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Whole Genome Sequencing of ESBL-producing Bacterial Isolates from Norwegian Aquatic Environmental Samples

Helgenomsekvensering av ESBL-produserende Bakterieisolater fra Norsk Vannmiljøprøver

Kayleigh Robson Moe
Chemical Engineering and Biotechnology

Preface

This 30-point master thesis was written as the final assessment for a master´s in Chemical Engineering and Biotechnology degree at the Norwegian University for Life Science in Norway. The laboratory work was performed in the research laboratory for Dairy Technology and Food Quality at the Faculty of Chemistry, Biotechnology and Food Science, with the help, guidance and supervision of head supervisor Professor Bjørn-Arne Lindstedt and senior laboratory engineer Ahmed Abdelghani. The duration of the thesis was February to November 2021. Laboratory access was somewhat limited due to the Corona virus pandemic and subsequent restrictions. Delivery issues also somewhat hindered the progress of the project. After a long year, I am contended in completing this thesis on such an important and relevant topic.

Thank you to Bjørn for the opportunity, your time, help, enthusiasm and especially your kind and motivating words. Thank you to Ahmed for all the helpful tips, suggestions and your positive and friendly attitude in the laboratory. Thank you to Guro for an enjoyable time in the laboratory and for all your help in the beginning. Finally, thank you to my friends, family and partner for all your help and support, not only during this master project but whenever it is needed. I would not be in the position I am today if it were not for my incredibly supportive aunts and sister, and for that I am eternally grateful. Brage, my best friend and partner, thank you for all that you do and all that you are. My time in Ås would not have been nearly as enjoyable without you.

After five years of studying, I am incredibly excited to start my professional career in molecular biology and biotechnology. As Bjørn told me, sequencing a whole bacteria genome was an incredible achievement some years ago. Now, a student can perform whole genome sequencing during a master thesis project on a portable sequencing machine. This emphasises the incredible advancements in biotechnology over the recent years, and I look forward to the journey in the years to come!

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Kayleigh Sharon Robson Moe

Abstract

β-lactams are among the most used antibiotics in Norway and have several associated resistance mechanisms. Among the most concerning is the production of extended-spectrum β-lactamases (ESBLs) and carbapenemases. Antibiotic resistance is intricate, complexed by evolution and dissemination of resistance genes and mechanisms. Research prospects include studying antibiotics and how they work (modes of actions) and studying bacteria´s ability to fight back (resistance mechanisms). Surveillance gives insight into resistance mechanisms, bacteria that harbour them and their dissemination. While Norwegian surveillance provides insight into ESBL prevalence in clinical settings and agriculture, environmental studies are not as vast. The aim here was to investigate ESBL-containing and carbapenem-resistant strains from Norwegian aquatic environmental samples, applying micro- and molecular biological methods including selective screening, 16S rRNA Sanger sequencing, MIC tests and multiplex PCR. ESBL, carbapenem and other resistance genes were characterized utilizing Illumina and Nanopore whole genome sequencing. Complete hybrid assemblies were obtained for isolates KA0, KA5 and KB3 which were characterized as non-identical but very similar *Rahnella variigena* strains (100%) and *Herbasprillium huttiense* (82%), respectively. Nanopore assemblies were obtained for isolates KA4, KA7 and KB8 which were characterized as *R. variigena* (100%), *Pseudomonas laurentiana* (100%) and *Herbsapirillum aquaticum* (70%), respectively. Class A β-lactamase genes were found in all isolates except *Pseudomonas* isolate KA7. *bla*$_{\text{RAHN}}$ was plasmid harboured in all *Rahnella* isolates, which may be further environmentally disseminated. This study indicated that ESBL-encoding genes are prevalent in the Norwegian aquatic environment and pose the risk of spreading.

Sammendrag

B-laktamer er blant de mest brukte antibiotikaene i Norge og har flere tilknyttede resistensmekanismer. Blant de mest bekymringsfulle er produksjonen av utvidet spektrum β-laktamaser (ESBLS) og karbapenemaser. Antibiotika resistens er innviklet og blir mer komplisert ved evolusjon og spredning av resistensgener og mekanismer. Forskningsmuligheter inkluderer studier av antibiotika og hvordan de virker (modus for handlinger), i tillegg til bakteriens evne til å bekjempe de (resistensmekanismer). Overvåkning gir innsikt i resistensmekanismer, bakterier som bærer dem og hvordan de sprer seg. Norsk overvåkning gir innsikt i ESBL-prevalens klinisk og i landbruk, men der er færre miljøtestudier. Målet her var å undersøke ESBL-inneholdende og karbapenemresistente stammer fra norske akvatiske miljøprøver ved bruk av mikro- og molekylærbiologiske metoder inkludert selektiv screening, 16S rRNA Sanger-sekvensering, MIC prøver og Multiplex PCR. ESBL, karbapenem og andre resistensgener ble karakterisert ved å benytte Illumina og Nanopore helgenomsekvensering. Komplett hybrid *assemblies* ble oppnådd for isolater KA0, KA5 og KB3 som ble karakterisert som to ikke-identiske men svært liknende *Rahnella variigena* stammer (100%) og *Herbasprillium huttiense* (82%), respektivt. Nanopore-*assemblies* ble oppnådd for isolater KA4, KA7 og KB8 som ble karakterisert som *R. Variigena* (100%), *Pseudomonas Laurentiana* (100%) og *Herbasprillium aquaticum* (70%), respektivt. Klasse A β-laktamasegener ble funnet i alle isolater unntatt *Pseudomonas* isolat KA7. *bla*$_{\text{RAHN}}$ var plasmid lokalisert i alle *Rahnella* isolater, som kan være ytterligere spredd i miljøet. Denne studie viser at ESBL-kodingsgener er utbredt i det norske akvatiske miljøet og kan spre seg.

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Abbreviations

6-APA - 6-animopenicillanic acid
ABC - ATP-binding cassette (efflux transporter family)
ABRicate – mass screening of contigs for antimicrobial and virulence genes (tool)
AM – Ampicillin (antibiotic)
AMR – Antimicrobial resistance
Ara4N - 4-amino-4-deoxy-L-arabinose
ARG – Antibiotic resistance genes
ATP - Adenosine triphosphate (energy carrying molecules in cells)
BHI – Brain Herat Infusion (broth)
bla – gene encoding for β-lactamases
BLAST – Basic Local Alignment Search Tool
bp – base pairs
C - Carbon
Canu assembler - tool for assembling error-prone long reads such as ONT
CARD – Comprehensive Antibiotic Resistance Database
CDC – Centers for disease Control and Prevention
CI – Ciprofloxacin (antibiotic)
CLSI - Clinical and Labratory Standards Institute
CPE - Carbapenem producing Enterobacteriacea
CRE - Carbapenem resistant enterobacteriacea
CRT – cyclic reverse termination
CT – Cefotaxime (antibiotic)
ddNTPs - diseoxynucleotide triphosphates
DNA – Deoxyribonucleic Acid
dNTPS - deoxynucleotide triphosphates
ds DNA – double-stranded DNA
ESBL - Extended-spectrum β-lactamase
ESBL$_A$ - ESBL mechanism classification consisting of traditional class A ESBLS
ESBL$_{CARBA}$ - mechanism resluting in carbapenem resistance
ESBL$_M$ - Miscellaneous ESBLs, consisting of plasmid-mediated AmpC and OXA-ESBLs
EUCAST - European Committee on Amtimicrobial Susceptibility Testing
F – Forward (primer or strand)
FHI – Folkehelseinstituttet (NIPH – Norwegian Institute of Public Health)
Galaxy – an open, web-based platform for accessible, reproducible and transparent computational biological research, used in this project for sequence data analysis
gDNA – genomic DNA
GLASS - Global Antimocrobial Resistance Surveillance System (WHO-supported)
HGT – Horizontal gene transfer
IP – Imipenem (antibiotic)
Kbp – kilo base pairs
KESC - Klebsiella, Enterobacter, Serratia and Citrobacter

LPS - lipopolysaccharide
M – Multiplex
MATE - Multidrug and toxif efflux (efflux transporter family)
MBL - metallo-β-lactamase
Mbp – million base pairs
MDA - Multiple displacement amplification
MDR – Multidrug-resistant
MF - Major facilitator (efflux transporter family)
MFP - Membrane fusion protein (protein class)
MGE - Mobile genetic elements
MIC – Minimum inhibition concentration
MLSA – multilocus sequence analysis
MP – Meropenem (antibiotic)
MSIS – Meldingssystem for smittsomme sykdomer/Norwegian Surveillance System for Communicable Diseases
NCBI – National Center for Biotechnology Information
NDM1 - New Delhi metallo--lactamase
NGS – Next generation sequencing
NIH – U.S. National Library of medicine
NMBU – Norges miljø- og biovitenskapelige universitet (Norwegian University of Life Sciences)
NORM - Norwegian Surveillance System for antimicrobial drug resistance
NORM-VET - Norwegian Surveillance System for antimicrobial drug resistance – Veterinary Medicine
NSC – Norwegian High Throughput Sequencing Center
OMF - Outer membrane factor (protein class)
ONT – Oxford Nanopore Technology
ORF - Open reading frame
OUS – Oslo universitetssykhus (Oso University Hospital)
PBP - Penicillin bininf protein
PCR – Polymerase chain reaction
PMF - Protein motive force
PMR – Polymyxin resistance
Porechop - tool for trimming ONT data
Prokka – Prokaryotic genome annotation (tool)
PubMLST – Public databases for molecular typing and microbial genome diversity
R – Reverse (primer or strand)
R* - Resistant (antibiotic)
R1 – Read 1
R2 – Read 2
RNA – Ribosomal ribonucleic acid
RND - Resistance-nodulation division (efflux transporter family)
rRNA – Ribosomal ribonucleic acid
SBS – sequencing by synthesis
MR - Small multidrug ressitance (efflux transporter family)

SPades - St- Petersburg genome assembler (tool)
ss DNA – single stranded DNA
Trimmomatic – read trimming tool (Illumina data)
tRNA – Transfer ribonucleic acid
UCH . Ubiquitin carboxyterminal hydrolase
Unicycler - tool for hybrid-assembly and correction of long and short reads
UTI - Urinary tract infection
UV – Ultraviolet
VFDB – Virulence Factor Database
WGS – Whole genome sequencing
WHO – World Health Organization

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1. BACKGROUND AND INTRODUCTION

Antibiotics are defined by Oxford Languages as medicines that inhibit the growth of or destroy microorganisms. In microbiology, this is not the most appropriate definition as antibiotics are more specifically used to treat, and sometimes prevent, bacterial infections (WHO, 2016). The broader term antimicrobials, which includes antibiotic, antiviral, antifungal and antiparasitic drugs, are used for bacterial, viral, fungal or parasitic infections, respectively. Antibiotic resistance and antimicrobial resistance (AMR) occur when bacteria or microorganisms display the ability to overcome such drugs, resulting in continued growth and survival. Consequently, antimicrobial infections become much harder to treat as the medicines become increasingly ineffective (WHO, 2016). This results in great burdens and financial costs for healthcare systems, as well as threats on human lives. As such, AMR has been declared by the World Health Organization (WHO) as a top ten global health threat for humanity (WHO, 2016).

While antibiotics have changed the world of medicine, saving countless lives by eradicating infections by sickness-causing bacteria (van Hoek et al., 2011), the use of antibiotics can also have negative effects on the normal and helpful microbiota of humans when not completely selective. This dilemma and predicaments associated with antibiotic resistance highlight the importance of sufficient characterization and understanding of the mechanisms of antibiotics. The outstanding advances in molecular biological methods in recent years, especially that of sequencing technologies, have been crucial in increasing focus on the role of antibiotics in bacteria antagonization, as well as the ability of bacteria to overcome the antibacterial drugs. Still, resistance mechanisms of bacteria persist, causing a major threat to human health. As both the prevalence of resistant bacteria and the modes of gaining resistance increase with time, understanding the antibiotic mechanisms that impede bacterial growth is pivotal in the ongoing quest of developing new antimicrobial drugs and finding new ways to fight microbial infections (Kohanski et al., 2010).

Enterobacteriaceae, a large family of Gram-negative bacteria including various pathogens such as *Klebsiella*, *Enterobacter*, *Citrobacter*, *Salmonella (KECS)*, *Escherichia coli*, *Shingella*, *Proteus* and *Serratia,* have become a key cause of nosocomial and community-acquired infections (Coque et al., 2008; Nordmann & Cornaglia, 2012). *E. coli* is a well-

studied example. While naturally present in human intestinal tracts, forming a normal part of the gut flora, they commonly cause urinary tract infections (UTIs), diarrhoea and may cause serious complications if spread to the bloodstream (Fratamico & Smith, 2006). Nosocomial infections or healthcare-associated infections are those acquired in the health care system, such as in hospitals or care facilities, post admission (Sikora & Zahra, 2021). Nosocomial infections demonstrate the easy transmission of Enterobacteriaceae infections, which spread easily through contact, contaminated food and water (Nordmann, Patrice et al., 2012). β-lactam antibiotics, mainly broad-spectrum cephalosporins and carbapenems, as well as fluoroquinolone antibiotics are top therapeutic choices for such infections (Coque et al., 2008).

β-lactams, mainly broad spectrum penicillins and cephalosporins, are some of the most used antibiotics in Norway (NORM/NORM-VET, 2020). An antibiotics spectrum refers to the substrate specificity, where extended spectrum is exactly that; an extended spectrum of substrate activity that allows for treatment of a broad spectrum of both Gram-positive and negative bacterial infections. Several resistance mechanisms are associated with β-lactam antibiotics, inactivating some of the main antibiotics currently used in infection treatments (Nordmann & Cornaglia, 2012). The most common, studied and concerning mechanism of resistance towards β-lactams is the production of β-lactam hydrolysing enzymes called extended-spectrum β-lactamases (ESBLs) (Nordmann, P et al., 2012). When ESBLs hydrolyse the β-lactam ring (see Figure 2.2) they destroy the antibiotic and render them ineffective. A rapid diffusion of ESBLs, mostly of type CTX and mostly in *E. coli*, has been observed over the 21st century (Zahar et al., 2009). The ESBL$_{CARBA}$ mechanisms results in resistance to carbapenems which are broad-spectrum β-lactams often used as a "last resort" treatment (America, 2011). The unique structure of a carbapenem coupled with a β-lactam ring confers protection against most β-lactamases, including metallo-β-lactamases (MBLs) and ESBLs (Codjoe & Donkor, 2018). While hydrolytic profiles of carbapenemases are somewhat variable, almost all hydrolyse carbapenems (Nordmann & Cornaglia, 2012). Literature differentiates between carbapenem-resistant Enterobacteriaceae (CRE) and Carbapenem-producing Enterobacteriaceae (CPE), where CPE are especially alarming as carbapenem production traits can be spread genetically (Nordmann et al., 2012). As such, resistance through the acquisition of carbapenemase genes, especially in resistant Gram-negative bacteria, is currently one of the most important clinical issues that needs to be

strictly controlled and studied (Nordmann, P et al., 2012). Non -fermenting opportunistic bacteria such as *Acinetobacter* and *Pseudomonas* species that can for example colonize the lungs and cause serious infections can also be ESBL-producers and are therefore also a healthcare concern (FHI, 2019).

A global action plan on AMR was endorsed at the World Health Assembly in May 2015, aiming to ensure prevention and treatment of infections (WHO, 2016). The plan highlights the importance of a "One Health approach", which recognises that public health is intricately connected to the health of animals and the shared environment. The aim is to design and implicate policies, legislation and research through the collaboration of multiple sectors including healthcare, agriculture and the environment, in order to achieve better public health outcomes. Five objectives were outlined: 1) improve awareness and understanding of AMR, 2) strengthen knowledge and evidence through surveillance and research, 3) reduce infection occurrence with sanitation, hygiene and infection measures, 4) optimize antimicrobial use and 5) develop an economic case for sustainable investments (WHO, 2016). The Norwegian Public Health Institute (NPHI/FHI) states that surveillance is necessary for health services to provide safe medical treatment in the years to come. Due to the limited treatment options, high mortality rates, and the threats of further spreading in the Norwegian healthcare system, only ESBL$_{CARBA}$ variants are included in the Norwegian Surveillance System for Communicable Disease (MSIS) which began in 2012 (FHI). The Global Action Plan report also emphasizes the need for research regarding transmission routes of resistance, including food, water and the natural environment. Aquatic environments, which play a major role in the development and dissemination of clinically significant resistant genes (Hooban et al., 2020), will be investigated in this study.

The ongoing emphasis on and surveillance of ESBL and CRE resistance underlines the importance and purpose of this study. The aim was to isolate, identify and characterize phenotypic and genotypic ESBL-producing and CRE bacterial strains from aquatic environmental samples in Norway. An organism´s phenotype refers to observable physical properties, characteristics or traits. In this study phenotypic resistance is demonstrated by the ability of isolates to grow on selective media plates containing antibiotics and a series of MIC tests. Phenotypes are determined by the genotype, the genes the bacteria carry, as well as environmental influences acting on these genes. As such, organisms with identical genotypes subjected to different environmental influences may express different phenotypical traits.

Genotypic resistance is a probable cause of phenotypic resistance and is here studied by whole genome sequencing of isolated strains and ensued sequence and bioinformatic analyses.

2. THEORY

2.1. Classification of Antibiotics

Antibiotics are broadly classified as either bactericidal which result in cell death or bacteriostatic which result in growth inhibition (Kohanski et al., 2010). Further, antibiotics are often classified based on molecular structure, mode of action or mechanism, activity spectrum and sometimes administration (Etebu & Arikekpar, 2016). A narrow activity spectrum antagonizes only a few, often similar bacterial organisms, while an extended or broad activity spectrum means the antibiotics work on a wider variety of bacteria. Administration routes includes topical, oral and injectable (Etebu & Arikekpar, 2016).

Antibiotics with similar structures, thereby belonging to the same structural class, often display comparable effectiveness, toxicity and allergy traits (Etebu & Arikekpar, 2016). Therefore, structural classification can be favourable in clinical settings. Common chemical and molecular structural classes of antibiotics are β-lactams, Macrolides, Tetracyclines, Quinolones, Aminoglycosides, Sulphonamides, Glycopeptides and Oxazolidinones (Etebu & Arikekpar, 2016). In 2020, the three most used antibiotics in Norway were β-lactamase sensitive penicillins, tetracyclines and extended-spectrum penicillins (NORM/NORM-VET, 2020). In research, antibiotics are often classified based on their mode of action. This is logical as understanding the mechanisms by which drugs impede bacterial growth is essential in the quest to find and develop new ways to fight infections.

2.2. Mechanisms and Modes of Actions of Antibiotics

Antibiotics are often directed towards a specific target; a unique bacterial structural feature or a specific metabolic process (Etebu & Arikekpar, 2016) that is essential to the bacteria´s survival. An illustrative summary of the most common target sites for different antibiotics is presented in Figure 2.1. Modes of antibiotic mechanisms can be summarised into five major groups (Etebu & Arikekpar, 2016; Shaikh et al., 2015) by interference with or inhibition of 1) cell wall synthesis, 2) protein synthesis, 3) nucleic acid synthesis, 4) metabolic pathways and finally 5) the cell membrane. These are succinctly presented below.

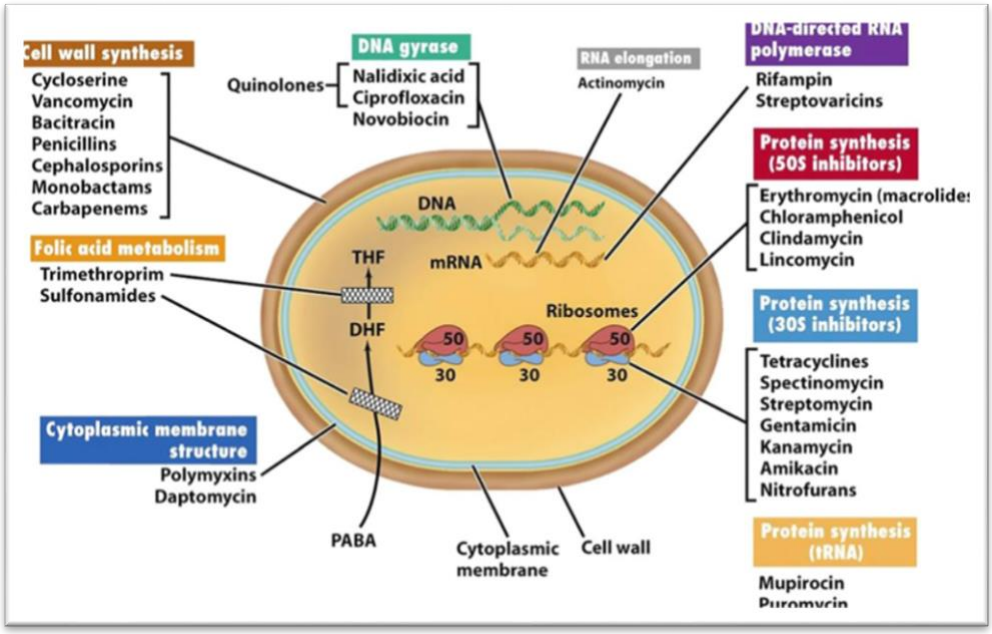


Figure 2.1. Madigan, T (2006). An illustrative summary of the common target sites for different antibiotics, as presented in Brock Biology of Microorganisms, 11th edition. Antibiotic modes of action can be summarised into five major groups, targeting cell wall synthesis, protein synthesis, nucleic acid synthesis (DNA and RNA), metabolic pathways and the cellular membrane.

2.2.1. Interference with cell wall synthesis

Bacterial cells are generally enclosed by a rigid, protective peptidoglycan layer in the cell wall. Peptidoglycan consists of cross-linked peptide bonds formed by for example transpeptidase and carboxypeptidase enzymes. These enzymes, which can bind β-lactam antibiotics, are termed penicillin-binding proteins (PBPs) (Heesemann, 1993). β-lactams, such as penicillin, target PBPs and so inhibits the enzymes responsible for forming the peptidoglycan layer (Benton et al., 2007). β-lactams, a focus of this study, are 2.3 belowdescribed in section 2.3 below. In Gram-negative cells, the antibiotics must penetrate through porins in the outer membrane to access the PBPs (Heesemann, 1993), providing opportunity for resistance.

2.2.2. Interference with nucleic acid synthesis

Rifampicin affects RNA synthesis by impeding a DNA-directed RNA polymerase. Quinolones inhibit DNA synthesis by interfering with type II and IV topoisomerases, as well as DNA gyrase, causing double-stranded (ds) breaks during replication (Strohl, 1997) , the process whereby dsDNA is copied to produce two identical replicas of the original DNA molecule.

2.2.3. Inhibition of protein synthesis

The most recent antibiotic class, Oxazolidinones, interact with the bacterial ribosome A site, affecting the placement of aminoacyl-tRNA. Tetracyclines and aminoglycosides bind to the 30S ribosomal subunit, weakening the ribosome-tRNA interaction and inhibiting protein synthesis initiation, respectively. Macrolides and chloramphenicol bind to the 50S ribosomal subunit, inhibiting elongation of the developing polypeptide chains and blocking peptidyl transferase reactions, respectively (Leach et al., 2007; Shaikh et al., 2015).

2.2.4. Inhibition of a metabolic pathway

Sulfonamides and trimethoprim both block key steps in the synthesis of folate, a cofactor in nucleotide biosynthesis (Strohl, 1997).

2.2.5. Disorganization of the cell membrane

The primary site of action for disorganization of the cell membrane is the cytoplasmic membrane or the inner membrane of Gram-positive and Gram-negative bacteria, respectively. The hypothesis is that polymyxins deploy inhibition by increasing the permeability of bacterial membranes, resulting in leakage of the cell. Rapid bacterial activity is induced by cyclic lipopeptide daptomycin through calcium-dependent binding to the cytoplasmic membrane, which oligomerizes the membrane and results in a potassium efflux from the cell and ultimately, cell death. (Shaikh et al., 2015; Straus & Hancock, 2006).

2.3. Beta-lactams

This structural class of antibiotics contain a highly reactive 3-carbon (C) and 1-nitrogen β-lactam ring (see Figure 2.2) that interferes with essential proteins and enzymes required for forming the peptidoglycan layer during cell wall synthesis (Benton et al., 2007). The antibiotics hinder peptidoglycan synthesis by binding to PBPs, so weaking the cell wall, resulting in lysis and ultimately cell death (Heesemann, 1993). The β-lactams, namely penicillins, cephalosporins, monobactams and carbapenems, are grouped by structure (see Figure 2.2). β-lactams also include β-lactamase inhibitors which inhibit β-lactamase hydrolysing activity.

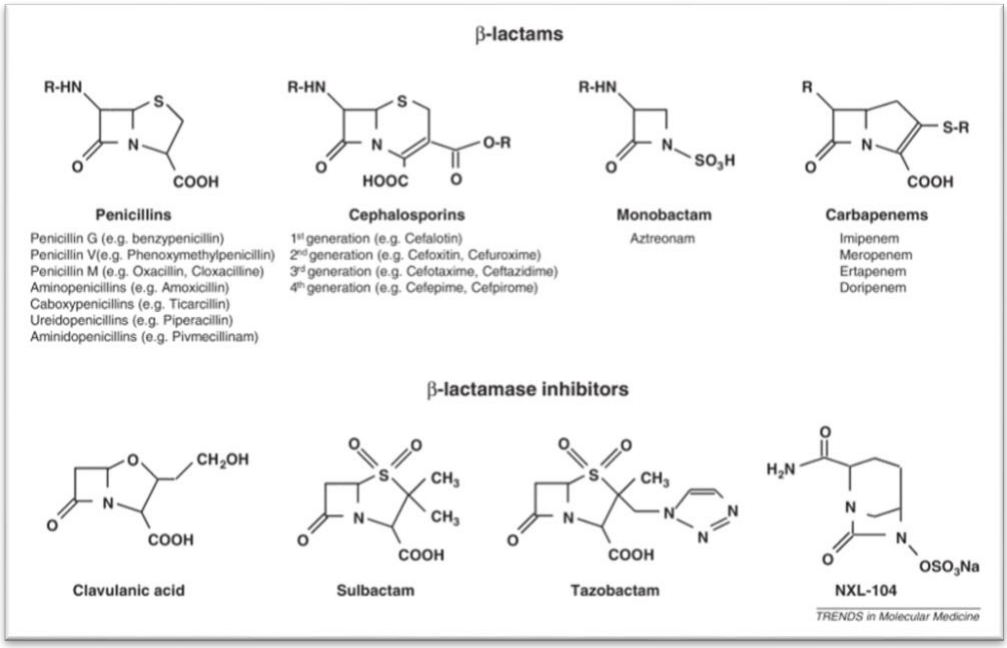


Figure 2.2. Nordmann et al. (2012). Chemical structures of the main β-lactam antibiotics and the clinically available β-lactamase inhibitors. The structural class of β-lactams includes penicillin and derivatives, cephalosporins, monobactams, carbapenems and β-lactamase inhibitors.

2.3.1. Penicillins

Penicillins are a diverse β-lactam group that contain a nuclease of 6-animopenicillanic acid (6-APA) ring as well as side chain rings. Penicillin G, the first produced antibiotic originally discovered by Alexander Flemming, is a narrow-spectrum antibiotic whereby only Gram-positive, some Gram-negative and meningococci display susceptibility (Etebu & Arikekpar, 2016). To increase the spectrum of susceptibility in Gram-negative bacteria, some penicillins, namely ampicillin, carbenicillin and amoxicillin have been semi-synthetically developed to contain different side chains. These side chains allow the drugs to bypass resistance mechanisms by degrading defensive enzymes produced by bacteria and helping to evade efflux across outer membranes of cell walls (Etebu & Arikekpar, 2016).

2.3.2. Cephalosporins

Cephalosporin antibiotics are structurally like penicillin and have similar modes of action. They contain a nuclease of 7-aminocephalosporanic acid and 3,6-dihydro-2 H-1,3-thiazine ring side chains. The group is divided into first to fifth generations based on target organisms and activity spectrum (van Hoek et al., 2011), where later generations are increasingly effective against Gram-negative pathogens. Various side chains allow for binding to different PBPs and bypassing the blood-brain barrier, as well as breakdown by penicillinase-producing

strains, thus aiding subspeciality of Gram-negative cells. Treatment profiles include infections of Penicillinase-producing, Methicillin-susceptible *Staphyl-* and *Streptococci* and *Proteus mirabilis*. Some *E. coli*, *Klebsiella pneumonia*, *Haemophophilus influenza*, *Enterobacter aerogenes* and *Neisseria* infections can also be treated. Second and third generation Cephalosporins such as ceftriaxone, cefotaxime, cefuroxime and ceftazidime can be administered in cases of extreme infection where non-cephalosporins would not suffice but the patient is allergic to penicillin (Pegler & Healy, 2007).

2.3.3. Carbapenems

Carbapenems are defined by their 4:5 fused lactam ring of penicillins and the double C2 and C3 bond with a sulphur substitution at C1. Carbapenem antibiotics, such as imipenem and meropenem, are considered broad spectrum β-lactams since they easily diffuse in bacteria and display the greatest potency against both Gram-positive and negative bacteria (Etebu & Arikekpar, 2016; Papp-Wallace et al., 2011). As such, this class is often considered as a "last resort" in serious infections or in cases of multidrug resistant (MDR) bacteria. They are distinctive to other β-lactams in that they are relatively resistant to β-lactamase hydrolysis, where their inhibition of β-lactamases provides reasonable grounds for class expansion (Papp-Wallace et al., 2011). Thienamycin, the reportedly first considered carbapenem, provides the standard for other antibiotics in this class (Papp-Wallace et al., 2011) which now consists of over 80 drug compounds.

2.3.4. Monobactams

These antibiotics, unlike other β-lactams, do not contain a nuclease with a fused ring but rather a ring that stands alone (van Hoek et al., 2011). The only commercial monobactam, Aztreonam, is not effective against Gram-positive or anaerobic bacteria. It is used to treat pneumonia and UTIs caused by Gram-negative *Neisseria* and *Pseudomonas* species.

2.3.5. Beta-lactam inhibitors

These inhibitors contain a β-lactam ring but display limited independent antimicrobial activity. They were introduced into clinical practice to overcome resistance mediated by β-lactams (Drawz & Bonomo, 2010). They are administered in conjunction with β-lactam antibiotics to treat infections by bacteria that produce penicillin-inactivating β-lactamases (Drawz & Bonomo, 2010; van Hoek et al., 2011). β-lactam inhibitors are reversible or

irreversible, where irreversible inhibitors eventually render enzymatic activity destruction and are therefore deemed more effective. As such, the clinically used inhibitors, namely clavulanic acid, sulbactam and tazobactam, are irreversible.

2.4. Antimicrobial Resistance

Antibiotic resistance and AMR occur when microbes display the ability to survive despite the use of antimicrobials. Multidrug-resistant (MDR) microbes are those that are resistant to more than one antimicrobial drug (Fisher and Mobashery, 2010). Antibiotic resistance research has traditionally been focused on pathogenic bacteria (Wright, 2010) which are bacteria that can cause disease. Davies, however, discovered in 1973 that antibiotic resistance is not restricted to pathogens (Benveniste & Davies, 1973). In fact, non-pathogenic, antibiotic-producing and opportunistic pathogens are often highly resistant compared to those typically associated with disease. Genome sequencing has shown that even bacteria with only 0,58 million base pairs (Mbp) contain genes encoding receptors, pumps and enzymes to chemically modify compounds in response to foreign cytotoxic molecules such as antibiotics (Wright, 2010). Furthermore, bacteria´s ability to share such genes provides a potential resistance gene pool that can be spread to other microorganisms (Allen et al., 2010; Wright, 2010). Antibiotic resistance is often thought of to be driven by the increasing over-use of antibiotics, as shown through multiple studies of increased antibiotic use contributing to resistance emergence in different bacteria (van Hoek et al., 2011). However, antibiotic use is not the sole nor the first driver. In fact, the first β-lactamase was identified in *E. coli* in 1940 before penicillin was publicly released in 1941, indicating that antibiotic resistance emerged prior to the introduction and wide-use of penicillin (Abraham & Chain, 1940). Resistance appears to be a natural phenomenon, but selection appears to have driven exposure in healthcare, the environment and agriculture (Holmes et al., 2016). Further, onward transmission and infection is driven and affected by for example infection control, sanitation and travel (Holmes et al., 2016). This is yet another reason the One Health approach is so important.

Resistance in bacteria can be displayed through intrinsic or acquired mechanisms. Intrinsic resistance is the result of inherent structural or functional characteristics (Blair et al., 2015) and involves genes naturally occurring in the bacteria´s genome, including those encoding various efflux pumps conferring MDR. Various genes responsible for intrinsic resistance

towards for example β-lactams and fluoroquinolones have been identified in *Staphylococcus aureus*, *E. coli* and *P. aeruginosa* using high-throughput screens of mutant genome libraries created by targeted insertion and random transposon mutagenesis (Blair et al., 2015; Blake & O'Neill, 2013; Liu et al., 2010). Some Gram-negative bacteria are intrinsically resistant to many compounds because the compounds cannot cross the outer membrane (Blair et al., 2015).

Acquired mechanisms are exactly that: acquired, usually by mutations of antibiotic targets or transfer of resistance determinants by mobile genetic elements (MGE) (Alekshun & Levy, 2007). MGEs are segments of DNA that can translocate to different parts of a genome or between genomes and are often acquired by transformation and conjugation during horizontal gene transfer (HGT) (Partridge et al., 2018). In HGT, MGE are often conjugative elements that contain genetic information required for transfer and mobilization elements that use conjugation functions of plasmids or transposons for transfer. MGE are presented in section 2.5.1 below.

The evolution of antibiotic resistance was initially thought unlikely due to assumed negligible frequency of resistance-resulting mutations. The adaptive ability of bacteria leading to a variety of resistance mechanisms and their ability to interchange genes through HGT was underestimated and unexpected (van Hoek et al., 2011). Over the years, bacteria have displayed resistance through a variety of biochemical mechanisms which can be summarized as follows (van Hoek et al., 2011):

1) **antibiotic inactivation** through enzymatic modifications or degradation
2) **target restriction** through target modification, target enzyme overproduction or permeability changes in the cell wall
3) **bypass** through active efflux from the cell or acquisition of alternative metabolic pathways to those inhibited.

2.5. Genetic Aspects and Dissemination of Acquired Resistance

Resistant phenotypes can be acquired and disseminated genetically by altering existing proteins through chromosomal **DNA mutations**, the formation of mosaic proteins through **transformation** and the exchange of genes or plasmids between bacteria of similar or

different species and genera through **HGT** (Dever & Dermody, 1991). Further, dissemination can also be attributed to clonal spread of a resistant strain. A strain harbouring genes for resistance may be preferentially selected and transferred within a population when faced with selective pressure of antibiotics (Hong et al., 2019).

Most resistance genes found in pathogens have been acquired by HGT of MGEs such as plasmids (Wright, 2010). The three main mechanisms for HGT, namely transformation, transduction and conjugation, are illustrated in Figure 2.3.

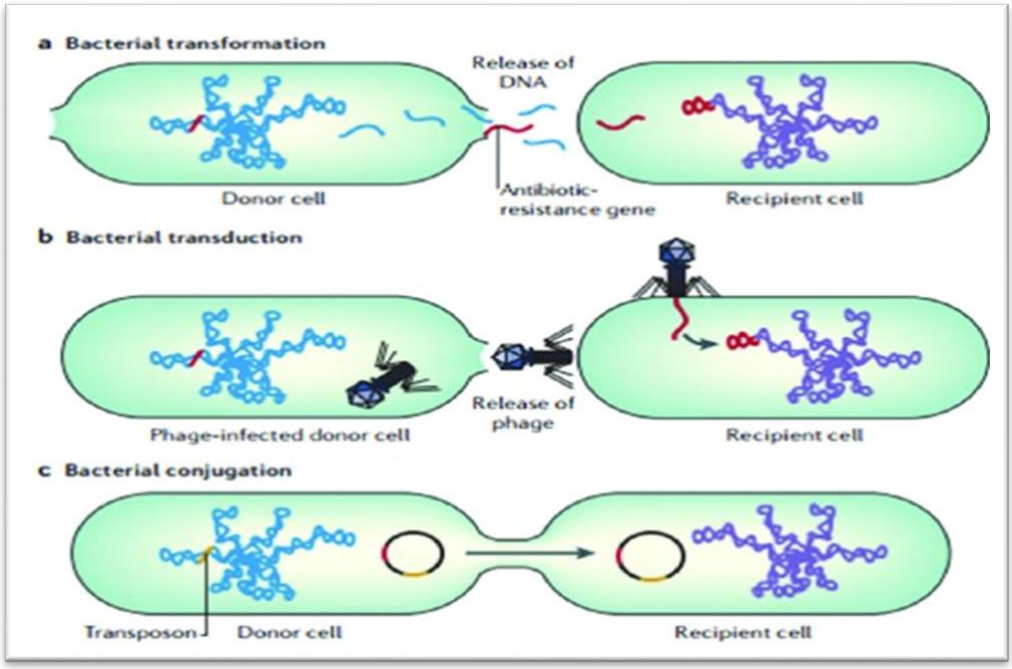


Figure 2.3. Kiros & Workineh (2019). The three main mechanisms of horizonal gene transfer; transformation, transduction and conjugation. Transformation is the uptake of naked DNA from the environment by component bacteria. Transduction entails the spread of DNA between bacteria by bacteriophages. Conjugation is the transfer of mobile genetic elements between adjacently located bacteria.

Transformation is the process where bacteria incorporate naked DNA from the environment into their own genomes through homologous recombination or transposition. Bacteria with this ability are termed competent. Bacteriophages can spread DNA between bacteria through transduction, the process whereby bacterial DNA is contained within a phage head and injected into a receiving bacteria (van Hoek et al., 2011). Conjugation, the transfer of MGE between adjacently located bacteria, occurs via plasmids and conjugative transposons (Thomas & Nielsen, 2005).

2.5.1. Mobile Genetic Elements (MGEs)

Integrons are genetic elements that can acquire and rearrange open reading frames (ORF) in a collection of genes called gene cassettes and confer them to functional genes. They were first identified as a Gram-negative bacterial mechanism to accumulate resistance genes and synergically express multiple resistance phenotypes with transposons (Cambray et al., 2010). Thus, mobile integrons are responsible for the integration and rearrangement of resistance determining gene cassettes (Koczura et al., 2016) and can transfer genes within a single exchange (Alekshun & Levy, 2007). Integrons consist of an integrase gene, *attL*; the primary recombination site and P_C, a promoter that directs integrated gene transcription. The integrase gene sequence determines the integron class. The most frequent class, class 1, seems to play a key role in resistance gene emergence and dissemination.

Plasmids are extra chromosomal dsDNA elements varying in length and depend on their hosts but also replicate independently (Couturier et al., 1988). They contain genes essential for maintenance such as replication initiation and control, and many carry genes that are otherwise useful to the host such as resistance genes and virulence factors. Such genes are often transposon located, rendering vast plasmid variation and flexibility. Plasmids can be transferred by conjugation or transduction. Plasmids containing conjugation genes are called conjugative while plasmids that only contain an origin of transfer are called mobilizable and can use conjugative plasmids to transfer to new hosts (van Hoek et al., 2011). ESBL and carbapenemase genes are often associated with plasmid harbouring, allowing them to disseminate (Hooban et al., 2020). Identification and classification of plasmids can be clinically relevant, aiding to trace infection sources and spreading.

Transposons can occur on plasmids or integrate into other transposons and host genomes. Transposons generally contain terminal regions involved in recombination and protein specification for incorporation facilitation. Conjugative transposons have plasmid-like qualities and can facilitate plasmid transfer between organisms. (Alekshun & Levy, 2007)

2.6. Resistance towards beta-lactams

Broad-spectrum β-lactams are the main therapeutics for nosocomial and community-acquired infections by human pathogenic Enterobacteriaceae such as *E. coli* and the KECS group (Nordmann, Patrice et al., 2012). Bacteria evade β-lactam antibiotics through four primary mechanisms (Drawz & Bonomo, 2010):

1) Production of β-lactamase enzymes that hydrolyse the β-lactam-ring and inactivate the antibiotic.
2) Decreased affinity for β-lactams and thereby increased resistance by changing the active sites of PBPs. Natural transformation, recombination and HGT have been shown to aid resistance development in *Neisseri* and *Streptococcus* species (Drawz & Bonomo, 2010).
3) Decreased expression of outer membrane proteins (OMPs) resulting in carbapenem resistance in some Enterobacteriaceae. β-lactams must diffuse through or traverse porin channels in the outer membrane of Gram-negative cell walls to reach the targeted PBPs. The loss of OprD is associated with imipenem resistance and reduced meropenem susceptibility in *P. aeruginosa*. CarO OMP loss in clinical *Acinetobacter baumannii* isolates is associated with imipenem and meropenem resistance. Point mutations and insertions of genes encoding porins can result in altered proteins with decreased function and lower permeability, but this does not always sufficiently produce a resistant phenotype and is generally not combined with β-lactamase mechanisms.
4) Efflux pumps capable of exporting a variety of substrates from the periplasm to the environment. Efflux pumps confer resistance in many Gram-negative pathogens, especially *P. aeruginosa* and *Acinetobacter* species. Decreased susceptibility to penicillins, cephalosporins, quinolones, tetracycline and chloramphenicol in *P. aeruginosa* is attributed to upregulation of the MexA-MexB-OprD system in combination with low outer membrane permeability. AdeABC, an RND-type efflux pump in *A. baumannii* can be attributed to carbapenem resistance of catalytically poor β-lactamases.

Resistance mechanisms against carbapenems include carbapenemase β-lactamases, efflux pumps, as well as genetic mutations that affect porin and PBP expression and functionality. Species such as *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* use a combination of these,

resulting in high resistance levels (Papp-Wallace et al., 2011). Carbapenem resistance in Gram-positive cocci generally result from amino acid substitutions in existing PBPs or acquisition of new PBPs. In Gram-negative rods, resistance is attributed to β-lactamase production, efflux pumps, loss of porins and PBP alterations (Papp-Wallace et al., 2011). Carbapenemase producers, mostly *K. pneumoniae* or *E. coli*, are viewed from a healthcare perspective as much more important than carbapenem-resistant bacteria (Nordmann, P et al., 2012). This is because, unlike most carbapenemase genes, the carbapenem resistance trait of CRE is not transferable. A study (Tamma et al., 2016) comparing the outcomes of patients with CPE and non-carbapenemase-producing CRE found that CPE may be more virulent than CRE. CPE was associated with poorer outcomes and higher mortality. Only ESBLs and efflux pumps, which can be detected through genome analysis, will be investigated in this study.

2.6.1. ESBL

The primary, most common mechanism towards β-lactams that has been most thoroughly studied (Papp-Wallace et al., 2011) is the expression of β-lactamases; periplasmic enzymes which hydrolyse β-lactams. It was proposed by Davies (1994) that pathogenic bacteria likely obtained ESBL resistance determinants from a resistance gene pool resulting from other microbials, including those that naturally produced antibiotics. These resistance genes were subsequently integrated into different naturally occurring gene expression cassettes through site-specific recombination. The integrons were then dispersed through microbial populations by gene transfer mechanisms (Davies, 1994).

There are over 2000 identified natural β-lactamases, each with unique amino acid sequences and characteristic hydrolysis profiles (Bonomo, 2017). Novel families are still being discovered owing to huge advancements in sequencing technologies. Rapid replication rates and high frequencies of mutations are speculations for the evolution of these enzymes. They have been discovered in clinically relevant enteric bacteria, including species of *Klebsiella*, *Enterobacter* and *P. aeruginosa*. The most clinically important β-lactamases include class A penicillinases, ESBLs, the AmpC cephalosporinases and serine and metallo-enzyme groups of carbapenem-hydrolysing enzymes (Bonomo, 2017).

