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Genotypic and phenotypic antimicrobial resistance in the opportunistic pathogen *Stenotrophomonas maltophilia*

Anne Steffensen

M-Biotechnology

Faculty of Chemistry, Biotechnology and Food Science

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Anne Steffensen, Oslo 2018

Abstract

Stenotrophomonas maltophilia is an environmental bacterium and an opportunistic pathogen that may be associated with several clinical syndromes, primarily in immunocompromised patients. This bacterium shows high levels of intrinsic and acquired resistance to many antimicrobial agents, resulting in that the treatment of infections by *S. maltophilia* is difficult and dramatically reduces the antibiotic options available for treatment. At present, only one antimicrobial agent (trimethoprim-sulfamethoxazole) is available for determining susceptibility in a clinical laboratory setting.

Presented in this thesis are 99 isolates of *S. maltophilia* collected from Oslo University Hospital, from 1989 to 2017. They are isolates from patients with cystic fibrosis, patients from the Intensive care unit, patients from the Department of Haematology and environmental isolates collected from patient rooms in the hospital. The isolates were examined for phenotypic resistance using four different methods for antimicrobial susceptibility testing. A core genomic resistome of these isolates was proposed on the basis of whole genome sequencing (WGS) data examined with several bioinformatic tools. Known resistance genes that were identified were linked to their phenotypic resistance profile.

Phenotypic susceptibility testing of the *S. maltophilia* isolates in this thesis showed several differences between the methods performed. Some resistance genes were detected that correlated their phenotypic resistance profile. However, since this bacterium comprises many intrinsic resistance genes perhaps not found in this thesis, further work should be done to define the whole core resistome for this species. A larger quantity of isolates would be needed for this.

In conclusion, our study supports earlier studies that show that phenotypic susceptibility testing of *S. maltophilia* for antibiotics other than trimethoprim-sulfamethoxazole is difficult and inconstant. Some resistance genes were found that can support their phenotypic resistance profiles. The main goal in a larger setting, is to find a method, either phenotypic, genotypic or a combination of both, to accurately predict antimicrobial susceptibility for other antimicrobial agents than trimethoprim-sulfamethoxazole in *S. maltophilia*. The work in this thesis leaves several ideas for further research on this matter.

Sammendrag

Stenotrophomonas maltophilia er hovedsakelig kjent som en miljøbakterie, men innehar også den egenskapen å være opportunistisk patogen. Hos pasienter er bakterien ofte assosiert med kolonisering, men kan sporadisk skape infeksjon, særlig hos immunsupprimerte pasienter. Denne arten kjennetegnes for iboende antibiotikaresistens, men kan også inneha flere ervervede resistensgener. En konsekvens av dette er færre behandlingsmuligheter for infeksjoner assosiert med *S. maltophilia*. Per idag finnes det kun godkjente brytningspunkter for fenotypisk resistensbestemmelse for ett antibiotikum, trimetoprim-sulfametoxazol.

Presentert i denne studien, er 99 isolater av *S. maltophilia* samlet inn fra Oslo Universitetssykehus i tidsrommet mellom 1989 og 2017. Samlingen isolater kommer fra pasienter med cystisk fibrose, intensivavdelingen, avdeling for blodsykdommer og isolater innhentet fra vannkilder tilhørende pasientrom på sykehuset. Isolatene ble testet med fire ulike metoder for å finne et fenotypisk resistensmønster per isolat. Et kjerne-resistom av isolatene er foreslått her, basert på data fra helgenomsekvensering prosessert med flere bioinformatiske verktøy. Kjente resistensgener som ble identifisert ble også koblet opp mot isolatenes fenotypiske resistensprofil og vurdert.

Den fenotypiske resistenstestingen av *S. maltophilia*-isolater avdekket flere diskrepanser mellom metodene. Noen resistensgener kunne kobles opp mot deres fenotypiske resistensprofil, mens andre ikke. Siden bakterien innehar mange iboende resistensgener, som muligens ikke er funnet i denne studien, bør videre arbeid utføres før en endelig konklusjon av et kjerne-resistom for denne arten foreslås. Et større utvalg isolater bør involveres før dette kan konkluderes.

Studien presentert her,støtter tidligere studies som viser til at fenotypisk resistenstesting for antibiotika andre enn trimetoprim-sulfametoxazol er vanskelig for denne arten, og gir sprikende resultater. Hovedmålet, sett i en større sammenheng, er å finne en metode, fenotypisk, genotypisk eller en kombinasjon, som kan brukes til å sikrere korrekt resistensbestemmelse for *S. maltophilia*. Videre studier på dette området må utføres.

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1. Introduction

1.1. The opportunistic pathogen *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia is an opportunistic pathogen found in various environmental settings like soil and aquatic environments [1]. The genus *Stenotrophomonas* is phylogenetically classified as part of the group Gammaproteobacteria and is an aerobic, non-fermentative Gram-negative bacillus [2]. Currently, this genus is comprised of eight species: *Stenotrophomonas acidaminiphila*, *Stenotrophomonas chelatiphaga*, *Stenotrophomonas humi*, *Stenotrophomonas koreensis*, *Stenotrophomonas rhizophilia*, *Stenotrophomonas terrae*, *Stenotrophomonas nitrireducens* and *Stenotrophomonas maltophilia* [3].Originally *S. maltophilia* was named as a member of the genus *Pseudomonas* [4], then assigned to the *Xanthomonas* genus [5]. However, in 1993 it was reclassified as *Stenotrophomonas* [6].

1.2. Colonization and infection

Stenotrophomonas maltophilia is an environmental microbe, but as an opportunistic pathogen it may also be associated with many clinical syndromes, such as urinary infections, endocarditis, and respiratory infections like pneumonia [7, 8]. Infections caused by *S. maltophilia* are primarily seen in immunocompromised patients, e.g. cancer patients, and in patients with cystic fibrosis [9, 10].

The pathogenic factors and virulence associated with infection caused by *S. maltophilia* include the production of proteases and elastases, but also the ability to adhere to synthetic materials. *S. maltophilia* forms biofilm which is a serious virulence factor, making it possible to adhere avidly to medical implants and catheters. Biofilms are constituted by an assemblage of surface-associated community of cells that is enclosed in an extracellular matrix composed of polysaccharides and proteins [11]. This gives the microbe protection against host immune defences, and several antimicrobial agents [11, 12].

Vidipò et al investigated the adherence and penetrations of epithelial respiratory cells by *S. maltophilia* in 2001. Their research showed that *S. maltophilia* mainly associates with

intercellular junctions, and only a small percent of the bacterial cells penetrate and survive within membrane-bound vacuoles [13]. Studies show compelling results that a SMF-1 fimbriae is involved in adherence to mammalian cells, but also in hemagglutination and biofilm formation in *S. maltophilia* [14].

1.3. Clinical breakpoints of an antimicrobial agent

Antimicrobial susceptibility testing is a daily task in clinical microbiology laboratories worldwide to categorize microorganisms as clinically susceptible, intermediate or resistant to an antimicrobial agent. This is based on clinical breakpoints where the minimum inhibitory concentration (MIC) value is a corner stone. There are different organizations that publish agreed upon breakpoint values and tables, the most widely used standards are made by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) for Europe (and other parts of the world) and Clinical and Laboratory Standards Institute (CLSI; www.clsi.org) for the U.S. [15].

According to EUCAST, the definition of susceptibility is "a microorganism is defined as susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success." Resistance is, on the other hand, defined as "a high likelihood of therapeutic failure."

Setting breakpoints for an antimicrobial agent is based on multiple factors and studies such as:

- Evaluations of the efficiency of a drug in preclinical studies
- Evaluations of the efficiency of a drug in clinical studies
- Derived breakpoints from the pharmacodynamic target
- Monte Carlo simulations to estimate exposures of the antimicrobial agent in a patient population
- Wild-type population-studies of the microorganisms
- Resistance mechanism studies

Clinical outcome is dependent on the relationships between the MIC for the microorganism, exposure of the drug to the microorganism in the patient as well as the efficiency of the drug. Drug exposure of the microorganism in the patient is also dependent on the pharmacokinetic (PK) properties of the drug, meaning the drug concentration over time. Pharmacodynamic (PD), meaning the drug effect over time, is the relationship between the concentrations of the drug and clinical effect [16, 17].

EUCAST considers all this information before setting a clinical PK/PD breakpoint to ensure reliable antimicrobial susceptibility testing in a clinical setting. A schematic description of the process is displayed in figure 1.

If clinical studies and/or PK/PD studies are lacking, a clinical breakpoint cannot be set. In these cases, MIC values must be used as guidance. For *S. maltophilia*, the antimicrobial agent trimethoprim-sulfamethoxazole is the only drug with clinical breakpoint.

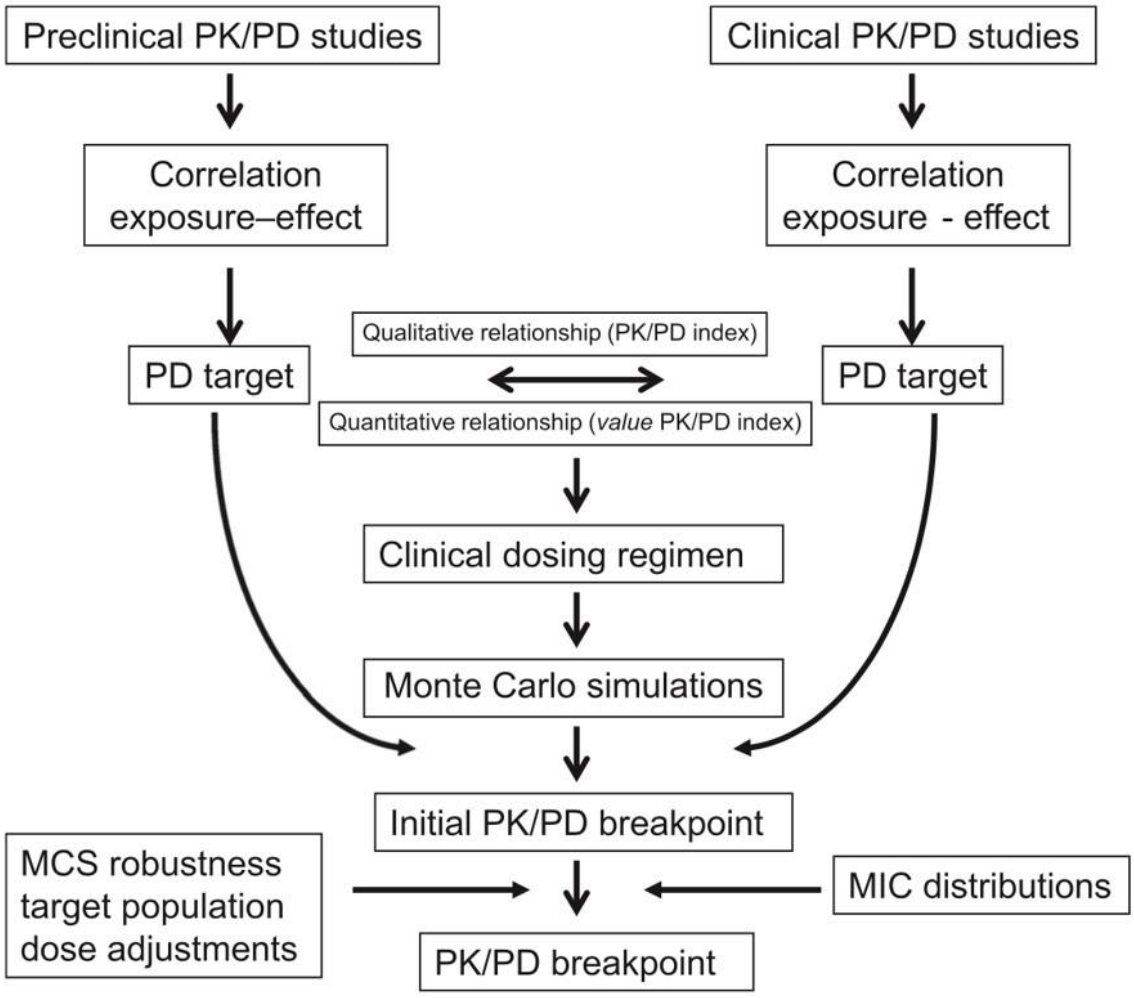

Figure 1: Process of setting clinical pharmacokinetic/pharmacodynamic (PK/PD) breakpoints by EUCAST [16]. Reprinted with permission through Elsevier.

1.4. Antimicrobial susceptibility testing in a laboratory setting

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit visible growth of a microorganism after overnight incubation, usually reported as mg/L. Minimum bactericidal concentration (MBC) is defined as the lowest concentration of an antimicrobial that will prevent the growth of an organism after sub cultivation on to antibiotic-free media. This thesis focuses on MICs only, as MIC is usually what is used by diagnostic laboratories to confirm antimicrobial susceptibility [18].

The aim of antimicrobial susceptibility testing is to determine the MIC value that, under defined test conditions, inhibits the visible growth of the bacterium being investigated.

Broth dilution is for most bacteria considered the gold standard for antimicrobial susceptibility testing [19, 20]. Figure 2 illustrates the method: First, one inoculates a certain concentration of bacteria (often 0,5 MF) into several tubes with liquid growth medium. Antibiotics are added in increasing concentrations; two-fold dilution series. Growth is assessed after incubation for a defined period of time, normally 16-24 hours. The MIC value is read at the first tube with no visible growth. [21].

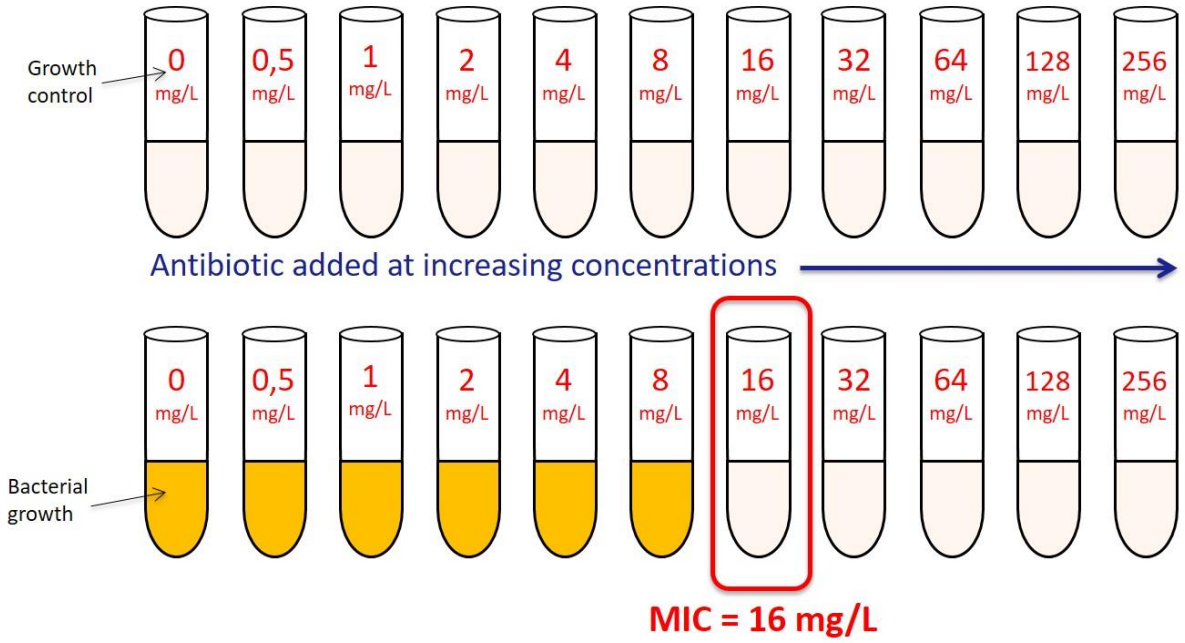

Figure 2: Describes minimum inhibitory concentration with the use of the broth dilution method.

Besides broth agar other methods for antimicrobial susceptibility testing are agar dilution, disk diffusion, MIC gradient strip tests and automated systems [18, 19].

Antimicrobial susceptibility testing of *S. maltophilia* is difficult because the results are significantly affected by several factors like incubation temperature, the choice of culture medium and different methods.

Susceptibility test results for agents other than trimethoprim-sulfamethoxazole should be treated with caution as there are no clinical PK/PD breakpoints for other agents, and therefore no data to support a relationship between susceptibility testing results and clinical outcome for *S. maltophilia* infection [22, 23].

