VF0087)] [Pseudomonas aeruginosa PAO1]
lasI	VFDB	98.84	100.00	NP_250123	Autoinducer synthesis protein LasI [Quorum sensing (VF0093)] [Pseudomonas aeruginosa PAO1]
lip1	VFDB	99.57	100.00	NP_248770	Lipoprotein [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
mbtH-like	VFDB	100.00	100.00	NP_251102	MbtH-like protein from the pyoverdine cluster [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
motA	VFDB	99.53	100.00	NP_253641	Flagellar motor protein [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
motB	VFDB	98.85	100.00	NP_253640	Flagellar motor protein [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
motC	VFDB	99.19	100.00	NP_250151	Flagellar motor protein [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
motD	VFDB	97.98	100.00	NP_250152	Flagellar motor protein [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
motY	VFDB	98.86	100.00	NP_252216	Probable outer membrane protein precursor [Deoxyhexose linking sugar 209 Da capping structure (AI138)] [Pseudomonas aeruginosa PAO1]
mucA	VFDB	99.14	100.00	NP_249454	Alkaline metalloproteinase precursor [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
mucB	VFDB	98.84	100.00	NP_249455	Anti-sigma factor MucA inhibitor of alg gene expression [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
mucC	VFDB	99.34	100.00	NP_249456	Negative regulator for alginate biosynthesis MucB [Alginate (VF0091)] [Pseudomonas aeruginosa PAO1]
mucD	VFDB	99.16	100.00	NP_249457	Serine protease MucD precursor [Alginate regulation (CVF523)] [Pseudomonas aeruginosa PAO1]
mucE	VFDB	97.04	100.00	NP_252722	Small envelope protein MucE [Alginate regulation (CVF523)] [Pseudomonas aeruginosa PAO1]
mucP	VFDB	98.37	100.00	NP_252339	Metalloprotease protease [Alginate regulation (CVF523)] [Pseudomonas aeruginosa PAO1]
pchA	VFDB	98.25	100.00	NP_252921	Salicylate biosynthesis isochorismate synthase PchA [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchB	VFDB	99.02	100.00	NP_252920	Salicylate biosynthesis protein PchB [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchC	VFDB	98.55	100.00	NP_252919	Pyochelin biosynthetic protein PchC [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchD	VFDB	98.72	100.00	NP_252918	Pyochelin biosynthesis protein PchD [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchE	VFDB	98.03	100.00	NP_252916	Dihydroaeruginoic acid synthetase PchE [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchF	VFDB	97.92	100.00	NP_252915	Pyochelin synthetase PchF [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchG	VFDB	98.29	100.00	NP_252914	Pyochelin biosynthetic protein PchG [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]

pchH	VFDB	97.90	100.00	NP_252913	ABC transporter ATP-binding protein [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchI	VFDB	98.67	100.00	NP_252912	ABC transporter ATP-binding protein [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pchR	VFDB	99.21	100.00	NP_252917	Transcriptional regulator PchR [Pyochelin (VF0095)] [Pseudomonas aeruginosa PAO1]
pcr1	VFDB	99.28	100.00	NP_250390	Type III secretion system protein Pcr1 [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcr2	VFDB	98.92	100.00	NP_250391	Type III secretion system protein Pcr2 [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcr3	VFDB	97.81	100.00	NP_250392	Type III secretion system protein Pcr3 [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcr4	VFDB	98.79	100.00	NP_250393	Type III secretion system protein Pcr4 [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcrD	VFDB	98.68	100.00	NP_250394	Type III secretion system protein PcrD [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcrG	VFDB	96.97	100.00	NP_250396	Type III secretion system cytoplasmic regulator PcrG [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcrH	VFDB	98.62	100.00	NP_250398	Type III secretion system regulatory protein PcrH [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcrR	VFDB	98.85	100.00	NP_250395	Type III secretion system regulatory protein PcrR [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pcrV	VFDB	98.08	100.00	NP_250397	Type III secretion system hydrophilic translocator needle tip protein PcrV [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
phzA1	VFDB	98.57	100.00	NP_252899	Phenazine biosynthesis protein PhzA [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzA1	VFDB	95.94	90.59	NP_252899	Phenazine biosynthesis protein PhzA [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzB1	VFDB	97.34	100.00	NP_252900	Phenazine biosynthesis protein PhzB [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzB1	VFDB	89.19	88.96	NP_252900	Phenazine biosynthesis protein PhzB [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzC1	VFDB	99.26	100.00	NP_252901	Phenazine biosynthesis protein PhzC [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzD1	VFDB	99.04	100.00	NP_252902	Phenazine biosynthesis protein PhzD isochorismatase [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzE1	VFDB	98.99	100.00	NP_252903	Phenazine biosynthesis protein PhzE [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzF1	VFDB	99.40	100.00	NP_252904	Phenazine biosynthesis protein PhzF isomerase [Phenazines biosynthesis (CVF536)] [Pseudomonas aeruginosa PAO1]
phzM	VFDB	99.10	100.00	NP_252898	Phenazine-specific methyltransferase PhzM (adenosylmethionine dependent methyltransferase) [Pyocyanin (VF0100)] [Pseudomonas aeruginosa PAO1]
phzS	VFDB	98.59	100.00	NP_252907	Flavin dependent hydroxylase PhzS [Pyocyanin (VF0100)] [Pseudomonas aeruginosa PAO1]