β-lactamase nomenclature is reviewed by Jacoby (2006). Names were originally designated by the strain or plasmid by which they were produced. This has evolved to include name designation originating from substrates, biochemical properties, location of discovery or the gene, bacterial strains, patients and researchers (Jacoby, 2006). Classification of β-lactamases is based on two major systems; the Ambler classes characterized by amino acid sequence homology and the Bush-Jacoby system characterized by substrate hydrolysis and inhibitor profiles. A comparison of these systems is summarized in Table 2.1, as presented by Bonomo (2017).

Table 2.1. Bonomo (2017). Comparison of the Bush-Jacoby and Ambler systems for β-lactamase characterization.

Bush-Jacoby	Ambler	Defining substrates	Inhibited by EDTA	Inhibited by clavulanic acid or tazobactam	Representatives
1	Class C	Cephalosporinases Cephamycinases	(-)	No	P99 FOX-4
2	Class A		(-)	Yes	
2a		Penicillins		Yes	PC1
2b				Yes	TEM-1, SHV-1
2be		Cephalosporins		Yes	TEM-10, SHV-2
2br				No	TEM-30
2ber				No	TEM-50
2ce				Yes	RTG-4
2d	Class D	Penicillins	(-)	Variable	OXA-1
2de		Cephalosporins			OXA-11
2df		Carbapenems			OXA-23
2e				Yes	CepA
2f		Carbapenems		Variable	KPC-2
3	Class B	Carbapenems	(+)	No	
3a	B1				NDM-1, VIM-2, IMP-1
3b	B2				CphA
4a	B3				L1

In Norway, ESBL-containing bacteria are divided into three classes (FHI, 2019), namely $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$, based on the classification proposal by Giske et al. (2009) (see Table 2.2). $ESBL_A$ consists of classical class A ESBLs. Miscellaneous ESBLs, $ESBL_M$, consist of plasmid-mediated AmpC and OXA-ESBLs. Carbapenemases fall under $ESBL_{CARBA}$ along with MBLs and OXA-carbapenemases (Giske et al., 2008). $ESBL_A$ and $ESBL_M$ bacteria generally display resistance towards penicillins and most cephalosporins but not carbapenemases. $ESBL_{CARBA}$ bacteria are generally resistant towards all β-lactams, including carbapenemases. ESBL-producing bacteria are often also resistant to other antibiotics, namely fluroquinolones and aminoglycosides, while Gram-negative bacteria often

display resistance to the polymyxin antibiotic colistin (FHI, 2019). As such, $ESBL_{CARBA}$ bacteria that are MDR have limited treatment options and are therefore extremely worrisome.

Table 2.2. Giske et al. (2008). Classification of ESBLs in Norway, as proposed and presented by Giske et al. (2008)

	$ESBL_A$	$ESBL_M$	$ESBL_{CARBA}$
β-lactamase class	High prevalent $ESBL_A$ TEM-ESBLs SHV-ESBLs VEB PER	$ESBL_{M-C}$ (Plasmid-mediated AmpC) CMY FOX MIR MOX DHA LAT BIL ACT ACC	$ESBL_{CARBA-A}$ KPS GES-2, -4, -5, -6, -8 NMC SME IMI-1, -2
	Low prevalent $ESBL_A$ GES-1, -3, -7, -9 SFO-1 BES-1 BEL-1 TLA IBC CMT	$ESBL_{M-D}$ (OXA) OXA-10 group PXA-13 group OXA-2 group OXA-18 OXA-45	$ESBL_{CARBA-B}$ (MBL) IMP VIM SOM-1 GIM-1 SIM-1 AIM-1
			$ESBL_{CARBA-d}$ (OXA-carbapenemase) OXA-23 group OXA-24 group OXA-48 OXA-58 group
Operational definition	Non-susceptibility to extended spectrum cephalosporins AND Clavulnate synergy	Non-susceptibility to extended spectrum cephalosporins AND Phenotypic detection ($ESBL_{M-C}$) OR Genotypic detection ($ESBL_{M-D}$)	Non-susceptibility to extended spectrum cephalosporins AND $ESBL_{CARBA}$ detected with phenotypic/genotypic methods

2.6.2. $ESBL_{CARBA}$

Carbapenemases are able to hydrolyse almost all β-lactams, deeming them the most concerning (Nordmann, P et al., 2012). Carbapenemase producers are MDR, and the internationally observed spread of carbapenemase traits in Enterobacteriaceae is especially concerning (Nordmann, P et al., 2012). Detection of carbapenem resistant isolates is increasing worldwide (see Figure 2.4). The increase could of course somewhat be attributed to increased surveillance. In Enterobacteriaceae the increase could further be attributed to possible porin deficiencies that decrease permeability of the outer membrane combined with

overexpression of β-lactamases with weak carbapenemase activity, as well as the expression of carbapenemases (Nordmann et al., 2011; Nordmann, P et al., 2012). Approaches to prevent further dispersion include detection and surveillance. Detection is especially important clinically regarding infected patients and in research where colonizing strains, for example from the environment, are screened as possible carriers.

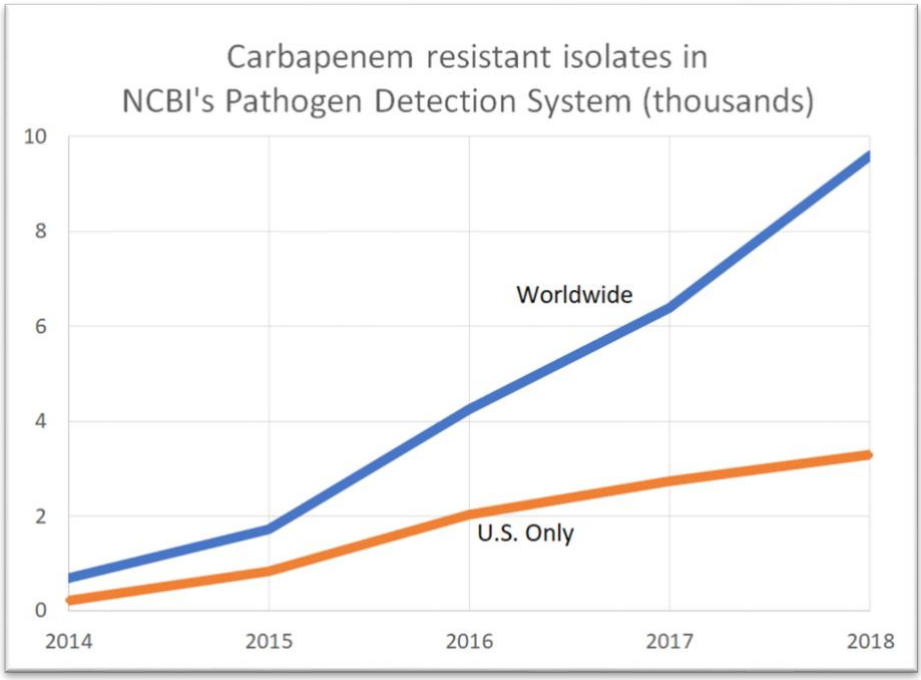


Figure 2.4. NDARO (2004-2018). Carbapenem resistant isolates detected in NCIB´s Pathogen Detection System. The graph indicates a clear increase in isolates (x axis, in thousands) from 2004 to 2018 (y-axis), both worldwide and in the U.S.

Carbapenemases are mostly of types KPC, VIM, IMP, NDM and OXA-48. Three classes of carbapenemase β-lactamases have been identified in Enterobacteriaceae: the Ambler classes A, B and D (Nordmann, P et al., 2012). Enterobacteriaceae-produced, rare chromosomally encoded cephalosporinases belonging to Ambler class C/AmpC may display slight extended activity towards carbapenems but with debated clinical significance (Nordmann et al., 2011). The KPC type of Ambler class A carbapenemases are of most clinical significance as they can hydrolyse all β-lactams. Their activity is inhibited by boronic acid and somewhat by clavulanic acid and tazobactam (Nordmann, P et al., 2012). Carbapenemase types IMP, VIM and NDM in Enterobacteriaceae belong to class B β-lactamases which display the highest carbapenemase activity (Nordmann, P et al., 2012). These enzymes display a broad hydrolytic activity spectrum of all penicillins, cephalosporins and carbapenems, where activity is not inhibited by commercial β-lactamase inhibitors (Nordmann et al., 2011).

Activity can be inhibited by EDTA and dipicolinic acid, explained by the β-lactam and zinc ion(s) dependant interaction on the active site during hydrolysis of B class enzymes (Nordmann, P et al., 2012). Ambler class D β-lactamases with carbapenemase activity are mostly types OXA-48 and OXA-181 (Nordmann et al., 2011). These enzymes can hydrolyse cefotaxime at low levels, do not hydrolyse ceftazidime and resist clavulanic acid-tazobactam inhibition (Nordmann et al., 2011). Clinical isolates often also contain other β-lactamases, especially ESBLS, which result in broader resistance profiles, MDR and phenotypic resistance that is not only attributed to carbapenemase expression (see Table 2.3) (Nordmann et al., 2011; Nordmann, P et al., 2012). Detection of CPE can therefore be problematic.

Table 2.3. Nordmann et al. (2011). Phenotypic resistance resulting from carbapenemase-expression reported in Enterobacteriaceae, with* and without ESBL expression.

	AMX	AMC	TZP	CTX	CAZ	IMP	ETP	MER	ATM
KPC	R	S/I	R	R	R	S/I/R	I/R	S/I/R	R
KPC*	R	I/R	R	R	R	I/R	I/R	I/R	R
IMP/VIM/NDM	R	R	I/R	R	I/R	S/I/R	I/R	S/I/R	S
IMP/VIM/NDM*	R	R	I/R	R	R	I/R	R	S/I/R	R
OXA-48/OXA-181	R	R	S/I/R	S/I	S	S/I	S/I	S/I	S
OXA-48/OXA-181*	R	R	I/R	R	R	I/R	I/R	I/R	R

*ESBL expression
AMX: amoxycillin, AMC, amoxycillin-clavulanic acid; TZP, piperacillin-tazobactam; CTX, cefotaxime, CAZ, ceftazidime; IMP, imipenem; ETP, ertapenem; MER, meropenem; ATM, aztreonam.

2.6.3. Efflux pumps

Efflux pumps are proteins that transport substrates, including antibiotics, outside the cell. They are found in both antibiotic susceptible and resistant Gram-negative and positive bacteria and can be specific or nonspecific. The nonspecific pumps with wide substrate specificity are often associated with MDR (Blair et al., 2015). There are five major efflux transporter families in prokaryotes (Lomovskaya et al., 2001; Webber & Piddock, 2003): major facilitator (MF), multidrug and toxic efflux (MATE), resistance-nodulation division (RND), small multidrug resistance (SMR) and ATP-binding cassette (ABC) efflux pumps. The proton motive force (PMF) provides energy for all the systems, except the ABC family where efflux is driven by ATP hydrolysis. RND superfamily transporter pumps in Gram-negative bacteria are the best characterized, clinically relevant MDR efflux pumps and are associated with two other protein classes, the outer membrane factor (OMF) protein family and periplasmic adaptor proteins in the membrane fusion protein (MFP) family. Overexpression of RND pumps confer high levels of clinically relevant MDR and have extremely broad substrate specificity. RND pumps such as AcrB in *E. coli* and MexB in *P.*

aeruginosa are inner membrane residing homotrimers that form tripartite complexes with periplasmic adaptor proteins AcrA and MexA, respectively, and an outer membrane channel such as TolC or OprM (Blair et al., 2015). AcrB contains two binding pockets; distal and proximal, which accommodate substrates of varying size and properties. Mutations within the binding pockets have provided further understanding of substrate binding (Blair et al., 2015).

While many bacteria chromosomally carry multiple genes encoding MDR efflux pumps, some have been mobilized onto plasmids and can as such transfer between bacteria. For example, genes for a novel tripartite RND pump were found on a plasmid isolated from *Citrobacter freundii* also carrying a New Delhi MBL (NDM1), indicating the resistance mechanism is transmissible (Dolejska et al., 2013). Further, especially overexpression of efflux pumps is a common resistance mechanism in clinical isolates. Efflux encoding genes are transcriptionally controlled by both local and global regulators (Blair et al., 2015).

2.7. Surveillance of Antibiotic Resistance

This study will focus on Norwegian surveillance of antibiotic resistance. Antibiotic resistance is surveyed in three Norwegian Surveillance Systems (NORM/NORM-VET, 2020), namely that for Communicable Diseases (MSIS), for antimicrobial drug resistance in human pathogens (NORM) and for antimicrobial drug resistance in animals, food and feed (NORM-VET).

Based on the great concerns and limited treatment options for infections of $ESBL_{CARBA}$ variants, especially CPE, only these are included in the national Norwegian Surveillance System for Communicable Diseases (Meldingssystem for smittsomme sykdomer, MSIS) which began in 2012. Detection of Enterobacteriaceae, *Pseudomonas* and *Acinetobacter* isolates with detected $ESBL_{CARBA}$ genes, independent of meropenem sensitivity, should be reported to MSIS. Other such species with combined decreased sensitivity towards meropenems and phenotypical traits compatible with carbapenemase production but no detected carbapenemase genes should be biochemically investigated for carbapenemase production. Verified finds by validated biochemical assays should be reported. Figure 2.5 provides an illustration of carbapenemase-producing Gram-negative rod infections in Norway, reported to MSIS from 2012 to 2021. Increasing cases of outbreaks are reported

internationally and in Norway, where travel is deemed a probable major cause of global dissemination (FHI).

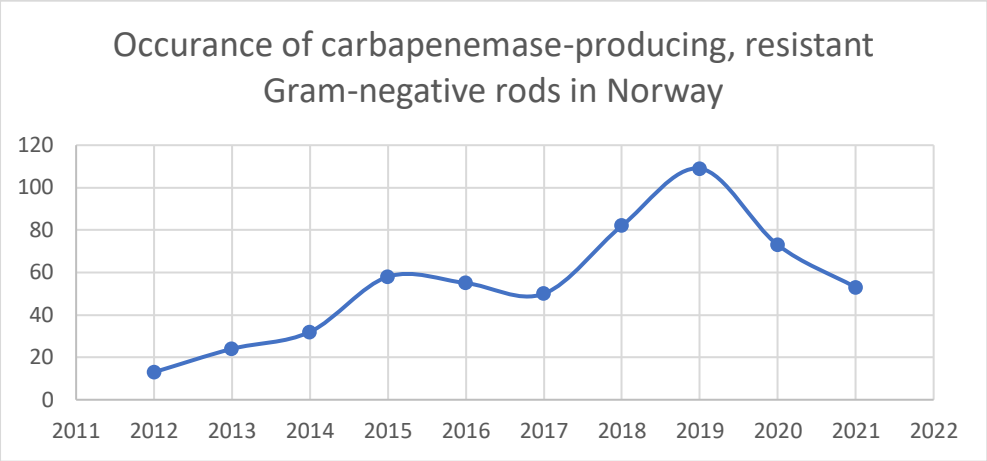


Figure 2.5. MSIS (2021). The occurrence of carbapenemase-producing Gram-negative rods in Norway from 2012 to 2021, as reported by MSIS (2021). Increasing cases of occurrence are being reported both internationally and in Norway. The decrease after 2019 is likely attributed to the extreme decrease in travel due resulting from the Corona virus pandemic and subsequent reduction in imported infections.

The NORM-VET report provides an annual status regarding antibiotic resistance occurrence as well as antibiotic use in food and agriculture in Norway. The 2020 report (NORM/NORM-VET, 2020) showed that antibiotic use in Norwegian agriculture continues to decrease and that the overall situation in Norway is considerably favourable due to relatively low occurrence of resistant bacteria. However, reports of ESBL-producing bacteria are increasing and this is of great concern. Reported cases of CRE and *Acinetobacter* spp. infections in 2020 decreased compared to 2019, from 75 to 57 and 23 to 10 for CRE and *Acinetobacter* species, respectively. Carbapenem-producing *P. aeruginosa* was stable at four reported cases. The massive decrease of international travel due to the Coronavirus pandemic is a likely explanation, highlighting the occurrence of imported infections.

The NORM and NORM-VET reports provide insight on the prevalence of ESBL-producing bacteria in Norwegian healthcare and agriculture, but occurrence and presence in the environment is not as well studied. Environmental studies of ESBL encoding genes aid insight into their origins and dissemination mechanisms, as well as allow for comparison studies of clinical and environmental ESBL-types. Environmental studies are also important for the One Health approach, which recognizes that public health is intricately connected to the heath of animals and the environment we share (WHO, 2016).

WGS is useful for AMR surveillance, shedding light on resistant strains, their genes and their mechanisms, as well as transmission and dissemination patterns. With WGS and subsequent phylogenetic trees, clonal lineages of different strains can be studied. Genes or mutations conferring resistance can be detected, as well as the MGE that carry them. This allows for the detection of high-risk or dangerous strains and is especially useful infectious disease epidemiology and surveillance. (Argimón et al., 2020)

2.8. Antibiotic Resistance in the Aquatic Environment

The evidence demonstrating that environmental isolates are highly drug resistant and that the environment provides a pool of resistance genes is growing (Wright, 2010). More alarming is the evidence that at least some clinically relevant resistant genes originated from environmental strains. Of note, the CTX-M ESBL type seems to have originated from chromosomal genes of environmental *Kluyvera* (Poirel et al., 2002). Forsberg et al. (2012) provided evidence for recent transmission and dissemination of AMR genes from soil bacteria to clinical pathogens via lateral exchange of various mobilisation sequences, also non-coding regions. They did so through a high throughput metagenomic approach combined with a pipeline for *de nova* assembly of short read sequencing data, and found resistance cassettes with perfect nucleotide identity to human pathogen genes (Forsberg et al., 2012). As such, studying environmental resistomes and the potential for transfer to clinical pathogens is another key in antibiotic research.

This study focuses on the spread of resistance in the natural aquatic environment. Literature has reviewed by for example Zhang et al. (2009) and Baquero et al. (2008). Resistance in aquatic environments, like in soil, can be intrinsic, genetically acquired or acquired in response to anthropogenic contamination from agricultural runoff or aquaculture antibiotic use (Wright, 2010). Approximately 70% of antibiotics used in agriculture are used to treat human infections and are therefore humanly relevant (Martin et al., 2015). This "nontheraputic use" is seen as driver of antibiotic resistance, where resistant strains are easily spread from animals to humans directly, through food or exposure through soils and manure (Martin et al., 2015). However, novel emerging resistance genes in the absence of external influences, for example runoff exposure, and the dissemination of these genes by environmental bacteria via MGE are not as well studied (Hooban et al., 2020).

Hooban at el. (2020) reviewed the role of the natural aquatic environment in the spread of ESBL and carbapenemase genes, which are commonly associated with plasmid harbouring and dissemination. Their results exposed the presence of clinically significant resistance genes in water bodies without direct contamination discharge, demonstrating that anthropogenic contamination may not be the sole contributor to dissemination of resistance genes (Hooban et al., 2020). Water environments also play a major role in the dissemination of integrons (Koczura et al., 2016) and thereby resistance genes.

3. METHODOLY

3.1. Phenotypic resistance

3.1.1. Sample Collection

The location of sample collection was decided with the aims and purpose of the study in mind; to survey ESBL-producing and CRE bacteria in the Norwegian aquatic environment without direct points of contamination or discharge. Two graphically close and connected water bodies on the park grounds of a university campus were chosen (see Figure 3.1).

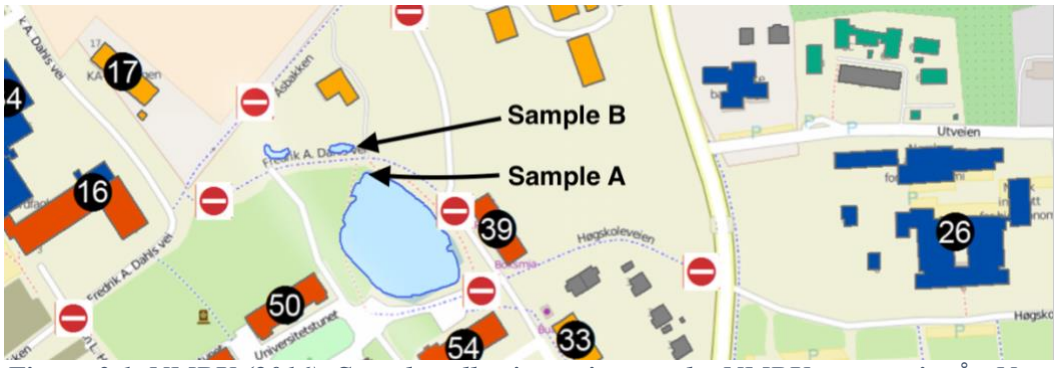


Figure 3.1. NMBU (2016). Sample collection points on the NMBU campus in Ås, Norway. Sample A (Svanedammen) and Sample B (Niagara) were obtained from two proximal and connect water bodies, with no apparent point of contaminations.

The Swan Pond ("Svanedammen"), more commonly known as the Duck Pond ("Andedammen") is a natural water body that was previously used for livestock drinking water and a reserve in case of fire (RedaksjonenNMBU, 2014). Contrary to the original name, there are in fact few swans to be found, but many ducks during summer months and other bird species. The sample was taken from the edge of water as seen today, in the area

where a drainage system is found. This mouth leads to the second water body, Niagara, where the small drainage system provides opportunity for somewhat of a bottle neck. Niagara, “the world´s smallest waterfall”, is not a natural body but was built on a slope north of Svanedammen in the late 1930s. Niagara takes on a miniature form of the well-known Niagara Falls in Canada. The stream was under reconstruction from 2015 to 2018. (NLA)

3.1.2. Selective screening for Antibiotic Resistance

By plating environmental aquatic samples directly onto plates selective for antibiotic resistance, one can select for only strains that exhibit phenotypic resistance and therefore likely contain resistance genes. Strains that are antagonised by the antibiotics are deemed sensitive and will obviously not grow. Selective plates are often chromogenic, containing enzyme substrates that change colour when hydrolysed by bacterial enzymes, allowing for the presumptive differentiation and identification of bacteria based on colony colourisation (Perry & Freydière, 2007). Selective chromogenic screening with antibiotic plates is especially useful in clinical settings, allowing for timely presumptive identification and thus targeted treatments.

Two chromogenic antibiotic resistance selective plates by Oxoid (Hampshire, United Kingdom) were used in this study; *Brilliance* ESBL and *Brilliance* CRE for the selection of ESBL-producing and CRE bacteria, respectively. The user guides for presentative detection based on colony colour are summarized in Table 3.1.

Table 3.1. Oxoid (2010). Summary of result detection for Brilliance ESBL and Brilliance CRE selective plates.

Brilliance ESBL (white plates)	
Blue/pink	*E. coli*
Green	KESC
Colourless	*Salmonella*
Straw	*Pseudomonas*
Brow halo	*Proteus, Morganella, Providencia*
Brilliance CRE plates (blue plates)	
Pale pink	*E. coli*
White/colourless	*Acinetobacter* or other
Blue	KESC

Brilliance ESBL agar is selective for ESBL mechanisms, but colonization through other resistance mechanism can occur and may be clinically relevant (Oxoid, 2010). It contains cefpodoxime, a third-generation cephalosporin, as well as antimicrobial agents that inhibit most non-ESBL Enterobacteriaceae and supresses other non-ESBL and AmpC organism growth (Oxoid, 2010). The plates are chromogenic, specifically targeting two enzymes: galactosidase and glucuronidase. Galactosidase-positive KESCs produce green colonies, galactosidase and glucuronidase-positive *E. coli* give blue colonies and β-galactosidase negative *E. coli* grow in pink colonies. *Proteus*, *Morganella* and *Providencia* deaminate tryptophan, growing tan-coloured, brown-haloed colonies.

Brilliance CRE agar contains a Clinical and Laboratory Standards Institute (CLSI) recommended amount of a modified carbapenem and two chromogens. *E. coli* grows pink while the KESC group grows blue. Other non-*Enterobacteriaceae* but carbapenem-resistant growth, for example *Acinetobacter*, may occur. Such colonies are white or naturally pigmented colonies and easily distinguishable (Oxoid, 2016).

3.1.3. Minimum Inhibition Concentration (MIC) Tests

Antibiotic Etest strips can be used to measure the level of phenotypic resistance to specific antibiotics by reading the Minimum Inhibition Concentration (MIC) value; the minimum antibiotic concentration required to inhibit bacterial growth. Bacteria are considered susceptible if the MIC value is smaller or equal to the breakpoint. Breakpoints are chosen concentrations of antibiotics that result in susceptibility to successful treatment (Mitka, 2012). An isolate culture diluted in sterile salt water is plated onto agar plates before placing the strip in the middle. Post incubation, an ellipse appears on the plates that intersects the MIC value scale. The MIC value in μg/mL is determined by reading the intersected value on the test scale where the inhibition of growth stops. Table 3.2 shows a range of carbapenem MIC values for tested Enterobacteriaceae clinical isolates expressing the main carbapenemases types.

Table 3.2. Nordmann et al. (2012). Range of carbapenem MICs in clinical Enterobacteriaceae expressing the main carbapenemase types.

	MIC (mg/L)		
	Imipenem	**Meropenem**	**Ertapenem**
KPC	0.5 to >32	0.5 to >32	0.5 to >32
IMP/VIM/NDM	0.5 to >32	0.5 to >64	0.38 to >32
OXA-48/OXA-181	0.25 to 64	0.38 to 32	0.38 to >32

Two breakpoint standards are often used, those by CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). CLSI lowered the carbapenem breakpoints in 2010 to allow for sufficient detection of carbapenem resistance in Enterobacteriaceae.

3.2. Genotypic Resistance Aspects

3.2.1. Genomic DNA (gDNA)

In order to study an organism´s genotype we need to study the genes encoded in its DNA, which must first be extracted from the cell. Widely available DNA extraction kits have made this somewhat easy. Cells must be lysed in order to retrieve the DNA within, where especially the thick peptidoglycan layer in Gram-positive cells needs to be sufficiently deteriorated. The GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MI, United States) used in this study lyse the cells in a salty chaotropic solution to ensure denaturation and includes an optional Gram-positive lysis solution. Ethanol is added to the lysate, causing DNA to bind to a silica membrane when spun through, eliminating the need for expensive resins and hazardous organic compounds, as well as alcohol precipitation. Contaminants are removed by washing before the DNA is eluded.

Extracted DNA concentration and purity can be measured based on fluorescence and spectrochemical principles, respectively. The Qubit fluorometer (Invitrogen) is based on specific labelling of targets with fluorescent dyes. It is much more sensitive and accurate than traditional ultraviolet (UV) spectroscopy (Invitrogen, 2016). The DNA specific dyes display low fluorescence until bound to the target DNA and is so more specific and better for concentration determination. Nanodrop is based on UV-absorbance, where the natural absorbance of light at 260 nm is spectrophotometrically measured. More absorbance is correlated with higher concentrations. Nucleic acids have absorbance maxima of 260 nm, but all molecules that absorb at 260 nm, including nucleotides, ds and ss DNA as well as RNA, will contribute to the total measured absorbance (Motlock, 2015). The $A_{260/280}$ product ratio provides an indication of purity and should be approximately 1,8 for DNA. Lower ratios indicate protein, phenols or other contaminants that absorb at 280 nm. The $A_{260/230}$ product ratio provides a secondary measurement for nucleic acid purity and is often somewhat higher than $A_{260/280}$, between 2,0 and 2,2. Contaminants that absorb at 230 nm, such as EDTA, carbohydrates and phenol, can be detected by a lower ratio (Motlock, 2015). Nanodrop and

Qubit are often used in conjunction to assess purity and concentration, respectively (Invitrogen, 2016).

Once DNA has been extracted and assessed, it can be directly sequenced for genome analyses. Although this technology has expanded greatly over recent years, replacing many traditional molecular biology techniques, it is still somewhat expensive compared to traditional methods. In clinical settings polymerase chain reaction (PCR) is often used to amplify specifically targeted genes of interest for faster and cheaper detection. While more convenient, PCR is not as accurate or comprehensive as direct sequencing and is limited by for example primer design.

3.2.2. Polymerase Chain Reaction (PCR)

This biochemical, *in vitro* technique amplifies targeted double stranded (ds) DNA segments. It involves DNA polymerase, deoxynucleotides and two synthetic single stranded (ss) oligonucleotide primers; one complementary to the 5´end of one target DNA strand and one complementary to the 5´end of the opposite strand. In the first cycle, the template DNA is denatured and annealed to the respective primers. DNA polymerase then extends the two primers by adding deoxynucleotides to the 3´ends of the ss templates, generating ds DNA on both strands and producing two copies of the template. In the next cycle the DNA is again denatured and this time primers can anneal to the template and newly synthesized strands, generating four copies. When primers anneal to newly synthesized strands, DNA polymerase extends the primer to the end of the template before falling off. As such, the region between the two primers is amplified geometrically over multiple cycles. (Watson, 2014)

3.2.3. 16S rRNA Sanger Sequencing

The gene that encodes for 16S ribosomal RNA (16S rRNA) is an ancient, functionally constant, universal gene that is found in almost all bacteria. It contains highly conserved regions that provide information regarding higher taxa classification as well as variable and hypervariable regions that make it possible to differentiate between closely related species. As such, it is the most widely used target for identification of clinical and environmental isolates and for phylogenetic relationship studies (Church et al., 2020). Limitations include primer selection, insufficient sequence data and high homology degrees between several related genera and species. GenBank (NCIB) contains the most sequences but is not curated to ensure correct sequences and annotations. More curated databases often focus on reference

strains, limiting microbial diversity. Gaps in the sequence databases also limits analysis and interpretation. Still, 16S is the preferred target compared to other highly conserved genes that have not been as thoroughly studied. (Church et al., 2020)

16S rRNA genes in bacterial gDNA samples can be amplified through PCR utilizing universal primers that target the generally conserved domains. Laboratories often target the first 500 bp as analysis of this variable region is considered sufficient for genera and species identification, but identification of genetically similar human pathogens requires more specific forward and reverse primer pairs. (Cloud et al., 2002)

The average length of bacterial 16S rRNA molecule is approximately 1 500 nucleotides and the amplification product can be visualized by agarose gel electrophoresis. Sequencing must then be performed to entirely confirm the *16S* gene and thereby identify the bacterial genus. Chain termination Sanger sequencing was used in this study. After initial PCR with short oligonucleotide 16S primers to generate template amplicons, a secondary cycle sequencing is performed. DNA polymerase synthesizes DNA by adding deoxyribonucleotide triphosphates (dNTPs; dATP, dTTP, dGTP and dCTP) to the 3´end of the template strands. Individually fluorescent-labelled, chain terminating dideoxynucleoside triphosphates (ddNTPs; ddATP, ddTTP, ddGTP and ddCTP) are also added to the reaction mix, in a much lower concentration compared to dNTPs. The ddNTPs lack the 3´-hydroxyl group required to polymerise to the next nucleotide, preventing the addition of another dNTP once incorporated and thus terminating elongation. As such, the cycle sequencing generates a set of ss DNA fragments of different lengths with the same 5´end but different 3´ends, each marked with a fluorescent tag. This mix is electrokinetically loaded into a polyacrylamide gel to be separated by electrophoresis. A fluorometric detector generates an electropherogram trace using the fluorescent tags for base calling, assembling the different fragments into a derived DNA sequence. (Church et al., 2020; Watson, 2014)

3.2.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to confirm, visualize and estimate the size of isolated gDNA and PCR products. Gel electrophoresis separates DNA (and RNA) molecules according to size. When subjected to an electric field, the negatively charged DNA molecules migrate through the porous gel matrix that acts as a sieve. Larger molecules with a larger effective volume will migrate slower than smaller molecules. The separated products are

visualized with dyes and appear as bands. (Watson, 2014). Here, a nucleic acid binding dye, peqGREEN, was added to the cooled gel mix before setting to aid visualization. Gel size, percentage, run settings and run time were decided according to the Sub-Cell ® GT Agarose gel Electrophoresis Systems Instruction Manual (BIO-RAD).

3.2.5. Detection of ESBL and carbapenemase encoding genes

Genes encoding β-lactamases and carbapenemases can be detected through PCR-amplification and subsequent sequencing, but this requires prior knowledge of the gene sequences (Wright, 2010). Novel resistance genes have been discovered with phenotypic screening and combined functional metagenomics-based approach by Handelsman et al. (2004). Total DNA is isolated to construct expression libraries before antibiotic screening (Handelsman, 2004). The limitation, that gene expression is required for selection screening, can be overcome with pyrosequencing and hybridization of probes of known genes.

In this study a set of three multiplex PCR assays for the detection of ESBL and carbapenemase encoding genes designed by Dallenne et al. (2010) and Finton et al. (2020) were used. These target the most common β-lactamases, ESBLs and carbapenemases found in clinical settings, namely *bla*$_{\text{CTX-M}}$ (groups 1, 2 and 9), *bla*$_{\text{CMY}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SFC1}}$ and *bla*$_{\text{VIM}}$ (Dallenne et al., 2010; Finton et al., 2020).

3.3. Whole Genome Sequencing

Next generation sequencing (NGS), first introduced by Sydney Brenner (Brenner et al., 2000), has hugely expanded regarding both technologies and applications over the last twenty years. There are two main NGS sequencing standards, short read sequencing and long read sequencing. Short reads are often more accurate, reliable and cost less than typically error-prone long reads. While short reads are useful regarding research on the population level and clinical variant studies, long reads are useful in *de nova* genome assemblies (Goodwin et al., 2016). Two sequencing platforms are utilized in this study, Illumina short read sequencing and long read MinION sequencing by ONT.

3.3.1. Illumina MiSeq Seqeuncing

Illumina, the current leading sequencing technology, is based on the generation of vast numbers of PCR colonies on a chip. All colonies are then sequenced simultaneously, reading one nucleotide in each colony in each cycle. This is a sequence-by-synthesis (SBS) approach which utilizes cyclic reversible termination (CRT) (Goodwin et al., 2016). The Illumina CRT approach (see Figure 3.1) utilizes terminator molecules like those used in Sanger sequencing, where the ribose 3´-OH group is blocked and thus prevents elongation. A DNA template is primed by a sequence complementary to the adaptor. The adaptor initiates polymerase binding to the dsDNA. A mixture of four blocked, individually labelled dNTPs are added during each cycle. Once a dNTP has been incorporated to the elongating strands, dNTPs that have not bound are removed. Imaging with total internal reflection fluorescence microscopy is used to identify dNTP incorporation before the blocking group is removed and a new cycle begins. (Goodwin et al., 2016)

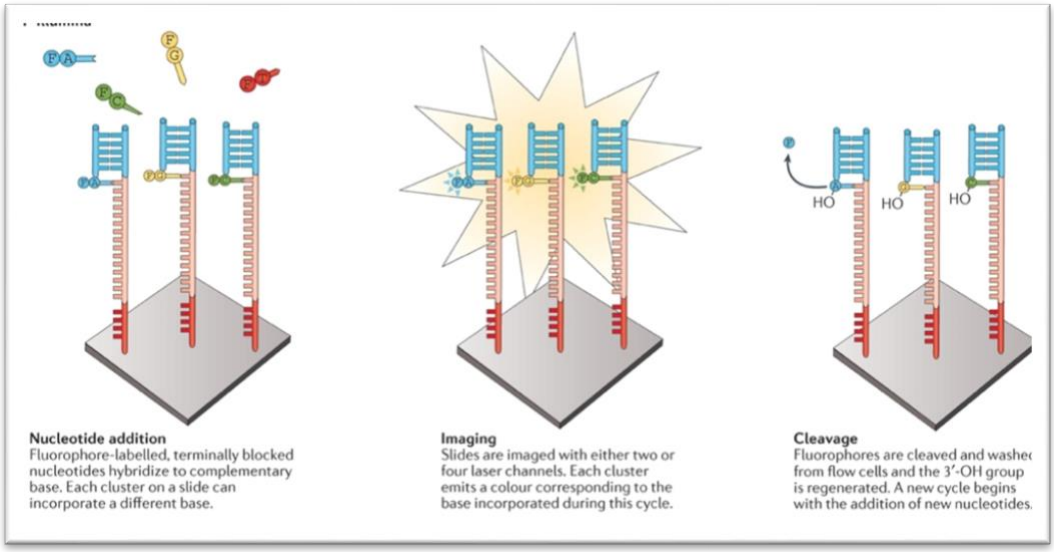


Figure 3.2. Goodwin et al. (2016). The principle of Illumina sequencing, utilizing sequencing-by-synthesis and cyclic reversible termination approaches. Fluorescent, terminally blocked nucleotides are incorporated during elongation. Slides are imaged to determine dNTP incorporation before incorporated dNTPs are cleaved and the cycle starts again.

3.3.2. Oxford Nanopore Technology (ONT)

Third generation Nanopore sequencing by Oxford Technologies (see Figure 3.2.) is based on engineered protein nanopores placed on membranes that block ionic current as single DNA strands pass through the pores (Jain et al., 2016). The current block creates a characteristic shift in current, called a squiggle, where each k-mer produces its own squiggle which can then be referenced against a library. The portable MinION nanopore DNA sequencer was

unveiled in 2012 and released in 2014 (Jain et al., 2016). Adapters are ligated to both gDNA ends prior to sequencing which facilitate the capture of the strands and concentrate substrates near the pores on the membrane surface. A processive enzyme, called a motor protein, at the 5´-end of one strand ensures the strands displaces in one direction. A hairpin adaptor connects the who strands and so allows both strands to be translocated through the pore and sequenced one after the other. The ionic current changes are recorded as squiggles as the strands translocates through the pores, which are interpreted using a library of graphical models. The reads of the two strands provide "1D" reads, which are combined to produce a complete "2D" read (Jain et al., 2016).

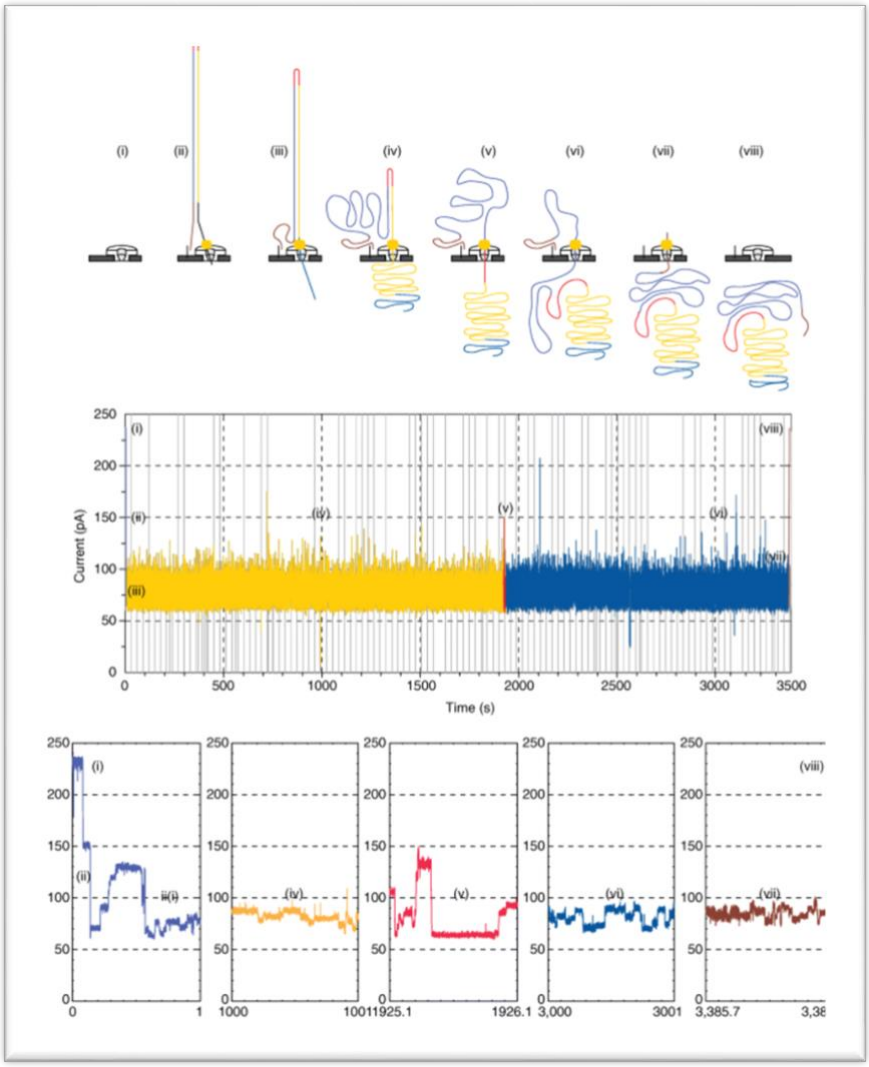


Figure 3.3. Jain et al. (2016). The principle of long read sequencing by Oxford Nanopore Technology. Single strands of DNA, connected by a hairpin adapter (red) are translocated through a protein nanopore. The strands are captured by the lead adaptor (blue) and the motor protein (orange). Capture is proceeded with translocation of the lead adaptor, template strand (gold), the hairpin adaptor, the complement strand (dark blue) and finally the trailing adaptor (brown). The raw current trance is recorded. Each current shift generates a unique squiggle that is used for base calling.

3.4. Bioinformatic Tools

3.4.1. BLAST

BLAST (Altschul et al., 1990; Altschul et al., 1997), Basic Local Alignment Search Tool, by the NCIB finds similar regions in biological nucleotide or protein sequences by comparing them to sequence databases and evaluating the statistical significance (NCBI). Ultimately the uploaded query sequence is compared to a huge database to find the most similar matches. As such, BLAST is commonly used to identify isolates by comparing them to previously sequenced organisms. Access to reference genomes and vast entries, in addition to easy bioinformatic comparison tools allow for simple and easily accessible analysis. Besides the match, BLAST provides an expected value (E value), percent identity and query cover. The E value tells how many matches can be expected by chance, given the size of the database. Lower E values means more significant matches. The percent identity indicates how similar the query and target sequences are. Higher present identities indicate more significant matches. The query cover indicates how covered the query sequence is by the target so gives an indication of the lengths relative to each other. (Fassler & Cooper, 2011)

3.4.2. Galaxy

Galaxy is a web-based platform for accessible, reproducible and transparent computational biomedical research (Afgan et al., 2018). The Galaxy ToolShed consists of over 5500 tools for genomic, proteomic and metabolomic analyses as well as imaging (Blankenberg et al., 2014). The Galaxy Community has created various tutorials and workflows for common genomic analyses, providing easy accessibility even for those with no programming experience. The Australian UseGalaxy server (https://usegalaxy.org.au) was used for the duration of this study, utilizing the tools described below.

Trimmomatic, (Bolger et al., 2014) a flexible and efficient read trimming tool designed to correctly handle paired-end data, was used to trim Illumina NGS data. The main algorithm is related to adaptor sequence identification and quality filtering through various processing steps. The SLIDINGWINDOW step performs a sliding window trimming that cuts when the average quality within the window is below a certain threshold.

Illumina reads were assembled utilizing SPAdes (Bankevich et al., 2012), the St. Petersburg genome assembler for standard isolates and single-cell multiple displacement amplification

(MDA) assemblies. SPAdes uses Bayes Hammer (Nikolenko et al., 2013) to correct reads by counting k-mers in reads and computed k-mer statistics that take base quality into account. Then a Hamming graph is constructed, where k-mers are nodes and edges connect nodes if the nucleotide number differences reach a certain threshold. Bayesian sub-clustering of this graph, which is central in the algorithm for error correction, determines the centre of each k-mer subcluster. Assumably error free, solid k-mers are derived from cluster centres and are mapped back to correct reads. The traditional handling of paired-end reads by SPAdes is slow and insufficient, leading to the emergence of Shovill (Seemann, 2017), a pipeline cored in SPAdes but with alternative, less time-consuming steps prior to and post primary assembly. Shovill was unavailable or too slow to be used in the duration of this study.