For some antimicrobial agents, intrinsic resistance mechanisms are known, and antimicrobial susceptibility testing is therefore unnecessary, although it may be performed as part of panels of test agents. In these cases, 'susceptible' results should be viewed with caution, as their MIC values are not based on clinical studies. In some cases, intrinsic resistance to an agent may be expressed at a low level, with MIC values close to the susceptible breakpoint, although the agent is not considered to be clinically active. There are also situations where the agent appears to be fully active in vitro (MIC values cannot be separated from those of the wild type) but is inactive in vivo. Results without clinical breakpoints should therefore be interpreted with caution [15].

1.5. Antibiotic resistance

S. maltophilia shows low susceptibility towards many antibiotics, including those one normally uses to treat the infections it may cause [24] [25]. Intrinsic resistance, as opposed to acquired and/or mutational resistance, is a characteristic found in nearly all isolates of a bacterial species [26].

Intrinsic resistance may be due to reduced outer membrane permeability or to multidrug efflux pumps. However, specific mechanisms of resistance such as aminoglycoside-modifying enzymes or the heterogeneous production of metallo-β-lactamase have contributed to the multidrug-resistant phenotype displayed by this pathogen [27]. Antibiotic pressure increases the sequence variability in resistance genes or related genes, like regulators. The use of quinolones in *S. maltophilia* allows the appearance of mutants overexpressing efflux pumps, first *SmeDEF,* and when this is not present, *SmeVWX*. In both cases the overexpression is mainly associated to mutations in their regulators, *SmeT* and *SmeRv*, respectively [1, 28]. *S. maltophilia* produces diverse drug-hydrolysing enzymes such as *L1* and *L2,* respectfully a zinc-dependent metalloenzyme, and a cephalosporinase. These enzymes can terminate important classes of β-lactams such as carbapenems and cephalosporins [29].

S. maltophilia possesses a great many antibiotic resistance mechanisms. Most of the genes involved were present in *S. maltophilia* before any use of antibiotics. For example, the efflux pump *SmeDEF* is associated with the ability of *S. maltophilia* to colonize plants, and its

regulator *SmeT* is induced by plant-produced flavonoids [24] thus, the main function of the genes encoding them is unlikely to be the provision of antibiotic resistance. Contribution of integrons, and *SmeABC and SmeDEF* efflux pumps to multidrug resistance in clinical isolates of *S. maltophilia [2]*.

Because of mutations in different outer-membrane proteins, *S. maltophilia* isolates have shown to develop resistance to fluoroquinolones. Different efflux-pumps are also the reason for different mechanisms of antibiotic resistance as shown in table 1.

Trimethoprim-sulfamethoxazole is the drug of choice for treatment of infections by *S. maltophilia*, and resistance against it has emerged in the last years. This resistance is due to a gene called *sul1* [30], and presumable also *sul2* [31].

Table 1: A summary of known resistance genes in *S. maltophilia.*

Gene(s)	Product	Antibiotic resistance phenotype	Intrinsic, acquired or not determined	Reference
smeABC	RND-type efflux pump	Aminoglycosides, β-lactams, and quinolones	Acquired	Li et al. 2012 [25]
smeDEF	RND-type efflux pump	Chloramphenicol, tetracycline and quinolones	Intrinsic and acquired	Alonso and Martinez, 2000 [26] Zhang et al., 2001 [27]
smeGH	RND-type efflux pump	Unknown	Not determined	Crossman et al., 2008 [28]
smeIJK	RND-type efflux pump	Aminoglycosides, tetracycline and ciprofloxacin	Intrinsic and acquired	Crossman et al., 2008 [28] Gould et al., 2013 [29]
smeMN	RND-type efflux pump	Unknown	Not determined	Crossman et al., 2008 [28]
smeOP	RND-type efflux pump	Aminoglycosides, nalidixic acid, doxycycline, macrolides	Intrinsic	Lin et al., 2014 [30]
smeVWX	RND-type efflux pump	Chloramphenicol and quinolones	Acquired	Chen et al., 2011 [31] Garcia-Leon et al., 2014 [24]
smeYZ	RND-type efflux pump	Aminoglycosides	Intrinsic and acquired	Crossman et al., 2008 [28] Gould et al., 2013 [29]
emrCABsm	MFS-type efflux pump	Nalidixic acid and CCCP	Acquired	Huang et al., 2013 [32]
smlt0032	MFS-type efflux pump	Unknown	Not determined	Crossman et al., 2008 [28]
smtcrA	MFS-type efflux pump	Tetracycline	Acquired	Chang et al., 2011 [33]
smrA	ABC-type efflux pump	Fluoroquinolones, tetracycline, doxorubicin	Acquired	Al-Hamad et al., 2009 [34]
macABCsm	ABC-type efflux pump	Macrolides, aminoglycosides and polymyxins	Intrinsic (Not determined if some is acquired)	Lin et al., 2014 [30]
L1	β-lactamase	β-lactams	Intrinsic and acquired	Hu et al., 2008 [35] Okazaki and Avison, 2008 [36]
L2	β-lactamase	β-lactams	Intrinsic and acquired	Hu et al., 2008 [35] Okazaki and Avison, 2008 [36]
aph (3')-IIc	Aminoglycoside phosphotransferase	Aminoglycosides	Intrinsic and acquired	Okazaki and Avison, 2007 [37]
aac (6')-Iz	N-Aminoglycoside acetyltransferase	Aminoglycosides	Intrinsic and acquired	Li et al., 2003 [38]
Smqnr	Pentapeptide Repeat Proteins	Quinolones	Intrinsic and acquired	Sanchez and Martinez, 2010 [39] Chang et al., 2011 [33]
Sul1	Dihydropteroate synthase	Trimethoprim-sulfamethoxazole	Acquired	Chung et al. 2015 [40]
Sul2	Dihydropteroate synthase	Presumable: Trimethoprim-sulfamethoxazole	Acquired	Hu et al., 2016 [41], Toleman et al., 2007 [42]

1.6. Clinical treatment

Since this opportunistic pathogen shows high levels of intrinsic and/or acquired resistance to many antimicrobial agents, the treatment of infections by *S. maltophilia* is difficult and dramatically reduces the antibiotic options available for treatment [2].

In addition to the different resistance genes mentioned in the previous chapter, the lack of standardized susceptibility testing and criteria to interpreted them (see section 1.3) also makes the choice of an adequate antibiotic treatment challenging. In vitro studies, some non-randomized trials and anecdotal experiences are the basis of the recommendation for antibiotic treatment. The drug of choice is trimethoprim-sulfamethoxazole, and this is the only drug with clinical breakpoints for this species by EUCAST. Several in vitro studies indicate that ticarcillin-clavulanic acid (no longer available on the market), minocycline, some of the new fluoroquinolones, and tigecycline may be useful antibiotics [2, 10].

Antimicrobial agents that may be relevant for susceptibility testing in *S. maltophilia isolates*:

- Ceftazidime is a third-generation cephalosporin, which has a broad spectrum of in vitro activity against Gram-positive and Gram-negative aerobic bacteria and is resistant to hydrolysis by several beta-lactamases and is bactericidal. Ceftazidime shows high in vitro activity against *Pseudomonas aeruginosa* [43, 44].
- Ciprofloxacin is a fluoroquinolone and has broad spectrum activity against Gram-positive and Gram-negative bacteria and is bactericidal. It works by blocking the DNA replication of the bacteria by binding to topoisomerases, either DNA gyrase or topoisomerase-IV, which are essential for DNA replication [45].
- Colistin, also known as polymyxin E, is an old antimicrobial agent that is effective against Gram-negative bacteria [46]. Resistance is rare, but observed, and colistin is often a last-resort for treating different multidrug-resistant bacteria [47, 48].
- Meropenem is a broad-spectrum antibacterial agent of the carbapenem family and is bactericidal to most bacteria by blocking cell wall-formation. The spectrum of action

includes many Gram-positive and Gram-negative bacteria (including *Pseudomonas* spp.) [49].

- Tigecycline belongs to the tetracycline-group of antibiotics; it is bacteriostatic and shows activity against many Gram-positive and Gram-negative organisms, including those with multi-drug resistance. It acts as a protein synthesis inhibitor by binding to the 30S ribosomal subunit of bacteria and blocking the interaction of aminoacyl-tRNA with their site on the ribosome [50].
- Trimethoprim-sulfamethoxazole is a combination of two agents, trimethoprim and sulfamethoxazole. It works by inhibiting the folate synthesis pathway of bacteria and is bacteriostatic. It is the treatment of choice for treating infections caused by *S. maltophilia* [51]. One of the side-effects is bone marrow depression, which is an unwanted effect in immunosuppressed patients. Also, allergies are quite common, and this is makes treatment difficult in many CF-patients [52].

1.7. The aim of this study

The main aim of this thesis was to map the core genomic resistome by using whole genome sequencing (WGS) in clinical isolates of *S. maltophilia*, and to compare the clinical resistome to an environmental resistome, with the assumption that there are differences between the clinical and environmental isolates; the clinical isolates have more resistance genes than the environmental isolates.

The second aim of the study was to use different methods for phenotypic antimicrobial susceptibility testing to map the phenotypic resistance patterns in the different *S. maltophilia* isolates. We wished to compare the different phenotypic methods to the presumed gold standard (broth dilution) and compare the phenotypic resistance profile to the genotypic resistance profiles found by WGS.

2. Materials

2.1. Growth media and agars

Two different agars for growth were used in this thesis. Lactose agar and human blood agar were used for inoculation and cultivation of the isolates. Mueller Hinton agar was used for antimicrobial susceptibility testing. There agars were produced by the Unit of Control and Production at the Department of Microbiology, following protocols from the manufacturer.

2.2. Laboratory equipment

Equipment	Supplier
96-well PCR plates	VWR
Eppendorf tubes 2 ml	Eppendorf
Gloves	VWR
Magnetic stand	Life technologies
Microseal for 96-well microtiter plates	Life technologies
Microtiter 96-well plates	Sarstedt
Multi-channel pipettes	Thermo Scientific
McFarland Standards Kit	Biomerieux
PCR tubes 0.2 ml	Sarstedt
Pipettes	Thermo Scientific
Pipette tips	VWR
Plastic loops	Sarstedt
Qubit 0.5 ml tubes	Life Technologies

2.3. Instruments

Instrument	Supplier
Densichek Plus	BioMérieux
Eppendorf centrifuge	Eppendorf
Heating block for 1.5 ml centrifuge tubes	Stuart Scientific
Illumina MiSeq	Illumnia
Incubator (36,5 °C)	Thermo Scientific
MagNA Lyser	Roche
MagNA Pure Compact	Roche
MALDI-TOF MS	Bruker Daltonics
Microplate centrifuge	Eppendorf
Microtiter plate shaker	VWR
NanoDrop One	Nanodrop Technologies
MasterCycler (PCR-machine)	Eppendorf
Vortex-Genie 2	Scientific Industries
VITEK 2	BioMérieux
Qubit 2.0	Life Technologies

2.4. Software

Software	Company
CARD/RGI	McMaster University
FastQC	Babraham Bioinformatics
FigTree	OMICtools
ResFinder/SpeciesFinder	DTU
Illumina Experiment Manager	Illumina

2.5. Kits

The protocols accompanying the various kits were utilized in their designated procedures, but usually with certain alterations. Thus, all procedures in the methods section will be described in their entirety.

Kit	Supplier
Agencourt AMPure XP 60 ml kit	Beckman Coulter
KAPA HyperPlus Kit	Roche
MiSeq Run Reagents (V3-600 cycles)	Illumina
MPC Nucleic Acid Isolation Kit I	Roche
Nextera XT Indexing Kit (96-indexes)	Illumina
NextFlex (24-indexes)	BioO Scientific

2.6. Chemicals and reagents

- α-cyano-4-hydroxycinnamic acid
- Ethanol
- Formic Acid
- NaOH
- Tris-HCl
- Tween 20

2.7. Antibiotics

Six different antibiotics were selected in this study, with for different methods.

The Liofilchem® MIC Test Strips were used for ceftazidime (CAZ 0.016-256), trimethoprim-sulfamethoxazole (SXT 0.002-32), tigecycline (TGC 0.016-256), Meropenem (MRP 0.016-256) and ciprofloxacin (CIP 0.002-32). For colistin, MIC gradient strips from BioMérieux were used, E-test (CO 0.002-32).

For broth microdilution, sensititre by Thermo Fischer, the panels NONAG04 and NONAG05 (Sensititre® plate: *Enterobacteriaceae* I & II) were used. For 20 isolates, NONAG06 (*Pseudomonas/Acinetobacter*) panel were used to get tigecycline results.

For VITEK 2 by BioMérieux, the panel AST-N222 (*Pseudomonas spp*.) were used.

2.8. Bacterial strains

The bacterial isolates in this thesis were collected from Oslo University Hospital, during the period from 1989 to 2017. Of the 100 strains, 75 of the strains were clinical samples and the remaining 25 were environmental samples collected from patient rooms in Oslo University Hospital. Of the 75 clinical strains, three different clinical wards/groups were chosen: 25 samples were from the Intensive Care Unit, 25 were from patients with Cystic fibrosis and the remaining 25 were from the Department of Haematology.

3. Methods

3.1. General methods in microbiology

3.1.1. Sterile working technique

Throughout the work implemented in this thesis, procedures with high risk of contamination were consistently carried out in sterile work benches accompanied by diligent use of gloves, ethanol and gas burners to avoid compromising the bacterial samples. Tools and equipment were autoclaved and/or sterilized with ethanol and gas burner prior to contact with samples.

3.1.2. Growth agar

Cultivation and experimentation with bacteria require suitable growth agars. These were assembled according to recipes provided by the manufacturer, followed by autoclaving and appropriate storage for later use. Agars were cooled to 50-60 °C and transferred to petri dishes, and again cooled to 25 °C before being used for streaking bacteria to single colonies. These protocols were followed out by the Unit of Control and Production at the Department of Microbiology. They also performed sterile controls of all the agars before sending them out of the production area.

3.1.3. Inoculation and cultivation of overnight pure cultures

To achieve single colonies which were later used for making pure cultures, bacteria from the freezing broths were plated on solidified lactose growth agar. This was executed by using sterile plastic loops and a streaking pattern aiming to dilute the bacteria to single colonies. The plates were incubated at 36, 5 °C overnight. Plates were subsequently stored at 4-8 °C for further use. Fresh pure cultures were attained by inoculating single colonies from agar plates using sterile plastic loops and transferred a new lactose agar, after overnight incubation at 36, 5 °C.

3.1.4. MALDI-TOF Mass Spectrometry for identification

In MALDI-TOF Mass Spectrometry, MALDI stands for “Matrix-assisted Laser Desorption/Ionization”, and TOF stands for “Time of Flight”. This ionization technique uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation [55]. The ratio of their mass to charge (m/z) is then measured [56].

MALDI-TOF spectra are used for the identification of microorganisms, in this case bacteria. The samples in this study were identified with this technique before library preparation to ensure correct identification.

The sample preparation for analysis om MALDI-TOF MS is first to pick the desired colony for identification and smear it onto a target plate. Then the sample is coated with a solution called a matrix, which is an energy-absorbent and organic compound. When this matrix dries, it is crystalized, and the sample entrapped within the sample also co-crystallizes. The sample within the matrix is ionized by the laser beam. With desorption and ionization singly protonated ions from analytes in the sample are created. With a fixed potential, the protonated ions are accelerated and separated from each other based of their different mass to charge ratio (m/z) [56]. These charged analytes are detected and measured by a mass analyzer by the time of flight (TOF). The m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube. Based on the time of flight, a characteristic spectrum is generated for the analytes in the sample. This is called a peptide mass fingerprint (PMF). The identification of microbes by MALDI-TOF MS is done by comparing the PMF of the sample with the database containing known microbes and their MS spectra. The typical mass range for species level identification is m/z of 2-20 kDa. Mainly ribosomal proteins and a few housekeeping proteins are represented for identification [57].

<u>Materials and instruments:</u>

α-cyano-4-hydroxycinnamic acid

Formic Acid

MALDI-TOF Mass Spectrometer

Protocol:

1. A portion of a colony of the microbe in question is placed onto the sample target and overlaid with formic acid for cell wall destruction.
2. After the formic acid has dried the matrix is placed on top of the fragmented colony on the plate.
3. A pulsed laser irradiates the sample, triggering ablation and desorption of the material in the sample and matrix.
4. The analyte molecules are ionized by being protonated or deprotonated in the hot plume of ablated gases and can then be accelerated into whichever mass spectrometer is used to analyse them.