pilB	VFDB	97.88	100.00	NP_253216	Type 4 fimbrial biogenesis protein PilB [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilC	VFDB	98.49	100.00	NP_253217	Still frameshift type 4 fimbrial biogenesis protein PilC [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilE	VFDB	99.06	100.00	NP_253246	Type 4 fimbrial biogenesis protein PilE [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilF	VFDB	98.95	100.00	NP_252494	Type 4 fimbrial biogenesis protein PilF [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilG	VFDB	99.51	100.00	NP_249099	Twitching motility protein PilG [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilH	VFDB	99.18	100.00	NP_249100	Twitching motility protein PilH [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilI	VFDB	99.81	100.00	NP_249101	Twitching motility protein PilI [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilJ	VFDB	99.41	100.00	NP_249102	Twitching motility protein PilJ [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilK	VFDB	98.63	100.00	NP_249103	Methyltransferase PilK [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilM	VFDB	99.34	100.00	NP_253731	Type IV pilus inner membrane platform protein PilM [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilN	VFDB	98.66	100.00	NP_253730	Type IV pilus inner membrane platform protein PilN [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilO	VFDB	99.20	100.00	NP_253729	Type IV pilus inner membrane platform protein PilO [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilP	VFDB	99.43	100.00	NP_253728	Type IV pilus biogenesis protein PilP [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilQ	VFDB	98.09	100.00	NP_253727	Type 4 fimbrial biogenesis protein PilQ [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilR	VFDB	98.73	100.00	NP_253237	Two-component response regulator PilR [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilS	VFDB	98.24	100.00	NP_253236	Two-component sensor PilS [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilT	VFDB	99.52	100.00	NP_249086	Twitching motility protein PilT [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilU	VFDB	99.30	100.00	NP_249087	Twitching motility protein PilU [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilV	VFDB	99.46	100.00	NP_253241	Type IV pilus biogenesis protein PilV [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilW	VFDB	91.64	100.00	NP_253242	Type IV fimbrial biogenesis protein PilW [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilX	VFDB	93.71	100.00	NP_253243	Type 4 fimbrial biogenesis protein PilX [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilY1	VFDB	93.04	99.43	NP_253244	Type 4 fimbrial biogenesis protein PilY1 [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
pilY2	VFDB	99.42	100.00	NP_253245	Type 4 fimbrial biogenesis protein PilY2 [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
plcH	VFDB	99.22	100.00	NP_249535	Hemolytic phospholipase C precursor [PLC (VF0092)] [Pseudomonas aeruginosa PAO1]
popB	VFDB	98.30	100.00	NP_250399	Type III secretion system hydrophobic translocator pore protein PopB [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
popD	VFDB	98.65	100.00	NP_250400	Type III secretion system hydrophobic translocator pore protein PopD [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]