Porechop (Wick et al., 2017), a tool for finding and removing adapters from ONT reads, was used to trim the ONT data. Adaptors are found by thorough alignments, even at low sequence identity. Adapters at the ends are trimmed off while internal adaptors in the middle of a read are treated as chimeric and cut into separate reads.

ONT reads were assembled utilizing Canu assembler (Berlin et al., 2015; Koren et al., 2017), an assembler optimized for long and error prone reads. The first phase, correction, improves base accuracy. The trimming phase removes remaining adapters and leaves reads appearing as high-quality. The final, assembly phase orders the reads into contigs, produces consensus sequences and generates alternative path graphs.

Hybrid-assembly and correction of Illumina and ONT reads was performed with Unicycler (Wick et al., 2016). Three modes can be chosen: conservative mode which is least likely to generate a full assembly but has lower misassemble risks, bold mode which most likely to generate a full assembly but with greater error risks and normal mode which is an intermediate. Unicycler uses SPAdes for error correction and assembly in a time-consuming step which can be skipped but will ultimately decrease the assembly quality.

ABRicate, mass screening of contigs for AMR and virulence genes (Seemann, 2016), was used to find antibiotic resistance genes in all resulting genome sequences. ABRicate makes use of several databases that can be selected by the user, but can only find acquired, not point mutated, genes. Incomplete gap reporting, heavily overlapping genes being reported on the

same locus and issues related to coverage calculation are other limitations of this tool. The databases used in this study include:

1) ResFinder by Center for Genomic epidemiology (Bortolaia et al., 2020)
2) AMRFinderPlus by NCIB (Feldgarden et al., 2019)
3) CARD; The Comprehensive Antibiotic Resistance Database (Alcock et al., 2020; Jia et al., 2017)
4) VFDB; The virulence factor database (Chen et al., 2016; Liu et al., 2018)

Finally Prokka (Seemann, 2014), prokaryotic genome annotation, was used to search for interesting resistance, resistance-related and virulence genes that were not picked up by ABRicate. Prokka uses various existing software tools to identify coordinates of genomic features within preassembled DNA contigs for reliable annotation. The putative gene product is then determined by hierarchical database comparison, starting with a small trustworthy database, moving on to domain-specific databases of medium size and finally to curated protein family models.

3.4.3. CGView

CGView (cgview.ca), a Java package for visual representation and navigation of circular and linear bacterial genomes, was used to create images of the isolates (Petkau et al., 2010). Prokka annotation can be performed on the server using the uploaded map sequence, which can then be added as a new track on the map.

4. MATERIALS AND METHODS

All methods were performed under the guidance of Professor Bjørn-Arne Lindsedth based on previous studies of similar scope and purpose (Finton et al., 2020).

4.1. Sample collection and Incubation

Two aquatic environmental samples, Samples A and B, were collected on Tuesday 09.02.2021 at 10.30 am from two water bodies at the Norwegian University of Life Sciences (NMBU) campus in Ås, Norway. It was a cloudy day with a temperature of -6^{o}C (felt like -12 ^{o}C), 1% precipitation, 59% humidity and 5ms^{-1} wind (according to yr.no). As it had been below freezing for some time before sample collection and both water bodies were frozen

solid, ice was smashed and collected in sterilized Schott Duran® Laboratory Bottles with blue caps. The ice samples were defrosted overnight at room temperature in the laboratory.

The defrosted water samples were coarsely filtered through Whatman® Grade 589/1 Filter Paper Circles for Coarse Precipitate Retention (ashless/Black ribbon, 150 mm diameter) by GE Healthcare Life Sciences (VWR™ Life Science, Pitsburg, USA) to remove earth and other unwanted particles. Filtered samples of 1mL were pipetted onto Thermo Scientific™ Brilliance™ CRE Agar (Catalog number PO1226) and Brilliance™ ESBL Agar plates. The water was spread evenly over the plates and allowed to dry before incubating the plates at 37^{o}C for 48-72 hours. Once substantial growth was achieved, individual colonies were selected based on colony colour as described in Table 3.1. These colonies were inoculated onto new plates using sterilized inoculation loops, aiming to produce isolated and pure colonies of single bacterial strains. Single colonies from these plates were then inoculated to overnight cultures in glass tubes containing 5mL Brain Heart Infusion (BHI) Broth (Code CM1135, Oxoid Ldt., Loughborough, Great Britain) to be used for DNA extraction, frozen glycerol stock preparation and MIC testing.

BHI Broth (Oxoid) was prepared according to the manufacturer's instructions: 37g of powder was dissolved in 1 litre distilled water and sterilized by autoclavation at 121^{o}C for 15 minutes. Prior to autoclaving, 5mL of medium was transferred to short-stemmed glass test tubes. The media tubes were stored in the fridge until needed and allowed to equilibrate to room temperature prior to use to avoid any thermal shock that may affect bacterial growth. Sterilized toothpicks were used to select single colonies from the agar plates, carefully picking up a colony, ensuring to choose those substantially distanced from neighbouring colonies. The toothpick was then dropped into a tube containing 5mL BHI before gently swirling. These tubes were incubated at 37^{o}C for 24 hours prior to DNA extraction, frozen stock preparation and MIC testing.

To prepare glycerol stocks for long-time storage at -80^{o}C, 333 µl 50% sterile glycerol was added to 666 µl overnight bacterial culture in marked screw-cap tubes.

4.2. DNA extraction

All DNA extractions were performed with GenElute™ Bacterial Genomic DNA Kit (NA2110-1KT, Lot # SLcc6973) by Sigma Life Science (Sigma Aldrich®, St. Louis, MI, USA) according to the GenElute™ Bacterial Genomic DNA Kit Protocol (NA2100, NA2110, NA2120). The optional Gram-positive lysosome step was included in the protocol to ensure sufficient lysis of these cells. The elution buffer included in the GenElute™ kit contained EDTA which would later interfere with subsequent sequencing. Therefore, another buffer, SequalPrep™ Normalization Elution Buffer (PIN 100003572, Lot No 1796066) by Oxoid was used instead. Assessment of DNA concentration and purity was determined with Qubit® 2.0 (Life Technologiesm Grand Island, NY, USA) and Nanodrop (Oxoid), respectively.

4.2.1. Qubit (dsDNA)

gDNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technologies). Two standards of 1 ng/µl and 10 ng/µl were prepared with 190 µl Qubit™ 1X dsDNA HS Working Solution (Invitrogen by Thermo Fisher Scientific Inc., Waltham, MA, USA) and 10 µl of the applicable Qubit™ 1X dsDNA HS Standard Solution (1 ng/µl or 10 ng/µl in TE buffer). Samples were prepared by mixing 198 µl Qubit™ 1X dsDNA HS Working Solution and 2 µl isolate gDNA. The standards and samples were incubated for two minutes prior to reading. Samples that were measured to be "too high" were diluted with a sample: RNase-free H_2O ratio of 1:9 before measuring again.

4.2.2. Nanodrop (Nucleic acid)

gDNA purity was measured using a Nanodrop™ 2000/2000C Spectrophotometer (Oxoid). A blank, 2 µl DEPC treated water, was measured prior to each reading. Samples of 2 µl DNA were then measured, carefully cleaning the node with filter paper between each measurement.

4.3. 16S rRNA

4.3.1. PCR

Once DNA was extracted, purified and measured, the 16S rRNA gene was targeted for subsequent sequencing identification. PCR amplification was performed with iProof™ High Fidelity DNA Polymerase Kit (Bio-Rad Laboratories Inc., Vilnius, Lithuania) following the manufacture´s protocol. A PCR multimix was prepared according to Table 4.1. 16S rRNA

PCR multimix preparation. Once prepared, 38 µl of the multimix was pipetted into 0,2 mL MicroAmp® 8-Tubes (Applied Biosystems™, Thermo Fisher Scientific) before adding 2 µl of isolate gDNA. The tubes were loaded into the Biosystems™ SimpliAmp Thermal Cycler (Applied Biosystems® by Life Technologies™, Foster City, MA, USA) and run on the pre-set 16S program presented in Table 4.2. Once PCR products were confirmed by electrophoresis as described in section 4.3.2 below, PCR clean-up was performed with GenElute™ PCR Clean-up Kit (Catalog Number: NA1020) by Sigma-Aldrich to remove any contaminants or primers. The manufacture´s protocol was followed, except eluding with 25 µl Elution Solution instead of 50 µl to limit dilution. Two 1,5 mL tubes, one forward and one reverse, were prepared for each isolate. These tubes were marked with barcodes provided by GATC Biotech (Eurofins GATC, Konstanz, Germany), the company that performed 16S rRNA Sanger Sequencing.

Table 4.1. 16S rRNA PCR multimix preparation.

	Initial Concentration	Final concentration	Volume/reaction (µl)
Iproof ™ HF Buffer	5x	1x	8
dNTP	10 mM	200 µM	0,8
16S Forward Primer AGAGTTTGATCMTGGCTCAG	5 µM	0,25 µM	2
16S Reverse Primer GYTACCTTGTTACGACTT	5 µM	0,25 µM	2
H_2O			2,8
DNA polymerase	2U/µl	0,02U/µl	0,4
DNA template	~50 ng/ul	(7,5 – 150 ng)	2
Total Volume			40

Table 4.2. Pre-set program for 16S rRNA PCR amplification.

	Temperature (°C)	Minutes : seconds	Cycles
DNA Polymerase Activation	98	00:30	1
Denaturing Primer annealing Primer extension	98 55 72	00:10 00:30 00:45	35
Final extension	72	10:00	1
Cooling	4	∞	

4.3.2. Electrophoresis

1% gels were prepared to visualize the 16S rRNA PCR amplification products by dissolving and boiling 0.5 g Seakem® LE Agarose (Lonza Rockland Inc., Rockland, USA) in 50 mL

TAE buffer (Bio-Rad Laboratories) and adding 1 µl peqGREEN (VWR™ Peqlab, UK) per 50 mL once sufficiently cooled. Loading samples of 8 µl were prepared with 2 µl PCR product, 2 µl Agarose Gel Loading Dye 6x (VWR™ Life Science, AMERSCO®, Solon, OH, USA) and 4 µl RNase-free water. The ladder mix of 6 µl was prepared with 1 µl 1KB ladder; 1 µl Agarose Gel Loading dye x6 and 4 µl water. The gel ran at 98V for approximately 40 minutes. Gel imaging was performed by Gel Doc™ XR+ with Image Lab™ Software (Molecular Imager®) by Bio-Rad.

4.3.3. Sanger Sequencing

Samples for 16S rRNA Sanger sequencing were prepared according to Table 9.1 and Table 9.2 in Appendix 9.1 based on the sample to primer ratio requested by GATC Biotech.

4.4. MIC Testing

ETEST® (bioMeriex, Marcy-l´Étoile, France) gradient antibiotic strips were used the measure the MIC values and thereby susceptibility of all isolates. Ampicillin (penicillin), cefotaxime (cephalosporin), ciprofloxacin (fluoroquinolone), imipenem and meropenem (carbapenems) ETEST® strips were tested. When frozen stocks were used, the isolates were plated on Mueller-Hinton (MH) agar plates (Thermo Fischer Scientific) and incubated at 37^{o}C overnight before inoculated into BHI broth. Depending on growth, between 300 and 500 µl was pipetted into a tube containing 9 mL sterile NaCl (8%). A sterile cotton swab was used to streak the culture-containing saltwater solution onto MH agar plates before the strip was placed in the middle of the plate with sterile tweezers. The plates were incubated at 37^{o}C overnight, before the MIC values were read.

4.5. ß-lactamase, ESBL and carbapenemase genes

4.5.1. Multiplex PCR

The multiplex PCR multimixes were prepared with Qiagen® Multiplex PCR Kit (QIAGEN®, Hilden, Germany) according to Table 4.3. The manufacture´s Quick-Start Protocol (March 2016) was followed but excluding the optional 5x QSolution and halving the recommended volume per reaction. Previous studies have shown that halving the volume did not majorly affect the outcome and was therefore economically and resourcefully

advantageous (Lindstedth, B.A, 2021). Three multiplex multimixes were prepared as presented in Table 4.4, each targeting specific ß-lactamase, ESBL and carbapenemase genes with specific primers by Invitrogen (ThermoFischer Scientific). Multiplex 4, targeting universal *16S* and *rpoB* genes, was used as a control. The pre-set protocols for the multiplex runs on PCR machine 2, Biosystems™ SimpliAmp Thermal Cycler (Applied biosystems), are presented in Table 4.5.

Table 4.3. QIAGEN. Multimix preparation for Multiplex PCR.

	Volume/Reaction (µl)	Final concentration
2X QIAGEN Multiplex PCR Master Mix 10x primer mix (2 µM of each primer) RNase-free H_2O	12.5 2.5 9	1x 0.2 µM of each -
Template DNA (sample)	1	≤ 1 µg / reaction
Total	25	

Table 4.4. Dallenne et al. (2010), Finton et al. (2020). Multiplex screening of ß-lactamase, ESBL and carbapenemase genes.

Target Genes	Primer Sequence (5´-3´)	Amplicon Size (bp)
Multiplex 1		
bla$_{OXA-48}$ (serine carbapenemase)	F - GCTTGATCGCCCTCGATT R - GATTTGCTCCGTGGCCGAAA	281
bla$_{CTX-M}$ (gr. 2)	F - CGTTAACGGCACGATGAC R - CGATATCGTTGGTGGTRCCAT	404
bla$_{OXA}$ (serine carbapenemase)	F - GGCACCAGATTCAACTTTCAAG R - GACCCCAAGTTTCCTGTAAGTG	564
bla$_{SHV}$	F - AGCCGCTTGAGCAAATTAAAC R - ATCCCGCAGATAAATCACCAC	713
Multiplex 2		
bla$_{CTX-M}$ (gr. 9)	F - TCAAGCCTGCCGATCTGGT R - TGATTCTCGCCGCTGAAG	561
bla$_{CTX-M}$ (gr. 1)	F - TTAGGAARTGTGCCGCTGYA R - CGATATCGTTGGTGGTRCCAT	688
bla$_{TEM}$	F - CATTTCCGTGTCGCCCTTATTC R - CGTTCATCCATAGTTGCCTGAC	800
Multiplex 3		
bla$_{NDM}$ (metallo-carbapenemase)	F - TGGCCCGCTCAAGGTATTTT R - GTAGTGCTCAGTGTCGGCAT	157
bla$_{VIM}$	F - ATAGAGCACACTCGCAGACG R - TTATTGGTCTATTTGACCGCGT	564
bla$_{KPC}$ (serine carbapenemases)	F - TCCGTTACGGCAAAAATGCG R - GCATAGTCATTTGCCGTGCC	460
Mulitplex 4		
rpoB	F - CAGGTCGTCACGGTAACAAG R - GTGGTTCAGTTTCAGCATGTAC	512
16S rRNA	F - AGAGTTTGATCMTGGCTCAG R - GYTACCTTGTTACGACTT	1505

Table 4.5. Pre-set protocol "ESBL" for Multiplex 1-4.

ESBL protocol for M 1-4	Temperature (°C)	Minutes : seconds	Cycles
Initial heat activation	95	15:00	1
Denaturing Primer annealing Primer extension	94 60 72	00:30 01:30 01:30	30
Final extension	72	10:00	1
Cooling	4	∝	

4.5.2. Electrophoresis

Large, 2 % gels were prepared for visual analysis of the multiplex PCR runs. 4.2g Seakem was dissolved in 210 mL TAE buffer before boiling, cooling and adding 4 µl peqGREEN. The ladder mix was prepared with 1 µl 100 bp ladder (New England Biolabs® Inc.), 4 µl dye, 25 µl TAE, giving 30 µl. Only 10 µl of the ladder mix was loaded into one well. Loading samples of 10 µl were prepared with 2 µl PCR product, 2 µl 6x dye and 6 µl TAE. Gels were run at 80V for approximately 90 minutes. Gel imaging was performed by Gel Doc™ XR+ with Image Lab™ Software (Molecular Imager®) by Bio-Rad.

4.6. Illumina MiSeq Sequencing

Four isolates; KA0, KA5, KB3 and KB8, were selected for Illumina MiSeq v3 WGS based on the presumptive identification, multiplex PCR results and MIC tests presented in RESULTS. The sequencing preparation (Nextera™ DNA Flex Tagmentation) and sequencing was performed by The Norwegian High Throughput Sequencing (NSC) Centre at The Department of Medical Genetics at Ullevål University Hospital (OUS) in Oslo, Norway. The Sample Information table requested by NSC can be found in Appendix 9.2. A sample of the elution buffer was also delivered along with the samples.

4.7. MinION sequencing

The same isolates that were sent to Oslo for Illumina Miseq sequencing were sequenced at NMBU with MinION ONT sequencing with the help and guidance of Professor Bjørn-Arne Lindstedt and head engineer Ahmed Abdelghani. The MinION Mk1C sequencing machine

was utilized following the manufacture´s protocol (Version: MKC_2005_v1_revJ_27Nov2019) with the R9 version Spot-ON Flow Cell (Product code FLO-Min106D, ONT). The Oxford Nanopore Rapid Barcoding Kit (Product code SQK-RBK004, Oxford Nanopore Technologies) was utilized following Rapid Barcoding Sequencing (SQK-RBK004) protocol (Version RBK9054 v2 revR 14Aug2019).

Samples of approximately 400 ng gDNA were prepared for ONT sequencing according to Formula 1and Table 9.3 (see Appendix 9.3).

4.8. Data analysis

The 16S rRNA sequences were uploaded to BLAST in order to determine possible species identification. The remaining sequence analysis was performed in Galaxy with guidance of Prof. Bjørn-Arne Lindstedth.

4.8.1. Illumina MiSeq Sequencing

All Illumina Miseq data was handled identically in Galaxy. The MiSeq FASTQ files were uploaded and trimmed with Trimmomatic (Bolger et al., 2014). “Paired-end (two separate files)” was selected with read 1 (R1) and read 2 (R2) as input. Once executed, the read pairing was retained in four filtered files. The “R1-paired” and “R2-paired” files, each containing one read from each pair where both have persisted, were used in further analysis. The two additional “unpaired” files consist of reads where one of the pair failed the filtering steps. Further, Spades (Bankevich et al., 2012) was executed, producing five report files. The “scaffolds (fasta)” files for each strain were used for further analysis. This sequence file was also uploaded to PubMLST for species identification.

ABRicate (Seemann, 2016) was executed to screen for any antibiotic resistance genes expressed in the isolates. The SPades “scaffolds (fasta)” file was selected as input. Four analyses were executed, selecting “ResFinder”, “CARD”, “NCBI Database” and “VFDB” databases under “Advanced options”, respectively.

Prokka (Seemann, 2014) was executed to annotate genomes, again selecting the SPades “scaffolds (fasta)” file as input. Here, the Genus name and Species name were selected based on the PubMLST results (see Table 5.4). To find any interesting antimicrobial and virulence

genes that were not picked up by ABRicate, the Prokka "gbk" files were opened and manually examined in Microsoft Excel. Key words, namely "lactamase", "antibiotic resist", "resist", "drug" and "virulence" were used to search entries. The sequences of positive hits were copied to BLAST for further identification and comparison.

Finally, the assemblies were uploaded to the CGView server. Once the maps were created, "Prokka annotation" under "Display" was started with default settings. Once the job was completed, the annotation was added to the map by selecting "add" and then selecting "features" under "track". All genes were selected before manually unselecting genes previously found by Prokka executed in Galaxy and then deleting all selected genes.

4.8.2. ONT Sequencing

The resulting ONT sequence files for each barcoded isolate were concatenated into a single FASTQ file for each isolate and zipped in the Mac coding Terminal using the "cat" and "gzip" commands. The resulting files were uploaded to Galaxy and trimmed with Porechop (Wick et al., 2017). Assembly was executed with Canu assembler (Koren et al., 2017), using the trimmed Porechop file as input. Better results were achieved when selecting "nothing" under Processing compared to selecting "trimmed". The estimated genome size was set to 6.0 m and all other settings were left as default. Canu produced six output files: report, contigs, unassembled, corrected reads and trimmed reads. The Canu contig files were uploaded to PubMLST for species identification. ABRicate, and Prokka were executed in Galaxy as described above, but using the Canu contig files.

5. RESULTS

5.1. Phylogenetic Resistance

5.1.1. Bacterial Growth and Isolation

Generally, more growth was observed on the ESBL plates compared to the CRE plates, as illustrated in Figure 5.1 and Figure 5.2. Sample B provided substantial growth on both ESBL and CRE plates, while sample A resulted in very little growth on the CRE plates. Note that the picture of sample A CRE plate (see Figure 5.1) was taken after the plate has been stored in the fridge at 4^{o}C for a few weeks. The cream-coloured colonies were not observable during original inspection and isolation. The very small, blue colonies resulting from sample A on

the ESBL plates were inoculated into overnight cultures before re-streaking as these were difficult to isolate and inoculate onto new plates.

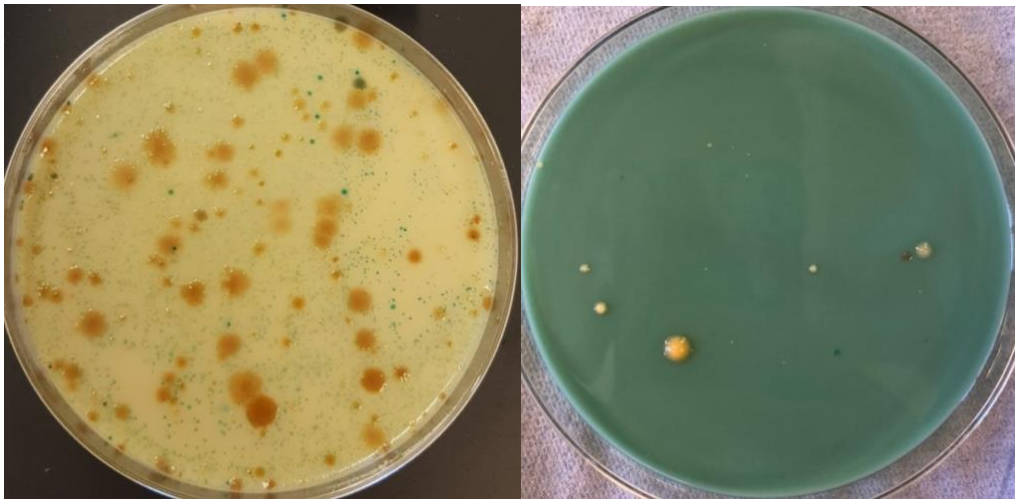


Figure 5.1. Sample A on Brilliance ESBL plates (left) and Brilliance CRE plates (right). Very small blue colonies and larger brown/cream-coloured colonies dominated on the ESBL plates. Almost no growth was observed on the CRE plate at the time of inspection and isolation.

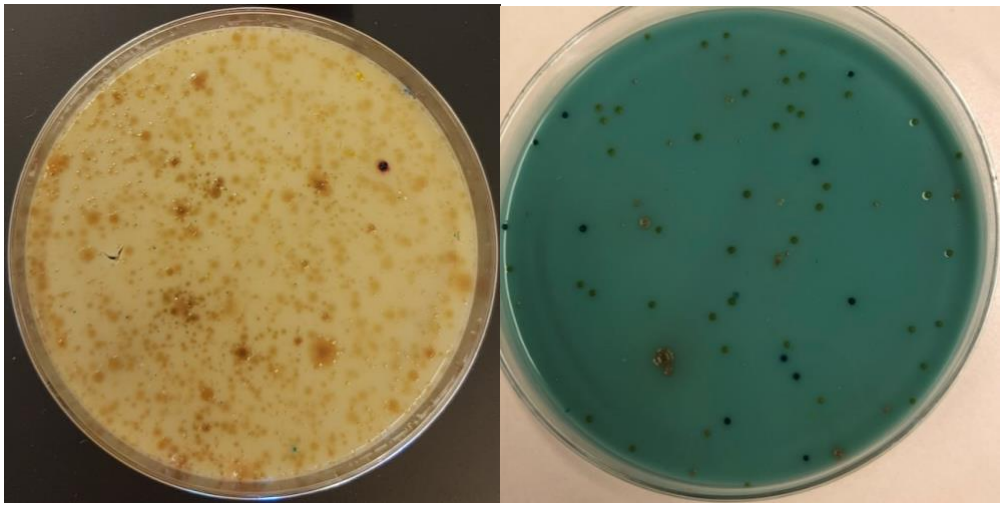
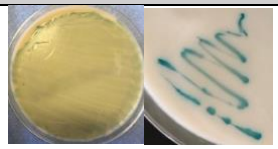
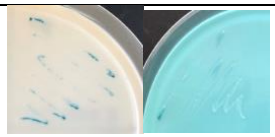
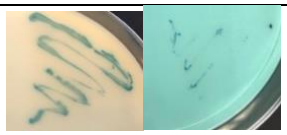
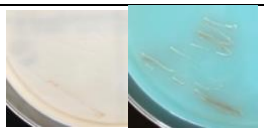
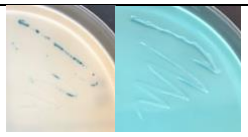


Figure 5.2. Sample B on Brilliance ESBL plates (left) and Brilliance CRE plates (right). Brown- and cream-coloured colonies dominated the Sample B ESBL plate. Some, very small blue colonies were observed on the ESBL plates, but these were difficult to isolate. Sample B on the CRE plates resulted in good growth of individual colonies of different colours.

Table 5.1 summarizes the presumptive identification based on colony colour on the chromogenic selective plates. The table first and foremost describes which plate (ESBL or CRE) the isolates were obtained from, the colour and the presumptive identification. Out of interest, the isolates were plated onto both plates. Isolates KB3 and KB8 only grew on the CRE plates, while isolates KA0, KA4, KA5 and KA6 only grew on the ESBL plates. Isolates KA1, KA2, KA7, KB2, KB5 and KB9 seemingly grew on both plates.

Table 5.1. Isolates obtained from aquatic samples A and B through selective screening.

	Plate	Description	Pressumptive Identification	Image
KA0	ESBL	Blue/green	*E. coli*	
KA1	ESBL	Blue	*E.coli*	
KA2	ESBL	Blue	*E.coli*	
KA4	ESBL	Cream/pink	*Proteus, Morganella or Providencia/E. coli*	
KA5	ESBL	Blue/green	*E.coli/KESC*	
KA6	ESBL	Blue/green	*E.coli/KESC*	
KA7	ESBL	Blue/green	*E.coli/KESC*	
KB2	ESBL	Green	*KESC*	
KB3	CRE	Pink	*E. coli*	
KB5	CRE	Blue	*KESC*	
KB8	CRE	Rust coloured	No identification	
KB9	ESBL	Blue	*E. coli*	

Many of the bacteria did not grow in the overnight culture medium, indicating that the BHI medium was not selective for those strains. Further, some of the cultures, namely KA1, KB2, KB5 and KB9, grew at the top of the liquid media in a biofilm fashion, which both limited growth within the medium and made it difficult to obtain a homologous solution which is essential for DNA extraction. As such, not all DNA extractions were successful. All DNA extractions and the subsequent Qubit and Nanodrop measurements are presented in Table 9.4 in Appendix 9.4. DNA extraction of the above-mentioned isolates resulted in little DNA that was sometimes sufficient for 16S rRNA PCR and subsequent Sanger sequencing, but not sufficient for Illumina or ONT sequencing.

5.2. Species Identification

The top BLAST hits for the 12 samples sent for 16S rRNA Sanger Sequencing is presented in Table 9.5 (see Appendix 9.5). Table 5.2 provides a summary of species identification based on 16S rRNA, Illumina MiSeq and ONT sequencing data. The 16S sequences of isolates KA6 and KB8 were of questionable quality and are as such coded with yellow. Isolates KA0, KA5, KB3 and KB8 were selected for Illumina sequencing based on the multiplex PCR and MIC tests presented in Section 5.6. KB8 failed on multiple attempts as illustrated with orange. Isolates KA1, KA2, KB2, KB5 and KB9 provided too little DNA under extraction to be ONT sequenced and are as such coded with yellow.

Table 5.2. Species identification of isolates based on 16S rRNA, Illumina Miseq and ONT sequencing, as determined by PubMLST.

Isolate	16S rRNA	Illumina	ONT	Hybrid
KA0	*Rahnella*	*Rahnella*	*	*Rahnella*
KA1	*Rahnella*			
KA2	*Rahnella*			
KA4	*Herbaspirillum*		*Ranella*	
KA5	*Herbaspirillum*	*Rahnella* (95%) *Herbaspirllum* (5%)	*	*Rahnella*
KA6	*Herbaspirillum*			
KA7	*Pseudomonas*		*Pseudomonas*	
KB2	*Rahnella*			
KB3	*Sphingomonas*	*Herbaspirillum*	*	*Herbaspirillum*
KB5	*Herbaspirillum*			
KB8	*Herbaspirillum*	Failed	*Herbaspirillum*	
KB9	*Herbaspirillum*			

* Isolates that were successfully sequenced with ONT, but where species identification was performed with hybrid Illumina and ONT assemblies which is assumed to be corrected, of better quality and thus more accurate.

5.3. Multiplex PCR

As seen in Figures 9.1 and 9.2 (see Appendix 9.6), many shadows were visible. This indicates unspecific primers and the presence of primer dimers. Further, the 1% gel and the ladders were not sufficient for gene size determination. Multiplex 1 (M1) indicated positive bands for isolates KA0, KA5, KA6 and KB3 between 500 and 1000 bp, possibly OXA (564 bp) or SHV (713) genes. Multiplex 2 (M2) indicated a positive band for isolate KA6 at approximately 500 bp, possibly a CTX-M group 9 β-lactamase. No positive bands of the target size were apparent for Multiplex 3 (M3). Most importantly, the control Multiplex 4 (M4) indicates insufficient DNA for isolates KA1, KA2, KB2 and KB9 as no 16S bands were observed. Strangely, no positive bands for *rpoB* were observed for isolates KB3, KB5 and KB8.

To better visualize the resulting bands, 2% gels were prepared. Only isolates with indication of positive bands on the 1% gels were loaded onto these gels, which are presented in Figure 5.3 (M1, M2 and M3) and Figure 5.4 (M4). The frozen PCR products from the original multiplex run were used, which may have degraded under thawing. Figure 5.3 shows that the positive bands for isolates KA0, KA5, KA6 and KA7 and KB3 seem to be approximately 800 bp. No genes of that length were target, but they could possibly be the SHV gene of 713 bp. A faint band can be seen for isolate KA6 just below 500 bp, possibly CTX-M group 2 (404 bp) or OXA (564). The positive band in M2 for KA6 at approximately 500 bp could be CTX-M group 9 (561 bp). Bands in M2 for isolates KA4 and KA6 between 700 and 800 bp could indicate CTX-M group 1 (688 bp) or TEM genes (800 bp). Bands over 1000 bp long were also observed for isolates KA4 and KB8, indicating unspecific primers. Bands over 1000 bp long were also observed for isolates KA0, KA5 and KB8 in M3. Bands were also observed just above 500 bp for isolates KA6 and KA7, possibly VIM gens (564 bp). Bands at approximately 400 and 500 bp were observed for isolate KB3, indicating the possible presence of VIM (564) or KPC (460 bp) genes. Figure 5.4 confirms little to no PCR products for isolates KA1, KA2, KB2 and KB9.

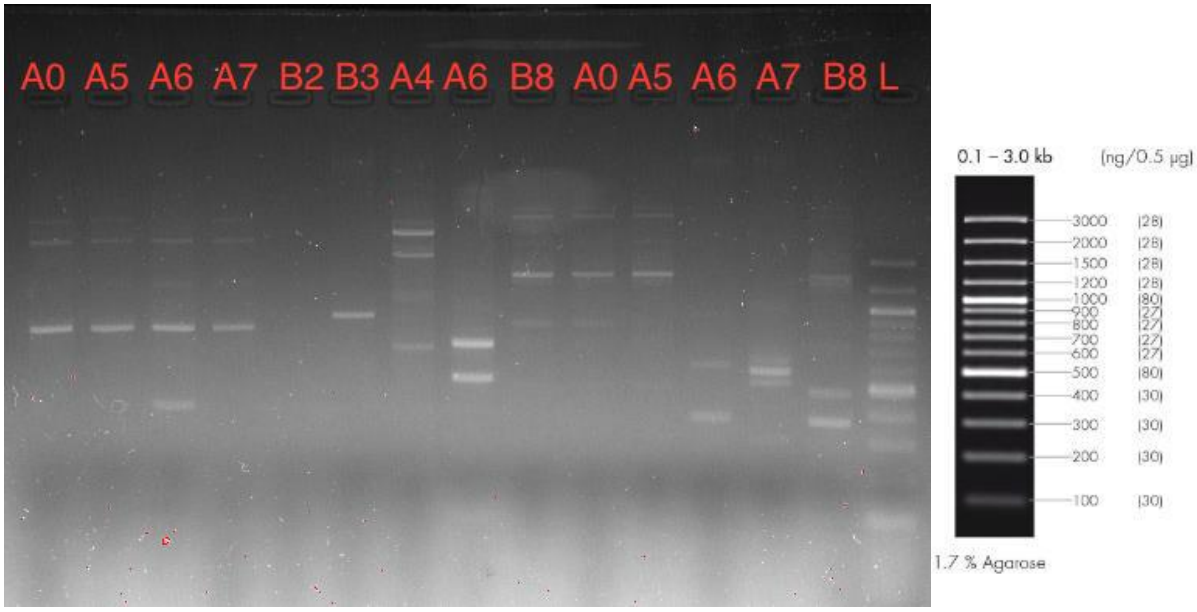


Figure 5.3. Gel image of multiplex 1,2 and 3 PCR products of selected isolate on 2% gel. Only isolates that gave indications of positive bands on previously run gels were loaded on this gel. Namely M1 products for isolates KA0, KA5, KA6, KB2 and KB3 were loaded in wells 1 to 6. M2 products for KA4, KA6 and KB8 were loaded in wells 7 to 9. M3 products for isolates KA0, KA6, KA7 and KB8 were loaded in wells 10 to 14. The ladder was loaded into the well furthest to the right. A ladder on 1,55% agarose gel is presented on the right for reference.

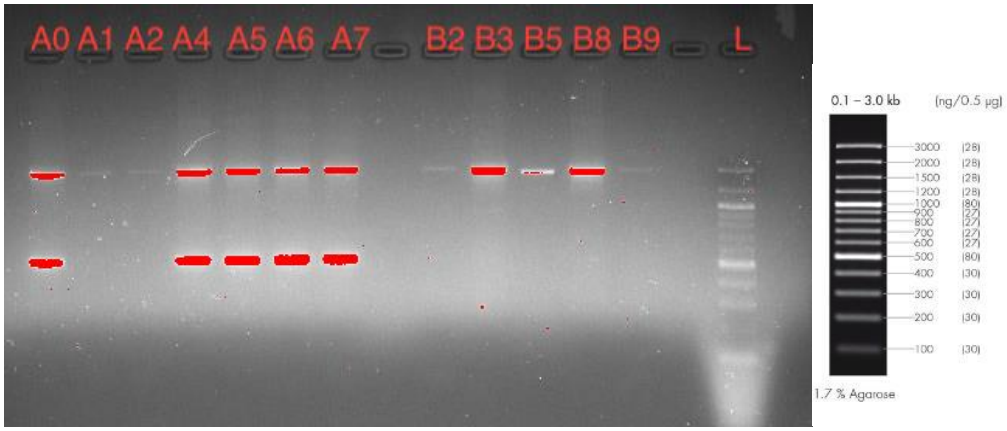


Figure 5.4. Gel image of Multiplex 4 PCR products of all isolates on 2% gel. The image reveals that isolates KA1, KA2, KB2, KB5 and KB9 did not provide enough DNA to be amplified and detected during PCR. Interestingly, no indication of the positive control gene, rpoB (512 bp) can be seen for isolates KB3 and KB8, but indications of the 16S gene bands are visible. The ladder was loaded into the well furthest to the right. A ladder on 1,55% agarose gel is presented on the right for reference.

Based on these results, isolates KA0, KA5, KB3 and KB8 were selected for Illumina WGS. Isolate KA6 was not selected based on the poor Sanger sequencing quality, indicating the DNA was not pure enough. Further, the very unspecific binding indicated by all the bands (Figure A.x) also indicated towards problematic DNA.

5.4. *de nova* Genome Assemblies

Isolates KA0, KA5 and KB3 were successfully sequenced with Illumina MiSeq technology, resulting in successful short read *de novo* genome assemblies. Isolate KB8 failed MiSeq sequencing on multiple attempts but was successfully sequenced with ONT at NMBU. This was the first time the MinION Mk1C sequencing machine was utilized at the NMBU Food Safety Research laboratory, and as illustrated in Figure 5.5, the sequencing run was successful with many active pores throughout the run.

Figure 5.5. The MinION Mk1C sequencing machine in action. As seen, many of the pores were active during sequencing, providing substantial reads of all included isolates.

Isolates K- A0, A4, A5, A7, B3 were also successfully sequenced with ONT. Where possible, namely for isolates KA0, KA5 and KB3, the long- and short- reads were hybrid assembled and corrected with Unicycler, and these assemblies were used for further analysis. Ultimately, WGS resulted in full, hybrid assembled and corrected circular genomes of isolates KA0, KA5 and KB3. Complete, circular genomes were obtained for KA4 and KA7 with ONT long reads. ONT sequencing of KB8 resulted in an assembly of a circular contig and a linear contig. The assembled contigs for all isolates are summarized in Table 5.3. Visual representations of the assemblies are illustrated in Figures 5.6-5.12 (see section 5.5.2).

The coverage of the Illumina reads was generally very high. The node lengths and coverage for isolate KA0 and KA5 are presented in Appendix 9.14 and Appendix 9.15, respectively. The coverage for the Nanopore corrected reads were 37,51 times, 35,14 times and 19,23 times for isolates KA4, KA7 and KB8, respectively (see Appendix 9.16).

Table 5.3. Summary of contigs obtained through Canu and Unicycler genome assemblies.

Canu Assembly of ONT long reads	
KA4	>tig00000001 len=4751705 reads=16876 class=contig suggestCircular=yes >tig00000002 len=699612 reads=2383 class=contig suggestCircular=yes >tig00000003 len=152022 reads=402 class=contig suggestCircular=yes
KA7	>tig00000001 len=5040205 reads=13724 class=contig suggestCircular=yes
KB8	>tig00000001 len=5579191 reads=9317 class=contig suggestCircular=yes >tig00000002 len=13119 reads=17 class=contig suggestCircular=no
Unicycler hybrid Assembly of Illumina short reads and ONT long reads	
KA0	>1 length=4733665 depth=1.00x circular=true >2 length=675034 depth=0.88x circular=true >3 length=102912 depth=0.25x circular=true
KA5	>1 length=4733803 depth=1.00x circular=true >2 length=675034 depth=0.91x circular=true >3 length=103045 depth=0.73x circular=true
KB3	>1 length=5976833 depth=1.00x circular=true

Isolates were identified using PubMLST Species ID. For clarity and comparison, the MiSeq resulting SPAdes "scaffolds" files, the ONT resulting Canu Assembly "contig" files, as well as the hybrid Unicycler "final assembly" files were uploaded to PubMLST. The resulting predicted taxa for all three instances are presented in Table 5.4, but the Canu predictions are not presented in instances with more accurate hybrid assemblies.

Table 5.4. Species taxa identification as predicted by PubMLST

Isolate	Assembly	Exact Matches	Taxon	Support (%)
KA0	SPAdes	46	*Rahnella variigena*	100
	Unicycler	46	*Rahnella variigena*	100
KA4	Canu	21	*Rahnella variigena*	100
KA5	SPAdes	47	*Rahnella variigena* *Herbaspirillum huttiense*	95 5
	Unicycler	46	*Rahnella variigena*	100
KA7	Canu	23	*Pseudomonas laurentiana*	100
KB3	SPAdes	32	*Herbaspirillum huttiense* *Herbaspirillum aquaticum*	81 8
	Unicycler	38	*Herbaspirillum huttiense* *Herbaspirillum aquaticum*	82 7
KB8	Canu	16	*Herbaspirillum aquaticum* *Herbaspirillum huttiense*	70 23

As seen in Table 5.3 and Table 5.4.4, WGS resulted in the identification of six isolates. Isolates KA0, KA4 and KA5 were identified as *R. variigena* with 100% support, based on Unicycler assemblies for KA0 and KA5 and a Canu assembly for KA4. Isolate KA5 was predicted as *R. variigena* with 95% support and *H. huttiense* with 5% support based on the SPAdes assembly. This is likely attributed to DNA contamination during Illumina sequence preparation or isolate contamination during early inoculation. All three *Rahnella* isolates contained three circular contigs, presumably a circular chromosome of approximately 4,7 mega base pairs (mbp), and two plasmids of approximately 675 kilo base pairs (kbp). The length of the Canu assembled isolate KA4 was somewhat longer than the more similar Unicycler assemblies of the KA0 and KA5 isolates, likely attributed to the lack of correction by hybrid assembly.

Further, Canu assemblies led to the predicted taxa identification of KA7 as *P. laurentiana* and KB8 as *H. aquaticum* with 100% and 70% support, respectively. The *Pseudomonas* isolate KA7 contained a circular contig of approximately 5 mbp. Both SPAdes and Unicycler assemblies of KB3 resulted in taxa predictions of *H. huttiense* (81 and 82%, respectively) and *H. aquaticum* (8 and 7%, respectively), with a minutely higher support of 1% for *H. huttiense* with the Unicycler assembly compared to Canu. The *Herbaspirillum* isolates KB3 and KB8 contained circular contigs of approximately 5,98 and 5,58 mbp, respectively. KB3 contained no other contigs while KB8 contained an additional, non-circular contig.

5.5. Resistance and Resistance-related Genes

5.5.1. Antimicrobial Gene Mass Screening

A summary of the antimicrobial and virulence genes found using ABRicate is presented in Table 5.5. A more detailed overview including product definitions is presented in Table 9.11 (see Appendix 9.12). The same genes were found in all *Rahnella* isolates despite assembly tools, as indicated below. The description of the AMR and virulence genes to follow are based on the information provided by ABRicate and within the respective databases.

Table 5.5. Summary of ABRicate results; mass contig screening for antimicrobial and virulence genes.

ISOLATE (ASSEMBLY)	DATA BASE	CONTIG	STRAND	GENE
Rahnella KA0 (U) KA4 (C) KA5 (U)	ResFinder	2	-	blaRAHN-1_1
		2	-	oqxB_1
	CARD	1	-	CRP
		1	-	H-NS
		2	-	oqxB
	NCIB	2	-	blaRAHN-1
		2	-	oqxB9
Pseudomonas KA7 (C)	CARD	1	-	MexF
	VFDB	1	+	flgC
		1	+	flgG
		1	+	flgI
		1	+	fliG
		1	+	fliM
		1	+	fliP
		1	+	fliQ
		1	+	fleN
		1	+	motC
		1	-	algU
		1	+	pilH

(U) – Unicycler assembly
(C) – Canu assembly

ABRicate found a plasmid-located RAHN β-lactamase encoding gene, $bla_{RAHN,}$ in the *Rahnella* isolates using the ResFinder and NCIB databases, but not with CARD. RAHN is listed in the CARD database, indicating that ABRicate is not able to find all resistance genes. RAHN is a class A ESBL found in *R. aquatilis* that confers resistance to cephalosporins by antibiotic inactivation (CARD, NCIB). *oqxB* family genes, encoding RND efflux pumps that confer resistance to diaminopyrimidine, fluoroquinolones, glycylcycline, nitrofuran and tetracyclines (CARD) were also found on the *Rahnella* isolate plasmids. *oqxB_1* confers resistance to nalidixic caid and ciprofloxacin (ResFinder) and oqxB9, a multidrug efflux RND transporter permease subunit, confers resistance to phenicol and quinolone (NCIB). CRP and H-NS protein encoding genes were found on the chromosome of the *Rahnella* isolates. CRP, a global regulator repressing MdtEF multidrug efflux pump expression, confers resistance to macrolides, penam and fluroquinolones (Nishino et al., 2008). H-NS, a histone-like protein involved in global gene regulation in Gram-negative bacteria, represses the membrane fusion genes *acrE, mdtE, emrK* and other genes of various RND multidrug exporters (CARD). H-NS belongs to the RND and MFS antibiotic efflux pump families and confers resistance to tetracycline, penam, macrolides, fluoroquinolones, cephamycin, and

cephalosporins (CARD). *Rahnella* was not listed under resistomes with sequence variants for oqxB, CRP nor H-NS in CARD, but had BLAST percent identities of roughly 80%, 83% and 84% to other *Rahnella* strains, respectively (see Appendix 9.9 and Appendix 9.10). As *Rahnella* is not typically considered humanly pathogenic, it is not surprising that the genus is not included in CARD.