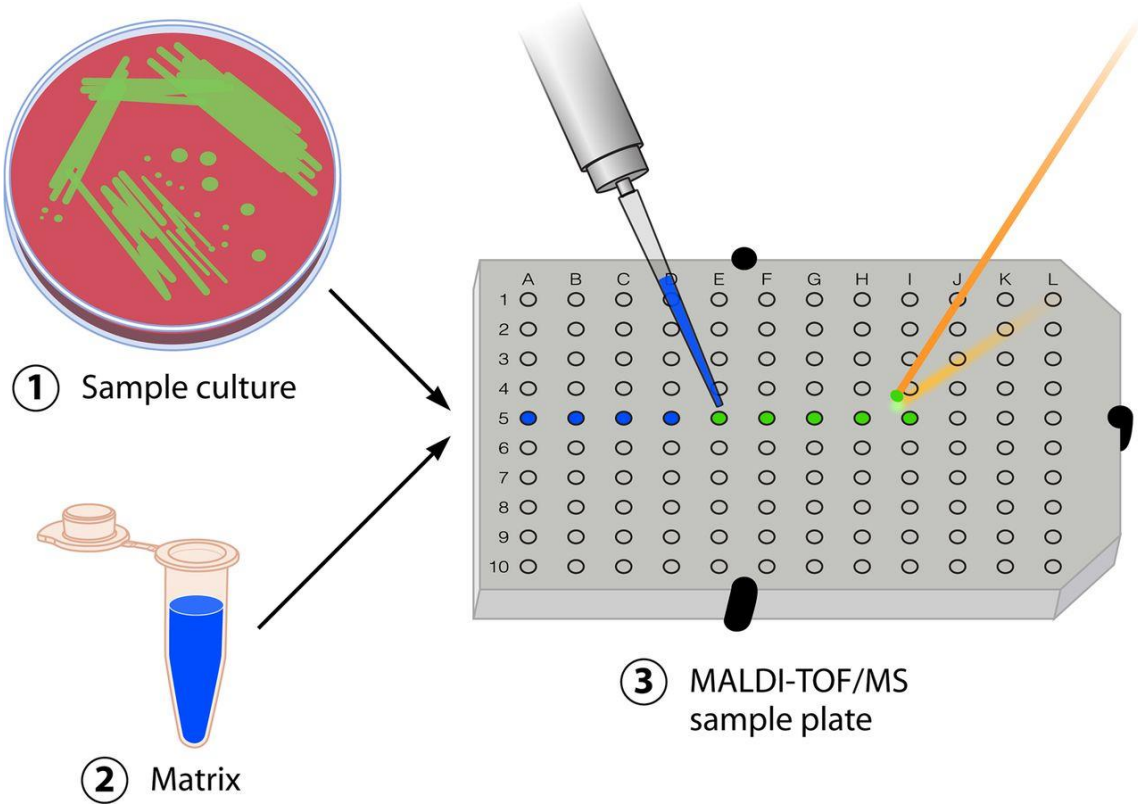

Figure 3: Inoculation of bacterial specimen onto a sample plate coated with matrix to run on MALDI-TOF Mass Spectrometer [53].

3.2. MIC determination

McFarland

All methods mentioned in the next chapters on antibiotic susceptibility testing are based on using a standard bacteria suspension, the McFarland standard. This is a measurement of the turbidity of the bacterial suspension, or optical density, so that the number of bacterial cells in the solution is within a given range to standardize the antimicrobial susceptibility testing. This is measured by a densitometer and calibrated with different McFarland standards.

Originally these standards were made by mixing specific amounts of barium chloride and sulfuric acid together to get a specific turbidity. The McFarland standards used to calibrate the densitometer nowadays are based on latex particles which is more stable for storage. The absorbance at wavelength 600 nm, is between 0.06 – 0.1 with the 0,5 McFarland standard. Use the direct colony suspension method to make a suspension of the organism in saline to the density of a McFarland 0,5 turbidity standard, approximately corresponding to 1-2 x 10^8 CFU/mL for *Escherichia coli* [18].

Table 2: Approximately cell density, transmittance (percentage) and absorbance at wavelength 600 nanometre at different McFarland standards [18].

McFarland Standard No.	0,5	1	2	3	4
Approx. cell density (1X10^8 CFU/mL)	1,5	3,0	6,0	9,0	12,0
% Transmittance	74,3	55,6	35,6	26,4	21,5
Absorbance	0,06 – 0,1	0,257	0,451	0,582	0,669

MIC determination by EUCAST

Since clinical breakpoint for *S. maltophilia* is only available for trimethoprim-sulfamethoxazole, breakpoints for *Pseudomonas spp*. were used as guidance to suggest susceptibility for the other antimicrobial agents. See table 3 and 4.

Table 3: Breakpoints for *S. maltophilia* by EUCAST, version 8.1

	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Group
	S ≤	R >		S ≥	R <	
Trimethoprim-sulfamethoxazole	4	4	1.25-23.75	16	16	Miscellaneous

Table 4: Breakpoints for *Pseudomonas spp*. by EUCAST, version 8.1

	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Group
	S ≤	R >		S ≥	R <	
Ceftazidime	8	8	10	17	17	Cephalosporins
Ciprofloxacin	0,5	0,5	5	26	26	Fluoroquinolones
Colistin	2	2		-	-	Miscellaneous
Meropenem	2	8	10	24	18	Carbapenems
Tigecycline	0,25	0,5		-	-	Tetracyclines

3.2.1. Agar disk diffusion

Agar disk diffusion is one of the oldest approaches to antimicrobial susceptibility testing and is also one of the most used methods of antimicrobial testing in routine clinical laboratories. Most bacterial pathogens are suitable for this testing method, which does not require special equipment and is versatile in the range of antimicrobial agents that can be used [58].

Colonies from the overnight culture from a non-selective media is collected with a sterile cotton swab and suspended in 0,9 % saline solution. The density of the suspension is adjusted to 0,5 McFarland by either adding saline or more bacteria.

A denser inoculum will result in reduced zones of inhibition and a decreased inoculum will have the opposite effect. A densitometer, here a densichek (BioMérieux), measures the optical density or turbidity of the solution.

<u>Inoculating agar plate</u>

1. Dip a sterile cotton swab into the suspension.
2. To avoid over-inoculation of Gram-negative bacteria, remove excess fluid by pressing and turning the swab against the inside of the tube

3. Make a cross on the agar and spread the inoculum evenly over the entire agar surface using an automatic rotator.
4. Apply disks within 15 min of inoculation. If inoculated plates are left at room temperature for long periods of time before the disks are applied, the organism may begin to grow, resulting in flawed reduction in sizes of inhibition zone diameters

Application of antimicrobial disks and incubation

1. Allow disks to reach room temperature before opening cartridges or containers used for disk storage. This is to prevent condensation, leading to decline of some agents.
2. Apply disks firmly to the surface of the inoculated agar plate within 15 minutes of inoculation. Disks must be in close and even contact with the agar surface and must not be moved once they have been applied as the initial diffusion of antimicrobial agents from disks is very rapid.
3. The number of disks on a plate should be limited to avoid overlapping of zones and interference between agents. 6 disks are used per plate. It is important that zone diameters can be reliably measured.
4. Invert agar plates and make sure disks do not fall off the agar surface. Incubate plates within 15 min of disk application. If the plates are left at room temperature after disks have been applied, pre-diffusion may result in erroneously large zones of inhibition.
5. Stacking plates in the incubator may affect results due to uneven heating. The efficiency of incubators varies and therefore the control of incubation, including appropriate numbers of plates in any one stack. Four plates are stacked in the incubator.
6. Incubation beyond the recommended time limits should not be performed as this may result in growth within inhibition zones and reporting isolates as false resistant. For *S. maltophilia* 20-24 hours of incubation is appropriate.

The 15-15-15-minute rule: use the inoculum suspension within 15 minutes of preparation, apply disks within 15 minutes of inoculation and incubate plates within 15 minutes of disk application.

After appropriate time of incubation, a circular zone around the disc is formed. The diameter from growth to growth surrounding the disk is read and translated into S-I-R, with the use of zone diameter breakpoints by EUCAST (see table 4 and 5).

3.2.2. MIC gradient strip test

Same procedure as with agar disk diffusion are used when it comes to McFarland standard and inoculating the plates. Some differences with adding the strip see below.

The MIC gradient strip is applied onto an inoculated agar surface; the preformed exponential gradient of antimicrobial agent is transferred into the agar matrix. After 18 hours incubation or longer, a symmetrical inhibition ellipse centred along the strip is formed. The MIC is read directly from the scale in terms of µg/mL, at the point where the edge of the inhibition ellipse intersects with the MIC gradient strip.

Part of the 15-15-15-minute rule: use the inoculum suspension within 15 minutes of preparation, apply MIC gradient strips within 15 minutes of inoculation and incubate plates within 15 minutes of application.

3.2.3. Broth microdilution

Broth microdilution is a method used to test the susceptibility of bacteria to antibiotics. Varying concentrations of different antibiotics are loaded in a plate, and the bacteria to be tested are then added to the plate. The results of the broth microdilution method are reported in MICs. Broth microdilution is highly accurate, and the accuracy of its results are comparable to broth dilution, the gold standard of susceptibility testing [19].

<u>INOCULATION AND INCUBATION</u>

1. Sweep the confluent portion of growth from growth on an agar plate with a swab. Emulsify in sterile saline (0,85 % NaCl) and adjust to a 0,5 McFarland Standard by using a densitometer.

2. Transfer 50 µl of the suspension into a tube of cation-adjusted Mueller-Hinton broth with TES buffer to give an inoculum of 5 x 10^5 cfu/mL, then mix on a vortexer.
3. Transfer 100 µL to each well of the AST plate with the Sensititre® autoinoculator, called AIM®. Replace the tube cap with a Sensititre® single-use dose head and insert into the AIM®.
4. Cover all wells with the adhesive seal. Press all wells firmly to assure adequate sealing. Avoid creases as these can lead to skips.
5. Incubate the plates in a non-CO_2 incubator for 20-24 hours at 36 °C.

Results can be read using the Sensititre® manual viewer or the Vizion®. It is not necessary to remove the adhesive seal, when reading the results. Growth appears as turbidity or as a deposit of cells at the bottom of a well. The MIC is recorded as the lowest concentration of antimicrobial that inhibits visible growth. Reading faint growth on Vizion® can be improved by use of bright indirect lighting against a dark background. The positive growth control wells should be read first. If any show no growth, results are invalid.

Growth can range from a few colonies with no turbidity to heavy growth comparable to positive growth control. The MIC is the lowest concentration that inhibits growth except for sulphonamides, where the MIC is read as the lowest concentration that inhibits 80 % growth compared to the positive control.

<u>The following points should be noted:</u>

- Contamination:
  Contamination may result in growth in a well surrounded by wells showing no growth. Such a single well contamination can be ignored, but if multiple well contaminants are suspected, the test should be repeated.
- Skips:
  Occasionally a “skip” may be seen - a well showing no growth surrounded by wells showing growth. There are variety of explanations including contamination, mutation, creased seal and wrong dosage. A single skip can be ignored. However, to

ensure effective antimicrobial therapy never read the skipped well as the MIC; always read the lowest well concentration above which there is consistently no growth.

- Mixed Cultures:

  Except as referred to in (a) above, if two end points are seen as a distinct “button” of cells followed by several wells of diffuse growth with the “button” no longer visible, there may be a mixed bacterial population. Purity should be checked by sub-culturing growth onto suitable agar. Test results are invalid if a mixed culture is detected.

3.3.4. VITEK 2

The VITEK 2 from BioMérieux is an automated microbiology system utilizing growth-based technology. The system uses colorimetric AST cards that are incubated in a cassette and interpreted automatically by the system.

Test Card Setup Procedure

1. Prepare inoculums from a pure culture, according to good laboratory practices. In case of a mixed culture, an isolation step is required. A purity check plate is used to ensure that a pure culture was used for testing.

2. Aseptically transfer 3,0 ml of sterile saline (0.45% NaCl) into a clear plastic test tube.

3. Use a sterile swab to transfer a sufficient number of colonies to the saline tube prepared in step 2. Prepare a homogenous organism suspension 0,5 McFarland standard using the DensiChek™. Place this tube in a VITEK cassette. NOTE: the age of the suspension before loading the instrument for AST testing must be less than 30 minutes.

4. Place a second plastic tube the cassette, scan the susceptibility card and place it in the tube.

3.3. DNA isolation and extraction for whole genome sequencing

3.3.1. MagNA Pure Compact Isolation Kit I

DNA extraction was done with the Nucleic Acid Isolation kit I by Roche on a MagNA Pure Compact Instrument. All of the nucleic acid isolation steps are performed automatically by the MagNA Pure Compact instrument, and is based on magnetic-bead technology. Description of the techology is presented in figure 4.

Magnetic beads techology involves seperation of nucleic acids with the use of magnet glass particles. Cell disruption and protein digestion is done by the addition of Lysis Buffer and Proteinase K. Then free DNA binds to the surface of Magnetic Glass particles added to the solution. Magnetic seperation of the DNA-coated glass particles, lets the system to wash cycles to remove the solution and cellular debris. Several washsteps are added to ensure a clean elution, before high temperature is set in to make DNA elute from the magnetic glass particles.

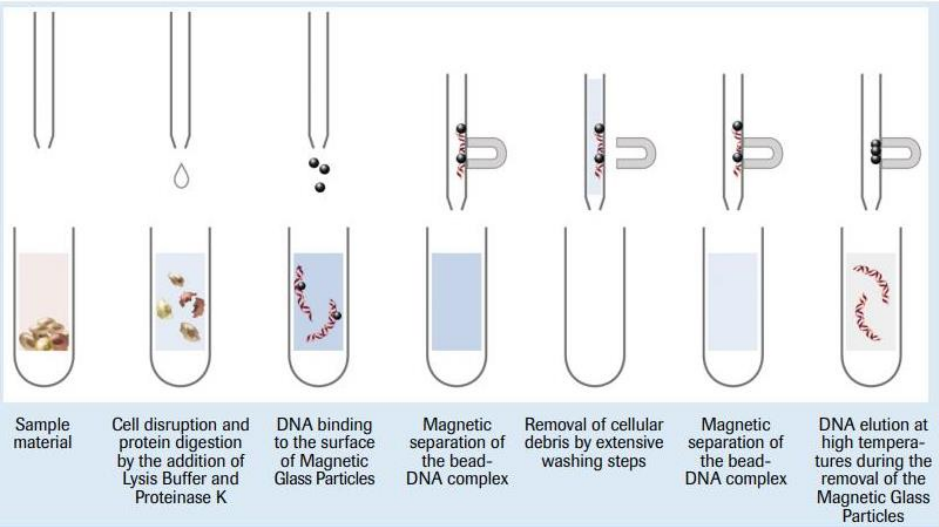

Figure 4: Schematic description of the magnetic beads technology used in MagNA Pure Compact by Roche [54].

Materials and instruments:

Elution tubes

MagNA Lyser

MagNA Pure Compact

MagNA Pure Compact Isolation Kit I

MagNA Lyser Green Beads

Phosphate-buffered saline (PBS)

Protocol

1. Colonies from fresh pure cultures on lactose agar are inoculated into a MagNA Lyser Green Bead-tube containing 500 µL of phosphate-buffered saline (PBS).
2. The tube is shaken on the MagNA Lyser for 30 seconds at 6500 rpm.
3. Resuspend 400 µL of the cell culture in a new elution tube. Avoid bubbles on top.
4. Add elution tube containing sample to the MagNA Pure Compact Machine, with MagNA Pure Compact Isolation Kit I and start the run.

This system allows for up to $2x10^6$ cultured cells per sample and the ouput is up to 100 ng/mL (MagNA Pure Compact Guide = referance). Each run can take up to 8 samples, and the duration is approximately 30 minutes.

3.4. DNA measurements

3.4.1. NanoDrop

NanoDrop One was used to obtain optical measurements of DNA-concentration and purity both after DNA isolation and PCR product clean-up. Elution buffer was employed for blanking the instrument, before 2 µl of DNA sample was applied to the pedestal. The absorbance was measured at 260 nm as this is the absorbance maximum of nucleic acids. The concentration was returned in ng/ml while the purity was emitted by the instrument as the 260/280 absorbance ratio. This ratio should be within the range of 1.8 and 2.0. Lower values indicate contamination by proteins (absorbance maximum at 280 nm), and higher values indicate that RNA is present in the sample.

3.4.1. Qubit

When working with gDNA for whole genome sequencing, a Qubit fluorometer was employed to evaluate the concentration of DNA. The Qubit in contrast to NanoDrop only measures DNA and is not influenced by protein contents or other contaminants.