popN	VFDB	98.39	100.00	NP_250389	Type III secretion system outer membrane protein PopN [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
ppkA	VFDB	98.71	100.00	NP_248764	Serine/threonine protein kinase PpkA [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
pppA	VFDB	99.04	100.00	NP_248765	Pseudomonas protein phosphatase PppA [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
pscB	VFDB	99.05	100.00	NP_250406	Type III secretion system protein PscB [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscC	VFDB	98.78	100.00	NP_250407	Type III secretion system secretin PscC [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscD	VFDB	97.77	100.00	NP_250408	Type III secretion system basal body protein PscD [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscE	VFDB	96.08	100.00	NP_250409	Type III secretion system cochaperone PscE for PscG [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscF	VFDB	99.61	100.00	NP_250410	Type III secretion system needle filament protein PscF [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscG	VFDB	98.28	100.00	NP_250411	Type III secretion system chaperone PscG for PscF [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscH	VFDB	97.69	100.00	NP_250412	Type III secretion system protein PscH [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscI	VFDB	99.11	100.00	NP_250413	Type III secretion system inner rod protein PscI [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscJ	VFDB	99.06	100.00	NP_250414	Type III secretion system inner MS ring protein [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscK	VFDB	97.45	99.04	NP_250415	Type III secretion system protein PscK [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscL	VFDB	98.30	100.00	NP_250416	Type III secretion systemt protein PscL [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscN	VFDB	98.41	100.00	NP_250388	Type III secretion system ATPase PscN [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscO	VFDB	98.53	100.00	NP_250387	Type III secretion system protein PscO [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscP	VFDB	94.05	96.22	NP_250386	Type III secretion system protein PscP [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscQ	VFDB	98.50	100.00	NP_250385	Type III secretion system protein PscQ [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscR	VFDB	99.23	100.00	NP_250384	Type III secretion system protein PscR [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscS	VFDB	99.62	100.00	NP_250383	Type III secretion system protein PscS [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscT	VFDB	99.11	100.00	NP_250382	Type III secretion system protein PscT [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
pscU	VFDB	99.33	100.00	NP_250381	Type III secretion system protein PscU [TTSS (VF0083)] [Pseudomonas aeruginosa PAO1]
ptxR	VFDB	98.83	100.00	NP_250948	Transcriptional regulator PtxR [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvcA	VFDB	99.09	100.00	NP_250944	Paerucumarin biosynthesis protein PvcA [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvcB	VFDB	98.06	100.00	NP_250945	Paerucumarin biosynthesis protein PvcB [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]

pvcC	VFDB	98.94	100.00	NP_250946	Paerucumarin biosynthesis protein PvcC [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvcD	VFDB	96.76	100.00	NP_250947	Paerucumarin biosynthesis protein PvcD [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdA	VFDB	98.87	100.00	NP_251076	L-ornithine N5-oxygenase PvdA [Pyoverdine (VF0094)] [Pseudomonas aeruginosa PAO1]
pvdE	VFDB	99.27	100.00	NP_251087	Pyoverdine biosynthesis protein PvdE [Pyoverdine (VF0094)] [Pseudomonas aeruginosa PAO1]
pvdF	VFDB	98.91	100.00	NP_251086	Pyoverdine synthetase F [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdG	VFDB	98.17	100.00	NP_251115	Pyoverdine biosynthesis protein PvdG [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdH	VFDB	98.86	100.00	NP_251103	Diaminobutyrate-2-oxoglutarate aminotransferase PvdH [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdI	VFDB	98.21	99.96	NP_251092	Peptide synthase [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdJ	VFDB	99.10	100.00	NP_251090	Pyoverdine biosynthesis protein PvdJ [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdL	VFDB	98.53	100.00	NP_251114	Peptide synthase PvdL [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdM	VFDB	98.96	100.00	NP_251083	Dipeptidase precursor [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdN	VFDB	98.75	100.00	NP_251084	Pyoverdine biosynthesis protein PvdN [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdO	VFDB	98.71	100.00	NP_251085	Pyoverdine biosynthesis protein PvdO [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdP	VFDB	99.33	100.00	NP_251082	Tyrosinase required for pyoverdine maturation [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdQ	VFDB	98.78	100.00	NP_251075	3-oxo-C12-homoserine lactone acylase PvdQ [pyoverdine (IA001)] [Pseudomonas aeruginosa PAO1]
pvdS	VFDB	99.11	100.00	NP_251116	Extracytoplasmic-function sigma-70 factor [Pyoverdine (VF0094)] [Pseudomonas aeruginosa PAO1]
rhlA	VFDB	98.54	100.00	NP_252169	Rhamnosyltransferase chain A [Rhamnolipid (VF0089)] [Pseudomonas aeruginosa PAO1]
rhlB	VFDB	99.30	100.00	NP_252168	Rhamnosyltransferase chain B [Rhamnolipid (VF0089)] [Pseudomonas aeruginosa PAO1]
rhlC	VFDB	98.26	100.00	NP_249821	Rhamnosyltransferase 2 [Rhamnolipid biosynthesis (CVF524)] [Pseudomonas aeruginosa PAO1]
rhlI	VFDB	98.02	100.00	NP_252166	Autoinducer synthesis protein RhlL [Quorum sensing (VF0093)] [Pseudomonas aeruginosa PAO1]
tagF/pppB	VFDB	98.97	100.00	NP_248766	Pseudomonas protein phosphatase PppB [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
tagQ	VFDB	99.23	100.00	NP_248760	Type VI secretiona ssociated protein TagQ outer membrane lipoprotein [HSI-1 (Hcp-secretion island 1) (SS178)] [Pseudomonas aeruginosa PAO1]
tagR	VFDB	99.71	100.00	NP_248761	Type IV secretion associated protein TagR positively regulates PpkA [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
tagS	VFDB	98.58	100.00	NP_248762	Type IV secretion associated protein TagS forming a stable inner membrane complex with TagT [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]