Several virulence associated genes were found in the *Pseudomonas* isolate KA7. These are included for clarity but are not the focus of this study. MexF, the multidrug inner membrane transporter of the MexEF-OprN complex, was also found in the *Pseudomonas* isolate. MexF is RND efflux pump that confers resistance to phenicol, diaminopyrimidines and fluoroquinolones (CARD).

5.5.2. Prokaryotic Genome Annotation

Manually searching for resistance-related genes in the Prokka files using search words resulted in far more hits than provided by ABRicate. These "hits" were then also searched for in the Prokka annotation provided by GCView. Only genes that are listed in CARD are presented here (see Table 5.6), but a systemized summary of all other resistance and resistance-related genes, their encoded products and the product functions is presented in Table 9.7 (see Appendix 9.8). Further, BLAST results using the protein sequences annotated by Prokka are presented in Appendix 9.9-11. BLAST searches were only executed for isolates with hybrid assemblies, namely isolates KA0, KA5 and KB3.

Figure 5.6 to 5.11, generated on the CGView server, provide a visual representation of the assembled genomes and the approximate location of the CARD-listed resistance genes. Note that it was not always possible to fit all genes within the image so the figures may not represent the presence of all CARD genes. Figure 5.12 shows all resistance and resistance-related genes, not only those listed in CARD, found on the plasmids of the *Rahnella* isolates KA0, KA4 and KA5.

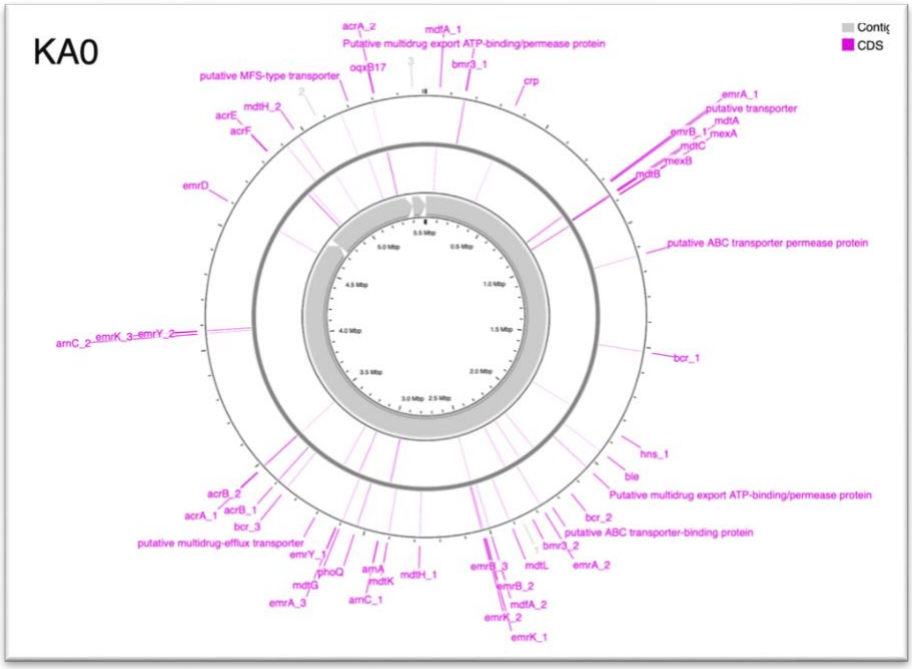


Figure 5.6. CARD listed resistance genes found in isolate KA0, R. variigena. Created on the CGView server, October 2021.

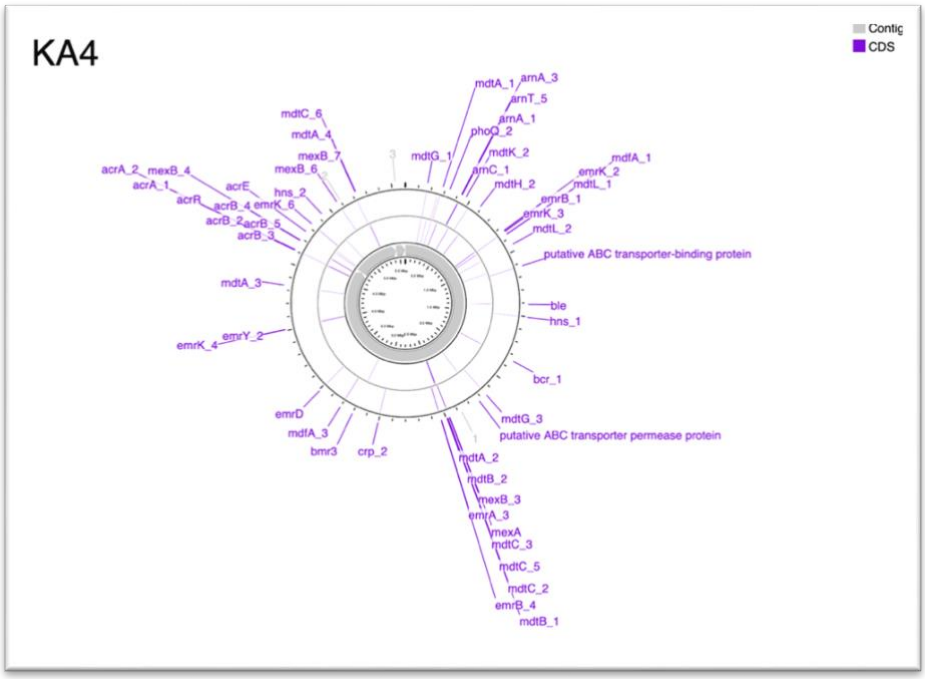


Figure 5.7. CARD listed resistance genes found in isolate KA4, R. variigena. Created on the CGView server, October 2021.

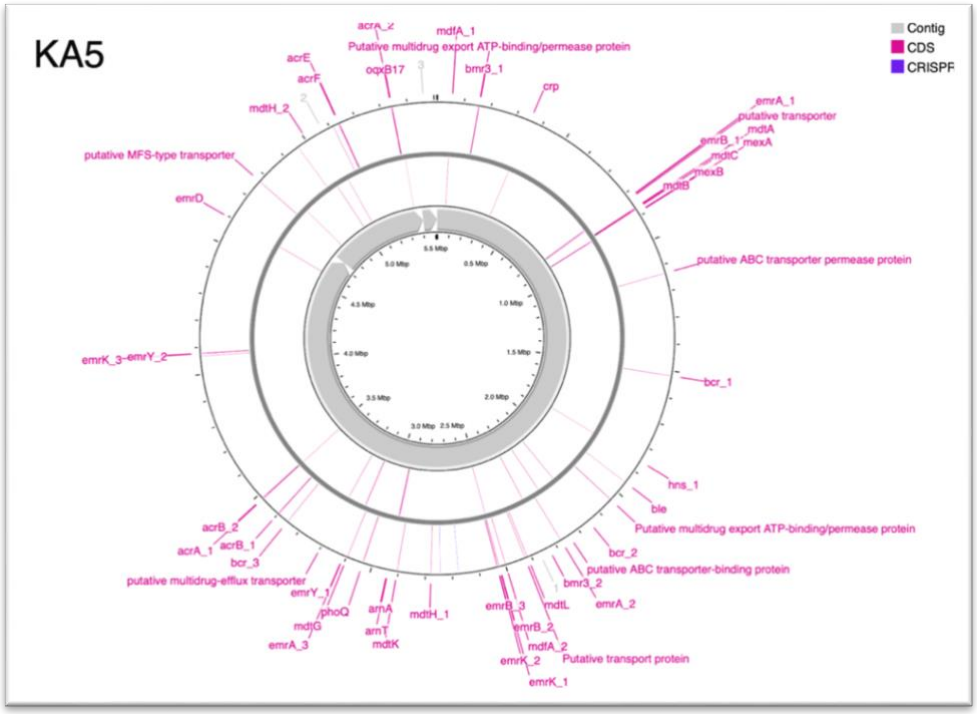


Figure 5.8. CARD listed resistance genes found in isolate KA5, R. variigena. Created on the CGView server, October 2021.

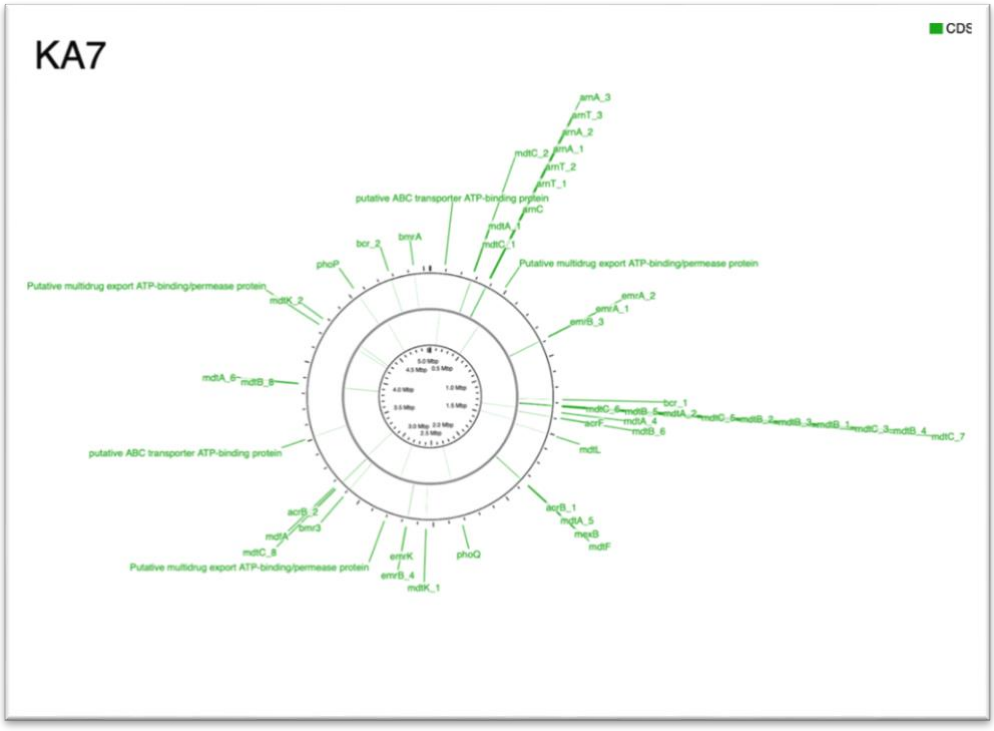


Figure 5.9: CARD listed resistance genes found in isolate KA7, P. laurentiana. Created on the CGView server, October 2021.

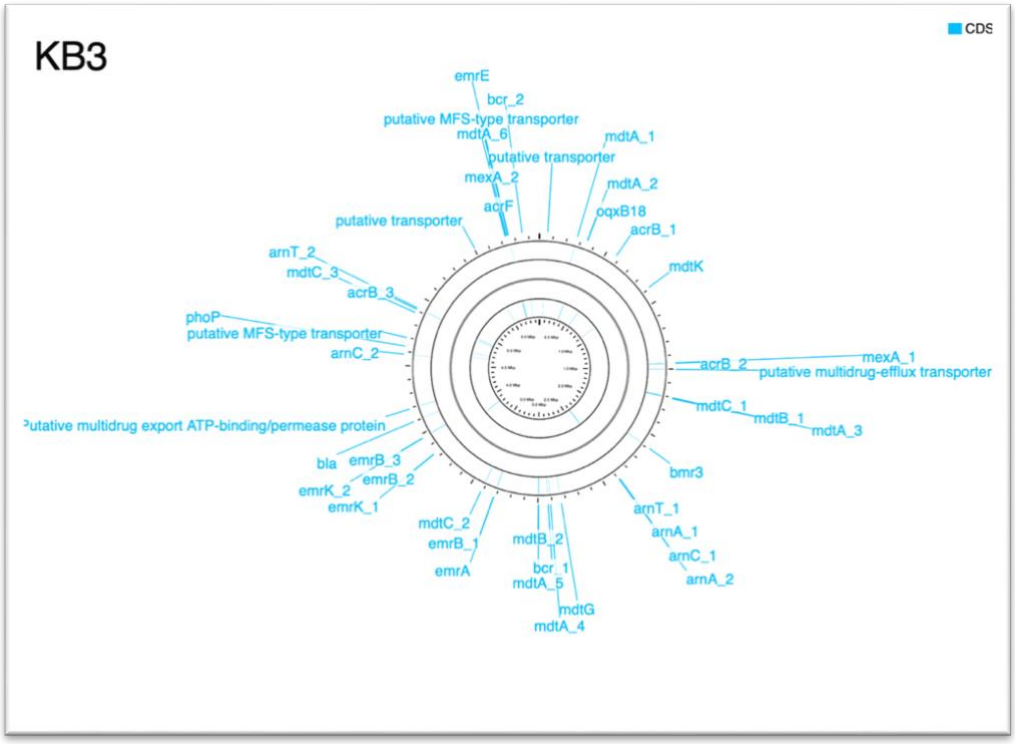


Figure 5.10. CARD listed resistance genes found in isolate KB3, H. huttiense. Created on the CGView server, October 2021.

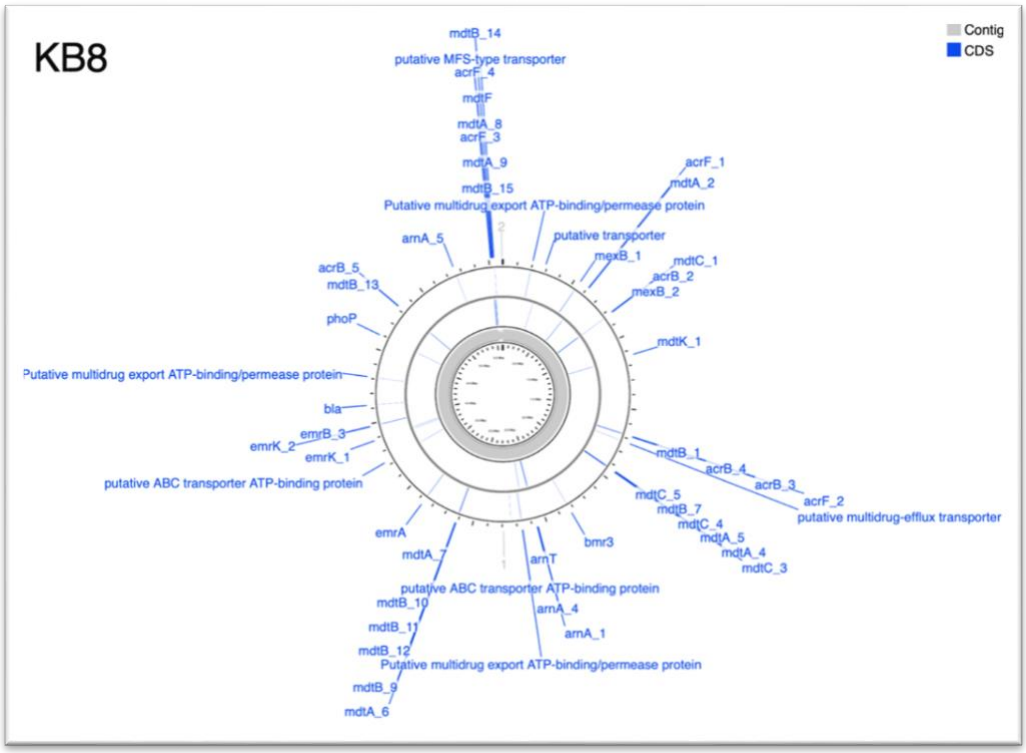


Figure 5.11. CARD listed resistance genes found in isolate KB8, H. aquaticum. Created on the CGView server, October 2021.

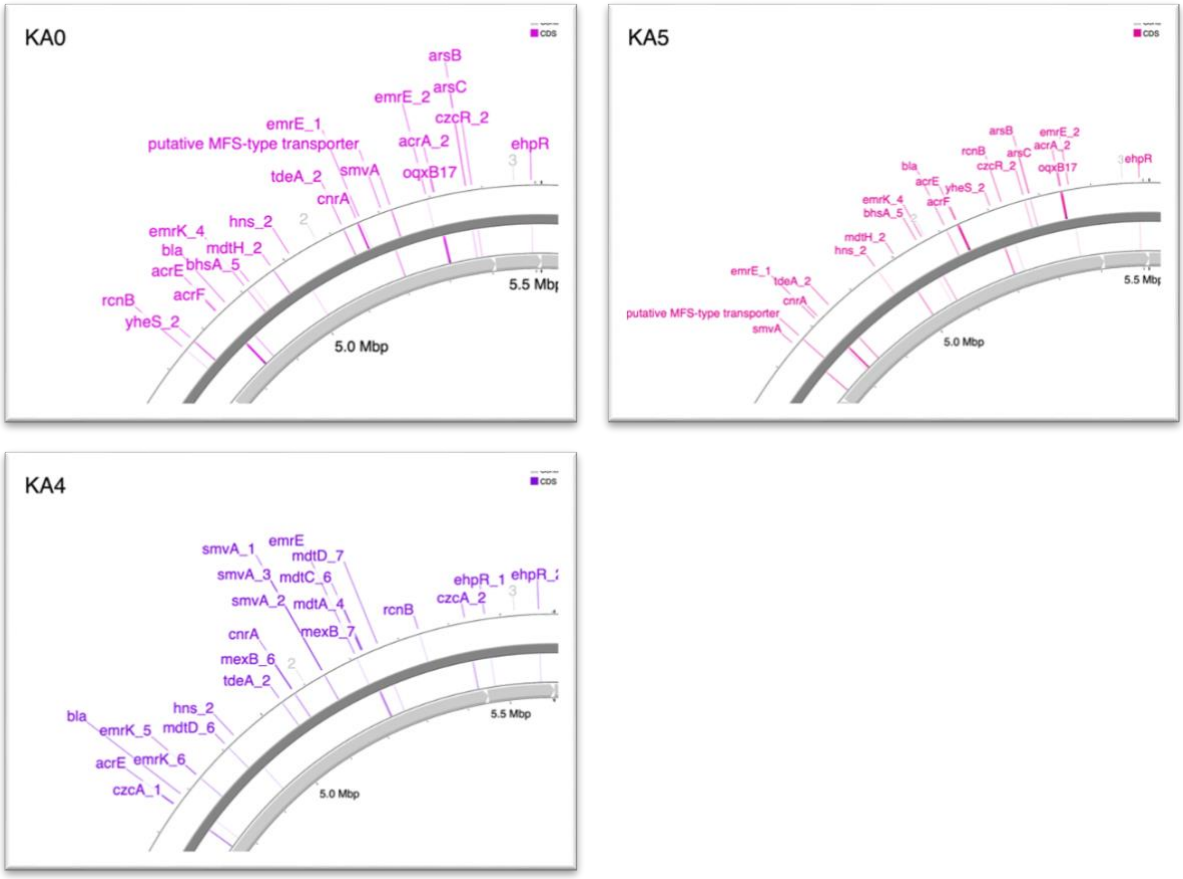


Figure 5.12. All resistance genes found on the plasmids of the Rahnella isolates KA0 (top left), KA4 (bottom left) and KA5 (top right). Created on the CGView server, October 2021.

Table 5.6. provides a summary of the resistance genes found by Prokka that are also listen in CARD, both by manually searching in the Prokka files generated by the Galaxy server and on the GView server. Note that executing Prokka in Galaxy and CGView provided slightly varying results, except for isolates KA0 and KA5.

Most notably, β-lactamase encoding genes were found in isolates except the *Pseudomonas* isolate KA7. As described above, the *Rahnella* isolates KA0, KA4 and KA5 harboured a plasmid harboured class A β-lactamase, bla_{RAHN}, annotated by Prokka as β-lactamase Toho-1, a β-lactamase with strong cefotaxime hydrolysis activity found in *E. coli* (Ishii et al., 1995). These were described by BLAST as class A β-lactamases with percent identities of 98,04% for KA4 and 99,66% for KA0 and KA5 to the *R. variigena* target sequence. Further, another class A β-lactamase gene was found in the *Herbaspirillum* isolate KB8. This gene was annotated by Prokka as $bla_{SHV\text{-}4}$. SHV-4 is an ESBL found in clinical isolates that confers resistance to carbapenem, cephalosporin and penem antibiotics (Péduzzi et al., 1989). The

protein sequence had a precent identity of 98,82% to a class A β-lactamase found in *Herbaspirillum*.

Besides the *oxqB* genes found in the *Rahnella* isolates found by ABRicate, *oxqB* genes were only found in *Herbaspirillum* KB3. An *oqxB* was found in *Rahnella* isolate KA4 by ABRicate but not by Prokka annotation.

The protein BRP is encoded by a MBL-associated *ble* gene. BRP expression confers resistance to bleomycin and bleomycin-like antibiotics through antibiotic inactivation (Dortet et al., 2012). A *ble* gene was found in all *Rahnella* isolates.

PhoPQ, a two component regulatory system, confers resistance to peptide and macrolide antibiotics through both target alteration and efflux (Groisman, 2001). phoP is phosphorylated by phoQ at low magnesium ion concentrations that activates the PmrA/B system repressor. *phoP* was found in all isolates while *phoQ* was found in the *Rahnella* isolates and *Pseudomonas* isolate KA7. phoPQ regulates arnA expression in *E. coli* and regulates OprH expression in *P. aeruginosa* (CARD).

The AcrAB-TolC, a RND efflux complex with broad substrate specificity, uses the PMF to export substrates (Okusu et al., 1996). It confers resistance to tetracyclines, penam, rifamycin, glycylcycline, cephalosporin, phenicol, triclosan and fluoroquinolones in Gram-negative bacteria (CARD). TolC is an OMP forming a subunit of several multidrug efflux pumps in Gram-negative bacteria (Koronakis, 2003). AcrA and AcrB were found in all *Rahnella* isolates, but only AcrB was found in the *Herbaspirillum* and *Pseudomonas* isolates. AcrR, a repressor of the AcrAB-TolC complex, was found in all isolates except KB3 and KA7. Mutations in this protein confer high levels of antibiotic resistance (Pradel & Pagès, 2002) by efflux and target alteration. The RND family MdtEF-TolC multidrug efflux complex in Gram-negative bacteria confers resistance to penam, macrolide and fluroquinolone antibiotics (CARD). MdtEF-TolC and AcrAB-TolC are structurally similar and confer resistance towards similar substrates (Schaffner et al., 2021), where MdtEF was limited to cationic amino-base biocides (Novoa & Conroy-Ben, 2019). Only MdtF was found in isolates KA7 and KB8, which is similar in homology and substrate specificity to AcrB (Novoa & Conroy-Ben, 2019).

Table 5.6. A summary of the resistance, resistance-related and virulence genes found using Prokka, prokaryotic genome annotation. This table only includes genes also listed in CARD.

Isolate	*Rahnella*						*Herbaspirillum*				Pseudomonas		Isolate
	KA0		KA5		KA4		KB3		KB8		KA7		
Gene	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	**Gene**
abaF	x		x		x		x				x (1-2)		*abaF*
abaQ					x		x		x (1-2)		x		*abaQ*
acrA	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)							*acrA*
acrB	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-5)	x (1-5)	x (1-3)	x (1-3)	x (1-7)	x (1-6)	x (1-2)	x (1-2)	*acrB*
acrE	x	x	x	x	x	x			x	x			*acrE*
acrF	x	x	x	x	x	x	x	x	x (1-4)	x (1-4)	x	x	*acrF*
acrR	x	x	x	x	x	x			x (1-2)	x			*acrR*
arnA	x	x	x	x	x (1-3)	x (1-3)	x (1-2)	x (1-2)	x (1-5)	x (1-5)	x (1-5)	x (1-5)	*arnA*
arnC	x (1-4)	x (1-2)	x (1-4)	x (1-2)	x	x (1-2)	x (1-2)	x (1-2)	x (1-3)	x (1-3)	x	x	*arnC*
arnT	x	x	x	x	x (1-5)	x (1-5)	x (1-2)	x (1-2)	x	x	x (1-5)	x (1-4)	*arnT*
bcr	x (1-3)	x (1-3)	x (1-3)	x (1-3)	x (1-2)	x (1-2)	x (1-2)	x (1-2)			x (1-2)	x (1-2)	*bcr*
bla	x	x	x	x	x	x	x	x	x	x			*bla*
ble	x	x	x	x	x	x							*ble*
crp	x	x	x	x	x (1-2)	x (1-2)							*crp*
emrA	x (1-4)	x (1-3)	x (1-4)	x (1-3)	x (1-6)	x (1-3)	x (1-2)	x	x (1-2)	x	x (1-3)	x (1-2)	*emrA*
emrB	x (1-3)	x (1-3)	x (1-3)	x (1-3)	x (1-5)	x (1-4)	x (1-2)	x (1-3)	x (1-4)	x (1-3)	x (1-3)	x (1-4)	*emrB*
emrD	x	x	x	x	x	x							*emrD*
emrE	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x	x	x	x	x			*emrE*
emrK	x (1-3)	x (1-4)	x (1-3)	x (1-4)	x (1-3)	x (1-6)	x	x (1-2)	x	x (1-2)		x	*emrK*
emrY	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-3)	x (1-3)							*emrY*
fsr	x	x	x	x	x (1-3)	x (1-2)	x	x	x	x	x (1-2)	x (1-2)	*fsr*

gntK	x	x	x	x	x	x							*gntK*
gyrA		x		x		x (1-2)		x		x (1-4)		x (1-3)	*gyrA*
gyrB		x		x		x (1-2)		x		x (1-4)		x (1-3)	*gyrB*
hns	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)							*hns*
mdfA	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-3)	x (1-3)					x	x	*mdfA*
mdtA	x (1-2)	x	x (1-2)	x	x (1-5)	x (1-4)	x (1-7)	x (1-6)	(1-10)	x (1-9)	x (1-7)	x (1-6)	*mdtA*
mdtB	x	x	x	x	x (1-9)	x (1-7)	x (1-2)	x (1-2)	(1-15)	x (1-15)	x (1-8)	x (1-8)	*mdtB*
mdtC	x	x	x	x	x (1-8)	x (1-6)	x (1-3)	x (1-3)	x (1-6)	x (1-5)	x (1-8)	x (1-8)	*mdtC*
mdtF										x		x	*mdtF*
mdtG		x		x	x (1-3)	x (1-3)		x					*mdtG*
mdtH	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)							*mdtH*
mdtK	x	x	x	x	x (1-2)	x (1-2)	x	x	x (1-3)	x (1-2)	x (1-2)	x (1-2)	*mdtK*
mdtL	x	x	x	x	x (1-5)	x (1-2)			x		x	x	*mdtL*
mexA	x	x	x	x	x	x	x (1-2)	x (1-2)	x	x			*mexA*
mexB	x	x	x	x	x (1-7)	x (1-7)			x (1-2)	x (1-2)	x	x	*mexB*
oqxB													*oqxB*
oxqB9													*oxqB9*
oqxB17	x	x	x	x									*oqxB17*
oqxB18								x					*oqxB18*
oqxB27							x						*oqxB27*
phoP	x	x	x	x	x (1-2)	x (1-2)		x	x	x	x	x	*phoP*
phoQ	x	x	x	x	x (1-3)	x (1-3)						x	*phoQ*

The AcrEF-TolC RND efflux pump system confers resistance to fluoroquinolones (ciprofloxacin), cephamycin, cephalosporin and penam antibiotics (Lau & Zgurskaya, 2005). AcrE and AcrF genes were found in all *Rahnella* isolates and the *Herbaspirillum* isolate KB8. Only AcrF was found in the *Herbaspirillum* KB3 and *Pseudomonas* KA7. Another RND multidrug efflux system in Gram-negative bacteria, MdtABC-TolC, was found in all isolates. The pump confers resistance to novobiocin and derivatives of bile salts. None of the components confer resistance when individually expressed, but MdtAC confers limited individual resistance to bile salt derivatives (Nagakubo et al., 2002).

The *Pseudomonas*-identified RND family MexAB-OprM multidrug efflux protein is associated with resistance to fluroquinolone, chloramphenicol, erythromycin, novobiocin and some β-lactam antibiotics. Over-expression is associated with colistin resistance. (Srikumar et al., 1998; Sugimura et al., 2008). Genes for the MFP, MexA, were found in all isolates except *Pseduomonas* KA7. Genes for the inner membrane transport protein MexB was found in all isolates except *Herbaspirillum* KB3.

Genes for AbaF and AbaQ, MFS antibiotic efflux pumps, were found in the *Rahnella* isolate KA4, as well as the *Herbaspirillum* and *Pseudomonas* isolates. Only AbaF was found in *Rahnella* isolates KA0 and KA5. *abaF* expression increases Fosfomycin resistance (Sharma et al., 2016). AbaQ mediates quinolone-type antibiotic resistance (Pérez-Varela et al., 2018).

The MFS multidrug efflux system EmrAB-TolC confers resistance fluoroquinolones nalidixic acid and thiolactomycin in *E. coli* (Lomovskaya et al., 1995). EmrA is the linker and EmrB is the transporter powered by an electrochemical gradient (CARD). Both were found in all isolates. A homolog of EmrAB, EmrKY, confers resistance to tetracyclines. EmrK was found in all isolates while EmrY was only found in the *Rahnella* isolates. EmrD, a MFS multidrug transporter primarily found in *E. coli*, was found in all *Rahnella* isolates. EmrD couples efflux of amphipathic compounds and proton import across the plasma membrane (Saidijam et al., 2006). EmrE, a SMR transporter (Bay et al., 2008), was found in all isolates except *Pseudomonas* KA7.

Genes for bcr, a MFS antibiotic efflux pump, were found in all isolates except *Herbaspirillum* KB8. This transmembrane protein exports bicyclomycin from the cell and so

confers resistance (Fonseca et al., 2015). The MFS antibiotic efflux pump bmr confers resistance to fluroquinolone, nucleosides and phenicol antibiotics (Neyfakh et al., 1991). bmr3 genes were found in all isolates. Another MFS multidrug efflux pump, mdfA, confers resistance to tetracyclines and chloramphenicol in for example *E. coli* and *P. aeruginosa* (CARD). This gene was found in the *Rahnella* isolates and KA7, but not in the *Herbaspirillum* strains. Genes for MFS antibiotic efflux pumps MdtG, MdtH and MdtL were found in all *Rahnella* isolates. Overexpression of MdtG has been reported to increase resistance to Fosfomycin and deoxycholate (CARD). MdtH confers resistance to fluoroquinolones (CARD). The MdtG gene was also found in *Herbasprillium* KB3. Genes for MdtL, a putative/predicted multidrug MFS pump (CARD), were found in all isolates except *Herbaspirillum* KB3. A MATE family transporter, MdtK confers resistance to the fluroquinolone norfloxacin (CARD). Genes for this pump were found in all isolates.

Genes for antibiotic target alteration were also found. ArnA, a polymyxin resistance (pmr) phosphoethanolamine transferase, modifies lipid A in Gram-negative bacteria to confer resistance to catonic peptides and polymyxin (Gatzeva-Topalova et al., 2005). Lipid A is a component of the lipolysaccharide outer membrane of most Gram-negative cells (Raetz et al., 2007). The modification requires 4-amino-4-deoxy-L-arabinose (Ara4N). ArnT is involved in Ara4N synthesis (Bialek-Davenet et al., 2014) and ArnC is required for the synthesis and transfer of Ara4N to lipid A (Gunn et al., 1998). ArnA, ArnC and ArnT protein genes were found in all isolates.

DNA gyrase, consisting of two alpha and two beta subunits, is responsible for DNA supercoiling. Supercoiling can be inhibited by binding the beta-subunit (CARD). Point mutations in *gyrA* decrease susceptibility to triclosan antibiotics (Webber et al., 2017) in *E. coli* and fluroquinolones in *Mycoplasma genitalium* (Tagg et al., 2013). Point mutations in *gyrB* can result in fluroquinolone resistance in *Mycobacterium tuberculosis* (Maruri et al., 2012) and aminocoumarin resistance in *E. coli.* Point mutations of this gene were not investigated.

5.6. MIC Testing

The results from the MIC tests for all isolates are presented in Table 5.7.7. Where a range was observed, this is presented with " - " , where "*" indicates the MIC value where growth was completely inhibited. "R" is used to indicate no inhibition of growth and therefore resistance, and "(R*)" is used to indicate very little inhibition of growth and therefore intermediate susceptibility. Only the isolates that were WGS, namely isolates KA0, KA4, KA5, KA7, KB3 and KB8 will be further presented.

Table 5.7. MIC values for all isolates.

Sample	Ampicillin	Cefotaxime	Meropenem	Imipenem	Ciprofloxacin
A0	128 (R*)	6 - 4	0.047	0.38 - 0.25	0.094
A1	R/spots	R/spots	R/spots	R/spots	R/spots
A2 **	96 (R*)	4 - 3	0.047	0.025	0.094
A4	32	R	0.50* - 0.19	0.75* - 0.50)	0.064
A5	96 (R*)	4	0.094	0.38 – 0.25	0,047
A6	128 (R*)	3* - 2	0.023	0.125* - 0.047	0.047
A7	64	3* - 0.5	0.047 - 0.032	0.38* - 0.25	0.094* - 0.064
B2 **	0.023	R	0.094	0.064 - 0.047	0.064
B3	R	0.75* - 0.094	8* - 1,0	***	2* - 1.5
B3(b)	1.5 ***	0.125	0.064	0.064	0.5
B5 **	0.023	R	0.094 – 0.064	0.064	0.064
B8	32* - 8	0.19	0.047	0.064 – 0.094	3* - 0.50
B8(b)	6* - 0.23	***	0.094 – 0.064	0.064	0.50* - 0.064
B9	0.023	R	0.094 – 0.064	0.064	0.064

*clear
** failed test
*** not clear
(R*) very little inhibition of growth

Most resistance was observed towards ampicillin. Namely the *Rahnella* isolates KA0, KA4 and KA5, as well as the *Herbaspirillum* isolate KB3 displayed resistance to ampicillin. Further, resistance towards cefotaxime was displayed by the *Rahnella* isolate KA4, while the other *Rahnella* isolates displayed intermediate resistance with higher MIC values that observed for isolates KB3 and KB8. The MIC tests were performed twice for isolate KB3 and KB8 as the results were deemed interesting and should have been verified by another parallel.

6. Discussion

6.1. Isolation, Identification and Phylogenetic Analysis

It was not possible to genetically investigate the strains that grew towards the top of the tubes in a biofilm fashion. This could be attributed to exactly biofilm formation: the adhesion to and growth of microorganisms on a surface. Biofilm growth is often associated with resistance, and nosocomial infections are becoming increasingly important and studied in relation to mechanisms of biofilm resistance (Mah & O'Toole, 2001). Biofilms are often heterogeneous in nature, so multiple resistance mechanisms within a community is likely. General mechanisms include failed drug penetration through the biofilm, slower growth and stress response to antibiotics and quorum sensing (Mah & O'Toole, 2001). With more time in the laboratory, the applicable cultures would have been incubated on shaking tables, as the isolates may have also been extremely aerobic and therefore grew only at the top of the tubes.

As shown in

Table 5.1, presumptive identification based on the chromogenic plates was not necessarily easy. Further, the multiplex results were also problematic to analyse. The questionable multiplex results are likely attributed to unspecific primers or too high primer concentration and subsequent primer dimers (PDs). PDs are non-specific PCR products that result from template-independent primer interactions, often because high concentrations of primers result in weak interactions occurring between them (Brownie et al., 1997). With more time (and without a worldwide pandemic), these multiplex analyses should have been run again with better primers in lower concentrations. Singleplex PCR runs targeting specific resistance genes with more specific primers could also be investigated. Another major limitation of this study was the MIC tests. Firstly, substantial growth in the overnight cultures was assumed without testing turbidity or measuring the optical density. Further, only one test of each antibiotic for each isolate was performed, except when growth did not occur, the test failed in some other way or interesting results were to be verified. This was because of limited test strips that could not be delivered due to the worldwide Corona pandemic. At least two parallels should have been performed for each antibiotic, and the optical density should be

measured prior to plating. The MIC values obtained in this study are therefore not substantial to give an accurate indication of susceptibly or resistance of the isolates towards the tested antibiotics but provide somewhat of an indication. In incidences when isolates were MIC tested in two parallels, such as for isolates KB3 and KB8, the results were not reproducible. This is likely attributed to the general condition of the bacteria cell cultures. Some isolates were tested post 24 hours of incubation, others needed more time prior to plating. Some isolates were tested using the first liquid culture, while others were cultivated from frozen stocks. Stocks were not remade after thawing but refrozen, adding further uncertainty.

As such, and not surprisingly, the most reliable genome data in this study was the WGS data. The hybrid long and short read assemblies are clearly the most accurate and reliable. The Illumina reads are considered far more reliable than the error prone ONT reads, but the ONT sequencing was successful enough to provide a solid foundation of species identification and an insight into possible harboured resistance genes. While Prokka annotation allowed for the identification of various resistance and resistance-related genes in the studied isolates, the difference when executed in Galaxy versus GCView alone indicates that Prokka annotation is not completely reliable, especially when it comes to gene name designation. However, even though Prokka provides different names for genes, it is still able to find sequences similar enough to known sequences. This provides opportunity for easier, further investigation of the sequences, using for example BLAST.

6.2. The *Rahnella* Genus

The *Rahnella* genus in the Enterobacteriaceae family was first proposed in 1979 as a single species, *R. aquatilis*, containing similar strains isolated from French water samples (Brady et al., 2014). *R. aquatilis* later proved to be environmentally omnipresent in especially water samples, but also in soils, crop rhizospheres and snail intestines (Bellais et al., 2001; Stock et al., 2000). Occasional isolation from food and clinical samples are also observed. Human clinical samples include infected wounds, blood and acute gastroenteritis-resulting faeces, especially from immunocompromised patients (Kämpfer, 2015). *Rahnella* is described as facultatively anaerobic, chemoorganotrophic, oxidase negative, catalase positive and when grown at 4^{o}C, psychrotolerant (Kämpfer, 2015). The novel *R. variigena* species was first described by Brady et. al. (2014) as motile Gram-negative rods which form round, convex and smooth cream colored colonies on nutrient agar (Brady et al., 2014). Their study

consisted of 32 Gram-negative bacterial strains where *gyrB* sequences were most comparable to *R. aquatilis* and *Ewingella americana*, forming five *Rahnella* and one *E. americana* multilocus sequence analysis (MLSA) groups. Microplate and fluorometric DNA-DNA relatedness assays confirmed that the five *Rahnella* MLSA groups formed separate taxa which were then phenotypically differentiated from both each other as well as *R. aquatilis*. Subsequently, the previously phenotypically undistinguishable *Rahnella* genomospecies 2, isolated from water, clinical and snail intestine samples, was proposedly classified as *R. variigena* sp. nov. (CIP 105588^{T} = LMG 27711^{T}).

Bacteria commonly found in soil, such as *Rahnella,* confer resistance to relevant frontline antibiotics (see section 2.3). *Rahnella* was believed to confer resistance towards β-lactams through β-lactamases (Dever & Dermody, 1991) and able to spread this resistance to other, more pathogenic bacteria (D'Angelo & Larsen, 2018). Little data is available on the resistance patterns and mechanisms regarding *R. aquatilis* due to rare isolation, limited clinical studies and few antibiotic susceptibility studies of environmental isolates (Koczura et al., 2016). Antibiotic susceptibility was investigated by Stock et al. (2000) in a study of 72 clinical and environmental *R. aquatilis* strains, revealing resistance to amoxicillin, ticarcillin, cefuroxime and cephalothin. Intermediate susceptibility to cefotaxime and ceftriaxone and susceptibility to ceftazidime, imipenem as well as β-lactam combinations of amoxicillin-clavulanic acid and piperacillin-tazobactam were shown. These susceptibility patterns suggested mechanisms of an Ambler class A, clavulanic acid-inhibited β-lactamase (Stock et al., 2000).

A novel class A β-lactamase from *R. aquatilis* with an extended hydrolysis profile spectrum, RAHN-1, was characterized by Bellais et. al in 2001. Highest amino acid identity was shared with a chromosomal ESBL from *Serratia fonticola* (75,9%) and with plasmid-mediated β-lactamases CTX-M-2 (73,2%) and CTX-M-1 (71,5%). RAHN-1 was inhibited by clavulanic acid and displayed a hydrolysis spectrum spanning cephalosporins like cefotaxime and ceftriaxone but not ceftazidime. Chromosomal origin was indicated by positive signal detection in the chromosome migration position following a Southern transfer with a PCR-obtained internal $bla_{\text{RAHN-1}}$ labelled probe, whole cell *R- aquatilis* DON-1 and CIP reference strain DNAs (Bellais et al., 2001).

Ruimy et al. (2010) studied 51 *R. aquatilis* strains from two groups, G1 and G2, isolated from raw fruits and vegetables. Sequences of their *bla*$_{RAHN}$ from G1 and G2 showed <82,9 and >92% identity to *bla*$_{RAHN-1}$, respectively. Their novel chromosomal class A *bla*$_{RAHN-2}$ was deemed 89,8% identical to *bla*$_{RAHN-1}$ and 5-fold and 2,5-fold more efficient in ticarcillin and cefotaxime/cefuroxime hydrolysis, respectively (Ruimy et al., 2010). The specific activity of RAHN-1 was double that of RAHN-2, indicating differences in regulation and making phenotypic detection of RAHN-2 challenging. Note that the study by Ruimy et al. (2010) was executed before the proposed classification of *R. variigena* and therefore may have unknowingly included such isolates.

The first class 1 integron in an environmental *R. aquatilis* strain isolated from the Warta rover in Poland was described by Koczura et. al (2015). A PCR assay detected the Class 1 integrase gene. The integron was located on a 54-kbp plasmid. Sequencing of the variable region revealed a *drfA1-aadA1* gene cassette array, coding for dihydrofolate reductase confers trimethoprim resistance and aminoglycoside adenylyl transferase conferring to streptomycin and spectinomycin resistance, respectively. The relatively narrow resistance pattern indicated a typical MDR phenotype for bacteria carrying integrons. The studied strain´s resistance to aminoglycosides, trimethoprim, sulfamethoxazole and trimethoprim/sulfamethoxazole was related to the Class 1 integron. Penicillin-related resistance was determined by a *bla*$_{TEM}$ gene, but no ESBL was found. Their study supports that water environments play a substantial role in the spread of integrons and the consequent spread of AMR among different genera of bacteria (Koczura et al., 2016).