Materials and instruments:

Qubit 2.0

0.5 ml Qubit tubes

Qubit dsDNA HS Kit Q32854

The working solution for the instrument was prepared by mixing 199 µl buffer and 1 µl dye per sample to be measured (including two standards). Next, the working solution was utilized in the preparation of standard 1 and 2 to make a standard curve for the Qubit instrument, which later was used to make sample calculations. 190 µl working solution and 10 µl standard were mixed in Qubit tubes using standard 1 and 2 respectively. DNA samples were prepared by thoroughly mixing 198 µl working solution and 2 µl eluted DNA in Qubit tubes before reading the concentration.

3.5. Library preparation for whole genome sequencing

3.5.1. KAPA Hyper Plus Prep Kit

The first 10 samples were a pilot-run with both the KAPA Hyper Plus Prep kit and the Illumina MiSeq machine. This library preparation kit is suitable for 1 ng to 1 µg DNA.

Materials:

KAPA HyperPlus Prep Kit (96 samples)

BioO Adapters (24 indexes)

Agencourt AMPure XP 60 ml kit (beads)

MiSeq Run Reagents Kit (V3-600 cycles)

96-well PCR plates

96-well microtiter plates

MasterCycler

Ethanol absolute

Heating block

Magnetic stand

Microplate centrifuge

Microplate shaker

Microseal for 96-well PCR plates

NaOH

Step 1: Enzymatic fragmentation

After measuring the samples with NanoDrop and Qubit, 6 µL DNA and 29 µL Tris-HCl were mixed to get a fixed volume of 35 µL of each library. Each library was kept on ice prior to the reaction setup to ensure that the fragmentation process is kept on hold when adding KAPA Frag Buffer (10X) and the KAPA Frag Enzyme. The thermocycler was also pre-cooled to 4 °C before the reaction at 37 °C. The optimization range for 600 base pair fragments length is 3 – 10 minutes at 37 °C for this enzyme and in this pilot for 9 minutes.

Step 2: End Repair and A-Tailing

In the same plate in which enzymatic fragmentation was preformed, the end repair and A-tailing reaction occurs. Addition of End Repair & A-Tailing buffer and Enzyme Mix on ice to keep the reaction controlled before the thermocycler programme, at 65 °C for 30 minutes.

Step 3: Adapter-ligation

In the same plate in which end repair and A-tailing was preformed, the adapter-ligation reaction follows. The adapter stocks from BioO are diluted from 25 µM to 5 µM in PCR-grade water. The Ligation Buffer and DNA Ligase enzyme is mixed thoroughly with the adapters and DNA and centrifuged briefly before incubation at 20 °C in 15 minutes.

Step 4: Post-ligation clean-up

In the same plate in which adapter-ligation is performed, the bead-based post-ligation clean-up is obtained. It is important to pipette slowly and correctly when working with beads. Over-drying of the beads may result in reduced yield and caution when drying is important. After washing with 80 % ethanol you remove the beads from the magnet and resuspend the beads in elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). The supernatant is transferred to a new plate for amplification.

Step 5: Library amplification

Table 5: Displaying the library amplification program in the KAPA Hyper Plus library preparation protocol, preformed on MasterCycler (Eppendorf).

Step	Temp	Duration	Cycles
Initial denaturation	98 °C	45 secs	1
Denaturation	98 °C	15 secs	9*
Annealing*	60 °C	30 secs	
Extension	72 °C	30 secs	
Final extension	72 °C	1 min	1
HOLD	4 °C	∞	1

*Number of cycles may be changed based on the amount on DNA input.

Step 6: Post-amplification clean-up

In the same plate in which library amplification is performed, the bead-based post-amplification clean-up is obtained. It is important to pipette slowly and correctly when working with beads. Over-drying of the beads may result in reduced yield and caution when drying is important. After washing with 80 % ethanol you remove the beads from the magnet and re-suspend the beads in elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). The supernatant is transferred to a new plate for size selection.

Extra: Size selection

Size selection was performed with KAPA beads to ensure right size of the fragments. 0,7 X beads for the first cut and 0,9 X beads for the second cut.

Table 6: Displaying the possible modifications used for decreasing or increasing the size limit, either in first or second cut.

Upper size limit	Modification	Lower size limit	Modification
Increase	Decrease the ratio of the first cut	Increase	Decrease the ratio of the second cut*
Decrease	Increase the ratio of the first cut	Decrease	Increase the ratio of the first cut*

**The second size cut should be performed with at least 0.2 volumes of KAPA Pure Beads reagent and DNA recovery is dramatically reduced if the difference between first and second cuts is less than ~0.2 volumes.*

3.5.2. Nextera XT DNA Library Prep Kit

Sample preparation for MiSeq sequencing run:

The Nextera XT DNA Sample Prep Kit was used to prepare the 90 of the 100 samples for whole genome sequencing. The preparation procedure would result in a paired end library for each sample before library pooling and sequencing was initiated. The preparation for MiSeq sequencing is relatively lengthily and is described in five steps. Consistent replacement of pipette tips is crucial to avoid cross-contamination throughout the MiSeq sample preparation.

Materials and instruments:

Agencourt AMPure XP 60 ml kit (beads)

Nextera XT DNA Sample Prep Kit (96-samples)

Nextera XT Indexing Kit (96-indexes)

96-well PCR plates

96-well microtiter plates

96-well thermal cycler with heated lid (PCR machine)

Ethanol absolute

Heating block for 1.5 ml centrifuge tubes

Magnetic stand

Microplate centrifuge

Microplate shaker

Microseal for 96-well PCR plates

NaOH

Step 1: Tagmentation

After measuring DNA concentration by Qubit and diluting the samples to 0,2 ng/µl, a process termed tagmentation was initiated. This step exploits an engineered transposome which couples the process of fragmenting the DNA with tagging the ends with unique adapter sequences. The MiSeq can distinguish these adapter sequences. Thus, they provide a means of identification to differentiate reads from various isolates in the pooled sample. These unique adapter sequences are also the basis for primer annealing and initiating amplification of input DNA in the subsequent PCR (step 2).

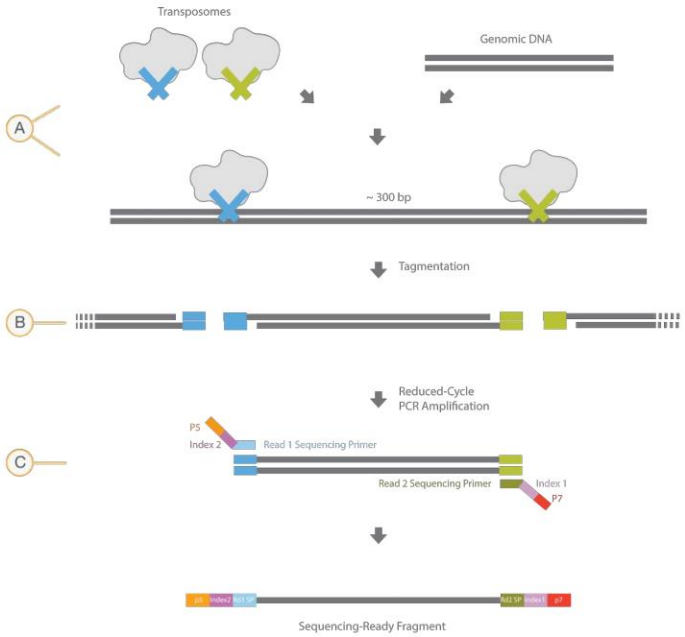

Figure 5: Visualization of the tagmentation process used in the Nextera XT DNA kit from Illumina. Enzymatic fragmentation by a transposase and adapter-ligation is happening in the same step on a thermocycler.

Step 2: PCR Amplification and indexing

For amplifying the tagged and fragmented DNA templates into larger quantities, a limited-cycle PCR program was used in correlation with indexing primers 1 (N7) and 2 (S5). For 90 samples, indexing primers S501-08 and N701-12 were used.

Table 7: PCR program for amplifying the tagmented DNA templates preformed on MasterCycler (Eppendorf) using the Nextera XT protocol.

Step	Temp	Duration	Cycles
Initial denaturation	72 °C	3 min	1
Initial denaturation	95 °C	30 secs	1
Denaturation	95 °C	10 secs	12
Annealing*	55 °C	30 secs	
Extension	72 °C	30 secs	
Final extension	72 °C	5 min	1
HOLD	10 °C	∞	1

Step 3: PCR Clean-up

The DNA paired end library now having been attained by PCR was purified in using magnetic AMPure XP beads for removal of both very small DNA fragments and left-overs from the PCR.

Step 4: Library Normalization

To ensure a more equal representation of each sample before pooling the libraries, the quantity of each library was normalized using normalization beads.

Step 5: Preparation of PhiX control:

In this case it was decided to add more diversity to the sample libraries to enhance the confidence in the sequencing run. This was attained by adding PhiX control. The PhiX is a control library consisting of fragments the MiSeq can recognize and differentiating from the samples. E.g. in 16S rRNA sequencing, the fragments are very similar to each other and it is common to add ~30% of PhiX. However, in whole genome sequencing using DNA libraries from various isolates and species, the fragments have much less similar sequences, making it unnecessary to add PhiX in such large quantities. PhiX increases diversity among the sequence fragments and can improve confidence in the MiSeq output. Also, it helps estimate error rates and determines if errors are likely due to sample preparation mistakes or the MiSeq.

Step 6: Library pooling and MiSeq sample loading

The MiSeq uses a pooled sample consisting of equal volumes of normalized sample libraries, which is diluted in hybridization buffer and heat denatured before sequencing can be engaged.

3.6. Whole genome sequencing on Illumina MiSeq

The Basics of NGS Chemistry

In principle, the concept behind NGS technology by Illumina is similar to Sanger sequencing. DNA polymerase catalyses the incorporation of fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand during sequential cycles of DNA synthesis. During each cycle, the nucleotides are identified by excitation of the fluorophores. This happens at the point of incorporation. The critical difference between the chemistries is that, instead of sequencing a single DNA fragment, NGS extends this process across millions of fragments in an immensely parallel way on a solid surface. The use of modified dNTPs containing a terminator allows for this, by blocking further polymerization from occurring. Only one single base can therefore be added by a polymerase enzyme to each growing DNA copy strand at a time.

The terminator also contains a fluorescent label, which a camera can detect. Only a single fluorescent colour is used, so each of the four bases must be added in a separate cycle of DNA synthesis and imaging. Following the addition of the four dNTPs to the templates, the images are recorded, and then the terminators are removed. This chemistry is called "reversible terminators". Lastly, another four cycles of dNTP additions are initiated. Since single bases are added to all templates in a even manner, the sequencing process produces a set of DNA sequence reads of even length [55].

NGS workflows include four basic steps:

1. Library Preparation: The sequencing library is prepared by random fragmentation of the DNA followed by 5'and 3' adapter ligation. Alternatively, "tagmentation" combines the fragmentation and ligation reactions into a single step that greatly increases the efficiency of the library preparation process. Both techniques are used in this thesis. Adapter-ligated fragments are then PCR amplified and purified.

2. Cluster Generation: For cluster generation, the library is loaded onto a flow cell where the fragments are captured on a lawn of surface-bound oligos complementary to the library adapters added in the library preparation step. Each fragment is amplified into distinct,

clonal clusters through bridge amplification. When cluster generation is complete, the templates are ready for sequencing.

3. Sequencing by synthesis: Illumina SBS technology uses a proprietary reversible terminator–based method that detects single bases as they are incorporated into DNA template strands. All four reversible terminator–bound dNTPs are present during each sequencing cycle, and natural competition minimizes incorporation bias and greatly reduces raw error rates compared to other technologies. The result is highly accurate base-by-base sequencing that virtually eliminates sequence context–specific errors, even within repetitive sequence regions and homopolymers.

4. Data Analysis: During data analysis and alignment, the newly identified sequence reads are aligned to a reference genome. Following alignment, many variations of analysis are possible, such as single nucleotide polymorphism (SNP) or insertion-deletion (indel) identification, read counting for RNA methods, phylogenetic or metagenomic analysis, and more. Each raw read base has an assigned quality score so that the software can apply a weighting factor in calling differences and generating confidence scores [55]

3.6.1 MiSeq

MiSeq Reagent Kits v3 enable the highest output of all MiSeq kits. The kits with a 600-cycle (2 x 300 bp) format were used in this project. The maximum number of reads is 25 million, and the maximum output is 15 Gb. The MiSeq can yield ~25 million forward and reverse reads of approximately 600 bases each in one run (per flow cell). Coverage of ~50 per isolate is normally required for good assembly.

Using a genome size of 5 Mb in this calculation, 50 genomes can theoretically be sequenced, but due to the error rate it should be less than this. Here it was decided that 44 samples would most likely be within the MiSeq limitations as the genome sizes were also expected to be less than 5 Mb. Commonly, 75 % of the reads will have an adequate error rate of less than 0.001 per base (>Q30). However, this is dependent on an optimal clustering of 1200K/mm2 to yield the necessary reads per flow cell, which again is affected by the DNA concentration and sample preparation.

MiSeq washing routines

After each run on the MiSeq, post-run washes are carried out using Tween 20. First, 5 ml 100 % Tween 20 is added to 45 ml of dH2O to attain a 10 % Tween 20 solution. Then, 25 ml of the 10 % Tween 20 is added to 475 ml dH2O constituting the MiSeq was solution. 350 ml of this 0.5 % Tween 20 wash solution is poured into the wash bottle and "Post-run wash" selected from the MiSeq interface.

Other washing procedures are also done regularly to uphold optimal performance of the MiSeq and include maintenance wash once a month and stand-by wash when the MiSeq will not be used for the next 7 days.

3.7. Analysis of sequencing data

3.7.1. FastQC

FastQC is a free software from the Babraham Bioinformatics Institute based on modular set of analyses which is used to give a quick impression of whether your data has any problems of which you should be aware before doing any further analysis. It provides basics statistics of you sequencing data, an overview of sequence and base quality scores, GC content and overrepresented sequences, like contamination or your PhiX control.

3.7.2. Trimming reads and alignment

A pipeline driven by Snakemake and based on several command-line based programmes running under Manjaro Linux were used to process the raw data.

Trimming of PhiX174 and adapters were carried out using BBmap. The reads for each isolate were mapped against the genome of the S. maltophilia reference strain K279a (RefSeq accession number NC_010943.1) and alignments were carried out using Bowtie 2, version 2.3.4.1, with default parameters.

Variation analyses, pileup and raw files of mapped reads were obtained with different SAMtools; like mpileup, bcftools, vcfutils (vcf2fq) and in the end PicardTools with raw files as outcome.

3.7.3. SpeciesFinder

SpeciesFinder is web-based method, which is based on identification by the 16S rRNA gene. When predicting the identification of a species with whole-genome sequencing data, the algorithm for this program is by BLAST hits, by ranking the output from the BLAST alignment by the best cumulative rank of coverage, percent identity, bit score, number of mismatches, and number of gaps. The highest ranked hit accumulates the species prediction [56]. Version 2.1 were used in this thesis.

3.7.4. The Comprehensive Antibiotic Resistance Database – CARD

The Comprehensive Antibiotic Resistance Database (CARD) provides data, models, and algorithms relating to the molecular basis of antimicrobial resistance. The CARD provides curated reference sequences and SNPs organized via the Antibiotic Resistance Ontology (ARO). Resistance Gene Identifier (RGI) is a tool that can be used for analysis the resistome of genome sequences.

Molecular sequences are imported into the CARD from GenBank using custom software developed specifically for the CARD. Only sequences available in GenBank with reviewed publications are included in the database. RGI targets, reference sequences, and significance cut-offs are updated regularly [57]. Open reading frames (ORF) are predicted using Prodigal and homolog detection by Diamond [58]. RGI version 4.2.0 and CARD 2.0.3 were used in this thesis.

3.7.5. ResFinder

ResFinder is a web-based method that uses BLAST for identification of acquired antimicrobial resistance genes in whole-genome data. As input, the method can use both pre-assembled, complete or partial genomes, or raw data sequence reads directly from the sequencing platform. The method was evaluated on 1862 GenBank files containing 1411 different resistance genes, as well as on 23 de-novo-sequenced isolates. The database is regularly updated, and the version used in this thesis is ResFinder 3.0 [59].

4. Results

4.1. Phenotypic resistance

In total, 100 strains were tested with four different methods towards six antimicrobial agents: ceftazidime, ciprofloxacin, colistin, meropenem, tigecycline and trimethoprim-sulfamethoxazole.

One of the isolates turned out to be another species, *Pseudomonas putida,* and was therefore eliminated in the presentation and comparison of the results.

For MIC determination, four different methods were used to collect a phenotypic resistance profile for all isolates. Broth microdilution method, MIC gradient strip test, VITEK 2 and Agar disk diffusion method. When comparing the four methods to each other, there are clear inconsistencies between the methods for the different antimicrobial agents. Number of isolates per method tested, MIC range in mg/L and differences between methods are presented in table 8, with broth microdilution being a gold standard.