tagT	VFDB	98.06	100.00	NP_248763	Type six secretion associated protein TagT ATP-binding component of ABC transporter [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
tse1	VFDB	99.78	100.00	NP_250535	Type VI secretion system effector Tse1 peptidoglycanhydrolase [HSI-1 (Hcp-secretion island 1) (SS178)] [Pseudomonas aeruginosa PAO1]
tse2	VFDB	98.32	100.00	NP_251392	Type VI secretion system effector Tse2 [HSI-1 (Hcp-secretion island 1) (SS178)] [Pseudomonas aeruginosa PAO1]
tse3	VFDB	99.02	100.00	NP_252174	Type VI secretion system effector Tse3 glycoside hydrolase [HSI-1 (Hcp-secretion island 1) (SS178)] [Pseudomonas aeruginosa PAO1]
vgrG1a	VFDB	99.22	100.00	NP_248781	Type VI secretion system substrate VgrG1 [HSI-I (VF0334)] [Pseudomonas aeruginosa PAO1]
vgrG1b	VFDB	98.43	100.00	NP_248785	Type VI secretion system substrate VgrG1b [HSI-1 (Hcp-secretion island 1) (SS178)] [Pseudomonas aeruginosa PAO1]
wzy	VFDB	97.11	100.00	NP_251844	O-antigen chain length regulator [LPS (VF0085)] [Pseudomonas aeruginosa PAO1]
wzz	VFDB	97.79	99.52	NP_251850	Positive regulator for alginate biosynthesis MucC [LPS (VF0085)] [Pseudomonas aeruginosa PAO1]
waaA	VFDB	98.67	100.00	NP_253675	Lipopolysaccharide core biosynthesis protein WaaP [LPS (VF0085)] [Pseudomonas aeruginosa PAO1]
waaC	VFDB	98.69	100.00	NP_253698	3-deoxy-D-manno-octulosonic-acid (KDO) transferase [LPS (VF0085)] [Pseudomonas aeruginosa PAO1]
waaF	VFDB	98.46	100.00	NP_253699	Heptosyltransferase I [LPS (VF0085)] [Pseudomonas aeruginosa PAO1]
waaG	VFDB	98.84	100.00	NP_253697	B-band O-antigen polymerase [LPS (VF0085)] [Pseudomonas aeruginosa PAO1]
waaP	VFDB	98.64	100.00	NP_253696	UDP-glucose:(heptosyl) LPS alpha 13-glucosyltransferase WaaG [LPS (VF0085)] [Pseudomonas aeruginosa PAO1]
xcpA/pilD	VFDB	98.74	100.00	NP_253218	Type 4 prepilin peptidase PilD [Type IV pili (VF0082)] [Pseudomonas aeruginosa PAO1]
xcpP	VFDB	99.01	100.00	NP_251794	Secretion protein XcpP [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpQ	VFDB	92.16	99.85	NP_251795	General secretion pathway protein D [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpR	VFDB	98.08	100.00	NP_251793	General secretion pathway protein E [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpS	VFDB	99.02	100.00	NP_251792	General secretion pathway protein F [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpT	VFDB	98.66	100.00	NP_251791	General secretion pathway protein G [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpU	VFDB	99.04	100.00	NP_251790	General secretion pathway protein H [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpV	VFDB	97.69	100.00	NP_251789	General secretion pathway protein I [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]

xcpW	VFDB	99.30	100.00	NP_251788	General secretion pathway protein J [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpX	VFDB	96.51	100.00	NP_251787	General secretion pathway protein K [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpY	VFDB	98.00	100.00	NP_251786	General secretion pathway protein L [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]
xcpZ	VFDB	99.05	100.00	NP_251785	General secretion pathway protein M [xcp secretion system (VF0084)] [Pseudomonas aeruginosa PAO1]

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