Another study (D'Angelo & Larsen, 2018) used *Rahnella* as a laboratory model to identify and better understand resistance mechanisms in more pathogenetic strains. The study resulted in the full sequencing of RAHN β-lactamase gene using primers designed based on a previously identified *Rahnella* β-lactamase. The predicted protein products showed high similarities to the previously identified *Rahnella* β-lactamase gene, but the individual sequences clustered into two groups distinct from the known gene. In contrast to Bellais et al. (2001) and Ruimy et al. (2010), but in agreement with this study, the Ambler class A β-lactamase gene was found within a 500,000 bp plasmid. Similarity discrepancies in the 16S amplification and β-lactamase amplification sequences could be attributed to uncomplicated transmission of genetic material and may explain how transmission occurred. Conferred

resistance by the gene was proven through further amplification, plasmid insertion and cloning.

Interestingly, the *ehpR* gene found on the third contig of the *Rahnella* isolates KA0 and KA5 was the only gene that did not provide *Rahnella* hits when the sequenced was investigated by BLAST. Rather, hits for *Lonsdalea britannuca, Pantoe alhagi* and *Klebsiella* hits were obtained with 100-99 % identities. EhpR was described by BLAST as a phenazine antibiotic resistance protein belonging to the vicinal oxygen chelate (VOC) family domain. The plasmid location of this gene indicates transfer.

Rahnella species antibiotic mechanisms are not necessarily specifically clinically significant (Koczura et al., 2016), highlighted by the few reported cases of serious infection. However, infections in humans are possible so it´s resistance mechanisms need to be investigated. Furthermore, the β-lactamase gene located within a plasmid that could possibly be transmitted to more pathogenic bacterial strains, providing reason for concern regarding the spread of resistance. It also provides an opportunity: studying resistance mechanisms using lesser pathogenic isolates as laboratory models may provide further insight (D'Angelo & Larsen, 2018) regarding the mechanisms, pathogenic isolates and the way forward in the ongoing battle of fighting resistance.

6.3. The *Herbaspirillum* Genus

Herbaspirillum are described as small, spiral-shaped bacteria from herbaceous, seed-bearing plants that do not produce persistent woody tissue (Baldani et al., 1986). The generally vibrioid but occasionally spirilloid, Gram-negative and motile cells are strictly respiratory, oxidase positive and catalase weak or variable. The *Herbaspirillum* genus was first proposed in 1984 by Baldani et al. (1986) during a survey of the *Azospirillum* species in cereal roots when some isolates could not be identified as previously described *Azospirillum* species. The *Herbaspirillum* genus is currently comprised of thirteen species, eight of them plant-associated and nitrogen-fixing (Matteoli et al., 2020). *Herbaspirillum* species have been isolated from various environments including plant tissue, water samples, soils, sediments as well as clinical samples, where some clinical isolates have been described as potential opportunistic humanly pathogenic (Matteoli et al., 2020). A literature review by Dhital et al. (2020) states that most cases of infections in humans have been reported in

immunocompromised patients diagnosed with cystic fibrosis or cancer. Specifically, the species has been recovered from the blood and phlegm in cystic fibrosis and pneumonia patients, as well as blood from leukaemia, aplastic amenia, multiple myeloma and cellulitis diagnosed patients. Infection has, however, also been reported patients with no apparent immunosuppression (Dhital et al., 2020). Dhital et al. (2020) summarized nine published studies of *Herbaspirillum* infection cases between 2005 and 2019 (see Appendix 9.13). These studies provide evidence that *Herbaspirillum* is an emerging and evolving pathogen, which may be more prevalent than previously thought due to phylogenetic and phenotypic similarities to organisms such as *Burkholderia cepacian* complex which result in misidentification. However, *Burkholderia cepacian* complex are usually MDR while *Herbaspirillum* species are not. As such, antimicrobial sensitivity tests could possibly be used for differentiation. In eight of the above-mentioned cases, the bacteria was easily eradicated with the appropriate antimicrobial medication (Dhital et al., 2020).

To better understand how *Herbaspirillum* bacteria migrated from the environment and evolved as opportunistic human pathogens, Faoro et al. (Faoro et al., 2019) sequenced two clinical isolate genomes obtained from cystic fibrosis patients and compared them to the environmentally obtained genome of an *Herbaspirillum seropedicae* isolates. Their genomic analysis study suggested that migration from environmental to opportunist resulted in the loss and gain of genes that allowed for survival of this transition. The loss of a gene cluster involved in *Herbaspirillum*-plant interaction, namely the *nif*-cluster involved in nitrogen fixation, and the acquisition of a lipopolysaccharide (LPS) biosynthesis-related gene cluster was seemingly the basis for human host colonization. In this acquisition, the addition of sialic acids allowed for the evasion of the immune system. A similar finding was reported in a genomic analysis of the genus *Klebsiella*, where the *nif* gene cluster was found in all environmental nitrogen fixing *Klebsiella* groups (phylogroup III), but only in one genome of phylogroup I, namely *K. pneumonia* which has been found to infect humans and other mammals (Holt et al., 2015). Two other coding sequences possibly involved in the transition proposed by Faoro et al. are CβG produced by CGS and the ubiquitin carboxyterminal hydrolase (UCH). CβG is related to both symbiotic and pathogenic bacteria-host interactions and has been shown to an essential virulence factor in both. All three possibly involved genomic regions were identified through a search for potential HGT regions and genomic islands. The study was also the first to describe and analyse a plasmid containing

Herbaspirillum strain, exhibiting possibilities for genetic material transfer. The *Herbaspirillum* analysed in this study, however, does not appear to contain a plasmid. Comparison studies like that of Faoro et al. (2019) are an exemplary example as to why environmental studies of resistance are important.

6.4. The *Pseudomonas* Genus

The *Pseudomonas* genus, consisting of over 191 species, is the largest Gram-negative group and is widely genotypically and phenotypically diverse (Rafikova et al., 2020; Wright et al., 2018). *Pseudomonas* representatives display broad metabolic capabilities and inhabit soils, water, plants and animals. The *Pseudomonas* genus is described (Palleroni, 2015) as Gram-negative, straight or slightly curved rods that are generally motile by one or more polar flagella. Respiration is aerobic with oxygen as the terminal electron acceptor. Anaerobic growth can occur if nitrate can be used as an alternate electron acceptor (Palleroni, 2015). *P. aeruginosa* is a well-studied pathogen that causes nosocomial infections (Strateva & Yordanov, 2009) and displays intrinsic resistance through AmpC β-lactamase production, overexpression of efflux pumps and low permeability of the outer membrane caused by OprD protein loss (Strateva & Yordanov, 2009). Resistance towards aminoglycoides and quinolone resistance is also conferred through aminoglycoside-modifying enzymes and structural alterations of topoisomerases II and IV, respectively (Strateva & Yordanov, 2009). Moreover, such mechanisms often occur simultaneously and result in MDR.

Wright et. al characterized the novel *P. laurentiana* species in 2018 (Wright et al., 2018). Their study aimed to investigate soluble ligand bound manganese (III) complexes (Mn (III)-L) from significant environmental bacteria such as *Pseudomonas*. One strain (GSL-101^T) was isolated from a 338m deep water sample in the St. Lawrence Estuary and genetically investigated through 16S sequencing, a neighbour-joining dendrogram and bootstrap analysis to determine tree topology and stability. Electron microscopy and Gram-staining revealed curved rod-shaped cells with lophotrichous flagella and Gram-negative cells, respectively. The genotypic and phenotypic results revealed a novel *Pseudomonas* species which was proposed as *P. laurentiana* sp. nov. (Wright et al., 2018). Another study by Rafikova et al. in 2020 resulted in the isolation of new *P. laurentiana* strains from activated sludge that were antagonistic to *Bipolaris sorokiniana*, a plant pathogenic fungi (Rafikova et al., 2020). 16S rRNA gene analysis, fatty acid compositions, and other physiological, biochemical and

morphological properties supported *P. laurentiana* classification. Characteristic properties of plant growth-promotion were shown, namely antifungal activity, phosphate decomposition and phytohormonal substance synthesis. Germination of cucumber, tomatoe and cabbage was benefitted by inoculation. Thus, *P. laurentiana´s* ability to stimulate plant growth and development was shown for the first time and the possibility of using the isolated *P. laurentiana* strains (ANT 17 and ANT 56) in biotechnology to increase agricultural activity was suggested (Rafikova et al., 2020).

The *Pseudomonas* species isolated in this study did not appear to contain any a β-lactamase genes but displayed phenotypic resistance. Several efflux encoding genes were detected. It is likely that resistance was conferred through these pumps or low permeability of the outer membrane (Strateva & Yordanov, 2009) resulting in poor target access. Again, the impressive adaptive ability of bacteria is displayed and shows that resistance through the production of ESBLs is not our only concern. It is also possible that the β-lactamase is uncharacterized or was not picked up by Prokka for some other reason.

6.5. Antibiotic Resistance and Dissemination

While carrying genes for resistance is undoubtably advantageous for bacteria's survival when faced with selection mediated by drugs, carrying these genes can also be costly. If this cost becomes too high one could expect the organism to displace resistant genes when not faced with antibiotic selection, possibly rendering the resistant strains eliminated with time (Lopatkin et al., 2017). Nonetheless, resistance persists, possibly attributed to cultivation of resistant genes through co-selection or evolutionary compensation of plasmid fitness costs. HGT of plasmids can also aid persistence if conjugation rates are sufficiently high. Lopatkin et al. (2017) showed, using a kinetic model, that common conjugal plasmids in *E. coli* are indeed maintained and transferred at sufficient rates even in the absence of drugs (Lopatkin et al., 2017). These results indicate that solely minimizing the use of antibiotics may not suffice to reverse resistance and show that the conjugation process needs to be inhibited for effective reversal. Another study by Stevenson et al. (2017) investigated the persistence of heavy metal resistance in *P. fluorescens* using a conjugative mercury resistant plasmid (pQBR57). Here, persistence was maintained when transferable by conjugation despite the lack of positive selection, whereas clonal expansion inheritance of vertical genes required positive selection (Stevenson et al., 2017). They also showed that under positive selection the spread of

mercury resistance was dominated by clonal expansion whereas infectious transfer dominated in mercury absence, indicating resistance genes are most likely spread by HGT when resistance is futile (Stevenson et al., 2017). As such, while it is assumably possible that bacteria isolated in this study lost resistant-related plasmids once inoculated into antibiotic-free media in the laboratory, the above-mentioned studies suggest otherwise. It also seems likely that the strains displaying resistance, especially those with plasmid harboured resistance genes, have likely gained these genes through HGT.

Carrying resistance genes does not necessarily confer complete resistance, and bacteria may still be susceptible to antibiotics. MIC testing is one indication but to really prove conferred resistance, identified genes must be further amplified, inserted into a plasmid and cloned into a bacterium. This approach could also provide insight to conferred resistance in other species.

6.6. The Way Forward

Understanding the mechanisms resulting in resistance and the resistance determinants, including lactamases, PBPs and efflux pumps, play a crucial role in the essential battle of talking antibiotic resistance. The most common and worrying mechanism of acquired resistance is the production of ESBLs. Possible strategies to preserve utility of β-lactam antibiotics include new β-lactams that can evade inactivation by β-lactamases and β-lactamase inhibitors that allow the antibiotics to reach their target (Drawz & Bonomo, 2010). Regarding carbapenems, modifications can possibly be achieved through bypass of efflux pumps, alternative stereochemical inhibitors, and overcoming the loss of porins by increasing permeability through the outer membrane (Papp-Wallace et al., 2011). Studies on carbapenems have resulted in modifications of the carbapenem class that have improved chemical stability and decreased hydrolysis susceptibility. Studies into the stereochemistry has also aided hydrolysis resistance and improved activity spectrums (Papp-Wallace et al., 2011). Such work should continue.

There are studies strengthening the idea that resistance development may be intrinsic to the natural production of antibiotics, aiding the formation and spread of antibiotic resistance in the environment (Hooban et al., 2020). For example, genes conferring resistance to β-lactams, tetracyclines and aminoglycosides that potentially confer low level resistance has been identified in permafrost (Perron et al., 2015). Further, transmission mechanisms

undoubtably aid bacterial species in sharing and adapting resistance mechanisms. Better understanding of these transmission mechanisms on a molecular level may provide insight into possible inhibitors to hinder resistance. Wright (2010) states that quantitative studies to measure the rate of HGT between organisms as well as identification of environmental factors that contributes to HGT, other than antibiotic exposure, are lacking. Especially conditions that allow for HGT of resistance genes from environmental to clinical settings needs to be studied. Studies have shown that environmental isolates have various resistance mechanisms for different antibiotic classes, but only some appear clinically (Wright, 2010). As such, investigating why some genes are more dominant in pathogenic bacteria is yet another key in predicting and combatting resistance to come.

7. Concluding remarks

In this study, the presence of ESBL-producing bacteria in Norwegian aquatic environmental samples was shown. Moreover, the presence of such genes on MGEs was indicated and highlights bacteria´s ability to disseminate the genes. The presence of *Herbaspirillum* species, who´s transition from environmental to opportunistic shows potential of bacteria to transition and evolve for survival, was also detected. The *Pseudomonas* isolate did not appear to contain a β-lactamase encoding gene but displayed phenotypic β-lactam resistance, likely through efflux pumps. This again highlights the adaptive capability of bacteria.

Over the years we have seen the dissemination of acquired resistance to β-lactams mediated by ESBLs conferring resistance to all β-lactams besides carbapenems and cephamycins. While carbapenems and cephamycins are sometimes inhibited by β-lactamase inhibitors like clavulanic acid, we have also witnessed the increasing occurrence of carbapenem-resistance conferring carbapenemases such as MBLs. Many of these ESBLs and carbapenemase groups have expanded significantly. Further, a shift in the distribution of ESBL-types was observed in Europe, with a major increase of CTX-M variants (Coque et al., 2008). As such, it is not unreasonable to ponder if a similar trend of dissemination and expansion will be observed in the RAHN or other species-specific β-lactamases in the years to come. Furthermore, the potential for shift of certain bacteria from environmental to opportunistic adds more uncertainty. This uncertainty is especially concerning if such bacteria also harbour ESBL genes, more so if they are plasmid harboured.

Antibiotic resistance is intricate. Diverse mechanisms of resistance and dissemination of resistance genes complex the issue. Drivers include resistance selection driven by antibiotic exposure and other factors that globally and locally promote the dissemination of resistant bacteria and their resistance genes (McEwen & Collignon, 2018). Overcoming resistance by removing selective pressure relies on resistance being a fitness cost, which is not always the case. Overcoming resistance should therefore be directed towards antibiotic and resistance mechanisms parallel with new drug discovery and multidisciplinary research across healthcare, agriculture and environmental sectors.

Remarkable progress has been made regarding sequencing technologies since the first two bacterial genomes were sequenced in 1995. In 2021, a master student was able to obtain the full genome of several isolates. Society needs to take advantage of such exceptional technological advances, as well as other advances in molecular biology to continue to find innovative ways to overcome threats to the population.

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9. Appendix

Table 9.1. Sample and primer volume specifications utilized when for 16S rRNA Sanger sequencing preperation. The requested sample to primer ratio was slightly adjusted when sample concentrations were low.

Sample concentration (ng/µl)	Sample (µl)	5 µM Primer (µl)
> 80	3	7
20-80	5	5
< 20	7	3

Requested sample ratio for sequencing by Eurofins GATC
DNA: 80-100 ng/µl
PCR product: 20-80 ng/µl
Primer: 5 pmol/µl (5 µM)

Table 9.2. Sample to primer volume ratio prepared for 16S rRNA Sanger sequencing. The Forward and Reverse ID tags provided by Eurofins GATC are also included.

Strain	Concentration (ng/µl)	Sample (µl)	Primer (µl)	Forward ID	Reverse ID
KA0	31.2	5	5	63GE61	63GE62
KA1	35.5	5	5	63GE75	63GE76
KA2	43.2	5	5	63GE77	63GE78
KA4	14.2	7	3	63GE63	63GE64
KA5	14.1	7	3	63GE65	63GE66
KA6*	8.81	8	2	63GE67	63GE68
KA7	35.1	5	5	63GE73	63GE74
KB2	40.8	5	5	63GE79	63GE80
KB3	26.4	5	5	63GE69	63GE70
KB5	39.7	5	5	63GE81	63GE82
KB8*	8.73	8	2	63GE71	63GE72
KB9	32.5	5	5	63GE83	63GE84

*Poor quality 16S rRNA sequence

3. Sample Information table (Fields in red are mandatory)

	Sample Name (MAX 16 characters, only letters, numbers and hyphen allowed). No spaces.	Type*	Conc. (ng/µl) ⓘ	A260 /280	A260 /230	Volume provided (µl)	Total DNA / RNA (µg)	Index name (mandatory for libraries, can be left blank for DNA/RNA)	Index sequence (mandatory for libraries, can be left blank for DNA/RNA)	Approx no. Reads, Gb or lanes requested	Primers, Linkers or RE sites present? †	Comments
	Example-1 (i.e. NOT Example_1)	gDNA	21.0	1.79	1.79	95	2	N701	ATTACTCG	1 Lane	EcoRI digest. All reads start AATTC	Pool together with sample 2 in 1 lane
1	KA0	gDNA	125	1.97	2.11	20	2.5			ca. 13-15 Gb		
2	KA5	gDNA	114	1.94	1.90	20	2.28			ca. 13-15 Gb		
3	KB3	gDNA	75.4	1.90	1.84	15	1.13			ca. 13-15 Gb		
4	KB8	gDNA	58.7	1.91	1.75	20	1.17			ca. 13-15 Gb		

Formula 1

$$n = c * v \rightarrow v = \frac{n}{c}$$

Table 9.3. Sample specifications for ONT sequencing preparation.

Strain	Concentration (ng/µl)	V_{sample} (µl)	V_{H20} (µl)	Barcode (2.5 µl)
KA0	156	2.56	4.94	RB01
KA5	114	3.51	3.99	RB02
KB3	96.2	4.32	3.18	RB03
KB8	58.7	6.8	0.7	RB04

Table 9.4. Qubit and Nanodrop measurements for all DNA extractions performed throughout the study.

	Qubit (ng/ul)				Nanodrop 02.03.21			Nanodrop 02.03.21		
	02.03.21	**21.04.21**	**29.04.21**	**12.05.21**	**ng/ul**	**260/280**	**260/230**	**ng/ul**	**260/280**	**260/230**
KA0	137	163	125	156	184,5	1,86	2,05	170,5	1,97	2,11
KA1	<0,50	<0,50			10,9	2,78	0,69			
KA2	0,055	<0,50			17,3	2,97	0,72			
KA4	270	279			363,9	1,89	2,03			
KA5	77,2	56,7	114	77,1	79,2	1,76	1,41	140,5	1,94	1,9
KA6	179	157	84,6		190,2	1,51	0,74	79,3	1,88	1,78
KA7	29,2	63,7			93,3	2	1,45			
KB2	3,76	3,77			74,9	2,37	1,63			
KB3	131		75,4		165,8	1,88	1,94	62	1,9	1,84
KB5	3,18	3,77			20,4	2,63	0,82			
KB8	160		58,7	96,2	185	1,89	2,07	69,7	1,91	1,75
KB9	0,243	0,284			38,1	2,95	0,73			

The percent identity indicates how similar the query sequence was compared to the targets, giving an indication of possible identification based on 16S rRNA sequencing. The family was included for clarity and subsequent comparison to NGS results.

Table 9.5. Top BLAST hits for the 16S rRNA Sanger sequences

Sample	Top BLAST hits	% Identity	Taxonomy (Family)
KA0	*Rahnella*	99,7	Enterobacteriaceae
	Ewingella americana	99,4	Yersiniaceae
	Yersinia	98,3	Yersiniaceae
	Serratia	98	Yersiniaceae
KA1	*Rahnella*	99,85	Enterobacteriaceae
	Ewingella americana	98,53	Yersiniaceae
	Yersinia	98,4	Yersiniaceae
	Serratia	98,2	Yersiniaceae
KA2	*Rahnella woolbedingensis*	98,95	Enterobacteriaceae
	Rahnella bruchi	99,77	Enterobacteriaceae
	Rahnella Victoriana	99,47	Enterobacteriaceae
	Ewingella	98,53	Yersiniaceae
	Yersinia	98,46	Yersiniaceae
KA4	*Herbaspirillum huttiense*	99,92	Oxalobacteraceae
	Herbaspirillum aquaticum	99,62	Oxalobacteraceae
	Herbaspirillum chlorophenolicum	99,02	Oxalobacteraceae
KA5	*Herbaspirillum huttiense*	99,85	Oxalobacteraceae
	Herbaspirillum aquaticum	99,55	Oxalobacteraceae
	Herbaspirillum chlorophenolicum	98,94	Oxalobacteraceae
KA6	*Herbaspirillum chlorophenolicum*	99,64	Oxalobacteraceae
	Herbaspirillum huttiense	99,64	Oxalobacteraceae
	Herbaspirillum aquaticum	99,64	Oxalobacteraceae
KA7	*Pseudomonas qingdaonensis*	99,56	Pseudomonadaceae
	Pseudomonas plecoglossicida	98,97	Pseudomonadaceae
	Pseudomonas taiwanesis	98,97	Pseudomonadaceae
KB2	*Rahnella victoriana*	99,7	Enterobacteriaceae
	Rahnella woolbedingsensis	99,32	Enterobacteriaceae
	Ewingella americana	98,75	Yersiniaceae
	Hafnia psychrotolerans	98,31	Hafniaceae
KB3	*Sphingomonas*	100	Sphingomonadaceae
KB5	*Herbaspirillum huttiense*	99,85	Oxalobacteraceae
	Herbaspirillum aquaticum	99,56	Oxalobacteraceae
	Herbaspirillum chlorophenolicum	99,56	Oxalobacteraceae
KB8	*Herbaspirillum huttiense*	99,81	Oxalobacteraceae
	Herbaspirillum aquaticum	97,8	Oxalobacteraceae
	Herbaspirillum chlorophenolicum	97,2	Oxalobacteraceae
KB9	*Herbaspirillum huttiense*	99,78	Oxalobacteraceae
	Herbaspirillum aquaticum	99,78	Oxalobacteraceae
	Herbaspirillum chlorophenolicum	99,03	Oxalobacteraceae

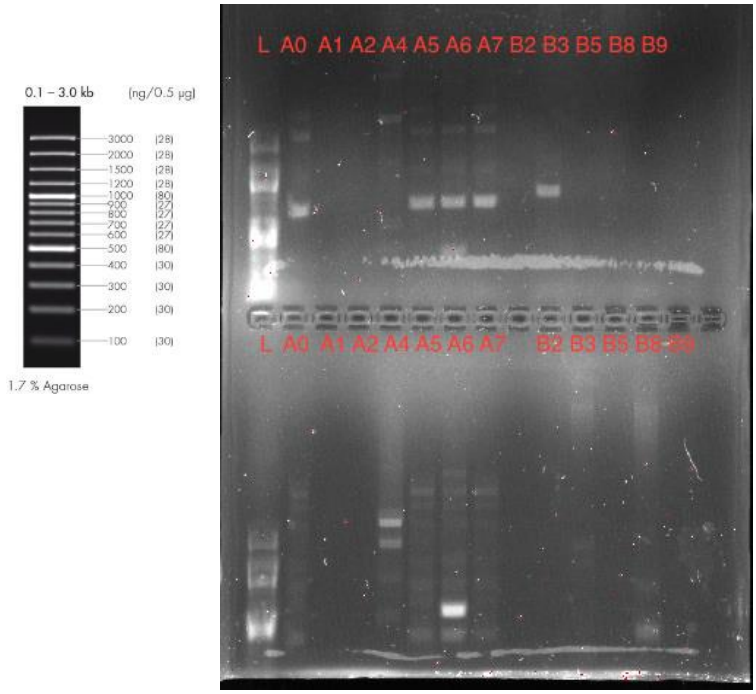


Figure 9.1. Multiplex 1 (top) and Multiplex 2 (bottom) PCR products of all isolates visualized on 1% agarose gel. As seen in the image, 1% gel was not sufficient for substantial resolution of the ladder. Further, many shadows are visible, indicating the formation of primer dimers. Again, isolates KA1, KA2, KB2, KB5 and KB9 did not provide sufficient DNA for multiplex analysis. The ladder was loaded in the first well, furthest to the left.

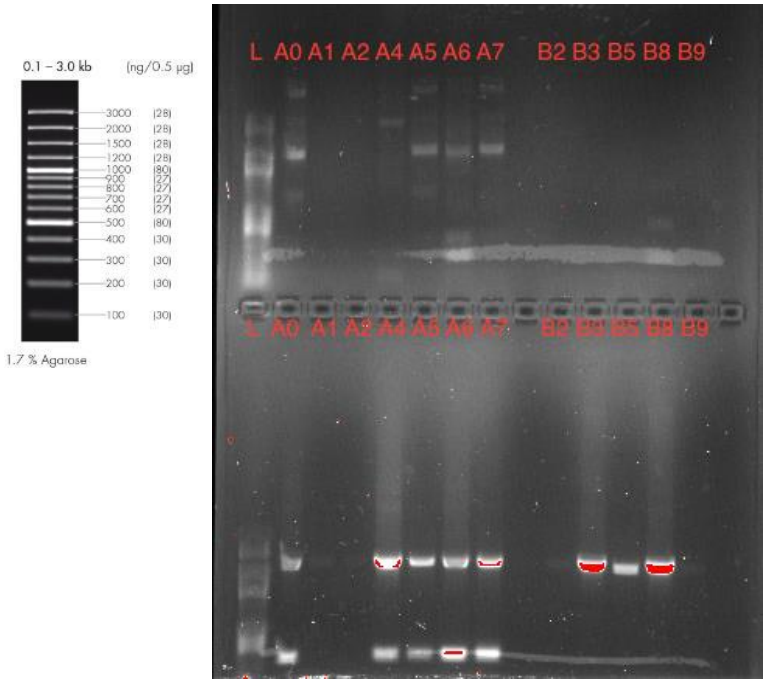
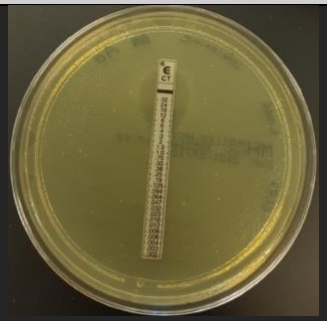
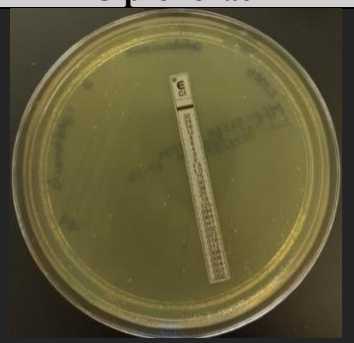
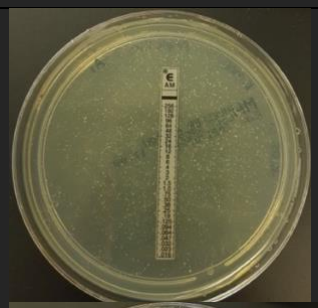
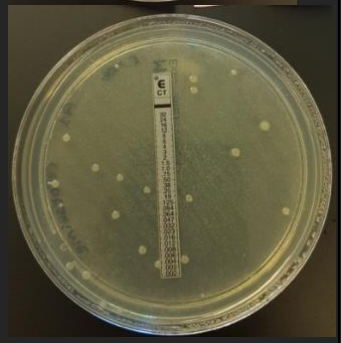
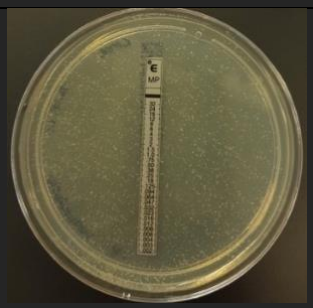
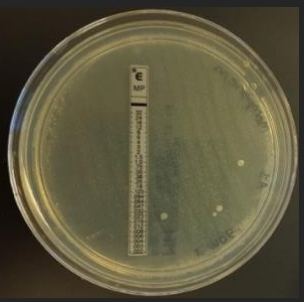
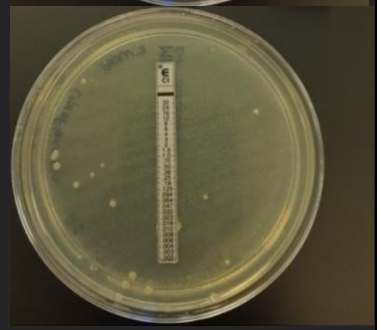
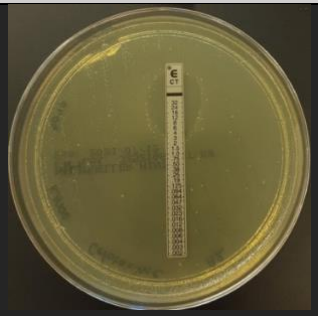
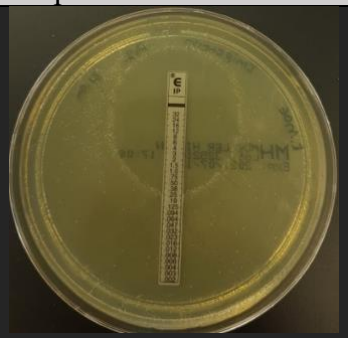
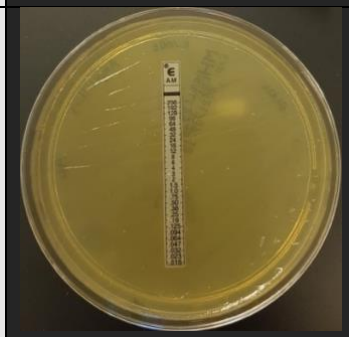
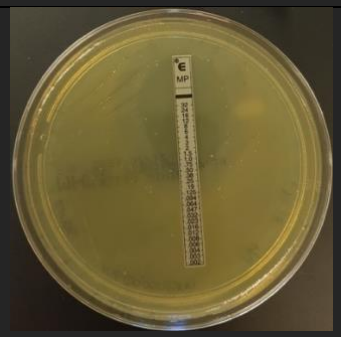
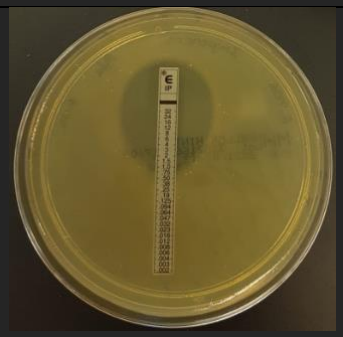
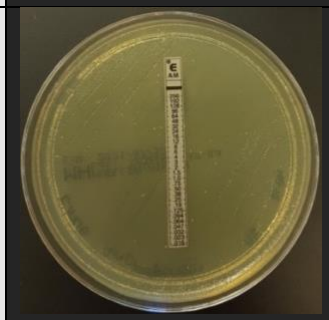
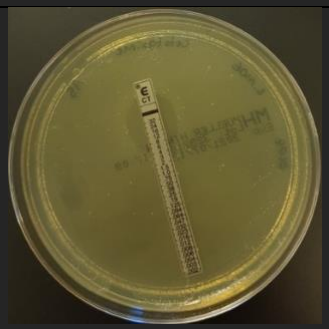
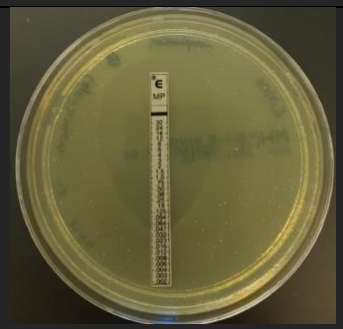
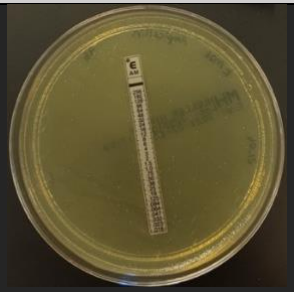
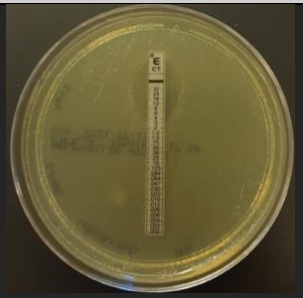
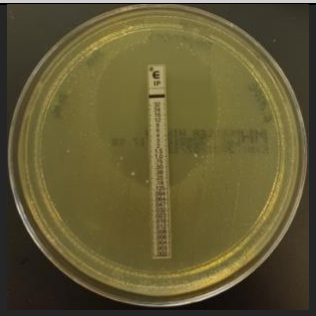
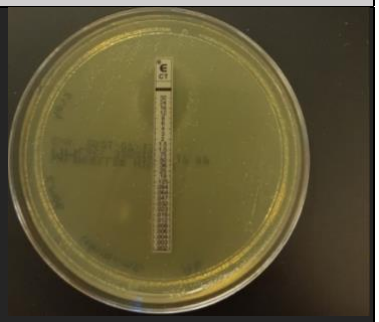
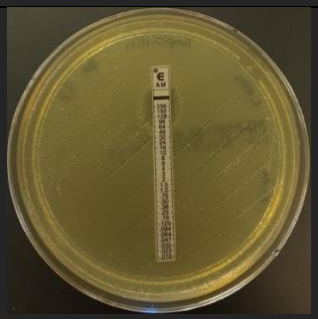
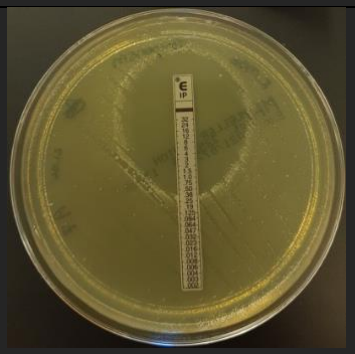
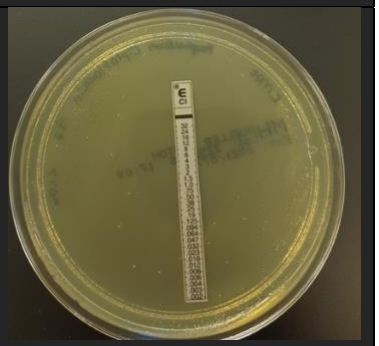


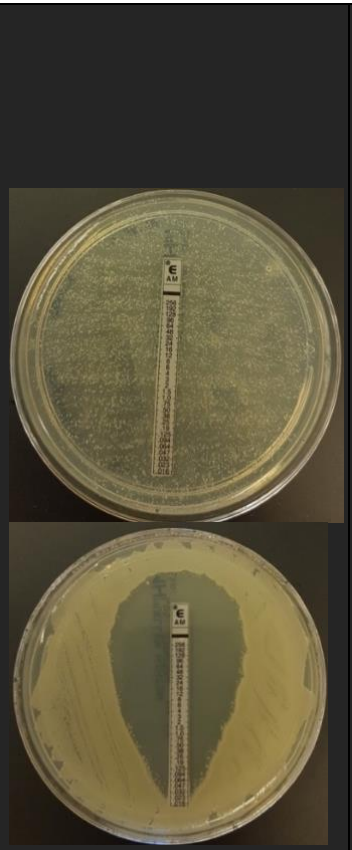
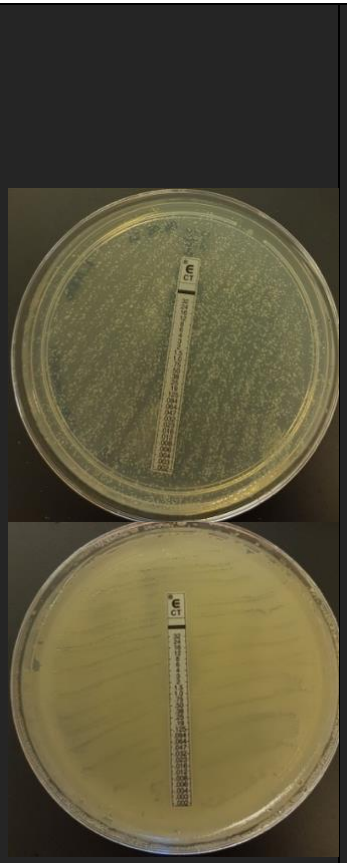
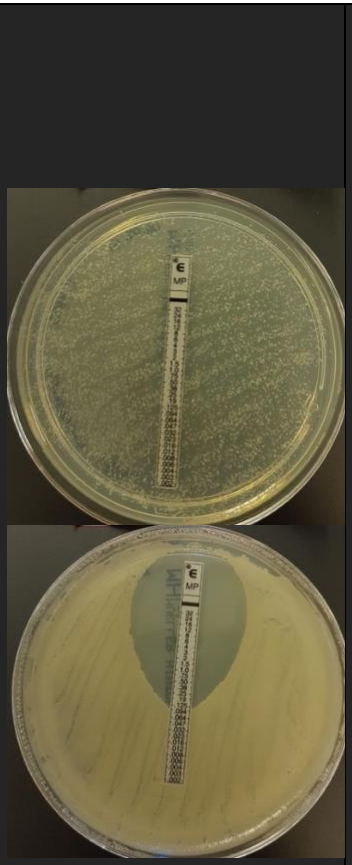
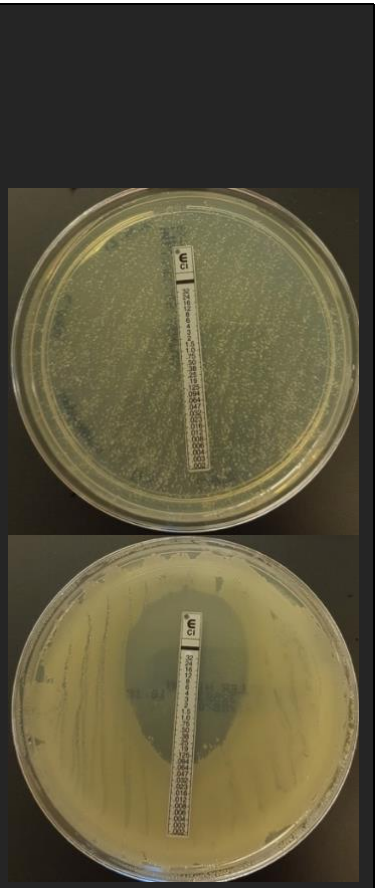
Figure 9.2. Multiplex 3 (top) and Multiplex 4 (bottom) PCR products of all isolates visualized on 1% agarose gel. As above, 1% gel was not sufficient for substantial resolution of the ladder. Further, many shadows are visible, indicating the formation of primer dimers. Again, isolates KA1, KA2, KB2, KB5 and KB9 did not provide sufficient DNA for multiplex analysis. Isolates KB2 and KB9 do not indicate the presence of rpoB. The ladder was loaded in the first well, furthest to the left.