Inconsistencies are defined as, very major error; when resistant by broth microdilution method and susceptible by one of the other the methods. A major error is defined as; susceptible by broth microdilution method and resistant by one of the other methods. Minor error is defined as, intermediate susceptible by the other methods when broth microdilution method is either susceptible or resistant – and when broth microdilution is intermediate susceptible, and the other method is either susceptible or resistant [60]. Minor error is only appropriate for meropenem, being the only antimicrobial agent in this panel with intermediate susceptibility breakpoints by EUCAST (table 3 and 4).

Table 8. Overview of the antimicrobial agents, tentative breakpoints (based on breakpoints for *P. aeruginosa* for four of the agents), methods, included in the study plus the errors between the methods based on the S-I-R system for 99 *S. maltophilia* isolates. Broth microdilution (BMD) is set as the gold standard for antimicrobial susceptibility testing, and the three other methods are compared to BMD. For results from VITEK 2, some isolates were terminated and gave no results.

				Broth microdilution		
Antimicrobial agent	Tentative breakpoint (mg/L) for resistance	MIC range (mg/L)	Method (no. of isolates tested)	Minor errors	Major errors	Very major errors
Ceftazidime	> 8	0,012 - 256	MIC gradient strip (99)	-	7	8
			VITEK 2 (97)	-	2	23
			Agar diffusion (99)	-	4	8
Ciprofloxacin	> 0,5	0,002 - 32	MIC gradient strip (99)	-	0	37
			VITEK 2 (97)	-	2	19
			Agar diffusion (99)	-	1	13
Colistin	> 2	0,002 - 32	MIC gradient strip (99)	-	8	22
			VITEK 2 (99)	-	18	20
			Agar diffusion (0) **	-	-	-
Meropenem	> 8	0,016 - 256	MIC gradient strip (99)	1	0	0
			VITEK 2 (91)	7	0	5
			Agar diffusion (99)	10	1	0
Tigecycline*	> 0,5	0,016 - 256	MIC gradient strip (99)	-	0	3
			VITEK 2 (0) *	-	-	-
			Agar diffusion (0) *	-	-	-
Trimethoprim-sulfamethoxazole	> 4	0,002 - 32	MIC gradient strip (99)	-	0	1
			VITEK 2 (98)	-	4	0
			Agar diffusion (99)	-	0	0

*No breakpoints for Tigecycline, not for *Pseudomonas spp*. or PK/PD for agar diffusion or VITEK 2.

**Colistin discs were not obtainable.

Phenotypic resistance

Tables 9 and 10, give an overview of the complete MIC-ranges found for each isolate with the MIC gradient strip test and broth microdilution method respectively. The MIC_{50} represents the MIC value at which ≥50% of the isolates in a test population are inhibited and is equivalent to the median MIC value. The MIC_{90} represents the MIC value at which ≥90% of the strains within a test population are inhibited; the 90th percentile.

All results for antimicrobial susceptibility testing are found in appendix 1.

Table 9: MIC50 and MIC90 values and percent antimicrobial resistance of clinical isolates of *S. maltophilia*, both clinical and environmental isolates (n = 99).

Antimicrobial agent	Breakpoint for resistance (mg/L)	MIC gradient strips: MIC values (mg/L)			
		% Resistance	Range	MIC_{50}	MIC_{90}
Ceftazidime	> 8	44,4 %	0,012 - 256	8	256
Ciprofloxacin	> 0,5	54,5 %	0,002 - 32	0,5	4
Colistin	> 2	27,3 %	0,002 - 32	1	12
Meropenem	> 8	95,9 %	0,016 - 256	128	256
Tigecycline	> 0,5	15,2 %	0,016 - 256	0,25	1
Trimethoprim-sulfamethoxazole	> 4	0 %	0,002 - 32	0,064	0,125

Table 10: MIC_{50} and MIC_{90} values and percent antimicrobial resistance of both clinical and environmental isolates, based on broth microdilution results (n=99).

Antimicrobial agent	Breakpoint for resistance (mg/L)	Broth microdilution: MIC values (mg/L)			
		% Resistance	Range	MIC_{50}	MIC_{90}
Ceftazidime	> 8	49,50 %	<2 - >128	16	64
Ciprofloxacin	> 0,5	89,90 %	<0,12 - >16	2	8
Colistin	> 2	40,40 %	<0,5 - >8	2	8
Meropenem	> 8	94,90 %	<0,5 - >32	>32	>32
Tigecycline	> 0,5	20,0%*	<0,12 - >16	0,25*	2
Trimethoprim-sulfamethoxazole	> 4	1 %	<0,25 - >32	0,25	1

*Tigecycline; only 20 isolates were investigated by broth microdilution.

The broth microdilution method, here considered the gold standard, displayed a higher percentage of resistance than the MIC gradient strip method for all antimicrobial agents other than meropenem. The isolates were resistant for colistin in 27,3 % of the isolates for the MIC gradient strip test compared with 40,4 % using the broth microdilution method. Isolates resistant to ciprofloxacin were also significantly higher with the broth microdilution

method (89,9 %) than with the MIC gradient strip method (54,5 %). One isolate (1 %) was resistant for trimethoprim-sulfamethoxazole with the broth microdilution method, which was not found with the MIC gradient strip method.

Clinical and environmental isolates

The box-plots below show the differences in MICs for clinical isolates and environmental isolates using MIC gradient strip and broth microdilution respectively. Each antimicrobial agent is shown in each box-plot, for clinical isolates in blue (n = 75), and for environmental isolates in orange (n = 24).

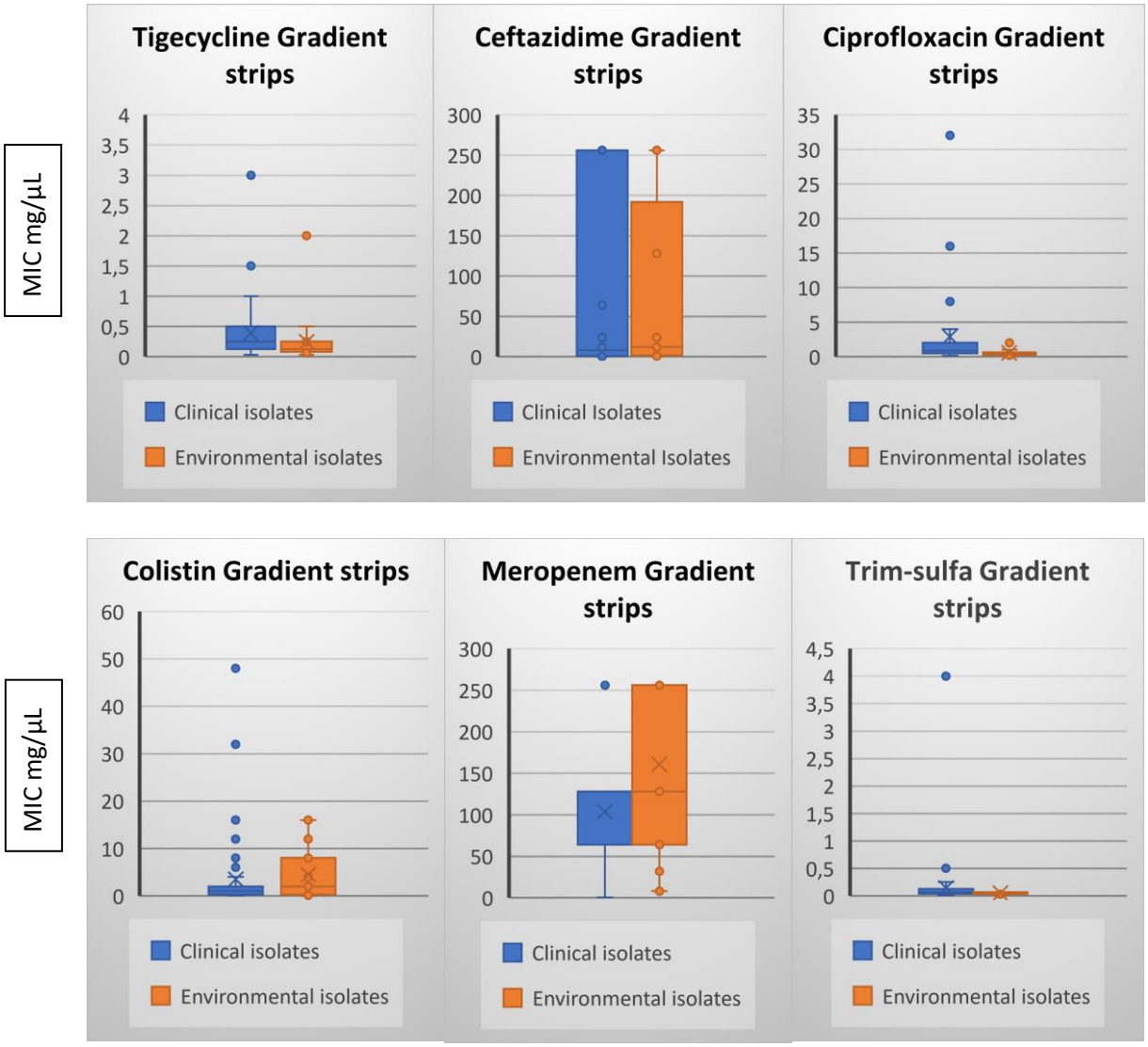

Figure 6: Meropenem displays generally higher MICs for the environmental isolates (n=24) than the clinical isolates (n =75). Ceftazidime displays higher MIC for the clinical isolates than the environmental isolates. All the isolates have some strays that display higher MIC for each antimicrobial agent.

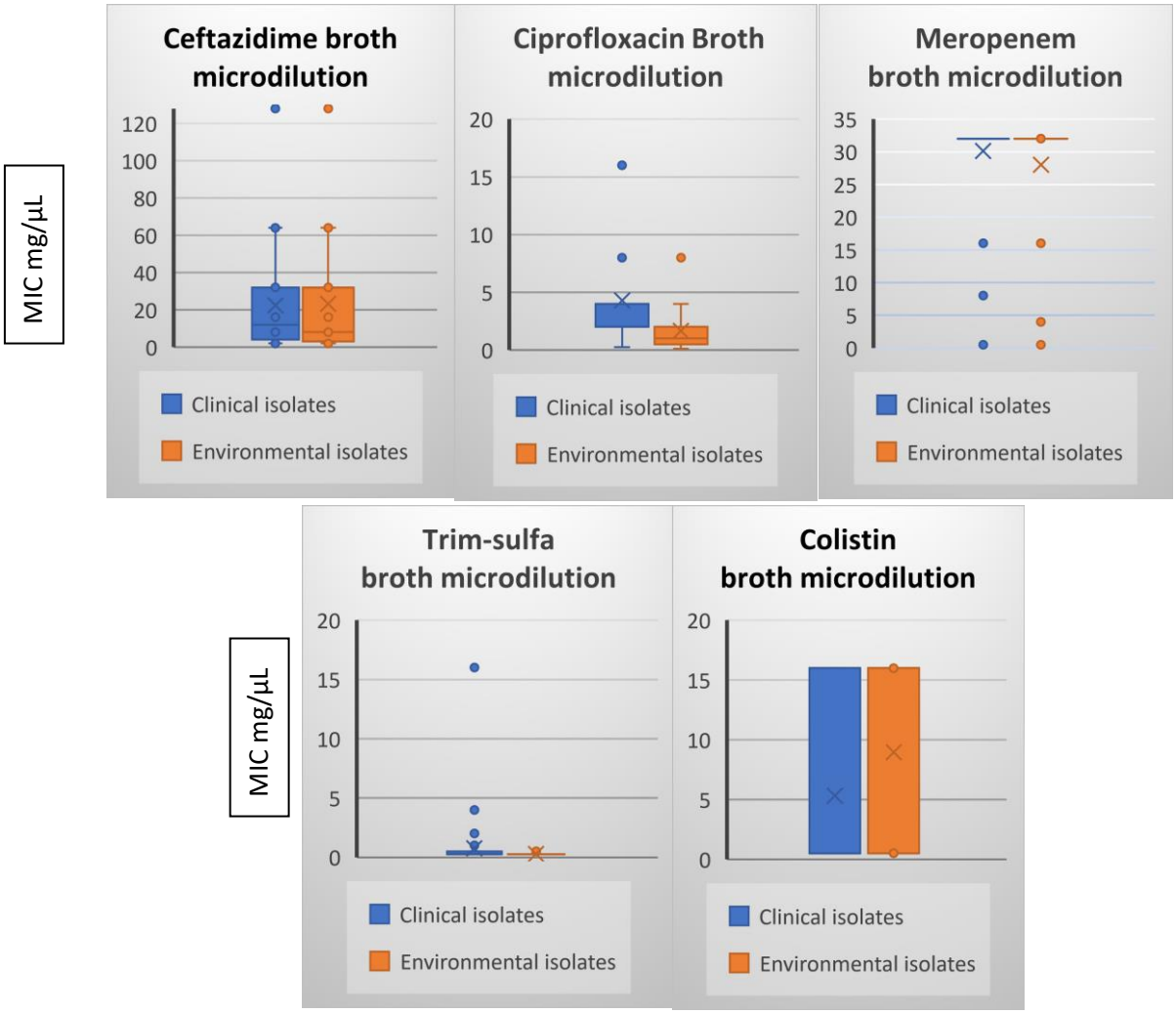

Figure 7: Displays clinical versus environmental isolates in boxplot, with average at a cross. Dots represent outliners. Tigecycline is not displayed because of limited numbers of test performed (n=20).

With broth microdilution method, there appears to be fewer differences that with the MIC gradient strip method. Ciprofloxacin displays generally higher MIC for the clinical isolates then the environmental isolates. The isolates resistant or with elevated MIC for trimethoprim-sulfamethoxazole belongs to clinical isolates.

4.2. Whole genome sequencing analysis

In general, the whole genome sequencing was very successful with high coverage assisting good assemblies to a reference genome. All dilution and DNA measurements that lead up to whole genome sequencing on Illumina MiSeq were adjusted the two kits used.

The quality check of the sequencing files before FastQC and trimming, were initially done with Illumina sequencing Analysis Viewer. The QScore distribution, data by lane, data by cycle and the chart of the Flow cell were checked to ensure that the sequencing was done right. The Q30-score is the most important, that shows the percentage of bases with a quality score of 30. As shown in figure 8, the Q30-score is 71,8 %, which is adequate.

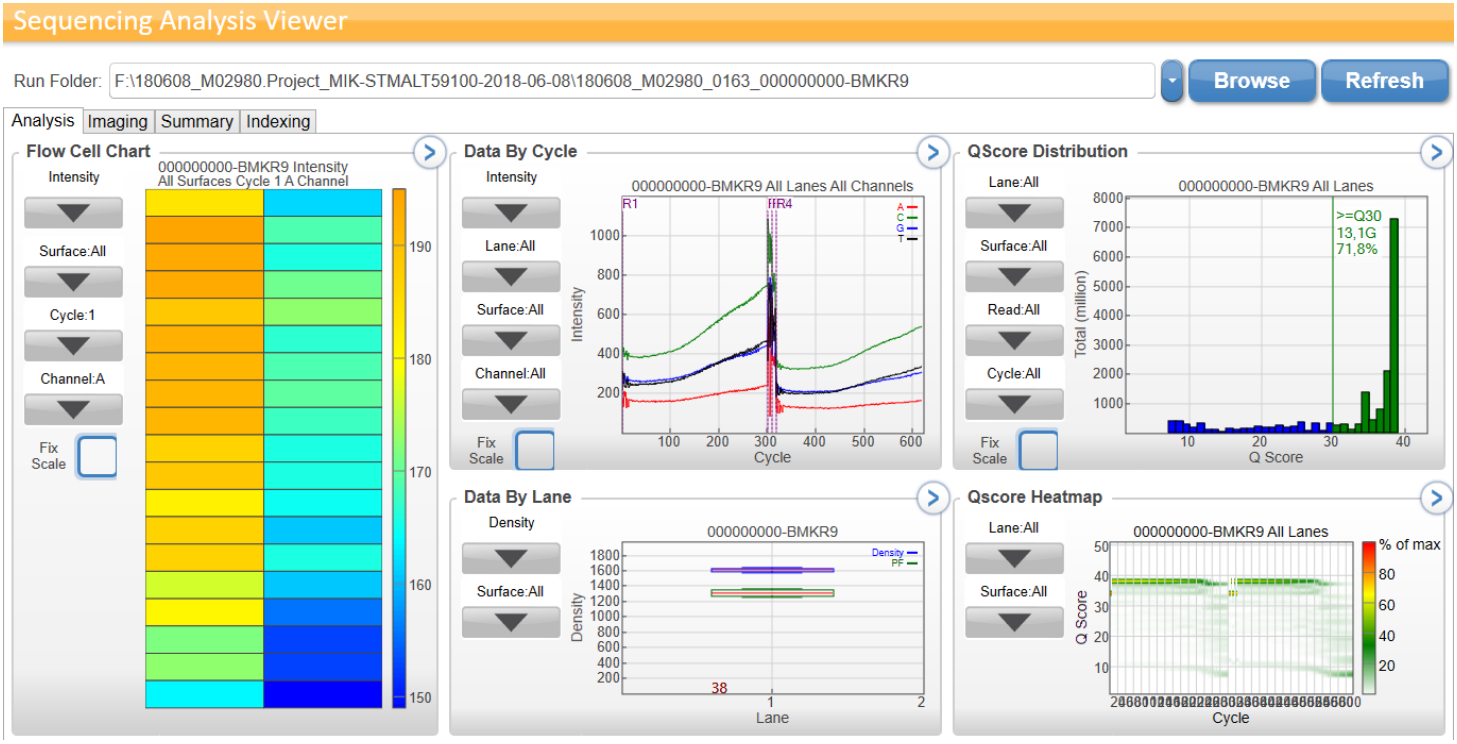

Figure 8: This picture shows the Sequencing Analysis Viewer by Illumina, with all the basic quality score of a sequencing run. This is run 3, with the sequencing quality of isolate 59-100.

The quality of the Q30-score on all the runs were acceptable, as shown in table 11. The first run displays the quality and number of clusters passing through filter on isolates 1-10. The library preperation on this run was done with the KAPA kit by Roche. The second run displays the quality of isolates 11-58, and the third run represents isolates 59-100, both libraries prepared with Nextera XT DNA kit by Illumina.

Table 11: Basic quality scores of all three Illumina Sequencing runs (isolate 1-100).

Project	PF cluster no	PF ratio	Raw cluster density(/mm2)	PF cluster density(/mm2)	Undetermined	AlignedPhiX	>=Q30	Quality
MIK-AnneSteffensen-2017-12-15 (Run 1)	11,450,593	0.95	480,487	454,970	3.60 %	0.90 %	76.45 %	Ok
MIK-AnneSteffensen-2018-03-11 (Run 2)	11,450,593	0.97	1 100,601	986,849	2.71 %	0.98 %	80.2 %	Ok
MIK-AnneSteffensen-2018-06-08 (Run 3)	11,450,593	0.96	1 899,272	1 563,091	1.19 %	0.96 %	71.8 %	Ok

4.2.1 FastQC

Quality controls of the sequences of all strains were checked using FastQC V0.11.7.

The sequence data delivered in FASTQ files was of sufficient quality and the data after trimming was of good. The alignments to the reference sequence gave average coverages up to 133. The average coverage for the 100 isolates was approximately 43. Isolates with low coverage showed low overall sequence length. Especially isolate 43 when compared with isolate 37, both with almost the same coverage depth. Isolate 91 showed in addition to short length an unusual G+C rate.

SpeciesFinder 1.2 identified sample 37 and 43 as *Stenotrophomonas maltophilia* (AM743169) and sample 91 as *Pseudomonas putida* (CP000712).

The reference sequence for the alignment was “NC_010943.1” *Stenotrophomonas maltophilia* K279a complete genome strain with a size of 4.851.126 sequence characters and a G+C content of 66,32 %. All samples gave around 3,2 – 4,8 mb of sequence each and their G+C content was around 67 %. See appendix 2 for coverage results of all sequences.

4.2.2. SNP tree

A phylogenetic tree was made to correlate the isolates to each other. No big clusters are showing in this tree based on single nucleotide polymorphisms (SNPs) with whole-genome sequencing data.

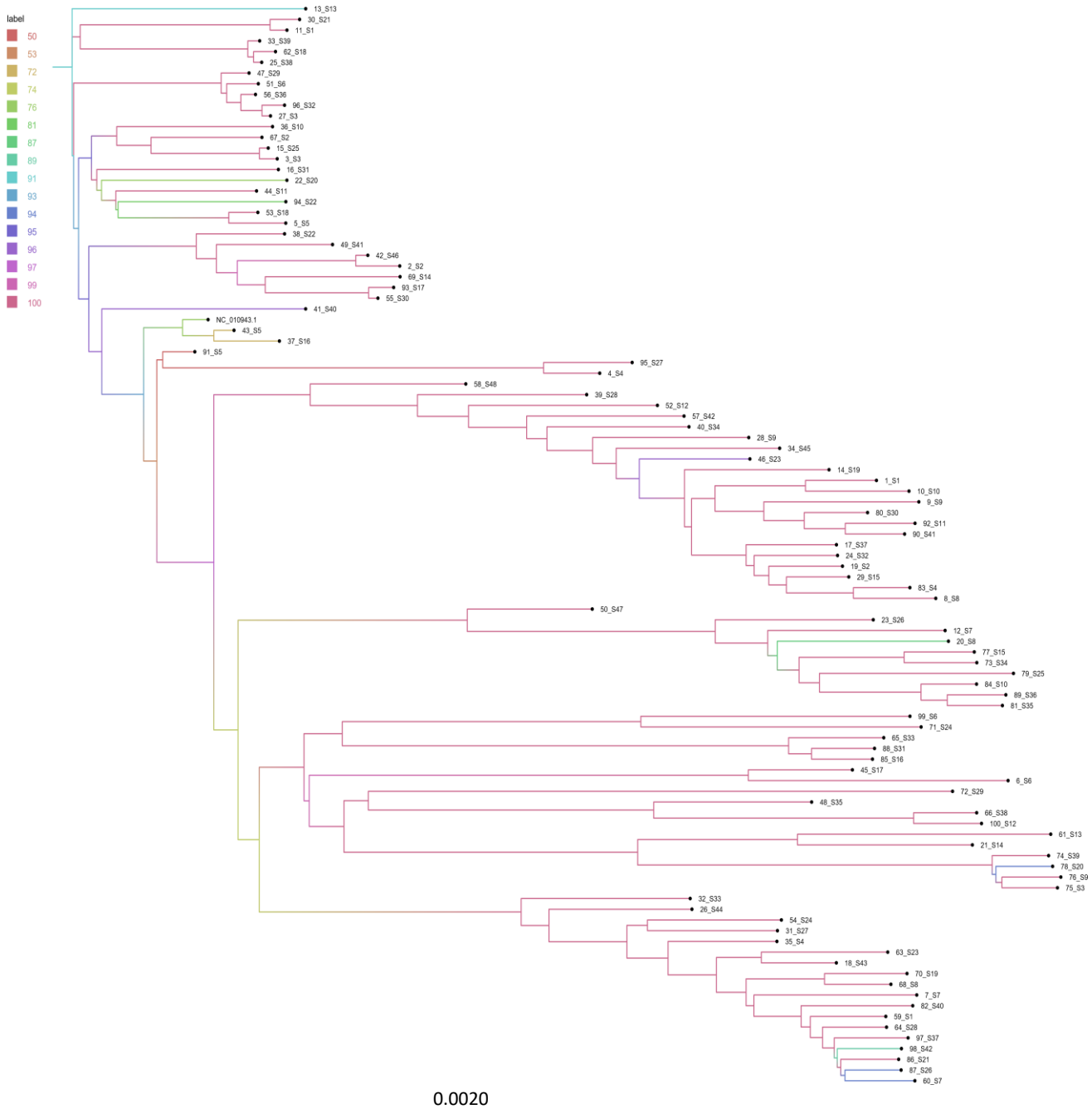

Figure 9: A phylogenetic SNP-tree based on whole-genome sequencing data of 99 *S. maltophilia* isolates, using the maximum likelihood method (bootstrap 100). Midpoint-rooted, with bootstrap shown in graded colors, with scale on the left.

4.2.3. MLST

All the isolates were grouped according to their sequence type (ST) based on multilocus sequencing typing (MLST). The seven genes selected for use with the MLST scheme were *atpD*, *gapA*, *guaA*, *mutM*, *nuoD*, *ppsA*, and *recA*. The MLST-tree usually shows greater clustering than the SNP-based tree, but still the association between sequence type and hospital ward/patient group was not obvious in this schematic tree (data not shown) and displayed the same uneven distribution as with the SNP-tree presented in figure 9.

4.3. Core-resistome

4.3.1. Acquired resistance genes

Acquired resistance genes were found using internet-based platforms, ResFinder and KmerResistance by Center for Genomic Epidemiology. Center for Genomic Epidemiology accepts FASTQ-files, great in size.

Searching simultaneously in both platforms using both FASTQ raw data, and FASTA files with aligned contigs, several resistance genes were obtained.

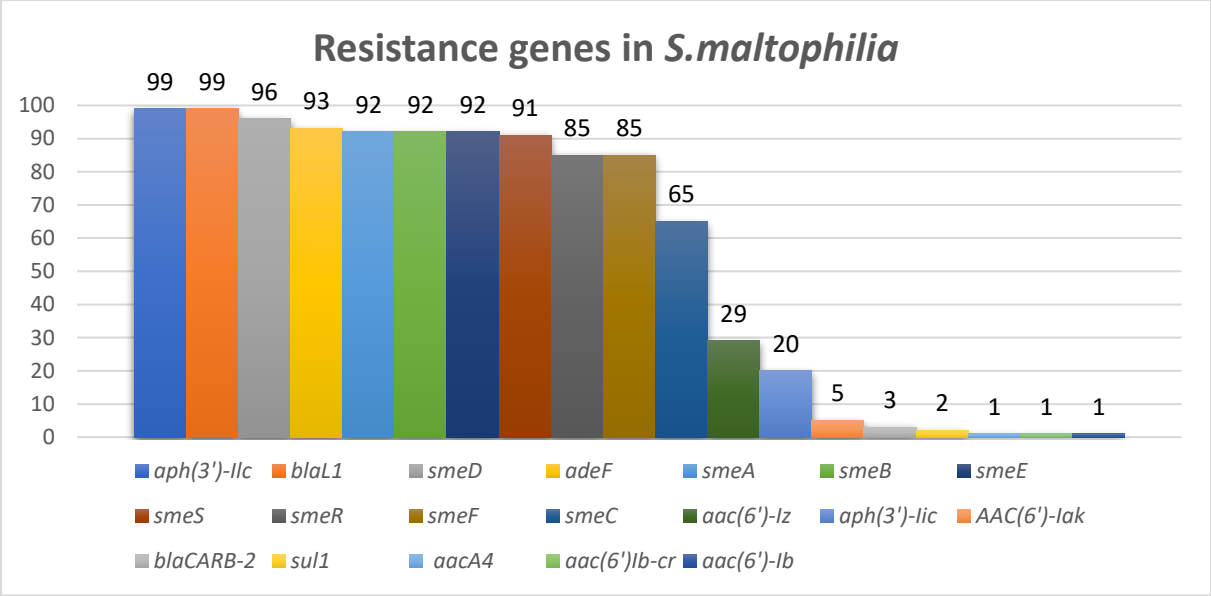

Figure 10: Shows an overview of different resistance genes and efflux pumps, and in how many *S. maltophilia* isolates they are found in (n=99).

A betalactamase were discovered in three different isolates, named *blaCARB-2*. All three genes were found in isolates from patients in the Intensive care unit (ICU). A search in the UniProtKB database showed that it has previously been discovered in *Burkholderia multivorans (*https://www.uniprot.org/uniprot/D2K8A5*).* Assuming the gene displays resistance towards carbapenems, the three isolates harbouring the gene were linked to their phenotypic resistance profile. Isolate 68 and 72 show resistance to meropenem with all four phenotypic methods. Isolate 58 shows a resistant phenotype with MIC gradient strip, broth microdilution and agar diffusion, but an intermediate result with VITEK 2.

Table 12: blaCARB-2: Three isolates contain *blaCARB-2* genes. Two were resistant to meropenem with all four methods, one with tree methods and intermediate on VITEK 2.

	Meropenem				
	MIC gradient strip	Broth microdilution	Vitek 2	Agar diffusion	Resistance genes
58	32 R	32 R	4 I	16 R	***blaCARB-2***
68	256 R	>32 R	>= 16 R	6 R	***blaCARB-2***
72	128 R	>32 R	>= 16 R	6 R	***blaCARB-2***

Another finding was two acquired *sul1* genes, a dihydropteroate synthase linked to sulphonamide-resistance. These two genes were found in isolates from two types of patients. Number 63 is from a patient with Cystic Fibrosis, and number 43 is from a patient in the ICU. Isolate 63 displays phenotypic resistance towards trimethoprim-sulfamethoxazole with three of the four methods. MIC gradient strip test displayed a MIC value of 4 mg/L and therefore considered susceptible. Isolate 43, however, does not indicate resistance using the phenotypic methods. MIC gradient strips, broth microdilution and agar disk diffusion all exhibited susceptible results, although VITEK 2 indicated an intermediate result. The susceptibility for agar diffusion and MIC gradient strip tests were retested two more times, with the same results.

Table 13: Two isolates containing sul1 genes. One is resistant to trimethoprim-sulfamethoxazole in three out of four methods, the other one is not. VITEK 2 shows higher MIC for this isolate, and broth microdilution is one level below resistance.

	Trimethoprim-sulfamethoxazole				
	MIC gradient test	Broth microdilution	Vitek 2	Agar diffusion	Resistance genes
63	S 4	R 16	R >= 320	R 6	***sul1***
43	S 0,5	S 2	I 160	S 17	***sul1***

4.3.2. Intrinsic resistance genes and efflux pumps

Intrinsic resistance genes and efflux pumps were predicted using the Comprehensive Antibiotic Resistance Database (CARD), and searches were done with trimmed data (FASTA).

An aminoglycoside resistance determinant named aph(3')-IIc and a betalactamase named *blaL1*, were found in all of the isolates (n=99).

As part of the multidrug efflux pump, *smeABC*, the genes *smeA* and *smeB* were detected in 92 of the isolates. In contrast, the gene *smeC* was only detected in 65 of the isolates.

Tabele 14: Displays genes that are a part of an efflux pumps found in *S. maltophilia* isolates.

Efflux pumps						
n = 96	n = 93	n = 92	n = 91	n = 88	n = 85	n = 65
smeD	*adeF*	*smeA*	*smeS*	*smeR*	*smeF*	*smeC*
		smeB				
		smeE				

When comparing the differences between the isolates containing the whole efflux pump *smeABC* with the ones lacking *smeC* with antimicrobial resistance phenotypes, there seems to be higher MIC_{50} for ceftazidime in the isolates containing *smeC*, although the numbers are small (table 15).

Table 15: Displaying MIC_{50} and MIC_{90} values of ceftazidime by isolates that harbouring a whole efflux system (*smeABC*) versus those who only harbour a part of if (*smeAB*).

Ceftazidime				
	MIC_{50}		MIC_{90}	
	Broth microdilution	MIC gradient strip test	Broth microdilution	MIC gradient strip test
Isolates harbouring *smeABC* (n=65)	16	10	64	256
Isolates only harbouring *smeAB,* not *smeC* (n= 27)	4	2	64	256

5. Discussion

5.1. MIC methods and drawbacks

As presented in the results, the correlation between the methods was less than satisfying and displayed many differences between the presented methods. These irregular results between methods are supported in previous studies [22, 23, 61]. Broth microdilution is considered a gold standard, even though there are differences between the CLSI and EUCAST. Antimicrobial susceptibility testing methods of *S. maltophilia* are not clearly standardized, and there is poor correlation between the different antimicrobial susceptibility methods [61]. Agar diffusion and VITEK 2 has been useful in our study in comparing the differences between the four methods presented and highlighting the problems in interpretation antimicrobial susceptibility testing for *S. maltophilia*. Given the difficulties to compare any results for the agar diffusion method and VITEK 2, other than S-I-R, the following discussion will focus on broth microdilution method and MIC gradient strip tests for discussion of MIC values.

A warning from EUCAST and CLSI states that broth microdilution is the only valid method for colistin susceptibility testing. This is due to colistin being a large molecule, and disk diffusion simply does not work with that size. The literature has questioned the validity of MICs obtained with MIC gradient strip tests. Currently available MIC gradient strip tests underestimate colistin MIC values and therefor may overlook resistance, and EUCAST has a warning stating MIC gradient strips should be avoided [62]. The results presented in this thesis similarly indicate that colistin-testing is the least predictable antibiotic, showing the lowest zero-error rate together with ciprofloxacin. The isolates are resistant for colistin in 27,3 % of the isolates for the MIC gradient strip test and 40,4 % with the broth microdilution method. Since the resistance rate for colistin is higher with the broth microdilution method than MIC gradient strip tests, this indicates that the latter is perhaps not a valid method for this antimicrobial agent and may present false susceptible results. In a clinical setting, this would result in several very major errors (false susceptible results), which in turn may result in inadequate treatment.

For ciprofloxacin, rate of resistance is higher using the broth microdilution method (89,9 %), then with the MIC gradient strip test (54,5 %). In a clinical setting, this is an important finding, indicating that MIC gradient strip tests perhaps should not be the method for determining MIC-values for *S. maltophilia*.

The broth microdilution method displays generally a higher percentage of resistance than with the MIC gradient strip test, for all antimicrobial agents other than meropenem, where all methods showed a high resistance rate. This again supports the fact that perhaps MIC gradient strip tests underestimate MIC values compared to the broth microdilution method for this microbe [18, 19, 63].

Trimethoprim-sulfamethoxazole-resistance was found in one isolate (isolate 63) by broth microdilution, but none using the MIC gradient strip test. Trimethoprim-sulfamethoxazole resistance in *S. maltophilia* has been associated with the genes *sul1* and *sul2*. These genes have been linked to the presence of class 1 integrons in plasmids in the main, but also in the chromosomal genome [42, 64]. Since *sul1* was found in this particular isolate, these results may indicate that the MIC gradient strip test also underestimates MIC values for trimethoprim-sulfamethoxazole compared to broth microdilution, which could lead to very major errors. However, since the numbers are low, we cannot draw any conclusions at this time.

Also, each MIC distribution should be repeated at least twice for each method, to try to reproduce each MIC value and calculate the differences between the MICs for each repetition. This was not possible in the time frame for this master thesis.

Clinical and environmental isolates

One issue with this set of isolates, when comparing them together, is the fact that they are not evenly distributed. The number of environmental were 1/3 of the clinical isolates and ideally, there should have been an equal number of isolates in each group. However, if we had found noticeable differences, it would have been interesting to study further on this matter. In this limited number of isolates tested, the only noteworthy difference was found between the isolates tested for trimethoprim-sulfamethoxazole. This is due to only one isolate from the clinical group is harbouring a resistance gene towards this antimicrobial

agent and displaying phenotypic antimicrobial resistance as well. Because of the limited number of isolates, it was decided statistical analyses of these results would not be relevant.

Another aspect to acknowledge is that the environmental bacteria are from a hospital environment, collected from water sources in patient rooms. In this study, it is not known if some of the patient-isolates are from the same rooms tested in the environmental isolates. It would be interesting to do a study and look for similarities between *S. maltophilia*-isolates from infections and *S. maltophilia*-isolates collected from the same patients hospital room and water source [65]. A phylogenetic tree was made to correlate the isolates to each other. No big clusters were showing in this tree based on single nucleotide polymorphisms (SNPs) with whole-genome sequencing data, either for the environmental and clinical isolates, or between the different hospital wards/patient groups.

5.2. Core-resistome

T The assessment of a core-resistome to a bacterial species is a difficult and complex task. The definitions are not well established, but some studies suggest that the genes presented should be homologues of greater than 80 % identity with the reference sequence and should appear in 95 % or more isolates [66]. Given these criteria, the core-resistome presented here would only contain three resistance genes; the aminoglycoside resistance determinant aph(3')-llc, the betalactamase *bla*L1 and part of an efflux-pump, *smeD.* This core resistome is based on the resistance genes found by ResFinder and CARD, and thus does not include all resistance genes in each isolate, only those well established and presented in each database. CARD possesses a large database of resistance genes and changing the percentage of homology in the search lower would have presented us with more resistance genes. However, this results in another problem, determining where the lower cut-off should be. One major difficulty with the web-interfaced version of CARD, is the fact that they only accept assembled genomes or contigs in a FASTA-format with a file-limit of 20 Mb. Like with ResFinder, the ideal search would be with FASTQ-files, containing raw data and therefor searching the true genome and not the reference-based one.

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The *S. maltophilia* isolate K279a was used as a reference genome. The ideal progress would have been to determine a core-resistome in isolates that have been assembled de novo, meaning without a reference genome. This would ensure that all genes, also the ones who are not present in the reference genome, could have been detected. De novo assembly is a big task, and require a lot of knowledge about bioinformatics, time and computer power, not feasible within the timeframe of this thesis.

5.3. Resistance genes linked to observed MIC-values

Several acquired resistance genes were found. Some could be linked to the phenotypic antimicrobial susceptibility results, whereas other could not. Amongst the latter is a cabapenamase, *blaCARB-2*. This resistance gene has been previously been found in *Burkholderia multivorans, Salmonella enterica* and *Klebsiella pneumoniae* [67-69], but we were not able to find published data on this gene in *S. maltophilia*. Assuming the gene displays resistance towards carbapenems, the three isolates harbouring this gene would

show in their phenotypic resistance profile. However, meropenem-resistance was present in all isolates with all methods except one intermediate result from Vitek 2. Thus, we could not find any link to phenotypic resistance. One interesting element about these results is however, the fact that all three isolates are from patients in the Intensive Care Unit (ICU). Given the fact that this department uses large amounts of carbapenems in their treatment, the antibiotic pressure may have selected this resistance gene in these three isolates somehow. Selective pressure is known to drive horizontal-gene-transfer [70, 71], and could be a valid theory.

Using broth microdilution, there were noticeable higher ceftazidime MIC values among the isolates harbouring *smeABC* than the ones harbouring just *smeAB*, and not *smeC*. The possible linkage between *smeC* and ceftazidime resistance is an interesting observation and some previous studies have found the same [27, 30, 72]. A study by Li et. Al (2012), suggested that the efflux pump *smeABC* does not function as a multidrug efflux system altogether, but that *smeC* plays a role in antimicrobial resistance independent of *smeAB* [72], possibly as the outer membrane factor component of a unidentified multidrug efflux system. *SmeABC* is known to involve acquired resistance to β-lactams, aminoglycosides and quinolones, but has no influence on intrinsic resistance. The deletion of the *smeC* gene affects susceptibility to several antibiotics [72] suggesting its possible relationship with other efflux pumps [25]. Other mechanisms might appear in the future, depending on antibiotic pressure, the emergence of mutations, and gene acquisition events. While resistance may benefit bacteria in the presence of antibiotics, in other situations it could impair growth. Alonso et al., 2004 described that the overexpression of the efflux pump *SmeDEF* [26] could impair growth. Further, the fitness cost of acquired resistance in *S. maltophilia* determines whether new mechanisms are kept.

It would be interesting to do a knock-out study on these isolates and include more β-lactams to support these findings. This thought is based on the studies done by Li et al. 2002 [72] and Lin et al. 2014 [30].

The same study proved that the deletion of *smeR* had an intermediate effect on β-lactamase activity, reducing it to a level below that of the MDR strain but above that seen for its parent. This same effect was not seen in the MIC values from isolates containing *smeR* provided in this thesis.

Another finding in this study, is the MIC value and resistance genes found for isolate 43. This isolate possesses the *sul1* resistance gene associated with trimethoprim-sulfamethoxazole resistance. However, the MIC values did not show phenotypic resistance towards this antimicrobial agent. Broth microdilution, MIC gradient strip and agar diffusion suggested this isolate to be susceptible towards trimethoprim-sulfamethoxazole, while Vitek 2 was intermediate. The isolate was retested twice with MIC gradient strip and agar diffusion, exhibiting the same result. This may indicate that the phenotypic resistance may be correct, and that maybe this isolate does not express this resistance gene. This needs to be explored further, and it would be interesting to investigate the origin of resistance in this isolate. In general, the presence of a certain gene does not necessarily mean that it is expressed. Expression-studies would therefore be interesting to look further on is this case as well.

A study has shown that the deletion of a porin named TolCsm can increase the susceptibility towards trimethoprim-sulfamethoxazole [32, 73], but further studies are required to determine whether other porins or efflux pumps also are involved.

5.4. Concluding remarks and prospects

In conclusion, our study supports earlier studies that show that phenotypic susceptibility testing of S. maltophilia for antibiotics other than trimethoprim-sulfamethoxazole is difficult and inconstant. We did find some resistance genes that can support phenotypic resistance profiles. The ultimate goal is to manage to find a method by either phenotypic, genotypic or a combination of both, to accurately predict antimicrobial susceptibility for other antimicrobial agents than trimethoprim-sulfamethoxazole in *S. maltophilia*. This leaves several future prospects.

As mentioned in the discussion, antimicrobial susceptibility testing should be performed with parallels tests, to see if the MIC distribution changes from time to time. This allows for statistical tests between results and will enhance the depth of the study. This should also include the agar dilution method which is considered a gold standard method for some bacteria.

De novo assembly of all the isolates would make it possible to asses and determine all resistance genes, both the resistance genes presented in this thesis and predicted resistance genes.

An expression study, would be exciting to perform in several of the isolates presented here, especially towards the isolates harbouring *smeC* and try to determine if this gene is involved in the elevated MIC towards ceftazidime.

Several aspects of these data could be interesting to look at from different angles. One way is to focus on environmental versus clinical aspects between the isolates. It would be fascinating to find out were these isolates come from originally - water source from the hospital environment, or maybe they are community-acquired? To be able to do this, one would have to know the patient identification and collect samples from the different rooms they stayed at during their *S. maltophilia*-infection.

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Appendix 1 – Antimicrobial susceptibility results

	Broth microdilution method					
Isolate no.	CAZ	CIP	CO	MRP	TGC	SXT
1	<2	2	2	>32		<0,25
2	64	2	>8	>32		0,25
3	<2	2	1	16		<0,25
4	8	2	4	>32		0,5
5	32	16	8	>32		0,25
6	32	2	8	>32		0,25
7	8	2	>8	>32		0,25
8	4	1	<0,5	>32		0,25
9	4	2	4	>32		0,25
10	4	8	4	>32		0,5
11	<2	0,25	8	32		<0,25
12	128	1	1	>32		0,5
13	16	2	>8	>32		0,25
14	4	2	2	>32		<0,25
15	4	2	2	>32		<0,25
16	16	4	>8	>32		<0,25
17	4	2	2	16		0,5
18	32	2	4	>32		1
19	2	0,5	<0,5	16		0,5
20	64	1	<0,5	>32		<0,25
21	32	1	1	>32		1
22	64	2	1	>32		2
23	64	1	<0,5	>32		0,5
24	<2	0,5	1	>32		0,5
25	64	8	4	>32		2
26	16	2	2	>32		0,5
27	>128	>16	2	>32		2
28	4	2	2	32		<0,25
29	4	2	2	>32		1
30	4	>16	<0,5	>32		2
31	16	4	8	>32		0,5
32	8	16	8	>32		0,5
33	64	4	2	>32		1
34	<2	1	1	>32		0,25
35	4	1	4	>32		0,5
36	16	2	4	>32		0,5
37	16	2	8	>32		0,5
38	16	2	1	>32		0,5
39	4	8	2	>32		0,5
40	4	1	>8	>32		0,25
41	16	4	4	>32		0,25
42	64	4	8	>32		0,25
43	64	4	>8	32		2
44	32	4	4	>32		0,25
45	16	>16	1	>32		0,25
46	<2	2	2	8		0,25
47	16	2	2	16		0,25
48	<2	8	8	32		1
49	16	2	2	>32		<0,25
50	<2	1	2	<0,5		0,25

	Broth microdilution method					
Isolate no.	CAZ	CIP	CO	MRP	TGC	SXT
51	16	2	4	32		0,5
52	<2	2	1	>32		<0,25
53	16	2	4	>32		0,25
54	8	8	1	>32		<0,25
55	16	2	8	>32		<0,25
56	>128	8	<0,5	>32		0,5
57	<2	1	8	>32		<0,25
58	<2	8	8	32		0,5
59	8	2	1	>32		0,25
60	16	2	8	>32		<0,25
61	64	4	1	>32		0,5
62	4	0,5	1	>32		<0,25
63	4	2	2	16		16
64	32	16	1	>32		<0,25
65	16	1	<0,5	>32		0,5
66	4	8	2	>32		0,5
67	8	2	8	>32		<0,25
68	<2	4	2	>32		<0,25
69	64	4	8	>32		0,5
70	4	2	4	>32		<0,25
71	4	1	<0,5	8		0,25
72	32	8	2	>32		0,5
73	8	0,5	2	>32		<0,25
74	<2	0,25	<0,5	<0,5		<0,25
75	32	2	<0,5	32		0,5
76	16	0,5	1	>32		<0,25
77	8	0,25	<0,5	4		<0,25
78	32	1	8	>32		<0,25
79	8	2	8	>32		<0,25
80	4	<0,12	1	16		<0,25
81	64	2	1	>32	0,5	<0,25
82	<2	1	2	>32	0,25	<0,25
83	<2	8	4	>32	0,5	<0,25
84	>128	1	<0,5	>32	0,25	1
85	16	2	<0,5	>32	<0,12	0,5
86	4	1	4	>32	0,25	<0,25
87	16	1	2	>32	0,25	<0,25
88	16	2	<0,5	>32	<0,12	0,5
89	64	4	1	>32	0,25	<0,25
90	<2	1	1	>32	<0,12	<0,25
91	4	0,5	1	2	1	8
92	<2	0,5	4	>32	0,25	<0,25
93	8	2	8	>32	0,5	<0,25
94	64	1	8	>32	1	0,25
95	32	2	2	>32	0,5	4
96	8	2	2	>32	0,25	<0,25
97	16	16	4	>32	2	0,25
98	8	16	1	>32	2	<0,25
99	16	4	2	>32	0,25	1
100	4	8	8	>32	2	0,5

Table 1.1: Antimicrobial susceptibility results in *S. maltophilia* with broth microdilution method.

CAZ = Ceftazidime CIP = Ciprofloxacin CO = Colistin

MRP = Meropenem TGC = Tigecycline SXT = Trimethoprim-sulfamethoxazole

	MIC gradient strip test					
Isolate no.	CAZ	CIP	CO	MRP	TGC	SXT
1	0,5	1	2	128	0,5	0,064
2	256	0,25	16	256	0,5	0,064
3	0,5	1	2	64	2	0,032
4	8	0,25	0,5	64	0,5	0,125
5	64	0,25	48	128	0,5	0,064
6	256	0,25	0,25	256	0,25	0,064
7	4	0,5	32	128	1	0,064
8	0,5	0,25	2	128	0,25	0,032
9	0,5	0,5	4	128	0,064	0,016
10	1	0,25	2	128	0,25	0,032
11	16	0,25	4	128	0,25	0,064
12	256	0,5	8	128	0,125	0,125
13	16	0,5	32	128	0,5	0,125
14	1,5	0,5	0,125	128	0,5	0,064
15	256	0,75	0,25	128	0,125	0,064
16	256	1	2	256	0,25	0,064
17	0,25	0,5	6	64	0,25	0,064
18	12	0,5	1	128	0,25	0,064
19	0,25	0,25	0,5	64	0,125	0,032
20	256	0,5	0,064	64	0,064	0,032
21	16	0,25	0,064	128	0,064	0,032
22	256	0,75	0,125	128	0,125	0,064
23	256	0,5	0,125	128	0,125	0,012
24	0,5	0,5	0,125	128	0,064	0,012
25	12	4	2	128	1	0,064
26	8	0,5	0,5	128	0,25	0,032
27	256	32	0,5	256	1	0,125
28	0,5	2	1	64	0,064	0,064
29	2	2	0,5	64	0,032	0,064
30	256	32	0,25	64	0,125	0,064
31	2	4	4	128	0,125	0,125
32	256	16	2	256	0,064	0,032
33	4	2	1	128	0,125	0,032
34	1	0,5	1	32	0,25	0,032
35	2	0,5	2	128	0,25	0,125
36	256	2	2	64	0,064	0,25
37	256	2	2	64	1	0,25
38	256	0,5	1	64	0,5	0,032
39	1	4	2	64	0,5	0,064
40	0,5	0,5	16	64	0,125	0,25
41	256	2	4	64	0,032	0,064
42	256	4	8	64	0,064	0,5
43	4	2	16	64	0,125	0,5
44	256	2	4	64	0,064	0,064
45	256	32	0,5	64	0,064	0,125
46	0,5	1	1	32	0,032	0,032
47	256	1	1	32	0,125	0,032
48	1	4	8	32	0,25	0,064
49	16	2	1	64	1	0,25
50	1	1	0,5	0,5	1	0,064

	MIC gradient strip test					
Isolate no.	CAZ	CIP	CO	MRP	TGC	SXT
51	16	2	0,25	32	0,5	0,125
52	1	2	0,5	64	0,25	0,064
53	24	0,5	4	128	1	0,064
54	2	4	0,25	64	1,5	0,064
55	0,5	1	4	64	0,125	0,064
56	256	8	0,125	64	0,125	0,064
57	1	0,25	0,25	32	0,25	0,032
58	0,75	2	12	32	3	0,064
59	6	0,5	1	128	0,5	0,064
60	1,5	0,75	1	128	1	0,064
61	256	0,5	0,125	64	0,25	0,064
62	4	0,25	1,5	64	0,032	0,064
63	1	0,5	4	32	1	4
64	8	2	0,125	128	0,032	0,064
65	8	0,25	0,5	128	0,064	0,064
66	1	1,5	0,25	128	0,5	0,125
67	8	0,5	0,125	64	0,064	0,016
68	1	2	0,125	256	0,5	0,032
69	256	1	0,25	256	0,25	0,064
70	4	1	0,125	256	0,25	0,064
71	1	0,5	0,125	8	0,064	0,064
72	32	4	0,5	128	0,25	0,5
73	4	0,25	0,25	64	0,125	0,064
74	32	0,5	0,016	8	0,125	0,064
75	32	0,5	0,25	32	0,064	0,064
76	256	0,125	0,125	256	0,064	0,012
77	2	0,25	0,125	8	0,064	0,032
78	128	0,75	8	32	0,25	0,064
79	2	1	16	128	0,25	0,125
80	128	0,2	8	32	0,125	0,032
81	256	0,25	16	256	0,25	0,032
82	0,5	0,5	2	128	0,125	0,064
83	0,125	2	2	128	0,25	0,064
84	256	0,25	4	256	0,032	0,064
85	12	0,75	2	128	0,094	0,032
86	1	0,5	0,25	128	0,064	0,032
87	2	0,5	0,125	256	0,125	0,032
88	24	0,25	0,125	256	0,064	0,032
89	256	0,25	8	256	0,25	0,032
90	0,5	0,25	0,5	256	0,25	0,064
91	2	0,25	0,5		0,25	2
92	1	0,5	12	256	0,125	0,064
93	8	1	4	256	0,25	0,064
94	256	0,125	2	32	0,25	0,032
95	8	0,5	1	64	0,25	0,125
96	256	0,5	2	128	0,125	0,094
97	4	4	0,064	64	0,75	0,064
98	8	8	0,125	64	1,5	0,125
99	64	0,75	0,5	64	0,25	0,064
100	4	4	0,25	32	1	0,125

Table 1.2: Antimicrobial susceptibility results in *S. maltophilia* with MIC gradient strip tests.

CAZ = Ceftazidime CIP = Ciprofloxacin CO = Colistin

MRP = Meropenem TGC = Tigecycline SXT = Trimethoprim-sulfamethoxazole

	VITEK 2				
Isolate no.	CAZ	CIP	CO	MRP	SXT
1	<= 1	1	<= 0.5	TRM	<= 20
2	16	1	>= 16	>= 16	<= 20
3	<= 1	0.5	<= 0.5	1	<= 20
4	2	1	<= 0.5	>= 16	<= 20
5	4	0.5	<= 0.5	>= 16	<= 20
6	16	1	<= 0.5	>= 16	<= 20
7	4	1	>= 16	>= 16	<= 20
8	<= 1	0.5	<= 0.5	TRM	<= 20
9	<= 1	1	>= 16	>= 16	<= 20
10	4	2	<= 0.5	>= 16	<= 20
11	4	<= 0.25	TRM	>= 16	<= 20
12	16	0.5	TRM	>= 16	80
13	8	1	<= 0.5	>= 16	<= 20
14	<= 1	0.5	<= 0.5	>= 16	<= 20
15	4	1	<= 0.5	>= 16	<= 20
16	16	2	>= 16	>= 16	<= 20
17	<= 1	0.5	<= 0.5	1	<= 20
18	4	1	2	>= 16	40
19	<= 1	<= 0.25	<= 0.5	TRM	<= 20
20	<= 1	<= 0.25	<= 0.5	TRM	<= 20
21	16	0.5	>= 16	>= 16	<= 20
22	32	1	>= 16	>= 16	<= 20
23	8	0.5	<= 0.5	>= 16	<= 20
24	<= 1	0.5	<= 0.5	TRM	<= 20
25	16	>= 4	<= 0.5	>= 16	<= 20
26	<= 1	1	<= 0.5	>= 16	<= 20
27	>= 64	>= 4	<= 0.5	>= 16	>= 320
28	<= 1	0.5	<= 0.5	1	<= 20
29	4	1	>= 16	>= 16	<= 20
30	8	>= 4	<= 0.5	>= 16	>= 320
31	<= 1	1	>= 16	>= 16	<= 20
32	4	>= 4	<= 0.5	>= 16	80
33	4	2	>= 16	>= 16	<= 20
34	<= 1	0.5	<= 0.5	8	<= 20
35	<= 1	0.5	<= 0.5	>= 16	<= 20
36	4	1	<= 0.5	>= 16	<= 20
37	4	1	<= 0.5	>= 16	<= 20
38	8	1	<= 0.5	>= 16	<= 20
39	4	2	<= 0.5	>= 16	<= 20
40	<= 1	<= 0.25	TRM	>= 16	<= 20
41	4	2	>= 16	>= 16	<= 20
42	16	2	>= 16	>= 16	<= 20
43	>= 64	>= 4	>= 16	>= 16	160
44	4	2	>= 16	>= 16	<= 20
45	16	>= 4	>= 16	>= 16	>= 320
46	<= 1	1	<= 0.5	2	<= 20
47	4	1	<= 0.5	>= 16	<= 20
48	<= 1	>= 4	<= 0.5	>= 16	<= 20
49	16	1	<= 0.5	>= 16	<= 20
50	<= 1	0.5	>= 16	<= 0.25	<= 20

	VITEK 2				
Isolate no.	CAZ	CIP	CO	MRP	SXT
51	4	1	<= 0.5	>= 16	<= 20
52	<= 1	0.5	<= 0.5	1	<= 20
53	4	2	>= 16	>= 16	<= 20
54	2	>= 4	<= 0.5	>= 16	80
55	4	1	>= 16	>= 16	<= 20
56	TRM	TRM	TRM	TRM	TRM
57	<= 1	0.5	<= 0.5	8	<= 20
58	4	>= 4	>= 16	4	160
59	16	1	<= 0.5	>= 16	<= 20
60	2	2	<= 0.5	8	<= 20
61	>= 64	2	<= 0.5	>= 16	80
62	2	1	4	>= 16	<= 20
63	4	1	<= 0.5	4	>= 320
64	16	>= 4	<= 0.5	>= 16	80
65	16	1	<= 0.5	>= 16	80
66	<= 1	>= 4	<= 0.5	>= 16	160
67	8	1	>= 16	8	<= 20
68	<= 1	2	<= 0.5	>= 16	80
69	16	2	<= 0.5	>= 16	<= 20
70	<= 1	1	<= 0.5	>= 16	<= 20
71	<= 1	1	<= 0.5	1	<= 20
72	32	>= 4	<= 0.5	>= 16	>= 320
73	2	<= 0.25	<= 0.5	8	<= 20
74	16	1	<= 0.5	>= 16	<= 20
75	32	>= 4	<= 0.5	TRM	TRM
76	4	<= 0.25	<= 0.5	>= 16	<= 20
77	TRM	TRM	TRM	TRM	TRM
78	2	0.5	>= 16	8	<= 20
79	<= 1	1	>= 16	>= 16	<= 20
80	8	0.5	>= 16	>= 16	<= 20
81	<= 1	0.5	>= 16	>= 16	<= 20
82	<= 1	0.5	>= 16	>= 16	<= 20
83	<= 1	>= 4	>= 16	>= 16	<= 20
84	16	1	>= 16	>= 16	<= 20
85	4	1	<= 0.5	>= 16	<= 20
86	<= 1	1	>= 16	>= 16	<= 20
87	<= 1	1	>= 16	>= 16	<= 20
88	4	1	<= 0.5	>= 16	80
89	4	0.5	>= 16	>= 16	<= 20
90	<= 1	0.5	<= 0.5	>= 16	<= 20
91	4	<= 0.25	<= 0.5	4	80
92	<= 1	0.5	>= 16	>= 16	<= 20
93	2	1	<= 0.5	>= 16	<= 20
94	16	0.5	<= 0.5	>= 16	<= 20
95	2	1	>= 16	>= 16	80
96	8	1	>= 16	>= 16	<= 20
97	2	>= 4	>= 16	>= 16	80
98	4	>= 4	<= 0.5	8	<= 20
99	2	1	<= 0.5	>= 16	80
100	2	>= 4	>= 16	>= 16	160

Table 1.3: Antimicrobial susceptibility results in *S. maltophilia* with VITEK2.

CAZ = Ceftazidime CIP = Ciprofloxacin CO = Colistin

MRP = Meropenem TGC = Tigecycline SXT = Trimethoprim-sulfamethoxazole

	Agar diffusion				
Isolate no.	CAZ	CIP	MRP	TGC	SXT
1	26	18	8	24	26
2	6	20	6	23	28
3	25	19	15	27	27
4	20	20	10	22	27
5	6	9	6	23	26
6	6	18	6	25	29
7	20	16	6	24	26
8	19	20	6	25	27
9	20	22	6	24	27
10	20	14	6	29	26
11	26	28	6	26	29
12	6	25	6	23	30
13	6	24	6	27	28
14	2	23	6	23	30
15	20	23	6	22	28
16	6	23	6	28	26
17	22	25	6	23	22
18	6	26	6	26	23
19	25	29	6	29	25
20	6	6	6	25	26
21	18	19	6	25	28
22	6	23	6	29	28
23	6	24	6	25	27
24	26	27	8	30	28
25	9	16	6	24	27
26	18	20	6	25	25
27	19	19	6	24	24
28	25	26	6	29	27
29	20	25	6	26	29
30	6	6	6	23	25
31	22	25	6	27	27
32	6	9	6	22	27
33	19	18	6	23	26
34	25	27	8	25	29
35	22	28	6	28	30
36	6	24	6	26	28
37	6	24	6	28	30
38	6	24	6	26	28
39	26	15	6	24	28
40	25	28	6	28	29
41	6	24	6	25	30
42	6	24	6	25	29
43	19	13	6	12	17
44	6	22	6	24	27
45	6	6	6	20	27
46	29	24	21	27	31
47	6	24	6	26	29
48	23	15	6	24	30
49	6	16	6	24	22
50	25	20	27	29	29

	Agar diffusion				
Isolate no.	CAZ	CIP	MRP	TGC	SXT
51	6	19	6	26	28
52	20	26	15	29	30
53	6	25	6	27	22
54	21	6	6	22	26
55	26	25	6	23	30
56	6	9	6	25	28
57	26	18	12	28	29
58	27	27	16	27	28
59	18	28	6	26	27
60	20	24	14	26	28
61	6	19	6	28	29
62	18	23	6	25	28
63	19	20	17	29	16
64	8	18	6	23	26
65	9	22	6	20	27
66	18	18	6	25	26
67	18	23	14	26	30
68	26	20	6	20	28
69	19	19	6	21	30
70	16	23	6	23	28
71	18	20	25	22	29
72	6	9	6	28	29
73	9	26	19	23	30
74	27	26	25	26	25
75	6	20	23	29	26
76	6	24	6	25	27
77	18	25	23	25	30
78	20	20	19	29	28
79	10	21	6	25	30
80	20	28	6	30	26
81	6	24	6	24	29
82	26	26	6	28	29
83	27	18	6	23	30
84	6	26	6	26	25
85	6	22	6	29	26
86	21	26	6	25	27
87	6	27	6	25	27
88	6	26	6	29	23
89	6	25	6	25	24
90	27	25	6	27	29
91	20	28	6	20	15
92	26	28	6	22	27
93	20	26	6	23	23
94	6	27	6	20	30
95	6	25	6	25	18
96	20	24	6	26	29
97	6	9	6	20	28
98	21	9	11	21	30
99	6	18	6	23	20
100	21	19	6	20	22

Table 1.4: Antimicrobial susceptibility results in *S. maltophilia* with Agar Disk Diffusion method.

CAZ = Ceftazidime CIP = Ciprofloxacin CO = Colistin

MRP = Meropenem TGC = Tigecycline SXT = Trimethoprim-sulfamethoxazole

Appendix 2 – Coverage depth

Isolate no.	Average coverage depth	Standard deviation	G+C content	Isolate no.	Average coverage depth	Standard deviation	G+C content
1	23,8401	11,7894	66,6	**51**	11,6177	5,50102	66,6
2	133,855	39,4678	66,9	**52**	20,5296	6,03857	66,7
3	103,083	26,4709	66,7	**53**	15,5546	6,69069	66,7
4	66,8545	54,6545	66,8	**54**	17,9612	10,272	66,8
5	115,528	30,1964	66,8	**55**	34,9433	12,7939	66,9
6	112,01	50,6763	66,3	**56**	22,8443	5,9734	66,7
7	81,6691	42,334	66,8	**57**	21,5837	16,53199	67,0
8	106,554	50,6005	66,9	**58**	36,12902	13,67837	66,9
9	119,685	63,6626	66,8	**59**	39,5109	21,7262	66,9
10	61,5151	28,3716	66,7	**60**	80,9216	43,0031	66,8
11	16,3632	7,11002	66,8	**61**	57,5811	26,313	66,9
12	30,0529	14,3961	66,7	**62**	74,5071	24,3069	67,0
13	20,595	8,75763	66,9	**63**	42,071	23,4567	66,8
14	23,6535	12,8489	66,6	**64**	42,6094	23,1679	66,7
15	36,2696	12,8244	66,6	**65**	52,2974	30,1757	66,8
16	39,7782	13,8705	66,6	**66**	47,0881	23,692	66,9
17	23,9219	12,9452	66,4	**67**	77,5306	24,3887	66,9
18	25,4733	14,4345	66,5	**68**	35,3008	18,5963	66,8
19	25,309	13,5025	66,9	**69**	86,1255	28,883	66,9
20	29,824	14,0617	66,8	**70**	82,1875	42,4827	66,9
21	27,4724	13,2209	66,8	**71**	79,2724	41,7448	67,0
22	22,5487	9,0352	66,8	**72**	54,2241	27,748	66,8
23	18,9721	10,1048	66,4	**73**	42,1486	18,8265	66,7
24	24,4493	13,8155	66,7	**74**	64,7685	29,3855	66,8
25	24,8271	9,94337	66,7	**75**	83,1059	38,3854	66,9
26	31,0723	6,57734	66,5	**76**	78,0733	36,449	66,9
27	25,1638	9,80397	66,7	**77**	36,7282	16,86	66,7
28	15,5257	8,44653	66,6	**78**	61,495	28,4518	66,8
29	26,5101	13,8499	66,9	**79**	74,5337	33,3894	66,8
30	33,8036	12,5407	66,9	**80**	21,7277	11,305	66,7
31	17,4495	9,96986	66,8	**81**	67,3146	32,3418	66,8
32	12,8834	7,32917	66,6	**82**	54,5592	29,8178	66,7
33	21,4791	8,4065	66,8	**83**	68,6396	35,3724	66,9
34	18,7791	10,4485	66,7	**84**	37,4747	18,0733	66,9
35	16,528	9,5862	66,7	**85**	60,7597	35,6283	66,8
36	16,1331	7,12124	66,7	**86**	63,052	31,9263	66,7
37	19,72205	1,00679	66,5	**87**	60,9578	31,3743	66,9
38	25,60044	3,09755	66,7	**88**	56,1259	31,8849	66,8
39	19,40637	5,42198	66,9	**89**	75,0644	33,8685	66,9
40	13,4094	7,46472	66,8	**90**	45,388	23,311	66,9
41	10,4359	5,15582	66,8	**91**	4,20661	14,1243	64,8
42	20,5357	9,0063	66,7	**92**	58,4876	29,531	66,8
43	19,5591	5,37562	66,3	**93**	71,4958	24,6131	66,8
44	12,0911	5,56476	66,5	**94**	81,7566	27,5433	67,0
45	16,9951	8,58282	66,6	**95**	45,6103	32,7107	66,9
46	14,1219	8,07615	66,5	**96**	86,0984	27,3399	66,9
47	10,018	4,87066	66,7	**97**	77,5294	40,9775	66,8
48	14,7837	7,9538	66,7	**98**	56,0431	29,9045	66,7
49	21,2301	5,1902	66,4	**99**	51,3071	27,1856	66,7
50	16,44336	7,77813	66,5	**100**	64,6534	31,3068	66,8

Table 2.1: Displaying coverage depth of all *S. maltophilia* isolates, sequenced and presented in this thesis.

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

Postboks 5003
NO-1432 Ås
Norway