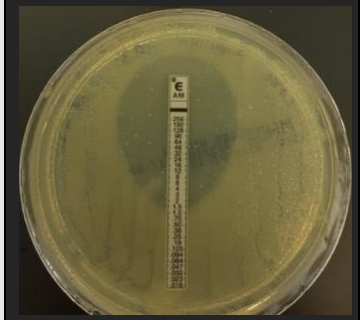
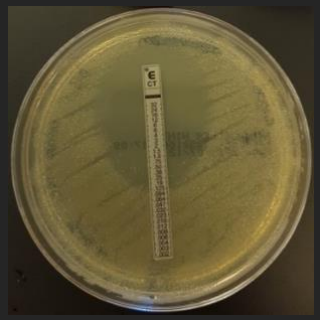
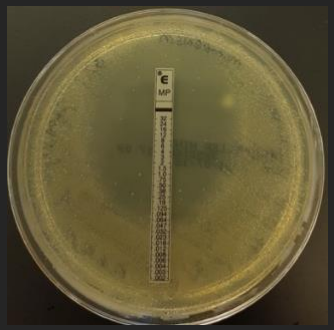
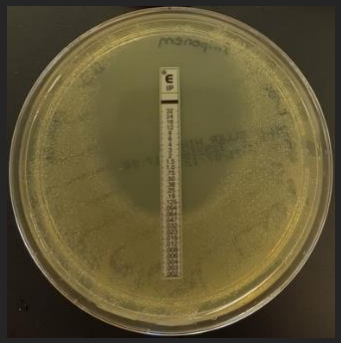
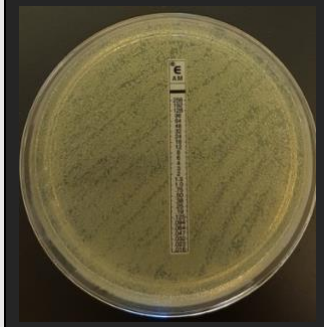
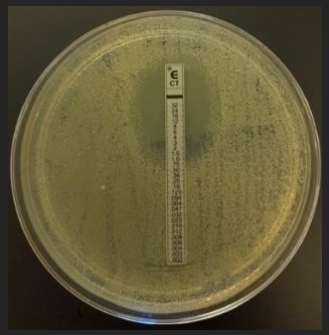
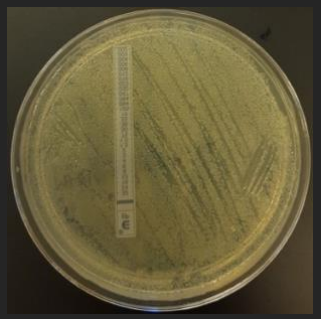
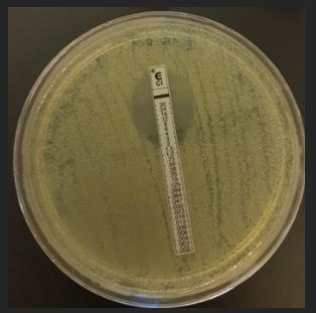
Table 9.6. Images of the MIC tests for all isolates

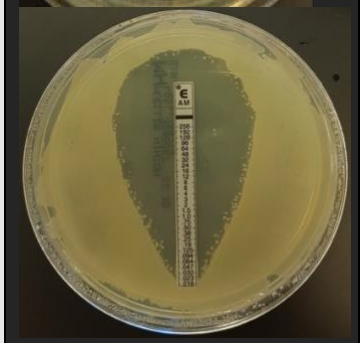
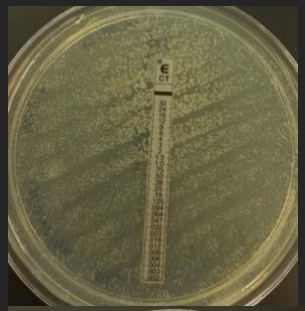
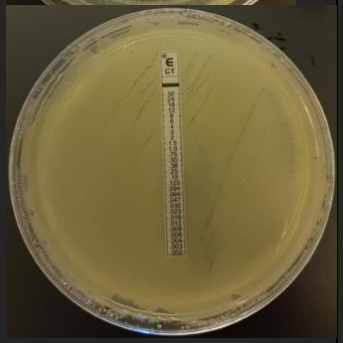
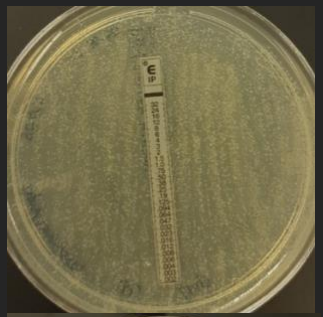
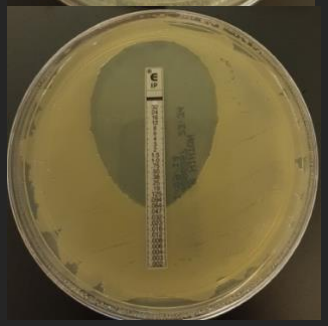
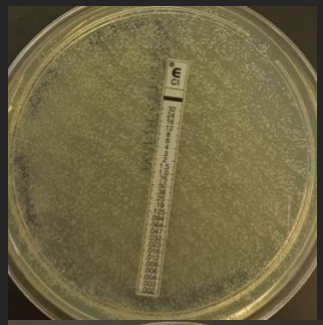
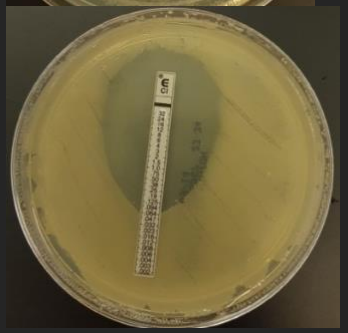
	Ampicillin	Cefotaxime	Meropenem	Imipenem	Ciprofloxacin
KA0					
KA1	 	 	 	 	 

	Ampicillin	Cefotaxime	Meropenem	Imipenem	Ciprofloxacin
KA2					
KA4					
KA5					

	Ampicillin	Cefotaxime	Meropenem	Imipenem	Ciprofloxacin
KA6					
KA7					

KB 2					
	Ampicillin	**Cefotaxime**	**Meropenem**	**Imipenem**	**Ciprofloxacin**

	Ampicillin	Cefotaxime	Meropenem	Imipenem	Ciprofloxacin
KB 3					
					

	Ampicillin	Cefotaxime	Meropenem	Imipenem	Ciprofloxacin
KB5	 	 	 	 	 

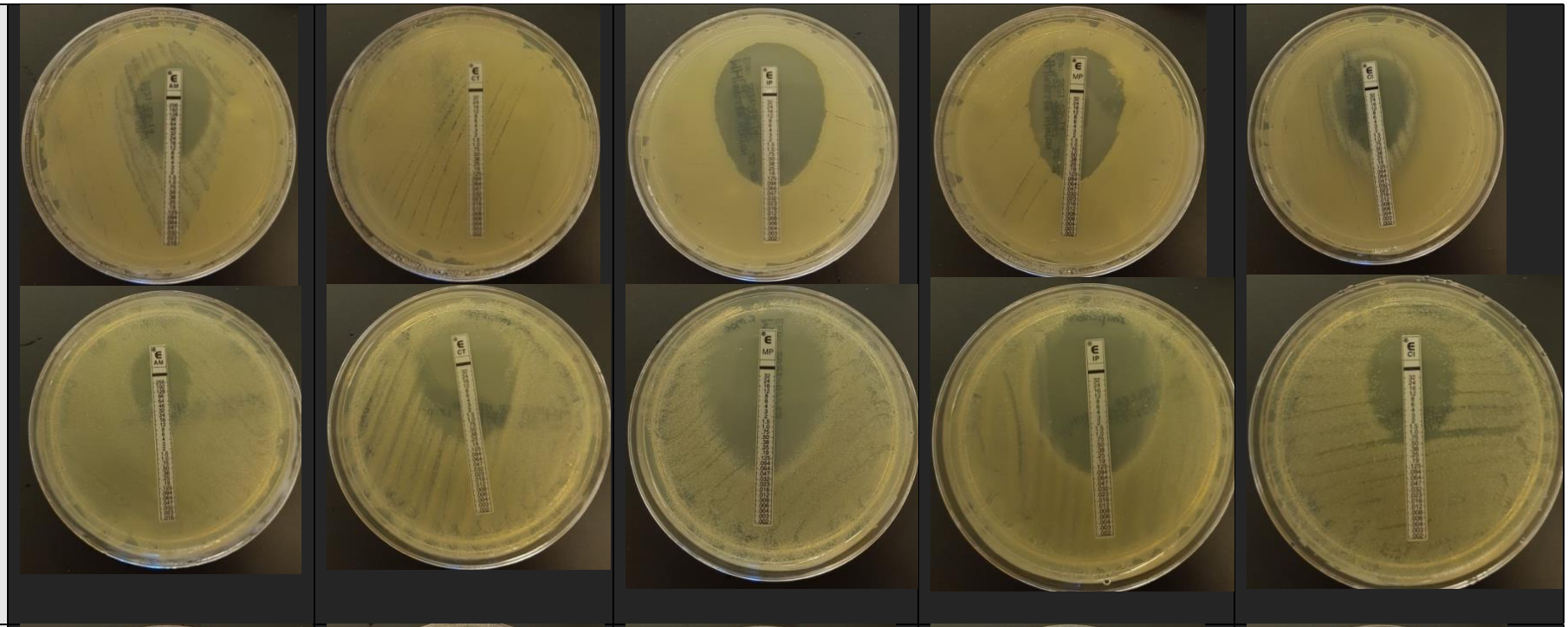
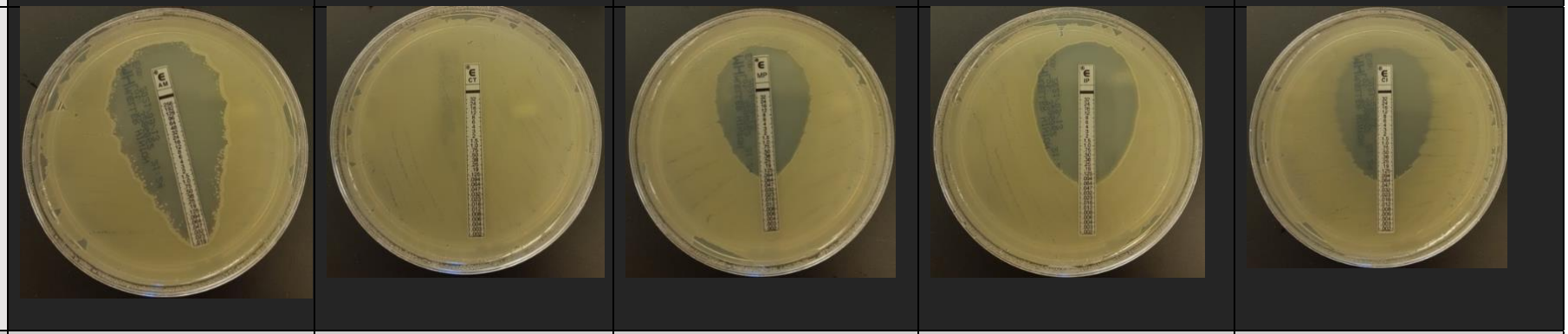
KB 8					
KB 9					
	Ampicillin	**Cefotaxime**	**Meropenem**	**Impinem**	**Ciprofloxacin**

Table 9.7. Summary of the resistance, resistance-related and virulence genes found in the isolates using Prokka, both when executed in Galaxy and on the CGView server. Only the genes not listed in CARD are listed here. A summary of the genes listen in CARD can be found in Table 5.6.

Isolate	***Rahnella***						***Herbaspirillum***				***Pseudomonas***		**Isolate**
	KA0		**KA5**		**KA4**		**KB3**		**KB8**		**KA7**		
Gene	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	**Gene**
acrZ	x	x	x	x	x	x							*acrZ*
ampD	x	x	x	x							x	x	*ampD*
ampE	x	x	x	x	x (1-2)	x (1-2)					x		*ampE*
ampG	x	x	x	x	x (1-2)	x (1-2)	x	x	x	x	x	x	*ampG*
ampH	x	x	x	x	x	x							*ampH*
ampR							x	x	x				*ampR*
arnB	x	x	x	x	x (1-4)	x (1-4)	x	x	x (1-3)	x (1-2)	x	x	*arnB*
arnD	x	x	x	x		x (1-2)	x	x	x (1-3)	x (1-2)		x	*arnD*
arnE	x	x	x	x	x	x			x (1-2)		x	x	*arnE*
arnF	x	x	x	x	x	x	x	x			x (1-2)	x (1-2)	*arnF*
arpB					x	x			x	x	x (1-2)	x (1-2)	*arpB*
arpC							x	x	x (1-2)	x (1-2)			*arpC*
arsA											x (1-3)	x (1-3)	*arsA*
arsB		x		x			x (1-3)	x (1-3)	x (1-3)	x (1-3)	x (1-2)	x	*arsB*
arsC		x		x			x (1-4)	x (1-4)	x (1-2)	x (1-2)			*arsC*
arsR							x						*arsR*
bhsA		x (1-5)		x (1-5)	x (1-4)	x (1-4)							*bhsA*
bmrA											x	x	*bmrA*
bvgs	x	x	x	x	x (1-2)	x (1-2)					x (1-8)	x (1-8)	*bvgs*
cnrA	x	x	x	x	x	x							*cnrA*

Isolate	*Rahnella*						*Herbaspirillum*				*Pseudomonas*		Isolate
	KA0		KA5		KA4		KA0		KA5		KA4		
Gene	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	Galaxy	CG view	Gene
copA	x	x	x	x	x (1-3)	x (1-3)	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-5)	x (1-5)	*copA*
copD							x	x	x		x	x	*copD*
corA	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x	x	x (1-2)	x (1-2)	x (1-2)	x (1-2)			*corA*
corC	x	x	x	x	x	x	x	x			x	x	*corC*
czcA					x (1-2)	x (1-2)	x (1-3)	x (1-3)	x (1-4)	x (1-4)			*czcA*
czcB							x	x	x	x			*czcB*
czcC							x	x	x	x			*czcC*
czcD							x	x	x	x	x	x	*czcD*
czcI							x	x					*czcI*
czcR	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x	x	x (1-3)	x (1-3)	x (1-2)	x (1-2)	x	x	*czcR*
czcS	x	x	x	x	x	x	x (1-2)	x (1-2)	x (1-2)	x (1-2)			*czcS*
ehpR	x	x	x	x	x (1-2)	x (1-2)							*ehpR*
fsr	x	x	x	x	x (1-3)	x (1-2)	x	x	x	x	x (1-2)	x (1-2)	*fsr*
gntK	x	x	x	x	x	x							*gntK*
iprA	x (1-4)		x (1-4)		x (1-4)								*iprA*
LnrL							x		x				*LnrL*
mdlB	x	x	x	x	x (1-3)	x (1-3)							*mdlB*
mdtD	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-9)	x (1-7)	x	x	x (1-8)	x (1-8)			*mdtD*
ohrB	x	x	x	x			x	x	x	x	x (1-2)		*ohrB*
ohrR	x	x	x	x	x	x	x	x	x	x			*ohrR*
pasT	x	x	x	x	x	x	x	x					*pasT*
poxB		x		x		x		x		x			*poxB*
rcnB	x	x	x	x	x	x							*rcnB*

Isolate	*Rahnella*						*Herbaspirillum*				*Pseudomonas*		Isolate
	KA0		KA5		KA4		KA0		KA5		KA4		
Gene	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	Galaxy	CG viewer	**Gene**
smvA	x	x	x	x	x (1-3)	x (1-3)							*smvA*
stp	x	x	x	x						x	x	x	*stp*
tdeA	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x	x	x	x			*tdeA*
yaaA	x		x		x (1-2)		x		x		x		*yaaA*
ybhR	x		x		x								*ybhR*
yheI	x	x	x	x	x (1-2)	x (1-2)							*yheI*
yheH													*yheH*
yheS	x (1-2)	x (1-2)	x (1-2)	x (1-2)	x (1-3)	x (1-3)	x	x	x	x	x (1-2)	x (1-2)	*yheS*

Table 9.8. Genes annotated by Prokka and subsequent BLAST results for isolate KA0 (R. variigena)

PROKKA		BLAST						
Gene	**Product (Prokka)**	**BLAST Description**	**Genus**	**Strain**	**Query Cover**	**E value**	**% Identity**	**Accession (NCIB)**
abaF	Fosfomycin resistance protein AbaF	MULTISPECIES: shikimate transporter	*Rahnella*	*MULTI*	100	0,0	99,32	WP_129639779.1
			Rahnella	*variigena*	100	0,0	98,42	WP_120163760.1
acrA_1	Multidrug efflux pump subunit AcrA	MULTISPECIES: efflux RND transporter periplasmic adaptor subunit	*Rahnella*	*MULTI*	100	0,0	100	WP_112287530.1
acrA_2	Multidrug efflux pump subunit AcrA	Efflux RND transporter permease adaptor subunit	*Rahnella*	*variigena*	100	0,0	99,75	WP_192878667.1
acrB_1	Multidrug efflux pump subunit AcrB	MULTISPECIES: multidrug efflux RND transporter permease AcrD	*Rahnella*	*MULTI*	100	0,0	100	WP_112287592.1
acrB_2	Multidrug efflux pump subunit AcrB	MULTISPECIES: multidrug efflux RND transporter permease subunit AcrB	*Rahnella*	*MULTI*	100	0,0	100	WP_113876130.1
acrE	Multidrug export protein AcrE	Efflux RND transporter permease adaptor subunit	*Rahnella*	*variigena*	100	0,0	98,42	WP_120162247.1
acrF	Multidrug export protein AcrF	Efflux RND transporter permease subunit	*Rahnella*	*variigena*	100	0,0	99,02	WP_120162248.1
acrZ	Multidrug efflux pump accessory protein AcrZ	AcrZ family multidrug efflux pump-associated protein	*Rahnella*	*variigena*	100	8E-27	100	WP_120160415.1
ampD	1,6-anhydro-N-acetylmuramyl-Lalanine amidase AmpD	1,6-anhydro-N-acetylmuramyl-Lalanine amidase AmpD	*Rahnella*	*variigena*	100	5E-138	99,47	WP_120161913.1

ampE	Protein AmpE	MULTISPECIES: β-lactamase regulator AmpE	*Rahnella*	*MULTI*	100	0,0	99,65	WP_112286929.1
		β -lactamse regulator AmpE	*Rahnella*	*variigena*	100	0,0	99,65	WP_120161912.1
ampG	Anhydromuropeptide permease	MULTISPECIES: muropeptide MFS transporter AmpG	*Rahnella*	*MULTI*	100	0,0	99,80	WP_113876145.1
			Rahnella	*variigena*	100	0,0	99,39	WP_120160795.1
ampH	D-alanyl_D-alanine-carboxypeptidase/endopeptidase AmpH		*Rahnella*	*MULTI*	100	0,0	100	WP_112289449.1
			Rahnella	*variigena*	100	0,0	99,47	WP_159282246.1
arnA	Bifunctional polymyxin resistance protein ArnA	MULTISPECIES: bifunctional UDP-4-deoxy-L-arabinose formyltransferase/UDP-glucuronic acid oxidase ArnA	*Rahnella*	*MULTI*	100	0,0	99,85	WP_112287969.1
arnB	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	UDP-4-amino-4-deoxy-L-arabinose aminotransferase	*Rahnella*	*variigena*	100	0,0	100	WP_120161770.1
arnC_1	Undecaprenyl-phosphate-4-deoxy-4-formamido-L-arabinose transferase	MULTISPECIES: glycosyltransferase family 2 protein	*Rahnella*	*MULTI*	97	0,0	99,67	WP_157961022.1
arnC_2		Undecaprenyl-phosphate-4-deoxy-4-formamido-L-arabinose transferase	*Rahnella*	*variigena*	100	0,0	100	WP_120161771.1
arnC_3		Glycosyl transferase	*Rahnella*	*aquatilis*	100	0,0	98,75	RBQ35625.1
			Rahnella	*variigena*	100	0,0	98,43	WP_120349669.1
arnC_4		Glycosyl transferase	*Rahnella*	*variigena*	100	0,0	98,21	WP_120163697.1
arnD	Putative 4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase ArnD	4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase ArnD	*Rahnella*	*variigena*	100	0,0	100	WP_120161772.1
arnE	4-amino-4-deoxy-L-arabinose-phophoundecaprenol flippase subunit ArnE		*Rahnella*	*variigena*	100	3E-69	99,09	WP_129638542.1

arnF	4-amino-4-deoxy-L-arabinose-phophoundecaprenol flippase subunit ArnF		*Rahnella*	*variigena*	100	6E-85	99,22	WP_120161775.1
arnT	Undecaprenyl-phosphate-4-deoxy-4-formamido-L-arabinose transferase	Lipid IV(A) a-amino-4-deoxy-L-arabinose arabinosyltransferase	*Rahnella*	*variigena*	100	0,0	100	WP_120161773.1
bla	β -lactamase Toho-1	Class A- β -lactamase	*Rahnella*	*sp. AN3-3W4*	100	0,0	100	WP_112286530.1
ble	Bleomycin resistance protein	Bleomycin resistance protein	*Rahnella*	*variigena*	100	3E-87	98,40	WP_120163049.1
bcr_1	Bicyclomycin resistance protein	Multidrug efflux MFS transporter	*Rahnella*	*sp. AN3-3W4*	100	0,0	99,49	WP_112287617.1
bcr_2	Bicyclomycin resistance protein	Bcr/CflA family multidrug efflux MFS transporter	*Rahnella*	*variigena*	100	0,0	99,75	WP_120162037.1
bcr_3	Bicyclomycin resistance protein	Multidrug effflux MFS transporter	*Rahnella*	*variigena*	100	0,0	99,73	WP_120162503.1
bmr3_1	Multidrug resistant protein 3	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,80	WP_120164002.1
bmr3_2	Multidrug resistance protein 3	MFS transporter	*Rahnella*	*variigena*	100	0,0	100	WP_120163501.1
bvgS	Virulence sensor protein BvgS	Transporter substrate-binding domain-containing protein	*Rahnella*	*variigena*	100	0,0	98,42	WP_120163760.1
cnrA	Nickle and cobalt resistance protein CnrA	Efflux RND transporter permease subunit	*Rahnella*	*variigena*	100	0,0	99,51	WP_120163281.1
copA	Copper exporting P-type ATPase	Copper-exporting P-type ATPase CopA	*Rahnella*	*sp. AN3-3W3*	100	0,0	99,76	WP_112287552.1
			Rahnella	*JUb53*	100	0,0	99,41	WP_132965083.1
			Rahnella	*variigena*	100	0,0	99,29	WP_159281225.1

corA_1	Cobalt/magnesium transport protein CorA	Magnesium/cobalt transporter CorA	*Rahnella*	*variigena*	100	0,0	100	WP_120164028.1
corA_2	Magnesium transport protein corA	MULTISPECIES: magneisum/cobalt transporter CorA	*Rahnella*	*MULTI*	100	0,0	100	WP_095924204.1
		Magnesium/cobalt transporter CorA	*Rahnella*	*sp. H11b*	100	0,0	99,68	WP_217172345.1
		MULTISPECIES: magneisum/cobalt transporter CorA	*Yersiniaceae*		100	0,0	99,37	WP_104924058.1
corC	Magnesium and cobalt efflux protein CorC	MULTISPECIES: CNNM family magnesium/cobalt transport protein CorC	*Rahnella*	*MULTI*	100	0,0	100	WP_015698153.1
			Yersiniaceae		100	0,0	99,66	WP_101075684.1
crp	cAMP-activated global transcriptional regulator CRP	MULTISPECIES: cAMP-activated global transcriptional regulator CRP	*Yersiniaceae*		100	2E-154	100	WP_013573634.1
			Enterobacterales		100	5E-154	99,52	WP_005969523.1
czcR_1	Transcriptional activator protein CzcR	MULTISPECIES: heavy metal response regulator transcription factor	*Rahnella*	*MULTI*	100	9E-159	100	WP_120163935.1
			Rahnella	*woolbedingensis*	100	7E-157	99,55	WP_120131363.1
czcR_2	Transcriptional activator protein CzcR	MULTISPECIES: heavy metal response regulator transcription factor	*Rahnella*	*MULTI*	100	4E-160	100	WP_095921607.1
			Enterobacterales		100	3E-159	99,12	WP_014226766.1
		DNA-binding response regulator	*Rahnella*	*aquatilis*	100	3E-159	99,56	RBQ33042.1
czcS	Sensor protein CzcS	Heavy metal sensor histidine kinase	*Rahnella*	*variigena*	100	0,0	100	WP_120163938.1
ehpR	Phenazine antibiotic resistance protein EhpR	VOC family protein	*Lomsdalea*	*britannica*	99	7E-69	80	WP_085652388.1
emrA_1		HlyD family secretion protein	*Rahnella*	*variigena*	99	0,0	99,42	WP_120160586.1

	Colistin resistance protein EmrA	MULTISPECIES: HlyD family secretion protein	*Rahnella*		100	0,0	97,67	WP_129638468.1
emrA_2	Colistin resistance protein EmrA	HlyD family secretion protein	*Rahnella*	*variigena*	100	0,0	100	WP_120163888.1
emrA_3	Colistin resistance protein EmrA	HlyD family secretion protein	*Rahnella*	*variigena*	100	0,0	100	WP_120163888.1
emrA_4	Colistin resistance protein EmrA	HlyD family secretion protein	*Rahnella*	*variigena*	100	0,0	99,73	WP_129638767.1
emrB_1	Multidrug export protein EmrB	MULTISPECIES: multidrug efflux MFS transporter permease subunit EmrB	*Rahnella*	*MULTI*	100	0,0	100	WP_113877526.1
		Multidrug efflux MFS trasnporter permease subunit EmrB	*Rahnella*	*sp. AN3-3W3*	100	0,0	99,80	WP_112290465.1
emrB_2	Colistin resistance protein EmrB	DHA2 family efflux MFS transporter permease subunit	*Rahnella*	*sp. JUb53*	100	0,0	99,42	WP_132963968.1
emrB_3	Multidrug export protein EmrB	MULTISPECIES: DHA2 family efflux MFS transporter permease subunit	*Rahnella*	*MULTI*	100	0,0	99,81	WP_112290238.1
			Rahnella	*variigena*	100	0,0	99,43	WP_159282137.1
emrD	Multidrug ressitance protein D	Multidrug efflux MFS transporter EmrD	*Rahnella*	*aquatillis*	100	0,0	99,50	WP_113877618.1
emrE_1	Multidrug transporter EmrE	MULTISPECIES: multidrug DMT transporter	*Rahnella*	*MULTI*	100	8E-71	99,1	WP_113876495.1
emrE_2	Multidrug transporter EmrE	MULTISPECIES: QacE family quaternary ammonium compound efflux SMR transporter	*Rahnella*	*MULTI*	100	2E-68	100	WP_113878269.1
emrK_1	Putative multidrug resistance protein EmrK	Membrane fusion protein (multidrug efflux system)/multidrug resistance protein K	*Rahnella*	*sp. JUb53*	100	0,0	99,21	TCQ86898.1

emrK_2	Putative multidrug resistance protein EmrK	Membrane fusion protein (multidrug efflux system)/multdrug resistance protein K	*Rahnella*	*sp. JUb53*	100	0,0	99,21	TCQ86898.1
		HlyD family efflux transporter periplmasmic adapotir subunit	*Rahnella*	*sp. FC061912-K*	100	0,0	98,69	WP_217204451.1
			Rahnella	*aquatilis*	95	0,0	99,73	WP_113876402.1
			Rahnella	*variigena*	95	0,0	99,18	WP_120349702.1
emrK_3	Putative multidrug resistance protein EmrK	MULTISPECIES: HlyD family secretion protein	*Rahnella*	*MULTI*	100	0,0	100	WP_112290239.1
emrY_1	Putative multidrug resistance protein EmrY	MULTISPECIES: DHA2 family efflux MFS transporter permease subunit	*Rahnella*	*MULTI*	100	0,0	100	WP_113876270.1
emrY_2	Putative multidrug resistance protein EmrY	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,81	WP_120160584.1
fsr	Fosmidomycin resistance protein	MULTISPECIES: MFS transporter	*Rahnella*	*MULTI*	100	0,0	100	WP_120160753.1
hns_1	DNA-binding H-NS	MULTISPECIES: H-NS histone family protein	*Rahnella*	*MULTI*	100	0,0	100	WP_112287421.1
hns_2	DNA-binding H-NS	MULTISPECIES: DNA_binding transcriptional regulator H-NS	*Yersiniaceae*		100	1E-91	100	WP_013575950.1
			Rahnella		100	2E-91	99,26	WP_120130915.1
iprA_1	Inhibitor of hydrogen peroxide resistance	Winged helix-turn-helix transcriptional regulator	*Rahnella*	*variigena*	100	2E-109	99,35	WP_120160306.1
iprA_2	Inhibitor of hydrogen peroxide resistance	Winged helix-turn-helix DNA binding protein	*Rahnella*	*sp. JUb53*	100	4E-172	99,58	TCQ90629.1
		MULTISPECIES: helix-turn-helix domain-containing protein	*Rahnella*	*MULTI*	90	8E-157	100	WP_112287603.1
		Helix-turn-helix domain-containing protein	*Rahnella*	*variigena*	90	2E-157	99,53	WP_129639414.1

iprA_3	Inhibitor of hydrogen peroxide resistance	Helix-turn-helix domain-containing protein	*Rahnella*	*variigena*	100	2E-161	100	WP_120161898.1
iprA_4	Inhibitor of hydrogen peroxide resistance	Winged helix-turn-helix transcriptional regulator	*Rahnella*	*variigena*	100	1E-152	100	WP_120162504.1
mexA	Multidrug resistance protein MexA	Membrane fusion protein (multidrug efflux system)	*Rahnella*	*sp. NFIX50*	100	0,0	96,95	RAR90611.1
		CmeA	*Campylobacter*	*jejuni*	100	0,0	96,92	VTQ57772.1
		MULTISPECiES: efflux RND transporter periplasmic adaptor subunit	*Rahnella*	*MULTI*	96	0,0	97,43	WP_120163073.1
mexB	Multidrug resistance protein MexB	MULTISPECIES: efflux RND transporter permease subunit	*Rahnella*	*MULTI*	100	0,0	99,90	WP_132967302.1
mdfA_1	Multidrug transporter MdfA	DHA1 family multidrug/chloramphenicol efflux transport protein-like MFS transporter	*Rahnella*	*sp. JUb53*	100	0,0	100	TCQ86207.1
		MULTISPECIES: MFS transporter	*Rahnella*	*MULTI*	100	0,0	99,75	WP_113876873.1
mdfA_2	Multidrug trasnporter MdfA	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,26	WP_120162831.1
mdlB	Multidrug resistance-like ATP-binding protein	SmdB family multidrug efflux ABC transorter permease/ATP-binding protein	*Rahnella*	*aquatillis*	100	0,0	99,83	WP_113876137.1
mdtA_1	Multidrug resistance protein MdtA	Efflux RND transporter periplasmic adaptor subunit	*Rahnella*	*variigena*	100	0,0	99,45	WP_120163282.1
mdtA_2	Multidrug resistance protein MdtA	MdtA/MuxA family multidrug efflux RND transporter periplasmic adapter subunit	*Rahnella*	*variigena*	100	0,0	100	WP_159282974.1

mdtB	Multidrug resistance protein MdtB	Efflux RND transporter permease subunit	*Rahnella*	*sp. AN3-3W4*	100	0,0	99,72	WP_112290331.1
mdtC	Multidrug resistance protein MdtC	MULTISPECIES: efflux RND transporter permease subunit	*Rahnella*	*MULTI*	100	0,0	100	WP_129638894.1
mdtD_1	Putative multidrug resistance protien MdtD	MFS transporter	*Rahnella*	*variigena*	100	0,0	100	WP_120161090.1
mdtD_2	Putative multidrug resistance protien MdtD	Multidrug transporter subunit MdtD	*Rahnella*	*variigena*	100	0,0	99,59	WP_120161587.1
mdtH_1	Multidrug resistance protein MdtH	MULTISPECIES: multidrug MFS transporter MdtH	*Rahnella*	*MULTI*	100	0,0	100	WP_112288248.1
mdtH_2	Multidrug resistance protein MdtH	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,03	WP_120162928.1
mdtK	Multidrug resistance protein MdtK	MATE family efflux transporter	*Rahnella*	*variigena*	100	0,0	99,78	WP_120161793.1
mdtL	Multidrug resistance protein MdtL	DHA1 family bicyclomycin/chloramphenicol resistance-like MFS transporter	*Rahnella*	*sp. JUb53*	100	0,0	99,50	TCQ93660.1
		Multidrug effflux MFS transporter	*Rahnella*	*variigena*	99	0,0	99,75	WP_120162890.1
oqxB17	Multidrug efflux RND transporter permease subunit OqxB17	MULTISPECIES: efflux RND transporter permease subunit OqxB	*Rahnella*	*variigena*	100	0,0	100	WP_120163574.1
ohrB	Organic hydroperoxide resistance protein OhrB	MULTISPECIES: organic hydroperoxide resistance protein	*Rahnella*	*MULTI*	100	7E-96	100	WP_112291684.1
ohrR	Organic hydroperoxide resistance (transcriptional regulator)	MULTISPECIES: MarR famly transcriptional regulator	*Rahnella*	*MULTI*	100	2E-89	99,25	WP_112291682.1

pasT	Persistence and stress-resistance toxin PasT	MULTISPECIES: type II toxin-antitoxin system RatA family toxin	*Rahnella*	*MULTI*	100	1E-101	100	WP_013576738.1
phoQ	Virulence sensor histidine kinase PhoQ	MULTISPECIES: two-component system sensor histidine kinase PhoQ	*Rahnella*	*MULTI*	100	0,0	100	WP_120160656.1
rcnB	Nickel/cobalt homeostasis protein RcnB	RcnB family protein	*Rahnella*	*variigena*	100	4E-72	100	WP_120163659.1
smvA	Methyl viologen resistance protein SmvA	MFS transporter	*Rahnella*	*aquatillis*	100	0,0	99,8	WP_113876442.1
stp	Multidrug resistance protein Stp	MFS transporter	*Rahnella*	*variigena*	100	0,0	98,78	WP_120162731.1
tdeA_1	Toxin and drug export protein A	Efflux transporter outer memebrane subunit	*Rahnella*	*variigena*	100	0,0	99,78	WP_120163304.1
tdeA_2	Toxin and drug export protein A	Efflux transporter outer memebrane subunit	*Rahnella*	*variigena*	100	0,0	99,56	WP_120163304.1
yaaA	Peroxide stress resistance protein YaaA	Peroxide stress protein YaaA	*Rahnella*	*variigena*	100	0,0	99,61	WP_120161962.1
ybhR	Putative multidrug ABC transporter permease YbhR	ABC transporter permease	*Rahnella*	*aquatilis*	100	0,0	99,74	RBQ34680.1
yheI	Putative multidrug resistance ABC transporter ATP binding/permease YheI	SmdA family multidrug ABC transporter permease/ATP-binding protein	*Rahnella*	*variigena*	100	0,0	100	WP_120160786.1
Putative multidrug export ATP-binding/permease protein		ABC transporter ATP-binding protein/permease	*Rahnella*	*variigena*	100	0,0	99,84	WP_120163500.1

Putative multidrug export ATP-binding/permease protein	MULTISPECIES: ABC trasnporter ATP-binding protein/permease	*Rahnella*	*MULTI*	100	0,0	100	WP_120162994.1
Putative multidrug-efflux transporter	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,58	WP_120160465.1

Appendix 9.10. Prokka and BLAST Results for Isolate KA5

Table 9.9. Genes annotated by Prokka and subsequent BLAST results for isolate KA5 (R. variigena)

PROKKA	BLAST

Gene	Product	BLAST Description	Genus	Strain	Query Cover	E value	% Identity	Accesion (NCIB)
abaF	Fosfomycin resistance protein AbaF	MULTISPECIES: shikimate transporter	*Rahnella*	*MULTI*	100	0,0	99,32	WP_129639779.1
		Shikimate transporter	*Rahnella*	*variigena*	100	0,0	99,32	WP_120161349.1
acrA_1	Multidrug efflux pump subunit AcrA	MULTISPECIES: efflux RND transporter periplasmic adaptor subunit	*Rahnella*	*MULTI*	100	0,0	100	WP_112287530.1
acrA_2	Multidrug efflux pump subunit AcrA	Efflux RND transporter permease adaptor subunit	*Rahnella*	*variigena*	100	0,0	99,75	WP_192878667.1
acrB_1	Multidrug efflux pump subunit AcrB	MULTISPECIES: multidrug efflux RND transporter permease AcrD	*Rahnella*	*MULTI*	100	0,0	100	WP_112287592.1
		Multidrug efflux RND transporter permease AcrD	*Rahnella*	*variigena*	100	0,0	99,71	WP_159281215.1
acrB_2	Multidrug efflux pump subunit AcrB	MULTISPECIES: multidrug efflux RND transporter permease subunit AcrB	*Rahnella*	*MULTI*	100	0,0	100	WP_113876130.1
		Multidrug efflux RND transporter permease subunit AcrB	*Rahnella*	*sp. AN3-3W3*	100	0,0	99,81	WP_112287529.1
acrR	HTH-type transcriptional regulator AcrR	MULTISPECIES: multidrug efflux transporter transcriptional repressor AcrR	*Rahnella*	*MULTI*	100	6E-156	100	WP_112287531.1
			Rahnella	*sp. H11b*	100	7E-155	99,06	WP_217174190.1
acrE	Multidrug export protein AcrE	Efflux RND transporter permease adaptor subunit	*Rahnella*	*variigena*	100	0,0	98,42	WP_120162247.1
acrF	Multidrug export protein AcrF	Efflux RND transporter permease subunit	*Rahnella*	*variigena*	100	0,0	99,02	WP_120162248.1
acrZ	Multidrug efflux pump accessory protein AcrZ	AcrZ family multidrug efflux pump-associated protein	*Rahnella*	*variigena*	100	8E-27	100	WP_120160415.1
ampD	1,6-anhydro-N-acetylmurmamyl-L-alanine amidase AmpD		*Rahnella*	*variigena*	100	5E-138	99,47	WP_120161913.1

ampE	Protein AmpE	MULTISPECIES: β -lactamase regulator AmpE	*Rahnella*	*MULTI*	100	0,0	99,65	WP_112286929.1
			Rahnella	*variigena*	100	0,0	99,65	WP_120161912.1
ampG	Anyhromuropeptide permease	MULTISPECIES_ muropeptide MFS transporter AmpG	*Rahnella*	*MULTI*	100	0,0	99,8	WP_113876145.1
			Rahnella	*variigena*	100	0,0	99,39	WP_120160795.1
ampH	D-alanyl-D-alanine-carboxypeptidase/endopeptidase AmpH		*Rahnella*	*MULTI*	100	0,0	100	WP_112289449.1
			Rahnella	*variigena*	100	0,0	99,47	WP_159282246.1
arnA	Bifunctional polymyxin resistance protein ArnA	MULTISPECIES: bifunctional UDP-4-amino-4-deoxy-L-arabinose formyltransferase	*Rahnella*	*MULTI*	100	0,0	99,85	WP_112287969.1
arnB	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	UDP-4-amino-4-deoxy-L-arabinose aminotransferase	*Rahnella*	*variigena*	100	0,0	100	WP_120161770.1
arnC_1	Undecaprenyl-phosphate-4-deoxy-4-formamido-L-arabinose transferase	MULTISPECIES: glycosyltransferase family 2 protein	*Rahnella*	*MULTI*	97	0,0	99,67	WP_157961022.1
arnC_2		Undecaprenyl-phosphate-4-deoxy-4-formamido-L-arabinose transferase	*Rahnella*	*variigena*	100	0,0	100	WP_120161771.1
arnC_3		Glycosyl transferase	*Rahnella* *Rahnella*	*aquatilis* *variigena*	100 100	0,0 0,0	98,75 98,43	RBQ35625.1 WP_120349669.1
arnC_4		Glycosyl transferase	*Rahnella*	*variigena*	100	0,0	98,21	WP_120163697.1
arnD	Putative 4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase ArnD	4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase ArnD	*Rahnella*	*variigena*	100	0,0	100	WP_120161772.1
arnE	4-amino-4-deoxy-L-arabinose-phophoundecaprenol flippase subunit ArnE		*Rahnella*	*variigena*	100	3E-69	99,09	WP_129638542.1

arnF	4-amino-4-deoxy-L-arabinose-phophoundecaprenol flippase subunit ArnF		*Rahnella*	*variigena*	100	6E-85	99,22	WP_120161775.1
arnT	Undecaprenyl-phosphate-4-deoxy-4-formamido-L-arabinose transferase	Lipid IV(A) a-amino-4-deoxy-L-arabinose arabinosyltransferase	*Rahnella*	*variigena*	100	0,0	100	WP_120161773.1
bla	β -lactamase Toho-1	Class A β -lactamase	*Rahnella*	*AN3-3W3*	100	0,0	100	WP_112286530.1
			Rahnella	*variigena*	100	0,0	99,66	WP_112286530.1
ble	Bleomycin resistance protein	Bleomycin resistance protein	*Rahnella*	*variigena*	100	3E-87	98,40	WP_120163049.1
bcr_1	Bicyclomycon resistance protein	Multidrug efflux MFS trasnporter	*Rahnella*	*sp AN3-3W3*	100	0,0	99,49	WP_112287617.1
			Rahnella	*variigena*	100	0,0	99,49	WP_120160319.1
bcr_2	Bicyclomycon resistance protein	Multidrug efflux MFS transporter	*Rahnella*	*variigena*	100	0,0	99,26	WP_120162503.1
bcr_3	Bicyclomycin resistance protein	Bcr/CflA family multidrug efflux MFS transporter	*Rahnella*	*sp. H11b*	100	0,0	100	WP_217174952.1
			Rahnella	*variigena*	100	0,0	99,75	WP_120162037.1
bmr3_1	Multidrug resistance protein 3	MFS transporter	*Rahnella*	*variigena*	100	0,0	100	WP_120163501.1
bmr3_2	Multidrug resistance protein 3	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,80	WP_120164002.1
bvgS	Virulence sensor protein BvgS	Transporter substrate-binding domain-containing protein	*Rahnella*	*variigena*	100	0,0	98,42	WP_120163760.1
cnrA	Nickel and cobalt resitance protein CnrA	Efflux RNDS transporter permease subunit	*Rahnella*	*variigena*	100	0,0	99,51	WP_120163281.1
copA	Copper-exporting P-type ATPase	Copper-exporting P-type ATPase CopA	*Rahnella*	*sp. AN3-3W3*	100	0,0	99,76	WP_112287552.1
			Rahnella	*variigena*	100	0,0	99,29	WP_159281225.1

corA_1	Cobalt/magnesium transport protein CorA	Magnesium/cobalt transporter CorA	*Rahnella*	*variigena*	100	0,0	100	WP_120164028.1
corA_2	Magnesium transport protein CorA	MULTISPECIES: magnesium/cobalt transporter CorA	*Rahnella*	*MULTI*	100	0,0	100	WP_095924204.1
			Yersiniaceae	*MULTI*	100	0,0	99,68	WP_217172345.1
corC	Magnesium and cobalt efflux protein CorC	MULTISPECIES: CNNM family magnesium/cobalt transport protein CorC	*Rahnella*	*MULTI*	100	0,0	100	WP_015698153.1
			Yersiniaceae	*MULTI*	100	0,0	99,66	WP_101075684.1
crp	cAMP-activated global transcriptional regulator CRP	MULTISPECIES: cAMP-activated global transcriptional regulator CRP	*Yersiniaceae*		100	2E-154	100	WP_013573634.1
			Enterobacterales		100	5E-154	99,52	WP_005969523.1
czcR_1	Transcriptional activator protein CzcR	MULTISPECIES: heavy metal response regulator transcription factor	*Rahnella*	*MULTI*	100	9E-159	100	WP_120163935.1
			Rahnella	*woolbedingensis*	99	7E-157	99,55	WP_120131363.1
			Rahnella	*sp. AN3-3W3*	100	7E-157	99,11	WP_112289411.1
czcR_2	Transcriptional activator protein CzcR	MULTISPECIES: heavy metal response regulator transcription factor	*Rahnella*	*MULTI*	100	4E-160	100	WP_095921607.1
			Enterobacterales	*MULTI*	100	3E-159	99,12	WP_071434289.1
czcS	Sensor protein CzcS	Heavy metal sensor histidine kinase	*Rahnella*	*variigena*	100	0,0	100	WP_120163938.1
ehpR	Phenazine antibiotic resistance protein EhpR	VOC family protein	*Lonsdalea*	*britannuca*	99	7E-69	80	WP_085652388.1
			Pantoea	*alhagi*	99	2E-68	79,17	WP_085070533.1
			Klebsiella	*sp. RIT-PI-d*	99	3E-68	80	WP_049840381.1
emrA_1	Multidrug export protein EmrA	MULTISPECIES: multidrug efflux MFS transporter periplasmic adaptor subunit EmrA	*Rahnella*	*MULTI*	100	0,0	100	WP_113877527.1
			Rahnella	*MULTI*	100	0,0	97,67	WP_129638468.1
emrA_2	Colistin resistance protein EmrA	HlyD family secretion protein	*Rahnella*	*variigena*	100	0,0	100	WP_120163888.1
emrA_3		HlyD family secretion protein	*Rahnella*	*variigena*	100	0,0	99,73	WP_129638767.1
emrA_4		HlyD family secretion protein	*Rahnella*	*variigena*	100	0,0	100	WP_120163888.1

emrB_1	Multidrug export protein EmrB	MULTISPECIES: multidrug efflux MFS transporter permease subunit EmrB	*Rahnella*	*MULTI*	100	0,0	100	WP_113877526.1
emrB_2	Colostin resistance protein EmrB	DHA2 family efflux MFS transporter permease subunit	*Rahnella*	*sp. Jub53*	100	0,0	99,42	WP_132963968.1
			Rahnella	*variigena*	100	0,0	99,42	WP_159282139.1
emrB_3	Multidrug export protein EmrB	MULTISPECIES: DHA2 family efflux MFS transporter permease subunit	*Rahnella*	*MULTI*	100	0,0	99,81	WP_112290238.1
		DHA2 family efflux MFS transporter permease	*Rahnella*	*variigena*	100	0,0	99,43	WP_159282137.1
			Rahnella	*variigena*	100	0,0	99,51	WP_120163281.1
emrD	Multidrug resistance protein D	Multidrug efflux MFS transporter EmrD	*Rahnella*	*aquatilis*	100	0,0	99,50	WP_113877618.1
			Rahnella	*variigena*	100	0,0	99,50	WP_120163793.1
emrE_1	Multidrug transporter EmrE	MULTISPECIES: multidrug DMT transporter	*Rahnella*	*MULTI*	100	8E-71	99,10	WP_113876495.1
		Multidrug DMT transporter	*Rahnella*	*sp. AN3-3W3*	100	4E-70	98,20	WP_112289647.1
emrE_2	Multidrug transporter EmrE	MULTISPECIES: QacE family quaternary ammonium compound efflux SMR transporter	*Rahnella*	*MULTI*	100	2E-68	100	WP_113878269.1
emrK_1	Putative multidrug resistance protein EmrK	MULTISPECIES: HlyD family secretion protein	*Rahnella*	*Multi*	99	0,0	100	WP_112290239.1
emrK_2	Putative multidrug resistance protein EmrK	Multidrug export protein EmrA	*Rahnella*	*aquatilis*	100	0,0	100	RBQ33798.1
		MULTISPECIES: HlyD family efflux transporter periplasmic adaptor subunit	*Rahnella*	*MULTI*	100	0,0	100	WP_159281632.1
emrK_3	Putative multidrug resistance protein EmrK	Membrane fusion protein (multidrug efflux system)/multidrug resistance protein K	*Rahnella*	*sp. Jub53*	100	0,0	99,21	TCQ86898.1
		HlyD family efflux transporter periplasmic adaptor subunit	*Rahnella*	*sp. FC061912-K*	100	0,0	98,69	WP_217204451.1
			Rahnella	*variigena*	95	0,0	99,18	WP_120349702.1

emrY_1	Putative multidrug resistance protein EmrY	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,81	WP_120160584.1
emrY_2	Putative multidrug resistance protein EmrY	MULTISPECIES: DHA2 family efflux MFS transporter permease subunit	*Rahnella*	*MULTI*	100	0,0	100	WP_113876270.1
		DHA2 family efflux MFS transporter permease subunit	*Rahnella*	*sp AN3-3W3*	100	0,0	99,80	WP_112289461.1
			Rahnella	*variigena*	100	0,0	99,61	WP_120349665.1
fsr	Fosmidomycin resistance protein	MULTISPECIES: MFS transporter	*Rahnella*	*MULTI*	100	0,0	100	WP_120160753.1
		MFS transporter	*Rahnella*	*aquatilis*	100	0,0	99,75	WP_113876119.1
			Rahnella	*variigena*	100	0,0	99,75	WP_129638333.1
hns_1	DNA-binding protein H-NS	MULTISPECIES: H-NS histone family protein	*Rahnella*	*MULTI*	100	8E-93	100	WP_112287421.1
		H-NS histone family protein	*Rahnella*	*woolbedingensis*	100	2E-91	98,52	WP_120134793.1
hns_2	DNA-binding protein H-NS	MULTISPECIES: DNA.binding transcriptional regulator H-NS	*Yersiniaceae*	*MULTI*	100	1E-91	100	WP_013575950.1
			Rahnella	*MULTI*	100	2E-91	99,26	WP_120130915.1
			Rahnella	*unclassified*	100	2E-91	99,26	WP_112152537.1
			Ewingella	*americana*	100	7E-91	98,52	WP_034791036.1
iprA_1	inhibitor of hydrogen peroxide resistance	winged helix-turn-helix DNA binding protein	*Rahnella*	*sp. Jub53*	100	4E-172	99,58	TCQ90629.1
		MULTISPECIES: helix-turn-helix doamin-containing protein	*Rahnella*	*MULTI*	90	8E-157	100	WP_112287603.1
			Rahnella	*variigena*	90	2E-156	99,53	WP_129639414.1
iprA_2	inhibitor of hydrogen peroxide resistance	winged helix-turn-helix transcriptional regulator	*Rahnella*	*variigena*	100	2E-109	99,35	WP_120160306.1
iprA_3	Inhibitor of hydrogen peroxide resistance	helix-turn-helix domain-containing protein	*Rahnella*	*variigena*	100	2E-161	100	WP_120161898.1

iprA_4	inhibitor of hydrogen peroxide resistance	winged helix-turn-helix transcriptional regulator	*Rahnella*	*variigena*	100	1E-152	100	WP_120162504.1
mexA	Multidrug resistance protein MexA	Membrane fusion protein (multidrug efflux system)	*Rahnella*	*sp. NFIX50*	100	0,0	96,92	RAR90611.1
		MULTISPECIES: efflux RND transporter periplasmic adaptor subunit	*Rahnella*	*MULTI*	96	0,0	99,73	WP_120163073.
			Rahnella	*variigena*	96	0,0	99,20	WP_159282553.1
mexB	Multidrug resistance protein MexB	MULTISPECIES: efflux RND transoorter permease subunit	*Rahnella*	*MULTI*	100	0,0	99,90	WP_132967302.1
			Rahnella	*variigena*	100	0,0	99,81	WP_120163072.1
mdfA_1	Multidrug transporter MdfA	DHA1 family multidrug/chloramphenicol efflux transport protein-like MFS trasnporter	*Rahnella*	*sp. JUb53*	100	0,0	100	TCQ86207.1
		MULTISPECIES: MFS transporter	*Rahnella*	*MULTI*	100	0,0	99,75	WP_113876873.1
			Rahnella	*variigena*	100	0,0	99,49	WP_120163145.1
mdfA_2	Multidrug transporter MdfA	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,26	WP_120162831.1
mdlB	Multidrug resistance-like ATP-binding protein MdiB	SmdB family multidrug ellfux ABC transporter permease/ATP-binding protien	*Rahnella*	*aquatilis*	100	0,0	99,83	WP_113876137.1
mdtA_1	Multidrug resistance protein MdtA	Efflux RND transporter periplasmic adaptor subunit	*Rahnella*	*variigena*	100	0,0	99,45	WP_120163282.1
mdtA_2		MdtA/MuxA family multidrug efflux RND transporter periplasmic adaptor subunit	*Rahnella*	*variigena*	100	0,0	100	WP_159282974.1
mdtB	Multidrug resistance protein MdtB	Efflux RNDS transporter permease subunit	*Rahnella*	*sp AN3-3W3*	100	0,0	99,72	WP_112290331.1
			Rahnella	*sp. Jub53*	100	0,0	99,62	WP_132967311.1
			Rahnella	*aquatilis*	100	0,0	99,62	WP_113877558.1
			Rahnella	*variigena*	100	0,0	99,44	WP_159282973.1

mdtC	Multidrug resistance protein MdtC	MULTISPECIES: efflux RND trasnporter permease subunit	*Rahnella*	*MULTI*	100	0,0	100	WP_129638894.1
			Rahnella	*aquatilis*	100	0,0	100	WP_113877559.1
			Rahnella	*variigena*	100	0,0	99,71	WP_120163734.1
mdtD_1	Putative multidrug ressitance protein MdtD	MFS transporter	*Rahnella*	*variigena*	100	0,0	100	WP_120161090.1
mdtD_2		Multidrug transporter subunit MdtD	*Rahnella*	*variigena*	100	0,0	99,59	WP_120161587.1
mdtH_2	Multidrug resistance protein MdtH	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,03	WP_120162928.1
mdtK	Multidrug resistance protein MdtK	MATE family efflux transporter	*Rahnella*	*variigena*	100	0,0	99,78	WP_120161793.1
mdtL	Multidrug resistance protein MdtL	DHA1 family bicyclomycin/chloramphenicol resistance-like MFS transporter	*Rahnella*	*sp. JUb53*	100	0,0	99,50	TCQ93660.1
		Multidrug efflux MFS transporter	*Rahnella*	*variigena*	99	0,0	99,75	WP_120162890.1
oqxB17	Multidrug efflux RND transporter permease subunit OqxB17		*Rahnella*	*variigena*	100	0,0	100	WP_120163574.1
ohrB	Organic hydroperoxide resistance protein OhrB	MLUTISPECIES: organic hydroperoxide resistance protein	*Rahnella*	*MULTI*	100	7E-96	100	WP_112291684.1
			Yersiniaceae	*MULTI*	100	1E-95	99,29	WP_013573432.1
			Rahnella	*variigena*	100	3E-95	99,29	WP_129637735.1
ohrR	Organic hydroperoxide resistance transcriptional regulator	MULTISPECIES: MarR family transcriptional regulator	*Rahnella*	*MULTI*	98	9E-105	99,34	WP_112291682.1
			Rahnella	*variigena*	98	3E-104	98,68	WP_120163171.1
			Yersiniaceae		98	1E-103	98,03	WP_152323669.1
pasT	Persistance and stress-ressitance toxin PasT	MULTISPECIES: type II toxin-antitoxin system RatA family toxin	*Rahnella*	*MULTI*	100	1E-101	100	WP_013576738.1

phoQ	Virulence sensor histidine kinase PhoQ	MULTISPECIES: two-component system sensor histidine kinase PhoQ	*Rahnella*	*MULTI*	100	0,0	100	WP_120160656.1
rcnB	Nickel/cobalt homeostasis protein RcnB	RcnB family protein	*Rahnella*	*variigena*	100	4E-72	100	WP_120163659.1
smvA	Methyl viologen resistance protein SmvA	MFS transporter	*Rahnella*	*aquatilis*	100	0,0	99,8	WP_113876442.1
			Rahnella	*variigena*	100	0,0	99,6	WP_159281768.1
stp	Multidrug resistance protein Stp	MFS transporter	*Rahnella*	*variigena*	100	0,0	98,78	WP_120162731.1
tdeA_1	Toxin and drug export protein A	Efflux trasnporter outer memebrane subunit	*Rahnella*	*variigena*	100	0,0	99,78	WP_120162740.1
tdeA_2			*Rahnella*	*variigena*	100	0,0	99,56	WP_120163304.1
yaaY	Peroxide stress resitance protein YaaY	Peroxide stress protein YaaY	*Rahnella*	*variigena*	100	0,0	99,61	WP_120161962.1
ybhR	Putative multidrug ABC transporter permease YbhR	ABC transporter permease	*Rahnella*	*aquatilis*	100	0,0	99,74	RBQ34680.1
yheI	Putative multidrug resistance ABC transporter ATP-binding/permease protein YheI	SmdA family multridrug ABS trasnporter permease/ATP-binding protein	*Rahnella*	*aquatilis*	100	0,0	100	WP_120160786.1
Putative multidrug export ATP-binding/permease protein		ABC transporter ATP-binding protein/permease	*Rahnella*	*variigena*	100	0,0	99,84	WP_120163500.1
Putative multidrug export ATP-binding/permease protein		MULTISPECIES: ABC trasnporter ATP-binding protein/permease	*Rahnella*	*MULTI*	100	0,0	100	WP_120162994.1

Putative multidrug-efflux transporter	MFS transporter	*Rahnella*	*variigena*	100	0,0	99,58	WP_120160465.1

Appendix 9.11. Prokka and BLAST Results for Isolate KB3

Table 9.10. Genes annotated by Prokka and subsequent BLAST results for isolate KB3 (H. huttiense)

PROKKA		BLAST						
Gene	**Product**	**BLAST Description**	**Genus**	**Strain**	**Query Cover**	**E value**	**% Identity**	**Accesion (NCIB)**
abaF	Fosfomycin resistance protein AbaF	MFS transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	100	WP_134223223.1

abaQ	Quinolone resistance transporter	MFS transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,77	MAF05889.1
		Helix-turn-helix transcriptional regulator	*Herbaspirillum*	*huttiense*	*100*	0,0	99,32	MBN9357274.1
acrB_1	Multidrug efflux pump subunit AcrB	Multidrug efflux RND transporter permease subunit	*Herbaspirillum*	*sp.*	*100*	0,0	99,62	MAF02819.1
		Multidrug efflux pump	*Herbaspirillum*	*huttiense*	*100*	0,0	99,52	MBM7748879.1
		Efflux RND transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,43	WP_039789377.1
acrB_2	Multidrug efflux pump subunit AcrB	Efflux RNS transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,71	WP_134221742.1
acrB_3	Multidrug efflux pump subunit AcrB	Multidrug efflux RND transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,71	WP_039784065.1
acrF	Multidrug export protein AcrF	Hydrophobe/amphiphile efflux-1 family RND transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,90	MBO14352.1
		Efflux RND transporter permease subunit	*Herbaspirillum*	*sp. 1130*	*100*	0,0	98,81	WP_121041169.1
		Efflux RND transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,81	WP_134223864.1
ampG	Anhydromuropeptide permease	AmpG family muropeptide MFS transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,52	WP_039790490.1
ampR	HTH-type transcriptional activator AmpR	LysR family transcriptional regulator	*Herbaspirillum*	*huttiense*	*100*	0,0	99,3	WP_134223023.1
arnA_1	Bifunctional polymyxin resistance protein ArnA	MULTISPECIES: bifunctional UDP-4-keto-pentose/UDP-xylose synthase	*Herbaspirillum*	*MULTI*	*100*	0,0	100	WP_051057356.1
		Bifunctional UDP-4-keto-pentose/UDP-xylose synthase	*Herbaspirillum*	*huttiense*	*100*	0,0	99,15	WP_051156567.1
arnA_2	Bifunctional polymyxin resisatnce protein ArnA	Formyltransferase	*Herbaspirillum*	*huttiense*	*100*	0,0	100	WP_134222219.1

arnB	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	DegT/DnrJ/EryC1/StrS aminotransferase family protein	*Herbaspirillum*	*huttiense*	*100*	0,0	100	WP_134135257.1
arnC_1	Undecaaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	MULTISPECIES: glycosyltransferase	*Herbaspirillum*	*MULTI*	*100*	0,0	100	WP_034330258.1
arnC_2	Undecaaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	Glycosyl transferase	*Herbaspirillum*	*sp.*	*100*	0,0	99,61	MAF02336.1
		Glycosyl transferase family 2 protein	*Herbaspirillum*	*huttiense*	*100*	0,0	99,45	WP_039787296.1
arnD	4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase ArnD	Xylanase	*Herbaspirillum*	*huttiense*	*85*	0,0	99,68	MBN9358561.1
arnF	a-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippse subunit ArnF	MULTISPECIES: EamA family transporter	*Herbaspirillum*	*MULTI*	*100*	1E-78	100	WP_121039800.1
		EamA family transporter	*Herbaspirillum*	*huttiense*	*100*	5E-78	99,19	WP_039788410.1
arnT_1	Undecaprenyl phosphate-alpha-4-amino-4-deoxy-L-arabinose arabinosyl transferase	Glycosyltransferase family 39 protein	*Herbaspirillum*	*huttiense*	*100*	0,0	99,65	WP_039788408.1
arnT_2		Glycosyl trasnferase	*Herbaspirillum*	*huttiense*	*95,00*	0,0	99,66	WP_134223399.1
arpC	Antibiotic efflux pump outer membrane protein ArpC	RND transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,39	MAF06004.1
		Efflux transporter outer membrage subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	98,78	WP_134221104.1
arsB_1	Arsenical pump membrane protein	Arsenic transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,53	MBN9358022.1

arsB_2	Arsenical pump membrane protein	Arsenic transporter	*Herbaspirillum*	*robiniae*	*100*	7E-147	95,13	WP_088752394.1
		MULTISPECIES: Arsenic transporter	*Herbaspirillum*	*MULTI*	*100*	1E-145	94,25	WP_050470660.1
		Arsenic transporter	*Herbaspirillum*	*huttiense*	*100*	5E-121	80,09	MBN9358022.1
arsB_3	Arsenical pump membrane protein	MULTISPECIES: Arsenic transporter	*Herbaspirillum*	*MULTI*	*100*	0,0	99,06	WP_077236030.1
		Arsenic transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	94,13	WP_039784425.1
arsC_1	Arsenate reductase	Arsenate reductase (glutaredoxin)	*Herbaspirillum*	*huttiense*	*100*	1E-79	98,33	WP_134221288.1
arcC_2	Arsenate reductase	Arsenate reductase (glutaredoxin)	*Herbaspirillum*	*huttiense*	*100*	2E-98	99,29	WP_134221933.1
			Herbaspirillum	*huttiense*	*100*	9E-98	98,58	WP_039790389.1
arsC_3	Arsenate reductase	MULTISPECIES: arsenate reductase ArsC	*Herbaspirillum*	*MULTI*	*100*	8E-124	100	WP_008328786.1
arsC_4	Arsenate reductase	MULTISPECIES: arsenate reductase (glutaredoxin)	*Herbaspirillum*	*unclassified*	*100*	6E-96	96,48	WP_008330634.1
			Herbaspirillum	*MULTI*	*100*	2E-95	95,77	WP_088756039.1
		Arsenate reductase (glutaredoxin)	*Herbaspirillum*	*huttiense*	*97*	1E-84	88,49	WP_134221933.1
arsR	Arsenical ressiatnce operon repressor	Helix-turn-helix transcriptional regulator	*Herbaspirillum*	*sp. CAH-3*	*100*	2E-74	99,1	MRT29961.1
		MULTISPECIES: metalloregulator ArsR/SmtB family transcription factor	*Herbaspirillum*	*unclassified*	*100*	2E-74	99,1	WP_174616829.1
arsR		Metalloregulator ArsR/SmtB family transcription factor	*Herbaspirillum*	*huttiense*	*99*	4E-70	94,55	WP_039784426.1
bcr_1	Bicyclomycin resistance protein	DHA1 family 2-module integral membrane pump EmrD-like MFS transporter	*Herbaspirillum*	*sp. 1130*	*100*	0,0	99,3	MBP1313523.1
		Bcr/ClfA family drug resistance efflux transporter	*Herbaspirillum*	*sp.*	*98*	0,0	99,52	MAF00956.1
		Multidrug efflux MFS transporter	*Herbaspirillum*	*huttiense*	*98*	0,0	99,29	WP_051156371.1

bcr_2	Bicyclomycin resistance protein	Bcr/ClfA family drug resistance efflux transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	100	WP_134223927.1
bla	β -lactamase Toho-1	Class A β -lactamase	*Herbaspirillum*	*huttiense*	*100*	0,0	99,33	WP_134223024.1
bmr3	Multidrug resistance protein 3	MFS transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	100	MBN9356030.1
copA_1	Putative copper-importing P-type ATPase A	Heavy metal translocating P-type ATPase	*Herbaspirillum*	*huttiense*	*100*	0,0	98,93	WP_039787698.1
copA_2	Putative copper-importing P-type ATPase A	Copper-translocating P-type ATPase	*Herbaspirillum*	*sp.*	*100*	0,0	98,33	MAF05634.1
		Heavy metal translocating P-type ATPase	*Herbaspirillum*	*huttiense*	*100*	0,0	97,61	MBN9359470.1
copD	Copper resistance protein D	Putative copper resistance protein D	*Herbaspirillum*	*sp. 1273*	*100*	2E-139	96,23	MBP1329972.1
		Copper homeostasis membrane protein CopD	*Herbaspirillum*	*huttiense*	*100*	9E-135	92,92	WP_039785766.1
		Copper resistance protein CopD	*Herbaspirillum*	*sp.*	*100*	1E-134	92,92	MAF00911.1
corA_1	Magnesium transport protein CorA	MULTISPECIES: magnesium/cobalt transporter CorA	*Herbaspirillum*	*MULTI*	*100*	0,0	99,69	WP_039789235.1
			Herbaspirillum	*unclassified*	*100*	0,0	98,75	WP_008334694.1
corA_2	Cobalt/magnesium transport protein CorA	Magnesium and cobalt transport protein CorA	*Herbaspirillum*	*huttiense*	*100*	0,0	100	WP_039786094.1
corC	Magnesium and cobalt efflux protein CorC	Magnesium/cobalt efflux protein	*Herbaspirillum*	*sp.*	*100*	0,0	100	MAF04318.1
		CBS domian-containing protein	*Herbaspirillum*	*huttiense*	*100*	0,0	99,66	MBN9358591.1
czcA_1	Cobalt-zinc-cadmium resistance protein CzcA	cusA/CzcA family heavy metal efflux RND transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,81	WP_134222447.1

czcA_2	Cobalt-zinc-cadmium resistance protein CzcA	Cobalt-zinc-cadium resistance protein CzcA	*Herbaspirillum*	*huttiense*	*100*	0,0	99,21	MBM7750599.1
		cusA/CzcA family heavy metal efflux RND transporter	*Herbaspirillum*	*huttiense*	*99*	0,0	99,24	WP_134223747.1
czcA_3	Cobalt-zinc-cadmium resistance protein CzcA	Efflux RND transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,91	WP_039783041.1
czcB	Cobalt-zinc-cadmium resistance protein CzcB	Cobalt-zinc-cadmium resistance protein CzcB	*Herbaspirillum*	*sp. 1273*	*100*	0,0	97,39	MBP1330945.1
		Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	96,48	WP_134223748.1
czcC	Cobalt-zinc-cadmium resistance protein CzcC	TolC family protein	*Herbaspirillum*	*huttiense*	*100*	0,0	98,6	WP_134223749.1
		Cobalt-zinc-cadmium resistance protein	*Herbaspirillum*	*sp.*	*100*	0,0	98,36	MAF04061.1
		Cobalt-zinc-cadmium efflux system outer membrane protein	*Herbaspirillum*	*huttiense*	*100*	0,0	98,13	MBM7750597.1
		Cobalt transporter	*Herbaspirillum*	*sp. 1130*	*100*	7E-80	98,37	WP_209569395.1
czcD	Cadium, cobalt and zinc/H(+)-K(+) antiporter	Cation transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,69	MAF01615.1
		CDF family Co(II)/Ni(II) efflux transporter DmeF	*Herbaspirillum*	*huttiense*	*99*	0,0	99,69	WP_134222193.1
czcI	Cobalt-zinc-cadmium resistance protein CzcI	Cobalt transporter	*Herbaspirillum*	*sp. 1130*	*100*	7E-80	98,37	WP_209569395.1
		Cobalt transporter	*Herbaspirillum*	*huttiense*	*100*	1E-79	97,56	MBN9358891.1
czcR_1	Transcriptional activator protein CzcR	MULTISPECIES: heavy metal response regulator transcription factor	*Herbaspirillum*	*MULTI*	*100*	2E-157	100	WP_034331353.1
		Heavy metal response regulator transcription factor	*Herbaspirillum*	*huttiense*	*99*	2E-148	93,24	WP_134221039.1

czcR_2	Transcriptional activator protein CzcR	MULTISPECIES: heavy metal response regulator transcription factor	*Herbaspirillum*	*MULTI*	*100*	1E-164	99,57	WP_034341551.1
		Heavy metal response regulator transcription factor	*Herbaspirillum*	*huttiense*	*100*	5E-164	98,7	WP_134222503.1
czcR_3	Transcriptional activator protein CzcR	Heavy metal response regulator transcription factor	*Herbaspirillum*	*huttiense*	*100*	9E-159	99,55	WP_121042020.1
czcS_1	Sensor protein CzcS	Heavy metal sensor histidine kinase	*Herbaspirillum*	*huttiense*	*100*	0,0	99,57	WP_039784133.1
czcS_2	Sensor protein CzcS	Two-component sensor histidine kinase	*Herbaspirillum*	*sp.*	*100*	0,0	99,59	MAF03860.1
		Heavy metal sensor histidine kinase	*Herbaspirillum*	*huttiense*	*100*	0,0	99,18	WP_134222817.1
emrA_1	Multidrug export protein EmrA	Membrane fusion protein (multidrug efflux system)	*Herbaspirillum*	*huttiense*	*100*	0,0	99,76	MBM7749905.1
		MULTISPECIES: HlyD family efflux transporter periplasmic adaptor subunit	*Herbaspirillum*	*MULTI*	*100*	0,0	99,28	WP_121041638.1
emrA_2	Colistin resistance protein EmrA	HlyD family secretion protein	*Herbaspirillum*	*huttiense*	*100*	0,0	99,75	WP_039784835.1
		Multidrug ABC transporter permease	*Herbaspirillum*	*sp.*	*100*	0,0	99,25	MAF05618.1
		Membrane fusion protein (multidrug efflux system)	*Herbaspirillum*	*huttiense*	*100*	0,0	98,25	MBM7747812.1
emrB_1	Colistin resistance protein EmrB	Multidrug efflux MFS transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,81	WP_039784836.1
		DHA2 family multidrug resistance protein	*Herbaspirillum*	*huttiense*	*100*	0,0	99,43	MBM7747811.1
emrB_2	Multidrug export protein EmrB	DHA2 family MFS transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,24	MBN9358436.1
emrE	Multidrug transporter EmrE	MULTISPECIES: EamA family transporter	*Herbaspirillum*	*MULTI*	*100*	9E-70	100	WP_034335425.1

		MULTISPECIES: QacE family quaternary ammonium compound efflux SMR transporter	*Herbaspirillum*	*unclassified*	*100*	1E-68	99,09	WP_145605627.1
		EamA family transporter	*Herbaspirillum*	*huttiense*	*100*	1E-68	98,18	WP_039783026.1
emrK	Putative multidrug resistance protein EmrK	HlyD family efflux transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,51	WP_198516449.1
		EmrA/EmrK family multidrug efflux transporter periplasmic adaptor subunit	*Herbaspirillum*	*sp.*	*100*	0,0	99,27	MAF02677.1
fsr	Fosmidomycin resisatnce protein	FSR family fosmidomycin resistance protein-like MFS trasnporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,76	MBM7746126.1
		MFS transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,52	MAF04605.1
mdtA_1	Multidrug resistance protein MdtA	RND family efflux transporter MFP subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	97,42	MBM7749129.1
		Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*93*	0,0	99,45	WP_134224001.1
mdtA_2	Multidrug resistance protein MdtA	Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,00	WP_134221132.1
mdtA_3	Multidrug resistance protein MdtA	MdtA/MuxA family multidrug efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,43	MBN9356243.1
		Multidrug transporter subunit MdtA	*Herbaspirillum*	*sp.*	*100*	0,0	99,05	MAF05025.1
mdtA_4	Multidrug resistance protein MdtA	Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,44	WP_034336494.1
mdtA_5	Multidrug resistance protein MdtA	Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,74	MBN9356854.1

mdtA_6	Multidrug resistance protein MdtA	Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,52	WP_051156429.1
mdtA_7	Multidrug resistance protein MdtA	Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*sp.*	*100*	0,0	99,02	MBO14346.1
		RND family efflux transporter MFP subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	98,77	MBM7750383.1
		Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	98,77	WP_039783043.1
mdtB_1	Multidrug resistance protein MdtB	Multidrug transporter subunit MdtB	*Herbaspirillum*	*sp.*	*100*	0,0	99,52	MAF05026.1
		MdtB/MuxB family multidrug efflux RND transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,43	WP_134221858.1
		Multidrug efflux pump	*Herbaspirillum*	*huttiense*	*100*	0,0	99,62	MBM7746773.1
mdtB_2	Multidrug resistance protein MdtB	HAE1 family hydrophobic/amphiphilic exporter-1	*Herbaspirillum*	*sp. 1273*	*100*	0,0	99,81	MBP1329815.1
		Efflux RND transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,90	WP_134222502.1
		Acriflavine resistance protein B	*Herbaspirillum*	*sp.*	*100*	0,0	99,71	MAF02747.1
mdtC_1	Multidrug resistance protein MdtC	Multidrug transporter subunit MdtC	*Herbaspirillum*	*sp.*	*100*	0,0	99,63	MAF05027.1
		Caun (trimmed reads) of trimmed B8 - 39 exact matches found	*Herbaspirillum*	*huttiense*	*100*	0,0	99,63	MBN9356241.1
		Multidrug efflux pump	*Herbaspirillum*	*huttiense*	*100*	0,0	99,63	MBM7746774.1
mdtC_2	Multidrug resistance protein MdtC	MMPL family transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	100	WP_134222716.1
mdtC_3		RND transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,62	MAF01784.1

	Multidrug resistance protein MdtC	MMPL family transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,27	MBN9359326.1
mdtD	Putative multidrug resistance protein MdtD	DHA2 family methylenomycin A resistance protein-like MFS transporter	*Herbaspirillum*	*huttiense*	*90*	0,0	99,16	MBM7746140.1
		MFS transporter	*Herbaspirillum*	*huttiense*	*85*	0,0	99,78	WP_084320369.1
mdtK	Multidrug resistance protein MdtK	MATE family efflux transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,78	MAF02568.1
			Herbaspirillum	*huttiense*	*100*	0,0	99,57	WP_134221432.1
mexA_1	Multidrug resisatnce protein MexA	Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,49	WP_134221741.1
		Membrane fusion protein (multidrug efflux system)	*Herbaspirillum*	*huttiense*	*100*	0,0	98,98	MBM7746550.1
mexA_2	Multidrug resisatnce protein MexA	Membrane fusion protein (multidrug efflux system)	*Herbaspirillum*	*huttiense*	*97*	0,0	97,92	MBM7750388.1
		Efflux RND transporter periplasmic adaptor subunit	*Herbaspirillum*	*huttiense*	*91*	0,0	98,53	MBN9356534.1
LnrL	Linearmycin resisatnce ATP-binding protein LnrL	ABC transporter ATP-binding protein	*Herbaspirillum*	*huttiense*	*100*	1E-179	99,81	WP_134222320.1
ohrR	Organic hydroperoxide resistance transcriptional regulator OhrR	MULTISPECIES: MarR family transcriptional regulator	*Herbaspirillum*	*MULTI*	*100*	4E-101	99,35	WP_039786512.1
ohrB	Organic hydroperoxide resistance transcriptional regulator OhrB	MULTISPECIES: organic hydroperoxide resisatnce protein	*Herbaspirillum*	*MULTI*	*100*	2E-98	100	WP_039785041.1
oqxB27	Multidrug efflux RND transporter permease subunit OqxB27	MULTISPECIES: multidrug efflux RND transporter permease subunit	*Herbaspirillum*	*MULTI*	*100*	0,0	99,81	WP_088755841.1

		Multidrug efflux RND transporter permease subunit	*Herbaspirillum*	*huttiense*	*100*	0,0	99,81	WP_134221131.1
pasT	Persistence and stress-resistance toxin PasT	MULTISPECIES: type II toxin-antitoxin system RatA family toxin	*Herbaspirillum*	*MULTI*	*100*	2E-103	100	WP_034336959.1
		Type II toxin-antitoxin system RatA family toxin	*Herbaspirillum*	*huttiense*	*100*	4E-103	99,30	WP_039788242.1
tdeA	Toxin and drug export protein A	Efflux transporter outer membrane subunit	*Herbaspirillum*	*sp. 1130*	*98*	0,0	98,28	WP_121039737.1
		NodT family efflux transporter outer membrane facotr (OMF) lipoprotein	*Herbaspirillum*	*huttiense*	*98*	0,0	98,28	MBM7747322.1
		RND transporter	*Herbaspirillum*	*sp.*	*90*	0,0	98,28	MBO17616.1
yaaA	Peroxide stress resisatnce protein YaaA	MULTISPECIES: peroxide stress protein YaaA	*Herbaspirillum*	*MULTI*	*100*	0,0	100	WP_121041951.1
		Peroxide stress protein YaaA	*Herbaspirillum*	*huttiense*	*100*	0,0	99,61	MBN9355655.1
yheS	Putative ABC transporter ATP-binding protein YheS	ATP-binding cassette domain-containing protein	*Herbaspirillum*	*huttiense*	*100*	0	100	WP_134222257.1
Putative multidrug export ATP-binding/permease protein		ABC transporter	*Herbaspirillum*	*sp.*	*100*	0,0	99,84	MAF04762.1
		ATP-binding cassette domain-containing protein	*Herbaspirillum*	*huttiense*	*100*	0,0	99,67	MBN9357608.1
Putative multidrug-efflux transporter		MFS transporter	*Herbaspirillum*	*huttiense*	*100*	0,0	99,16	MBN9359609.1
Putative MFS-type transporter		MFS transporter	*Herbaspirillum*	*huttiense*	*99,00*	0,0	97,83	WP_134223251.1

Table 9.11. Definitions of genes and their products found by Prokka, defined by CARD or UniProtKB.

Gene/ Product	Information source (Accession)	Product Function	AMR/Transporter Family/Domain/Type	Resistome with perfect match (sequence variants)
abaF	UniProtKB – P0DPR5 CARD ARO:3004573	Efflux pump that mediates Fosfomycin resistance Likely involved in biofilm matrix secretion, contributing to pathogenicity (Sharma et al., 2016)	MFS	*Acinetobacter baumannii (Pseudomonas)*

abaQ	UniProt KB – P0DPR4 CARD ARO:3004574	Efflux pump that mediates quinolone-type antibiotic resistance (Pérez-Varela et al., 2018). It is mainly involved in quinolone-type drug extrusion in *A. baumanni*	MFS	*Acinetobacter baumannii (Pseudomonas)*
AcrA-ArcB-ArcZ-TolC complex ARO:3004082		Drug efflux protein complex with broad substrate specificity, using the PMF to export substrates Confers resistance to tetracyclines, penam, rifamycin, glycylcycline, cephalosporin, phenicol, triclosan and fluoroquinolones		
acrA	UniProtKB – P0AE06 CARD ARO:3000207	Protein subunit representing the periplasmic portion	RND antibiotic efflux pump (MFP family)	*E. coli (Pseudomonas)*
acrB	UniProtKB – P31224 CARD ARO:3000216	Protein subunit that functions as a herterotrimer that forms the inner membrane component responsible for substrate recognition and energy transduction via antiport	RND	*Escherichia coli (Pseudomonas)*
acrZ	UniProtKB – P0AAX1	Binds to AcrB. Required for efflux of some (not all) substrates, thus possibly influencing drug transport specify (Hobbs et al., 2012)	AcrZ family protein	*E. coli*
TolC	CARD - ARO:3000237	A OMP subunit of several multidrug efflux pumps in Gram-negative bacteria. Efflux regulation is often at this periplasmic opening by other complex components. Confers resistance to macrolides, penem, carbapenem and fluoroquinolones, amongst others.	Subunit in RND, MFS and ABC antibiotic efflux pump	
AcrEF-TolC tripartite CARD ARO:3004083		Efflux pump system that confers resistance to fluoroquinolones (ciprofloxacin), cephamycin, cephalosporin and penam (Lau & Zgurskaya, 2005). Efflux of indole and organic solvents (Kawamura-Sato et al., 1999)		
acrE	UniProtKB – P24180 CARDARO:3000499	Periplasmic MFP (Kawamura-Sato et al., 1999; Lau & Zgurskaya, 2005) similar to AcrA	RND (MFP family)	*E. coli*
acrF	UniProtKB – P24181 CARD ARO:3000502	Inner membrane transporter (Kawamura-Sato et al., 1999; Lau & Zgurskaya, 2005) similar to AcrB	RND family	*E. coli (Pseudomonas)*
arnE	UniprotKB - Q47377	Small transporter with similar topological features as EmrE (Larrouy-Maumus et al., 2012). Transports alpha-L-Ara4n-phosphoundecaprenol from the	ArnE family	*E. coli*
arnF	UniprotKB - P76474		ArnF family	*E. coli*

		cytoplasm to the periplasm and confers polymyxin resistance in *E. coli (Yan et al., 2007)*.		
arnT	CARD ARO:3005053	Involved in cell wall biosynthesis, specifically Ara4N, conferring resistance to peptide antibiotics (Bialek-Davenet et al., 2014)	PMR phosphoethanolamine transferase	*Klebsiella pneumonia*
arpABC operon		Pump that confers resistance to various antibiotics such as carbenicillin, chloramphenicol, novobiocin, streptomycin and tetracycline (Kieboom & de Bont, 2001)		
arpB	UniprotKB - Q9KJC2	Inner membrane component of an antibiotic efflux pump that confers resistance to various antibiotics such as carbenicillin, chloramphenicol, novobiocin, streptomycin and tetracycline (Kieboom & de Bont, 2001)	RND family	*Pseudomonas putida*
arpC	UniProtKB - Q9KJC1	Outer membrane component of an antibiotic efflux pump that confers resistance to various antibiotics such as carbenicillin, chloramphenicol and tetracycline (amongst others) (Kieboom & de Bont, 2001)	RND family	*Pseudomonas putida*
bcr	CARD ARO:3003801	Transmembrane protein that extrudes bicyclomycin from the cell, conferring resistance (Fonseca et al., 2015)	MFS	*P. aeruginosa* *P. fluorescens*
bmr3	CARD ARO:3003007	MFS pump that confers resistance to fluroquinolones, nucleosides and phenicols	MFS antibiotic efflux pump	*Bacillus subtillis* *Bacillus tequilensis*
crnA	UniProtKB - P37972	Products of genes crnA, cnrB and cnrC likely form a membrane protein complex that confers nickle and cobalt resistance via efflux	RND family	*Cupriavidus metallidurans*
corC	UniProtKB - P0AE78	Magnesium and cobalt efflux protein	UPF0053	*E. coli*
crp	CARD ARO:3000518	Global regulator that represses MdtEF multidrug efflux pump expression conferring resistance to macrolides, penam and fluoroquinolones (Nishino et al., 2008)	RND antibiotic efflux pump	*E. coli* *(P. aeruginosa)*
Czc proteins		Confer cobalt-zinc-cadmium resistance through efflux activity		

czcA	UniProtKB - P13511	Essential for the expression of cobalt, zinc and cadmium resistance. Effective efflux of zinc with CzcB alone (Diels et al., 1995)	RND family	*Cupriavidus metallidurams*
czcB	UniProtKB - P13510	Seems to funnel zinc cations to CzcA	MFP family	*C. metallidurams*
czcC	UniprotKB - P13509	Seems to modify the Czc system specificity, possibly by affecting the CzcB protein. The efflux system gains specifity for cadmium and cobalt when CzcC binds to CzcA and CzcB (Diels et al., 1995)	OMF/	*C. metallidurams*
czcD	UniprotKB - A0A0C7CYD1	The protein CzcD facilitates a metal cation efflux system. Mediates low level metal ion resistance likely my effluc from the cytoplasm into the periplasm. Mediates cobalt, cadmium and zinc resistance by regulation of the Czc system, possibly represses expression by exporting inducing cations. Binds and transports zinc, but can also bind cobalt, copper and nickel (Anton et al., 1999)	OMF/CDF transporter family	*P. aeruginosa*
czcI	UniprotKB - Q44009	Component of the Czc cation-efflux system with a possible regulatory function	Periplasmic protein	*C. metallidurams*
czcR	UniprotKB - Q44006	Transcriptional activator protein. The CzcS/CzcR regulatory system controls cobalt, zinc and cadmium homeostasis	Transmembrane protein	*C. metallidurams*
czcS	UniprotKB - Q44007	Sensor protein. Seems to activate CzcR by phosphorylation	Trnasmembrane protein	*C. metallidurams*
EmrAB-TolC CARD ARO:3000344		Multidrug efflux system in E. coli that confers resistance to fluoroquinolones (nalidixic acid and thiolactomycin) (Lomovskaya et al., 1995)	MFS	
emrA	CARD ARO:3000027	The linker	MFP/MFS	*E. coli*
emrB	CARD ARO:3000074	The electrochemical-gradient powered translocase	MFS	*E. coli*
emrD	CARD ARO:3000309	Multidrug transporter primarily found in E. coli that couples efflux of amphipathic compounds and proton import across the plasma membrane (Saidijam et al., 2006)	MFS antibiotic efflux pump	*E. coli*

ermE	CARD ARO:3000264	Functions as a homodimer where efflux of small polyaromatic cations from the cell and proton import down an electrochemical gradient is coupled (Ma & Chang, 2007)	SMR	*P. aeruginosa* *E. coli*
EmrKY_TolC CARD ARO:3000373		EmrKY is a homolog of EmrAB in E. coli. It forms a tripartite multidrug transporter with TolC, conferring tetracyclines	MFS	*E. coli*
emrK	CARD ARO:3000206	A MFP that is a homolog of EmrA (Tanabe et al., 1997)	MFS/MFP	*E. coli*
emrY	CARD ARO:3000254	A homolog of EmrB that transports substrates across the inner membrane of E. coli	MFS	*E. coli*
fsr	UniprotKB - P52067	Confers resistance to fosmidomycin (Ishii et al., 1995)	MFS	*E. coli*
DNA gyrase *gyr* CARD ARO:3004333		DNA gyrase consists of two alpha and two beta subunits and is responsible for DNA supercoiling		
hns	CARD ARO:3000676	A histone-like protein involved in Gram-negative bacterial global gene regulation. It represses MFP genes acre, mdtE and emrK and other closely located genes of other RND-type exporters. Confers resistance eto tetracyclines, penem, macrolidesm fluroquinolones, cephamycin and cephalosporin	RND and MFS antibiotic efflux pump	*E. coli* *P. aeruginosa*
MexAB-OprM CARD - ARO:3000386		Multidrug efflux protein associated with fluroquinolones, chloramphenicol, erythromycin, novobiocin and some β-lactamases. Over-expression is associated with colistin resistance. (Srikumar et al., 1998; Sugimura et al., 2008)	RND antibiotic efflux pump	*P. aeuroginoas*
mexA	CARD ARO:3000377	The membrane fusion protein (Akama et al., 2004)	MFP/RND	*P. aeruginosa* *P. fluorescens*
mexB	CARD ARO:3000378	Inner membrane transporter (Sennhauser et al., 2009)	RND antibiotic efflux pump	*(P. aeruginosa)* *(P. fluorescens)*
OprM	CARD ARO:3000379	OMF family protein that forms the channel (Lambert et al., 2005)	OMF/RND antibiotic efflux pump	*P. aeruginosa* *P. fluorescens*

mdfA (cmlA/ cmr)	CARD ARO:3001328	Multidrug efflux pump identified in E. coli. The system was originally identified as the Cmr/CmlA chloramphenicol exporter (Heng et al., 2015)	MFS antibiotic efflux pump	*E. coli (P. aeruginosa)*
mdlB	UniprotKB - P0AAG5	Multidrug resistance-like ATP binding protein (Allikmets et al., 1993)	ABC transporter superfamily, Drug exporter-2 family	*E. coli*
MdtABC-TolC CARD ARO:3000787		A Gram-negative multidrug efflux system, which confers resistance to aminocoumarin (Nagakubo et al., 2002)	RND antibiotic efflux pump	
mdtA (yegM)	CARD ARO:3000792	MFP		*E. coil (P. aeruginosa)*
mdtB (yegN)	CARD ARO:3000792	Forms a heteromultimer drug transporter complex with MdtC. The system has narrower drug specificity without MdtB resulting in loss of novobiocin resistance		*E. coil (P. aeruginosa)*
mdtC	CARD ARO:3000794	MdtC can form a homomultimer complex in the absence of MdtB, but with narrower drug specificity		*E. coil (P. aeruginosa)*
mdtD	UniprotKB - P36554	Putative multidrug resistance protein that is transcriptionally regulated by the response regulator BaeR (Nagakubo et al., 2002)	MFS (TCR/Tet family)	*E. coli*
mdtG (yceE)	CARD ARO:3001329	Appears to be a MFS transporter that is reported to increase Fosfomycin and deoxycholate resistance when over-expressed	MFS antibiotic efflux pump	*E. coli*
mdtH	CARD ARO:3001216	Multidrug resistance protein	MFS antibiotic efflux pump	*E. coil (P. aeruginosa)*
mdtK	CARD ARO:3001327	Multidrug and toxic extrusion transporter that confers resistance to fluroquinolones (norfloxacin, doxorubicin and acriflavine) (Nishino et al., 2006)	MATE transporter	*Salmonella enterica (E. coli)*
mdtL (YidY)	CARD ARO:3001215	Putative/predicated multidrug resistance protein	MFS antibiotic efflux pump	

oqxAB CARD ARO:3003921		A plasmid (E. coli) or chromosomally (K. pneuomonia) located encoded multidrug efflux pump complex identified *Enterobacteriaceae (Kim et al., 2009)* It has a wide substrate specificity and can be transferred between Enterobacteriaceae (Hansen et al., 2007). Reduced susceptibility for quinoxalines (chloramphenicol and carbadox), quinolones (nalidixic acid) , trimethoprim and fluroquinolones (flumequine, norfloxacin and ciprofloxacin) (Hansen et al., 2007). The oqxAB cassettes in E. coli were shown as contained within a transposon seemingly originating form K. pneumonia, demonstrating the plasmid facilitated lateral transfer between Enterobacteriaceae (Norman et al., 2008)		
oqxB	CARD ARO:3003923	Confers resistance to fluroquinolone, tetracyclines, glycylcycline, nitrofuran and diaminopyrimidine. T	RND efflux pump	*E. coli*
oqxB9	UniprotKB - A0A5E6WZJ0	Efflux membrane transporter	RND family	*P. fluorescens*
oqxB17	UniprotKB - A0A7U7EJG8	Multidrug efflux RND transporter permease subunit conferring quinolone resistance	RND family	*Pseudomonas*
oqxB27	UniprotKB - A0A5E6SYE8	Efflux membrane transporter	RND family	*P. fluorescens*
PhoP/Q CARD ARO:3000821		A two-component regulatory system that activates the PmrA/B system with downstream affects that confers resistance in various pathogens. phoP binds to and represses the macAB transporter. phoPQ regulates arnA expression in E. coli and OprH expression in P. aeruginosa (Groisman, 2001)		
phoP	CARD ARO:3000834	Directly represses the macAB efflux genes	Pmr phosphoethanolamine transferase and ABC antibiotic efflux pump	
phoQ	CARD ARO:3000835	Sensor protein PhoQ induced by low magnesium ion concentration. It donates a phosphate to activate PhoP once activated. It is associated with peptide and macrolide antibiotic resistance through efflux and target alteration (Lee & Ko, 2014)		*P. aeruginosa*
smvA	UniprotKB - D0ZXQ3	Methyl viologen resistance protein that acts as an efflux pump for acriflavine and methyl viologen resistance (Santiviago et al., 2002; Villagra et al., 2008)	MFS (TCR/Tet family)	*Salmonella typhimurium*

stp	UniprotKB - P9WG91	Multidrug resistance protein Stp that contributes to spectinomycin and tetracycline resistance (Ramón-García et al., 2007)	MFS (EmrB family)	*Mycobacterium tuberculosis*
tdeA	UniprotKB - Q2EHL7	A TolC-like Toxin and drug export protein A required for LtxA leukatoxin secretion and AMR (Crosby & Kachlany, 2007)	OMF family	*Aggregatibacter*
yaaA	UniprotKB - P0A8I3	Peroxide stress resistance protein YaaA. That o prevents oxidative damage to DNA and proteins by reducing unincorporated iron within the cell during hydrogen peroxide stress (Liu et al., 2011)	UDPF0246 family	*E. coli*

ABC – ATP-binding cassette, ACR – arsenical resistance, CDF – cation diffusion facilitator, MFP – membrane fusion protein, MFS – major facilitator superfamily, OMF – outer membrane factor, PMR – polymyxin resistance, RND – resistance-nodulation-cell division, SMR – small multidrug resistance

Appendix 9.13. Summary of Herbaspirillum Infections.

Table 9.12. Dhital et al. (2020). Summary of nine published cases of Herbaspirillum infections in humans between 2005 and 2019.

Author, year	Country	Age/ sex	History/ predisposing conditions	Immune suppression	Likely risk factor/inciting event	Initial antibiotics	Respone to initial antibiotics/subsequent antibiotics	Specimen source positive for Herbaspirillium

Lui, 2019	Korea	93/F	Hypertension, Advanced age	N/A	-	Empiric vancomycin + ceftriaxone for encephalitis	Changed to meropenem and colistin at 10 days and changed to ceftazidime, minocycline and trimehtoprim/sulfamethoxazole thereafter	Blood: *Herbaspirillium huttiense*. Sputum: *H. huttiense* (2 months later)
Abreu-di berardino, 2019	Spain	59/F	Aortic wall thrombosis, visceral and cerebal ischemic lesions, JAK2 + essential thromboytopenia, new DM	N/A	-	Piperacillin-tazobactam	Recovered completely after antibiotics	Sputum: *H. huttiense*
Chen, 2010	China	48/F	Acute lymphoblastic leukemia	Chemotherapy and G-CSF	Drank sugarcane juice before fever started	Cefmetazole and gatifloxacin	Improved	Blood: *H. huttiense*
Spiker, 2008	USA	26/M	Moderate to severe lung disease, pancreatic insufficiency, diabetes and liver didease	Recent multiple admissions for exacerbation of respiratory symptoms	-	Vancomycin, piperacillin-tazobactam, and tobramyicn x 20 days. Antibiotics discontinued at 20 days, after FEV1 started to improve	On hospital day 23: changed to intravenous ceftazidime and tobramycin and oral trimethoprim-sulfamethoxazole (TMP-SMX), levofloxacin and minocycline	Blood: (GNR, initially identified as *burkholderia cepacia* complex, BBC). Later identified as *Herbaspirillium* species
Author, year	**Country**	**Age/ sex**	**History/ predisposing conditions**	**Immune suppression**	**Likely risk factor/inciting event**	**Initial antibiotics**	**Respone to initial antibiotics/subsequent antibiotics**	**Specimen source positive for Herbaspirillium**

Tan, 2005	USA	49/M	Probable hepatic cirrhosis	-	Homeless. Jumped from a bridge into a freshawater canal in central Florida	Ampicilllin/sulbactam	Switched to cefepime and levofloxacin after initial blood culture results	Blood: (oxidase-positive, nonlactase-fermenting GNR, submitted to outside reference labratory) psoitve for *Herbaspirillium seropedicae*
Regunath, 2014	USA	46/M	Childhood asthmas, atypical pneuminia as a teenager, tonsillectomy	N/A	Farming in rural Missouri, close contact with cattle and turkeys, mold and possible rat excerta. Drenched in rain during a fishing trip.	Vancomycin, ceftriaxone and azithromycin	Ceftriaxone switched to piperacillin-tazabactam. Azithromycin switched to Doxyxyxline. The patient improved after 12 days of treatment and was extubated.	Blood (Day 1) (from referring facility) identified as BBC, later as *Herbaspirillum aquaticum* or *H. huttiense*. BAL (Day 3): GNR as *H. aquaticum* or *H. Huttiense*
Chemaly, 2015	USA							
Hospital-based cluster of Herbaspirillium sp infections intially misidentified as *B. cepacia*		48/F	Ovarian adenocarcinoma	Chemotherapy	Unkown source and mechanism of the cluster	Cefepime (5 patients), ceftazidime (1 patient), moxifloxacin (1 patient), meropendem (1 patient) initially. Followed by cefriaxone or fluroquinolone.	All patients improved with antibiotics and had negative repeat blood cultures. One patient had recurrence, which resolved once the port was removed.	Blood, Infusaport tip
		67/F	Leukemia	Chemotherapy				Blood
		58/M	Leukemia/HSCT	High-dose steroid				Blood
		55/F	Leukemia/HSCT	High-dose steroid				Blood
		2/M	Ependymoma	High-dose steroid				Blood
Author, year	**Country**	**Age/ sex**	**History/ predisposing conditions**	**Immune suppression**	**Likely risk factor/inciting event**	**Initial antibiotics**	**Respone to initial antibiotics/subsequent antibiotics**	**Specimen source positive for Herbaspirillium**

Three additional Herbaspirillium sp after continued surveillance (sporadic) Chemaly, 2015		66/F	History of recurrent pneumonia, lung cancer	Radiation therapy				Sputum
		18/M	Lymphoma	Chemotherapy				Blood
		51/F	Aplastic anemia/HSCT	Tacrolimus				Blood, PICC line tip
Suwant arat, 2015	USA	65/M	Multiple myeloma ESRD on hemodialysis	Steroids and lenalidomide		Vancomycin, cefepime, ciprofloxacin and micafungin	Changed to vancomycin, meropenem and gentamicin within 12 hours. The patient remained clinically unstable and died after 4 days.	BAL (oxidase-positive, nonlactose-fermenting GNR), initially identified as *Cupriavidus*, later and *H. seropedicae*.
Ziga, 2010	USA	2/F	Acute lymphoblastic leukemia	Chemotherapy HSCT	Uncertain. Lives with parents and grandparents on a large farm.	Cefepime	Gentamicin added after oxidase-positive, Gram-negative bacillus. Later switched to meropenem	Blood: *B. cepacia* complex. As susceptibility pattern was not consitent, further identification was persued: *Herbaspirillium* sp.

Appendix 9.14. Scaffold statistics for Isolate KA0 (R. variigena), including node lengths and coverage.

Node	Lenth	Coverage	Node	Lenth	Coverage	Node	Lenth	Coverage	Node	Lenth	Coverage
NODE_1	896266	245.482103	NODE_26	30673	297.886407	NODE_51	615	1.044643	NODE_76	517	1.062771

NODE_2	717167	201.146015	NODE_27	27034	292.136439	NODE_52	595	1.237037	NODE_77	515	1.054348
NODE_3	685771	216.168031	NODE_28	24083	173.191651	NODE_53	580	2.274286	NODE_78	509	0.944934
NODE_4	655692	209.224620	NODE_29	21826	66.585044	NODE_54	573	1.324324	NODE_79	506	0.982262
NODE_5	223639	280.309034	NODE_30	17391	305.914917	NODE_55	561	1.677866	NODE_80	505	1.008889
NODE_6	219333	191.825737	NODE_31	14443	297.702391	NODE_56	561	1.290514	NODE_81	503	1.017857
NODE_7	179906	270.278425	NODE_32	12058	328.481046	NODE_57	561	0.970356	NODE_82	503	0.944196
NODE_8	176369	247.593078	NODE_33	11585	226.291847	NODE_58	556	0.932136	NODE_83	501	0.964126
NODE_9	166481	285.051945	NODE_34	6295	263.982853	NODE_59	552	0.987928	NODE_84	501	0.952915
NODE_10	166432	260.311107	NODE_35	4781	309.440965	NODE_60	546	0.940937	NODE_85	500	1.058427
NODE_11	148541	180.796634	NODE_36	4480	280.028701	NODE_61	545	290.8775	NODE_86	498	1.002257
NODE_12	134187	296.014694	NODE_37	3247	1892.342732	NODE_62	544	0.969325	NODE_87	495	0.988636
NODE_13	128526	183.328199	NODE_38	2481	252.213932	NODE_63	544	0.942740	NODE_88	494	277.207289
NODE_14	114948	177.927541	NODE_39	1493	251.105702	NODE_64	540	0.940206	NODE_89	494	1.052392
NODE_15	98953	238.553318	NODE_40	1288	488.768856	NODE_65	540	0.936082	NODE_90	493	1.367580
NODE_16	84424	303.012469	NODE_41	1148	457.083257	NODE_66	538	0.973085	NODE_91	492	0.988558
NODE_17	72244	56.323997	NODE_42	904	2.020024	NODE_67	538	0.946170	NODE_92	490	903.358621
NODE_18	69535	180.750604	NODE_43	784	1932.379973	NODE_68	535	0.947917	NODE_93	489	0.963134
NODE_19	60403	292.126814	NODE_44	774	475.194715	NODE_69	534	0.983299	NODE_94	489	0.953917
NODE_20	56986	309.716481	NODE_45	773	629.796657	NODE_70	534	0.941545	NODE_95	488	1.120092
NODE_21	47410	304.526639	NODE_46	757	463.413105	NODE_71	533	0.930962	NODE_96	487	0.995370
NODE_22	40014	276.970695	NODE_47	683	1545.463376	NODE_72	526	1.044586	NODE_97	485	1.053488
NODE_23	39709	317.711378	NODE_48	681	1.028754	NODE_73	525	0.993617	NODE_98	483	1.205607
NODE_24	37668	200.071704	NODE_49	635	1.472414	NODE_74	522	0.959315	NODE_99	482	1.135831
NODE_25	37264	311.737590	NODE_50	631	1.411458	NODE_75	519	0.967672	NODE_100	482	0.992974
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_101	481	1.044601	NODE_125	459	1.074257	NODE_150	437	1.282723	NODE_174	414	1.292479
NODE_102	479	0.974057	NODE_126	458	1.220844	NODE_151	436	2.493438	NODE_175	413	1.192737

NODE_103	476	1.121140	NODE_127	457	1.189055	NODE_152	434	1.097625	NODE_176	412	1.221289
NODE_104	474	1.133652	NODE_128	456	1.226933	NODE_153	433	1.208995	NODE_177	408	1.390935
NODE_105	474	1.128878	NODE_129	456	1.147132	NODE_154	433	0.976190	NODE_178	408	1.373938
NODE_106	474	1.069212	NODE_130	456	0.960100	NODE_155	432	1.299735	NODE_179	407	0.957386
NODE_107	474	1.038186	NODE_131	455	1.230000	NODE_156	432	0.986737	NODE_180	406	1.282051
NODE_108	473	1.174641	NODE_132	454	1.210526	NODE_157	430	1.226667	NODE_181	404	1.762178
NODE_109	472	1.088729	NODE_133	454	1.147870	NODE_158	430	1.088000	NODE_182	404	1.309456
NODE_110	471	1.125000	NODE_134	454	1.102757	NODE_159	430	1.064000	NODE_183	401	1.693642
NODE_111	470	1.089157	NODE_135	454	1.072682	NODE_160	426	1.159030	NODE_184	400	0.953623
NODE_112	469	1.185990	NODE_136	453	1.118090	NODE_161	426	0.994609	NODE_185	399	1.427326
NODE_113	468	1.142857	NODE_137	453	1.108040	NODE_162	425	1.191892	NODE_186	399	1.404070
NODE_114	467	0.995146	NODE_138	448	1.620865	NODE_163	424	2.154472	NODE_187	397	1.315789
NODE_115	466	1.197080	NODE_139	447	1.076531	NODE_164	421	1.251366	NODE_188	394	1.247788
NODE_116	466	1.124088	NODE_140	446	1.140665	NODE_165	421	1.226776	NODE_189	394	1.168142
NODE_117	465	1.109756	NODE_141	445	0.997436	NODE_166	421	1.122951	NODE_190	394	1.115044
NODE_118	464	1.156479	NODE_142	445	0.958974	NODE_167	420	1.268493	NODE_191	393	1.449704
NODE_119	463	1.149510	NODE_143	444	0.969152	NODE_168	420	1.128767	NODE_192	393	1.174556
NODE_120	463	1.112745	NODE_144	443	1.198454	NODE_169	420	1.084932	NODE_193	392	1.412463
NODE_121	463	1.073529	NODE_145	443	1.152062	NODE_170	419	1.271978	NODE_194	391	1.458333
NODE_122	462	1.103194	NODE_146	442	1.245478	NODE_171	417	1.265193	NODE_195	389	1.098802
NODE_123	461	1.211823	NODE_147	439	1.278646	NODE_172	417	1.052486	NODE_196	388	1.474474
NODE_124	459	1.178218	NODE_148	438	0.911227	NODE_173	416	1.351801	NODE_197	388	1.474474
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_198	387	1.457831	NODE_221	369	1.560510	NODE_245	306	358.505976	NODE_269	165	283.536364
NODE_199	387	1.445783	NODE_222	368	1.568690	NODE_246	295	265.095833	NODE_270	165	209.609091

NODE_200	386	1.435045	NODE_223	367	301.141026	NODE_247	295	149.016667	NODE_271	162	236.551402
NODE_201	386	1.141994	NODE_224	367	1.362179	NODE_248	282	288.814978	NODE_272	161	239.160377
NODE_202	386	0.960725	NODE_225	366	1.543408	NODE_249	277	580.387387	NODE_273	160	212.990476
NODE_203	385	1.381818	NODE_226	366	1.463023	NODE_250	258	302.088670	NODE_274	159	286.730769
NODE_204	384	1.422492	NODE_227	366	1.437299	NODE_251	243	265.601064	NODE_275	158	200.553398
NODE_205	383	1.189024	NODE_228	365	1.490323	NODE_252	233	295.662921	NODE_276	157	150.696078
NODE_206	383	1.179878	NODE_229	363	1.587662	NODE_253	225	227.482353	NODE_277	156	298.425743
NODE_207	382	1.446483	NODE_230	363	0.957792	NODE_254	218	273.294479	NODE_278	156	228.663366
NODE_208	381	1.481595	NODE_231	361	1.366013	NODE_255	213	244.759494	NODE_279	155	280.570000
NODE_209	381	1.432515	NODE_232	360	1.242623	NODE_256	202	265.265306	NODE_280	153	217.408163
NODE_210	380	1.080000	NODE_233	359	1.421053	NODE_257	196	390.078014	NODE_281	149	236.000000
NODE_211	379	1.395062	NODE_234	359	1.417763	NODE_258	196	224.007092	NODE_282	148	224.473118
NODE_212	377	1.406832	NODE_235	358	1.356436	NODE_259	193	549.688406	NODE_283	145	219.933333
NODE_213	376	1.380062	NODE_236	358	1.217822	NODE_260	183	128.796875	NODE_284	145	200.333333
NODE_214	373	1.544025	NODE_237	357	1.390728	NODE_261	182	459.157480	NODE_285	143	195.227273
NODE_215	373	1.462264	NODE_238	357	1.281457	NODE_262	182	209.566929	NODE_286	139	293.047619
NODE_216	373	1.339623	NODE_239	345	290.431034	NODE_263	180	173.008000	NODE_287	138	264.891566
NODE_217	372	1.536278	NODE_240	334	629.440860	NODE_264	177	602.049180	NODE_288	138	208.578313
NODE_218	371	1.234177	NODE_241	327	279.727941	NODE_265	177	138.229508	NODE_289	137	897.219512
NODE_219	371	1.003165	NODE_242	324	574.204461	NODE_266	175	1334.350000	NODE_290	136	201.271605
NODE_220	370	1.434921	NODE_243	317	261.595420	NODE_267	168	289.539823	NODE_291	135	280.712500
NODE_221	369	1.560510	NODE_244	306	15682.988048	NODE_268	165	331.327273	NODE_292	134	275.810127
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_293	132	218.792208	NODE_317	111	294.607143	NODE_341	126	45.129630	NODE_365	106	185.470588
NODE_294	130	238.306667	NODE_318	111	278.589286	NODE_342	108	589.566038	NODE_366	106	164.450980

NODE_295	128	195.958904	NODE_319	111	276.678571	NODE_343	108	341.830189	NODE_367	105	277.180000
NODE_296	127	296.972222	NODE_320	111	272.357143	NODE_344	108	329.301887	NODE_368	105	235.060000
NODE_297	123	278.779412	NODE_321	111	251.857143	NODE_345	108	298.226415	NODE_369	105	136.940000
NODE_298	123	271.661765	NODE_322	111	222.160714	NODE_346	108	270.264151	NODE_370	104	306.877551
NODE_299	123	246.691176	NODE_323	111	215.910714	NODE_347	108	219.754717	NODE_371	104	200.265306
NODE_300	117	326.177419	NODE_324	111	196.303571	NODE_348	108	191.584906	NODE_372	102	559.553191
NODE_301	117	300.935484	NODE_325	111	195.839286	NODE_349	108	190.716981	NODE_373	100	661.933333
NODE_302	117	237.983871	NODE_326	111	194.142857	NODE_350	108	185.207547	NODE_374	100	544.800000
NODE_303	117	201.838710	NODE_327	111	190.160714	NODE_351	108	174.509434	NODE_375	100	528.555556
NODE_304	116	203.245902	NODE_328	111	187.714286	NODE_352	108	169.830189	NODE_376	99	286.477273
NODE_305	115	588.266667	NODE_329	111	173.053571	NODE_353	108	157.773585	NODE_377	99	243.318182
NODE_306	115	532.783333	NODE_330	111	157.696429	NODE_354	108	156.301887	NODE_378	98	879.000000
NODE_307	114	241.406780	NODE_331	109	1146.962963	NODE_355	108	155.113208	NODE_379	97	464.785714
NODE_308	111	1197.464286	NODE_332	109	381.148148	NODE_356	108	149.811321	NODE_380	96	294.000000
NODE_309	111	938.089286	NODE_333	109	321.629630	NODE_357	108	135.433962	NODE_381	95	16062.750000
NODE_310	111	832.678571	NODE_334	109	216.407407	NODE_358	108	133.735849	NODE_382	93	860.315789
NODE_311	111	799.910714	NODE_335	109	184.296296	NODE_359	108	124.471698	NODE_383	93	271.973684
NODE_312	111	535.285714	NODE_336	109	178.314815	NODE_360	108	98.792453	NODE_384	92	583.891892
NODE_313	111	455.428571	NODE_337	109	164.703704	NODE_361	107	262.846154	NODE_385	92	564.675676
NODE_314	111	396.482143	NODE_338	109	153.537037	NODE_362	107	194.903846	NODE_386	91	288.833333
NODE_315	111	306.750000	NODE_339	109	145.407407	NODE_363	107	186.019231	NODE_387	90	387.428571
NODE_316	111	295.517857	NODE_340	109	129.703704	NODE_364	107	130.326923	NODE_388	90	300.342857
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_389	90	151.371429	NODE_413	70	524.733333	NODE_437	62	376.714286	NODE_461	56	18489.000000
NODE_390	88	474.575758	NODE_414	70	470.066667	NODE_438	62	206.571429	NODE_462	56	18248.000000

NODE_391	88	311.454545	NODE_415	70	297.466667	NODE_439	61	887.166667	NODE_463	56	16945.000000
NODE_392	87	587.937500	NODE_416	69	810.357143	NODE_440	61	403.833333	NODE_464	56	16898.000000
NODE_393	85	854.366667	NODE_417	69	440.071429	NODE_441	60	592.200000	NODE_465	56	16882.000000
NODE_394	84	183.103448	NODE_418	69	367.142857	NODE_442	60	530.600000	NODE_466	56	16504.000000
NODE_395	83	346.107143	NODE_419	69	312.142857	NODE_443	60	434.400000	NODE_467	56	10468.000000
NODE_396	82	841.518519	NODE_420	69	289.357143	NODE_444	60	304.400000	NODE_468	56	9704.000000
NODE_397	82	546.629630	NODE_421	69	268.500000	NODE_445	59	812.500000	NODE_469	56	9643.000000
NODE_398	81	287.730769	NODE_422	69	264.857143	NODE_446	59	765.000000	NODE_470	56	1906.000000
NODE_399	81	253.961538	NODE_423	69	259.642857	NODE_447	59	756.500000	NODE_471	56	1020.000000
NODE_400	79	570.416667	NODE_424	68	860.384615	NODE_448	59	613.500000	NODE_472	56	904.000000
NODE_401	79	498.375000	NODE_425	67	574.583333	NODE_449	58	613.333333	NODE_473	56	710.000000
NODE_402	79	435.250000	NODE_426	66	1276.181818	NODE_450	58	504.333333	NODE_474	56	600.000000
NODE_403	79	276.125000	NODE_427	66	487.181818	NODE_451	58	434.333333	NODE_475	56	596.000000
NODE_404	78	871.043478	NODE_428	66	318.000000	NODE_452	58	397.333333	NODE_476	56	544.000000
NODE_405	78	216.782609	NODE_429	66	223.363636	NODE_453	57	611.500000	NODE_477	56	490.000000
NODE_406	77	676.909091	NODE_430	64	1166.666667	NODE_454	56	70374.000000	NODE_478	56	431.000000
NODE_407	77	276.500000	NODE_431	64	520.000000	NODE_455	56	65724.000000	NODE_479	56	119.000000
NODE_408	77	219.318182	NODE_432	64	293.555556	NODE_456	56	34234.000000			
NODE_409	76	401.666667	NODE_433	63	892.000000	NODE_457	56	32609.000000			
NODE_410	75	536.450000	NODE_434	63	392.625000	NODE_458	56	20354.000000			
NODE_411	74	580.736842	NODE_435	62	1167.714286	NODE_459	56	19481.000000			
NODE_412	72	480.705882	NODE_436	62	943.285714	NODE_460	56	19464.000000			

Appendix 9.15. Scaffold statistics for Isolate KA5 (R. variigena), including node lengths and coverage.

Node	Lenth	Coverage	Node	Lenth	Coverage	Node	Lenth	Coverage	Node	Lenth	Coverage
NODE_1	869397	238.920831	NODE_26	58454	217.392062	NODE_51	774	524.6620	NODE_76	605	1.116364

NODE_2	656601	259.729865	NODE_27	54033	301.596502	NODE_52	773	729.4192	NODE_77	603	1.531022
NODE_3	406561	278.348118	NODE_28	46810	357.943493	NODE_53	757	567.0683	NODE_78	601	1.069597
NODE_4	347717	319.716351	NODE_29	40014	331.125779	NODE_54	743	1.218023	NODE_79	599	1.678309
NODE_5	242745	305.105752	NODE_30	39950	356.574859	NODE_55	734	1.225331	NODE_80	599	1.376838
NODE_6	230730	252.881773	NODE_31	38878	347.422095	NODE_56	729	1.307122	NODE_81	597	1.474170
NODE_7	214177	262.321775	NODE_32	37264	370.291919	NODE_57	710	1.340458	NODE_82	590	0.919626
NODE_8	200947	235.537917	NODE_33	27034	350.189740	NODE_58	706	1.402458	NODE_83	586	1.256121
NODE_9	166481	343.939841	NODE_34	24064	242.890378	NODE_59	702	1.132921	NODE_84	568	1.329435
NODE_10	150294	277.247639	NODE_35	19884	346.252912	NODE_60	694	0.940532	NODE_85	566	1.780822
NODE_11	148286	219.042103	NODE_36	11480	205.259606	NODE_61	683	1913.199	NODE_86	562	0.966469
NODE_12	136295	327.035276	NODE_37	8283	272.702479	NODE_62	672	1.369530	NODE_87	557	0.974104
NODE_13	122536	297.045517	NODE_38	4519	462.796595	NODE_63	671	1.347403	NODE_88	557	0.950199
NODE_14	121863	366.530400	NODE_39	4480	338.717966	NODE_64	668	1.446982	NODE_89	549	1.135628
NODE_15	115673	331.656541	NODE_40	3247	2321.035088	NODE_65	668	0.988581	NODE_90	545	346.6367
NODE_16	114948	214.830373	NODE_41	3226	279.418795	NODE_66	668	0.933116	NODE_91	544	1.002045
NODE_17	110365	301.280881	NODE_42	2481	303.453009	NODE_67	666	1.466448	NODE_92	544	0.983640
NODE_18	99490	371.647669	NODE_43	1288	611.442822	NODE_68	661	1.466997	NODE_93	542	0.946612
NODE_19	99117	289.354505	NODE_44	1031	1.097336	NODE_69	651	1.122483	NODE_94	541	0.967078
NODE_20	93710	353.486861	NODE_45	884	1.104946	NODE_70	646	0.944162	NODE_95	541	0.927984
NODE_21	88192	337.509071	NODE_46	882	1.222491	NODE_71	644	1.455008	NODE_96	540	1.012371
NODE_22	75535	228.645707	NODE_47	825	1.249351	NODE_72	633	1.551903	NODE_97	536	2.690229
NODE_23	72244	201.887227	NODE_48	793	1.258808	NODE_73	630	1.633043	NODE_98	535	0.941667
NODE_24	71586	255.519076	NODE_49	784	2519.872428	NODE_74	626	1.630473	NODE_99	534	0.954071
NODE_25	61762	357.677379	NODE_50	776	1.020804	NODE_75	623	1.315141	NODE_100	533	1.108787
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_101	532	1.008386	NODE_125	509	1.605727	NODE_150	495	1.102273	NODE_174	487	0.960648
NODE_102	531	0.989496	NODE_126	509	1.072687	NODE_151	495	0.929545	NODE_175	486	1.136891

NODE_103	531	0.974790	NODE_127	508	1.083885	NODE_152	494	313.1708	NODE_176	486	0.974478
NODE_104	529	1.008439	NODE_128	508	0.986755	NODE_153	494	0.943052	NODE_177	485	1.081395
NODE_105	529	1.006329	NODE_129	508	0.940397	NODE_154	493	1.486301	NODE_178	484	1.081585
NODE_106	529	0.981013	NODE_130	507	1.086283	NODE_155	493	1.102740	NODE_179	484	0.995338
NODE_107	527	1.330508	NODE_131	507	1.053097	NODE_156	493	1.011416	NODE_180	484	0.976690
NODE_108	527	1.040254	NODE_132	507	0.955752	NODE_157	492	1.141876	NODE_181	483	1.149533
NODE_109	526	0.968153	NODE_133	505	1.033333	NODE_158	492	1.045767	NODE_182	483	0.969626
NODE_110	524	1.000000	NODE_134	504	1.082405	NODE_159	491	1.071101	NODE_183	482	1.131148
NODE_111	521	2.375536	NODE_135	504	1.051225	NODE_160	491	1.018349	NODE_184	481	0.995305
NODE_112	521	1.877682	NODE_136	503	1.049107	NODE_161	491	1.011468	NODE_185	480	1.080000
NODE_113	521	0.935622	NODE_137	503	0.944196	NODE_162	490	1131.777	NODE_186	478	1.659574
NODE_114	520	0.989247	NODE_138	501	1.062780	NODE_163	490	1.126437	NODE_187	478	0.962175
NODE_115	519	1.463362	NODE_139	501	0.993274	NODE_164	490	1.068966	NODE_188	477	1.000000
NODE_116	518	1.058315	NODE_140	500	1.026966	NODE_165	490	0.972414	NODE_189	476	1.011876
NODE_117	518	0.956803	NODE_141	500	0.946067	NODE_166	490	0.947126	NODE_190	475	1.171429
NODE_118	516	1.032538	NODE_142	500	0.934831	NODE_167	489	1.502304	NODE_191	475	1.157143
NODE_119	516	1.019523	NODE_143	498	1.015801	NODE_168	489	1.062212	NODE_192	475	1.066667
NODE_120	514	1.189542	NODE_144	498	1.000000	NODE_169	489	1.052995	NODE_193	474	1.073986
NODE_121	513	1.399563	NODE_145	498	0.966140	NODE_170	489	1.011521	NODE_194	472	1.146283
NODE_122	513	0.997817	NODE_146	497	1.110860	NODE_171	489	0.965438	NODE_195	472	1.033573
NODE_123	513	0.938865	NODE_147	497	0.988688	NODE_172	488	1.006928	NODE_196	472	0.952038
NODE_124	511	1.548246	NODE_148	497	0.984163	NODE_173	488	0.974596	NODE_198	470	1.040964
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_198	475	1.066667	NODE_221	463	1.036765	NODE_245	451	1.118687	NODE_269	434	1.612137
NODE_199	474	1.073986	NODE_222	462	1.144963	NODE_246	451	1.037879	NODE_270	433	1.084656

NODE_200	472	1.146283	NODE_223	462	1.004914	NODE_247	449	1.149746	NODE_271	433	0.939153
NODE_201	472	1.033573	NODE_224	460	2.256790	NODE_248	449	0.951777	NODE_272	432	1.689655
NODE_202	472	0.952038	NODE_225	460	1.175309	NODE_249	447	1.145408	NODE_273	432	1.397878
NODE_203	472	0.932854	NODE_226	460	1.120988	NODE_250	447	1.109694	NODE_274	432	1.305040
NODE_204	470	1.040964	NODE_227	460	1.044444	NODE_251	446	1.191816	NODE_275	432	0.925729
NODE_205	469	1.089372	NODE_228	459	1.339109	NODE_252	446	0.961637	NODE_276	431	1.231383
NODE_206	468	1.016949	NODE_229	459	1.136139	NODE_253	445	1.097436	NODE_277	431	1.223404
NODE_207	467	1.160194	NODE_230	459	1.044554	NODE_254	445	1.048718	NODE_278	430	1.144000
NODE_208	467	1.160194	NODE_231	459	1.032178	NODE_255	444	1.120823	NODE_279	430	0.912000
NODE_209	467	1.111650	NODE_232	458	1.121588	NODE_256	443	1.600515	NODE_280	429	1.759358
NODE_210	467	0.949029	NODE_233	457	1.119403	NODE_257	442	1.149871	NODE_281	429	1.288770
NODE_211	466	1.104623	NODE_234	457	1.009950	NODE_258	442	1.069767	NODE_282	429	1.227273
NODE_212	466	1.065693	NODE_235	457	1.007463	NODE_259	441	1.072539	NODE_283	429	1.155080
NODE_213	466	1.046229	NODE_236	456	0.990025	NODE_260	440	1.470130	NODE_284	427	1.295699
NODE_214	465	1.565854	NODE_237	455	1.127500	NODE_261	440	1.215584	NODE_285	427	1.239247
NODE_215	465	1.197561	NODE_238	455	1.122500	NODE_262	440	1.132468	NODE_286	427	1.201613
NODE_216	465	0.980488	NODE_239	454	2.110276	NODE_263	438	226.342037	NODE_287	424	1.837398
NODE_217	464	1.202934	NODE_240	452	1.443325	NODE_264	438	1.279373	NODE_288	424	1.146341
NODE_218	464	1.185819	NODE_241	452	1.136020	NODE_265	437	1.130890	NODE_289	424	0.978320
NODE_219	464	0.973105	NODE_242	452	1.130982	NODE_266	437	1.115183	NODE_290	423	1.225543
NODE_220	463	1.196078	NODE_243	452	1.100756	NODE_267	436	1.028871	NODE_291	423	1.225543
NODE_221	463	1.036765	NODE_244	452	0.974811	NODE_268	435	1.239474	NODE_292	423	1.146739
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_293	422	1.288828	NODE_317	414	1.005571	NODE_341	402	1.204611	NODE_365	394	1.271386
NODE_294	422	1.029973	NODE_318	414	0.933148	NODE_342	402	1.184438	NODE_366	394	1.256637

NODE_295	421	1.229508	NODE_319	413	1.036313	NODE_343	402	0.965418	NODE_367	393	1.343195
NODE_296	421	1.215847	NODE_320	412	1.285714	NODE_344	401	1.289017	NODE_368	391	1.383929
NODE_297	421	1.215847	NODE_321	411	1.328652	NODE_345	399	1.508721	NODE_369	391	1.125000
NODE_298	421	1.193989	NODE_322	411	1.039326	NODE_346	399	1.424419	NODE_370	390	1.298507
NODE_299	420	1.345205	NODE_323	410	1.245070	NODE_347	399	1.404070	NODE_371	390	1.277612
NODE_300	419	1.153846	NODE_324	410	1.214085	NODE_348	399	1.369186	NODE_372	390	0.988060
NODE_301	418	1.294766	NODE_325	410	1.073239	NODE_349	399	1.177326	NODE_373	388	1.450450
NODE_302	418	1.280992	NODE_326	409	1.319209	NODE_350	398	1.431487	NODE_374	388	1.447447
NODE_303	418	1.157025	NODE_327	409	1.302260	NODE_351	398	1.428571	NODE_375	388	1.414414
NODE_304	418	1.107438	NODE_328	409	1.223164	NODE_352	398	1.335277	NODE_376	388	1.267267
NODE_305	417	1.229282	NODE_329	409	1.203390	NODE_353	398	1.291545	NODE_377	388	1.267267
NODE_306	417	0.908840	NODE_330	409	1.014124	NODE_354	398	1.221574	NODE_378	387	1.358434
NODE_307	416	1.310249	NODE_331	408	1.337110	NODE_355	397	2.087719	NODE_379	386	1.317221
NODE_308	416	1.047091	NODE_332	408	1.152975	NODE_356	397	1.263158	NODE_380	385	1.481818
NODE_309	415	656.3055	NODE_333	407	1.394886	NODE_357	397	1.257310	NODE_381	385	0.969697
NODE_310	415	1.363889	NODE_334	407	1.392045	NODE_358	397	1.239766	NODE_382	384	1.379939
NODE_311	415	1.316667	NODE_335	407	1.213068	NODE_359	396	1.434018	NODE_383	384	1.300912
NODE_312	415	1.283333	NODE_336	406	1.182336	NODE_360	396	1.381232	NODE_384	383	1.496951
NODE_313	415	1.183333	NODE_337	405	1.280000	NODE_361	396	1.363636	NODE_385	383	1.420732
NODE_314	415	1.105556	NODE_338	404	1.275072	NODE_362	396	1.302053	NODE_386	381	1.963190
NODE_315	414	1.334262	NODE_339	403	1.060345	NODE_363	395	1.214706	NODE_387	381	1.196319
NODE_316	414	1.220056	NODE_340	403	0.982759	NODE_364	394	1.805310	NODE_388	380	1.510769
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_389	380	1.332308	NODE_413	366	1.167203	NODE_437	309	212.8700	NODE_461	168	365.407080
NODE_390	380	1.206154	NODE_414	365	1.493548	NODE_438	306	490.6494	NODE_462	168	259.265487

NODE_391	379	1.453704	NODE_415	364	0.912621	NODE_439	295	368.254167	NODE_463	167	314.919643
NODE_392	379	1.046296	NODE_416	363	1.519481	NODE_440	295	210.141667	NODE_464	165	351.745455
NODE_393	378	1.390093	NODE_417	363	1.506494	NODE_441	295	182.450000	NODE_465	165	313.072727
NODE_394	378	1.356037	NODE_418	363	1.477273	NODE_442	287	2310.780172	NODE_466	165	273.300000
NODE_395	378	1.331269	NODE_419	363	1.159091	NODE_443	282	378.132159	NODE_467	162	251.523364
NODE_396	377	1.388199	NODE_420	362	1.429967	NODE_444	277	683.279279	NODE_468	161	252.452830
NODE_397	377	1.111801	NODE_421	362	1.140065	NODE_445	258	340.620690	NODE_469	161	100.198113
NODE_398	376	1.202492	NODE_422	360	1.498361	NODE_446	243	339.829787	NODE_470	160	241.971429
NODE_399	374	1.539185	NODE_423	360	1.219672	NODE_447	241	197.043011	NODE_471	158	308.485437
NODE_400	374	1.003135	NODE_424	359	1.493421	NODE_448	237	387.439560	NODE_472	157	189.411765
NODE_401	373	1.427673	NODE_425	359	1.371711	NODE_449	225	219.064706	NODE_473	156	364.831683
NODE_402	373	1.421384	NODE_426	358	1.524752	NODE_450	218	316.662577	NODE_474	156	277.287129
NODE_403	373	2065.015	NODE_427	358	1.405941	NODE_451	202	318.585034	NODE_475	155	351.920000
NODE_404	370	1.555556	NODE_428	358	1.392739	NODE_452	196	472.120567	NODE_476	153	231.571429
NODE_405	370	1.453968	NODE_429	357	1.629139	NODE_453	196	267.687943	NODE_477	152	259.278351
NODE_406	370	1.320635	NODE_430	357	1.622517	NODE_454	182	494.834646	NODE_478	150	224.368421
NODE_407	368	1.476038	NODE_431	345	344.2068	NODE_455	182	271.228346	NODE_479	149	253.372340
NODE_408	368	1.392971	NODE_432	334	768.1433	NODE_456	180	211.944000	NODE_480	143	238.136364
NODE_409	367	366.8237	NODE_433	327	342.9926	NODE_457	178	859.008130	NODE_481	141	131.767442
NODE_410	367	1.445513	NODE_434	324	704.9851	NODE_458	177	714.647541	NODE_482	139	329.071429
NODE_411	366	1.456592	NODE_435	317	325.3931	NODE_459	172	101.675214	NODE_483	138	322.783133
NODE_412	366	1.453376	NODE_436	310	659.0980	NODE_460	168	391.283186	NODE_484	138	252.048193
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_485	137	1077.329268	NODE_509	111	966.339286	NODE_533	109	334.870370	NODE_557	108	113.905660
NODE_486	136	210.592593	NODE_510	111	785.785714	NODE_534	109	327.462963	NODE_558	108	104.905660

NODE_487	135	354.762500	NODE_511	111	739.732143	NODE_535	109	294.962963	NODE_559	108	103.509434
NODE_488	132	266.181818	NODE_512	111	580.785714	NODE_536	109	209.185185	NODE_560	108	102.528302
NODE_489	127	341.875000	NODE_513	111	389.125000	NODE_537	109	176.074074	NODE_561	108	98.283019
NODE_490	123	366.985294	NODE_514	111	383.196429	NODE_538	109	163.203704	NODE_562	108	91.037736
NODE_491	123	361.250000	NODE_515	111	358.142857	NODE_539	109	79.462963	NODE_563	108	70.207547
NODE_492	123	314.161765	NODE_516	111	313.678571	NODE_540	109	39.814815	NODE_564	108	38.622642
NODE_493	119	299.968750	NODE_517	111	296.750000	NODE_541	108	364.641509	NODE_565	107	188.980769
NODE_494	117	624.612903	NODE_518	111	278.000000	NODE_542	108	257.792453	NODE_566	107	181.846154
NODE_495	117	348.032258	NODE_519	111	271.517857	NODE_543	108	220.132075	NODE_567	107	176.000000
NODE_496	117	332.645161	NODE_520	111	268.642857	NODE_544	108	187.358491	NODE_568	107	142.576923
NODE_497	116	247.770492	NODE_521	111	262.767857	NODE_545	108	172.207547	NODE_569	107	115.730769
NODE_498	115	1051.266667	NODE_522	111	260.607143	NODE_546	108	167.301887	NODE_570	98	112.000000
NODE_499	115	694.700000	NODE_523	111	256.482143	NODE_547	108	156.603774	NODE_571	107	106.596154
NODE_500	115	233.100000	NODE_524	111	254.214286	NODE_548	108	152.207547	NODE_572	105	245.200000
NODE_501	115	191.150000	NODE_525	111	246.142857	NODE_549	108	149.962264	NODE_573	104	801.040816
NODE_502	114	245.372881	NODE_526	111	225.446429	NODE_550	108	145.037736	NODE_574	104	366.285714
NODE_503	112	134.614035	NODE_527	111	221.732143	NODE_551	108	140.547170	NODE_575	104	215.163265
NODE_504	111	1359.071429	NODE_528	111	220.589286	NODE_552	103	137.962264	NODE_576	100	802.644444
NODE_505	111	1261.660714	NODE_529	111	214.267857	NODE_553	108	130.471698	NODE_577	100	729.777778
NODE_506	111	1217.410714	NODE_530	111	190.589286	NODE_554	108	125.754717	NODE_578	100	714.666667
NODE_507	111	1081.232143	NODE_531	110	99.345455	NODE_555	108	125.207547	NODE_579	99	342.454545
NODE_508	111	1053.785714	NODE_532	109	1449.111111	NODE_556	108	117.603774	NODE_580	99	273.977273
Node	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**	**Node**	**Lenth**	**Coverage**
NODE_581	98	1084.023256	NODE_605	82	1052.148148	NODE_629	67	690.500000	NODE_653	59	737.000000
NODE_582	98	953.744186	NODE_606	82	728.481481	NODE_630	67	109.583333	NODE_654	59	196.250000

NODE_583	97	534.309524	NODE_607	82	195.555556	NODE_631	67	104.666667	NODE_655	58	784.333333
NODE_584	97	2.142857	NODE_608	81	370.769231	NODE_632	66	362.909091	NODE_656	58	515.666667
NODE_585	97	1.047619	NODE_609	81	350.500000	NODE_633	66	269.090909	NODE_657	57	1929.000000
NODE_586	96	365.658537	NODE_610	80	377.520000	NODE_634	65	1058.800000	NODE_658	56	69194.000000
NODE_587	93	2834.552632	NODE_611	79	725.291667	NODE_635	65	791.500000	NODE_659	56	64323.000000
NODE_588	93	372.842105	NODE_612	79	611.333333	NODE_636	64	1434.222222	NODE_660	56	33642.000000
NODE_589	92	709.513514	NODE_613	79	354.583333	NODE_637	64	656.888889	NODE_661	56	32738.000000
NODE_590	91	630.666667	NODE_614	78	1084.260870	NODE_638	64	331.666667	NODE_662	56	19635.000000
NODE_591	90	752.742857	NODE_615	78	245.434783	NODE_639	63	1044.250000	NODE_663	56	18929.000000
NODE_592	90	487.857143	NODE_616	77	348.818182	NODE_640	63	517.500000	NODE_664	56	18205.000000
NODE_593	90	334.857143	NODE_617	77	303.181818	NODE_641	62	1177.857143	NODE_665	56	16850.000000
NODE_594	90	194.285714	NODE_618	76	183062.095238	NODE_642	62	1145.857143	NODE_666	56	16562.000000
NODE_595	88	338.606061	NODE_619	76	475.285714	NODE_643	62	638.571429	NODE_667	56	16426.000000
NODE_596	88	321.242424	NODE_620	74	765.684211	NODE_644	62	629.142857	NODE_668	56	16369.000000
NODE_597	88	310.030303	NODE_621	70	683.466667	NODE_645	62	451.000000	NODE_669	56	12929.000000
NODE_598	88	213.787879	NODE_622	70	371.866667	NODE_646	61	1077.333333	NODE_670	56	12331.000000
NODE_599	87	685.312500	NODE_623	69	460.428571	NODE_647	61	534.000000	NODE_671	56	11211.000000
NODE_600	85	1038.166667	NODE_624	69	381.714286	NODE_648	60	672.600000	NODE_672	56	6975.000000
NODE_601	84	361.758621	NODE_625	69	363.642857	NODE_649	60	665.600000	NODE_673	56	6812.000000
NODE_602	84	248.896552	NODE_626	69	357.928571	NODE_650	60	356.200000	NODE_674	56	3739.000000
NODE_603	83	3531.000000	NODE_627	69	327.000000	NODE_651	60	295.200000	NODE_675	56	3717.000000
NODE_604	83	443.750000	NODE_628	69	291.714286	NODE_652	59	994.000000	NODE_676	56	2384.000000

Node	**Lenth**	**Coverage**	**Node**
NODE_677	56	914.000000	NODE_677
NODE_678	56	822.000000	NODE_678

NODE_679	56	791.000000	NODE_679
NODE_680	56	710.000000	NODE_680
NODE_681	56	694.000000	NODE_681
NODE_682	56	615.000000	NODE_682
NODE_683	56	437.000000	NODE_683
NODE_684	56	402.000000	NODE_684
NODE_685	56	325.000000	NODE_685

Appendix 9.16. Coverage of ONT sequences.

Isolate	Found reads	Found bases	Coverage
KA4	23888	225097789	37,51x
KA7	17242	210867700	35,14x
KB8	10518	115385298	19,23x

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway