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Genome-wide Antibiotic Resistance and Virulence Profiling of *Pseudomonas aeruginosa* Isolated from Clinical Samples in Lebanon

By

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To my loving parents, I dedicate this work…

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Cynthia P. Harb

ABSTRACT

Extensively drug resistant *Pseudomonas aeruginosa* (XDR) are a great worldwide public health concern. *P. aeruginosa* is a Gram-negative opportunistic pathogen associated with a broad range of infections. It causes acute skin, lung, urinary tract infections and sepsis. It can also cause chronic infections in the pulmonary passages of individuals with cystic fibrosis. *P. aeruginosa* is widespread in nature lurking on diverse habitats including animals, plants, soil, in-animate objects and hospital settings. In this study, 18 *P. aeurginosa* clinical isolates were collected from the American University of Beirut Medical Center in Lebanon and were used for identification, phylogenetic analysis and detection of porin (*oprL*, *oprI* and *oprD*), efflux pumps (*mexA*, *mexC* and *mexE*) and exotoxin genes (*exoU* and *exoS*). Illumina paired-end libraries for twelve representative isolates were prepared and sequenced. The initial assemblies produced an average genome size of 6.8 Mb, G+C% content of 65.90-66.30% and 116-396 contigs. *In silico* MLST typing revealed that the isolates belonged to six MLST types: ST-233, ST-235, ST-296, ST-654, ST-1182 and ST-1233. Antimicrobial susceptibility testing showed that all except PA43, were XDR. Genomic analysis revealed the presence of several β-lactamases encoding genes (*bla*$_{\text{PAO}}$, *bla*$_{\text{OXA-50}}$, *bla*$_{\text{OXA-4}}$, *bla*$_{\text{IMP-15}}$, *bla*$_{\text{GES-1}}$ and/or *bla*$_{\text{VIM-2}}$ genes) and the *aac(6')Ib-cr* gene conferring aminoglycoside resistance. Additionally, all the 18 isolates carried *mexC* and *mexE* conferring ciprofloxacin resistance. *exoS* and *exoU* toxing encoding genes were detected in 56% and 39% of the isolates, respectively, and phiCTX was the most common detected plasmid in this study. In this study the comparative analysis of *P. aeruginosa* genomes isolated from clinical samples in Lebanon,

revealed many antibiotic resistance and virulence factor genes, a large number of genes that are involved in regulation, catabolism, transport, and efflux of organic compounds contributing to the remarkable ability of this bacterium to adapt to a wide range of environmental niches. The emergence and spread of XDR *P. aeruginosa*, which constitute a therapeutic problem of serious concern, emphasize the importance of having effective laboratory detection systems and infection control measures.

Keywords: *Pseudomonas aeruginosa*, Next-generation sequencing, Genome wide analysis, Resistance, XDR, Virulence, Efflux pumps, Lebanon.

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LIST OF ABBREVIATIONS

AHLs: *N*-acyl-homoserine lactones

AK: amikacin

AQs: 2-alkyl-4 quinolones

asRNAs: antisense RNAs

ATM: aztreonam

AUBMC: American University of Beirut Medical Center

BEL-: Belgium metallo-β-lactamase

CAR: carbencilin

CAZ: ceftazidime

CC: clonal complex

CDS: coding sequence

CF: cystic fibrosis

CGE: Center for Genomic Epidemiology

CI: ciproflaxin

CLSI: Clinical and Laboratory Standards Institute

CST: colistin

ESBLs: extended spectrum β-lactamases

FDA: Food and Drug Adminstration

GM: gentamicin

HHQ: 2-heptyl-4-quinolone

HK: histidine kinase

IPM: imipenem

MBL: metallo-β-lactamase

MBLs: mannose-binding lectins

MDR: multiple drug resistance

MLST: multi-locus sequence typing

NCCLS: The National Committee for Clinical Laboratory Standards

ORF: open reading frame

OXA-: active on oxacillin metallo-β-lactamase

PA: *Pseudomonas aeruginosa*

PAO1: *Pseudomonas aeruginosa* strain O1

PCR: polymerase chain reaction

PDR: pan-drug resistance

PER-: *Pseudomonas* extended resistance metallo-β-lactamase

PMN: polymorphonuclear neutrophilic

PQS: 2-heptyl-3-hydroxy-4-quinolone

PSE: Pseudomonas-specific enzyme

QS: quorum sensing

rgRNAs: regulatory RNAs

RR: response regulator

SNP: single-nucleotide polymorphism

SPM-: São Paulo MBL metallo-β-lactamase

ST: sequence type

TCS: two-component regulatory systems

TE: tetracycline

TM: tobramycin

TSA: Tryptic soy agar

TX: ceftriaxone

TZP: piperacillin/tazobactam

T3SS: type III secretion system

VIM: Verona-integron-encoded

XDR: extensively drug-resistant

Chapter One

INTRODUCTION

Pseudomonas aeruginosa, a member of the family *Pseudomonadaceae*, is considered to be a leading opportunistic pathogen (Darch et al., 2015). *P. aeruginosa* is one of the most abundant microorganisms found in nature. Due to its widespread availability, it can increasingly interact with human hosts, which in turn results in countless serious infections in the hospital settings and environment (Snyder et al., 2013). This life-threatening Gram-negative bacterium can cause a wide range of acute infections such as burns, lungs, urinary tract and wound infections and can also contribute to chronic infections in the lungs of patients with cystic fibrosis (Wong et al., 2012; Snyder et al., 2013), which are linked to the high rate of mortality and morbidity associated with this disease (Marvig et al., 2013).

The switch from an acute infection to a chronic infection is regulated by various mechanisms. These mechanisms control the ability of *P. aeruginosa* to: grow, accumulate and colonize the airway passages, grow in the form of biofilms, and control host gene expression through quorum sensing (QS). It also resists various antibacterial drugs due to its acquired, adaptive and intrinsic resistance mechanisms, rendering associated infections to be difficult to combat and eradicate (Darch et al., 2015; Kim et al., 2011). Most *P. aeroginosa* strains are extremely resistant to the fourth-generation cephalosporins, β-lactam drugs and many others, with some strains still exhibiting susceptibility to colistin and amikacin (Kos et al., 2014).

Consequently, it is of major importance to determine and thoroughly study the antibiotic resistance profiles of *P. aeruginosa* local strains with the purpose of precisely identifying the antimicrobial drug of choice and treatment measures to combat pseudomonal infections. All genetic elements and factors pertaining to antimicrobial drug resistance must be identified via molecular tests (Bentzmann et al., 2011). In addition to antibiotic resistance control and surveillance, the high-

throughput whole genome sequencing (WGS) approach is useful in analyzing the potential adaptation and evolution of *P. aeruginosa* over time (Snyder et al., 2013; Koser et al., 2014).

The overall objectives of this study are stated as follows:

- To compare the differences that exist between the *P. aeruginosa* local isolates
- To determine the evolutionary dynamics of *P. aeruginosa* local strains in terms of genome plasticity
- To determine the presence of resistance (*mexA*, *mexC*, *mexE* and *oprD*) and virulence genes (*exoS* and *exoU*) through PCR assays and compare to those identified from high-throughput sequencing
- To identify intrinsic resistance including mutations in the *ftsK* gene, which affects cell division and *ampG* gene, which is responsible for β-lactamase production
- To detect the presence of the AmpR β-lactamase regulator for positive regulation of the QS virulence genes
- To screen the *P. aeruginosa* genomes for the presence of the type III secretion system effectors
- To correlate antibiotic resistance to the presence of *mexA*, *mexC* and *mexE* efflux pump genes
- To construct a pylogenetic tree using whole-genome sequencing data

Chapter Two

LITERATURE REVIEW

2.1. Overview on *Pseudomonas aeruginosa*

P. aeruginosa is a clinically important aerobic, rod-shaped Gram-negative bacterium. The length of this opportunistic pathogen varies from approximately 1 µm to 5 µm, and the width ranges from about 0.5 µm to 1 µm (Sedighi et al., 2015). It grows as a distinct greenish-blue colony due to the production of pycocanin pigment (Gellatly & Hancock, 2013), with high phenotypic diversity ranging from motile to non-motile and mucoid to non-mucoid (Workentine et al., 2013). It has the ability to thrive in a variety of habitats due to the fact that it requires nominal nutrient concentrations. It is ubiquitous in soil, plants, animals and on inanimate surfaces (Nasreen et al., 2015). It's also found but to a lower extent on the skin and in the gastrointestinal tracts of humans (Sedighi et al., 2015).

It is characterized by having a mosaic genome with 90% of its genes or more encompassing the conserved core region. Strains are differentiated by a specific set of genes disrupting the conserved core. These segments, which are strain-specific, are found in the genome plasticity regions at limited chromosomal positions. This suggests that the genome among diverse pseudomonal strains is highly conserved despite the fact that some genomic islands have been encountered (Bentzmann & Plesait, 2011). *P. aeruginosa* PAO1 was the first strain to be sequenced, and its genome is composed of a circular chromosome of 6.264 Mb and includes 5570 protein coding sequences. Subsequently, other strains were sequenced including: PA14, LESB58 and PA7 (Klockgether et al., 2011). *P. aeruginosa* PA7 whole genome sequence has been studied as a taxonomic outlier consisting of 6,588,339 bp. It contains 51 genome plasticity regions and several genomic islands, and showed 95% similarity match compared to other isolates (Roy et al., 2010).

2.2. Resistance

P. aeruginosa can be characterized as being a multi-drug resistant, extensively-drug resistant or pan-drug resistant bacterium having several intrinsic resistance mechanisms rendering several antibiotics to be ineffective. (MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories; XDR: non-susceptible to ≥1 agent in all but ≥2 categories; PDR: non-susceptible to all antimicrobial agents). (Bonomo et al., 2006; Livermore, 2002, Magiarakos et al., 2011).

2.2.1. Epidemiology of Resistance

In the past 20 years, *P. aeruginosa* has progressively become more resistant to carbapenem, fluoroquinolone and third-generation cephalosporin treatments (Flamm et al., 2004). Upon the beginning of anti-pseudomonal treatment, intubated patients in critical care units can become multi-drug resistant in a period as short as ten days. Also, strains that are found to be resistant to ciprofloxacin and piperacillin show increasing degrees of resistance to other antimicrobial drugs compared to strains that are sensitive to ciprofloxacin and piperacillin (Sun et al., 2011).

2.2.2. Intrinsic Resistance Mechanisms of *P. aeruginosa*

The most important resistance mechanism within *P. aeruginosa* is the low permeability of its outer membrane which functions as a selective barrier reducing antibiotic uptake. The outer membrane of *P. auerogino*sa is characterized by the presence of an insufficient number of its major small porin channels OprF, and inefficient number of porins specific for the uptake of antibiotics such as OprB and OprD (Gellatly & Hancock, 2013). There are additional pathways for antibiotic uptake across the cell such as: hydrophobic molecule uptake through the phospholipid bilayer and self-driven polycationic antibiotic passage (Breidenstein et al., 2011). Resistance could be also due to rapid efflux, which is caused by the upregulation of efflux pumps expression, and the production of AmpC β-lactamases benefiting from the slow passage as a result of lower outer membrane permeability (Gellatly & Hancock, 2013).

Another intrinsic resistance mechanism is the simultaneous involvement of numerous unrelated genes and various mutations in the resistome of *P. aeruginosa*. Intrinsic resistance to ciprofloxacin and β-lactams is prevalent in strains that have

mutations in the *ftsK gene* which affects cell division, while resistance to β-lactams is linked to mutations in the gene *ampG,* which is responsible for β-lactamase production. Therefore, the increased intrinsic resistance to most antibacterial drugs is attributed to the synchronized action of multiple mechanisms (Breidenstein et al., 2011).

2.2.3. Acquired Resistance

Acquired resistance is directly related to the reduced susceptibility of *P. aeruginosa* and is divided into two forms: horizontal gene transfer and mutational resistance. Antibiotic resistance genes can be obtained via conjugation, transformation and transduction, and are carried on mobile genetic elements such as: plasmids, transposons and prophages. The presence of multiple antibiotic resistance genes on plasmids can result in multi-drug resistant strains. This is mostly the case with resistance to aminglycosides and β-lactams. Mutational resistance is another type of acquired resistance. Additionally, agents damaging genetic material and bacterial proliferation in biofilms increase the frequency of mutations (Blair et al., 2015).

Mutations that result in antibiotic uptake reduction, antibiotic target modification, β-lactamase overproduction and efflux pump overexpression render *P. aeruginosa* insusceptible to antimicrobial treatment. One example of mutational resistance mechanism is associated with mutations in genes encoding *mexR* and *nfxB* resulting in efflux pumps MexAB-OprM and MexCD-OprJ derepression. A mutation in the gene encoding *mexZ* is involved in overexpression of MexXY-OprM resulting in resistance to aminoglycosides, fluoroquinolones and cefepimes. Resistance to the antibacterial drug imipenem results from mutation in OrpD, while resistance to a variety of antibiotics, including imipenem, is generated by *mexT* and *mexS* mutations, which boost the efflux pump MexEF-OprN and decrease OrpD expression. Mutational resistance is also linked to mutations in AmpD and AmpC β-lactamase effectors, which leads to the increase in β-lactamase production (Breidenstein et al., 2011). Finally, fluoroquinolone resistance can also be attributed to mutations in gyrases (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) (Salma et al., 2012).

Therefore, accumulation of mutations can cause increased resistance. Creeping baselines is a step-wise process that converts low-level resistance to increased high-

level resistance through the combination of multiple mutations in due course. For example, three mutations in three distinct genes revealed an increase in tobramycin resistance by two times. However, resistance was augmented to 16-fold with the addition of a forth mutation (Breidenstein et al., 2011). An example of this is seen in aminoglycoside resistance, which includes mutations in diverse genes that encode DNA duplication, energy metabolism and lipopolysaccharide production. Fluoroquinolone resistance, on the other hand, is mediated by mutations in genes that encode efflux pump regulators, iron transport, and prophage. Thus, acquired resistance to a set of antibacterial drugs can occur due to numerous mutations in distinct genes (Mortita et al., 2015; Poole & McKay, 2003).

2.2.4. Adaptive Resistance

Bacteria develop adaptive resistance as a consequence of prolonged antibiotic treatment or the presence of a stimulus. Adaptive resistance is attained by a vast number of stimuli and conditions including: anaerobiosis, antibiotics, biocides, biofilm formation, cations, carbon resources, polyamines and swarming (Gellatly & Hancock, 2013). Such factors act by influencing the genes that control efflux pumps, enzymes and lipopolysaccharides. Adaptive resistance is pivotal in *P. aeruginosa* because its genome consists of a large percentage of regulatory genes. It is capable of resisting antibiotic action due to sub-inhibitory antibiotic dosage and environmental stimuli that modulate its gene expression (Fernandez & Hancock, 2012).

Aminoglycosides and polymyxins belong to polycationic antimicrobials, which undergo self-driven uptake across the bacterial outer membrane by attaching to specific binding sites on the lipopolysaccharide (Breidenstein et al., 2011). Their passage is limited by the action of *arnBCADTEF* operon, which allows the binding of 4-aminoarabinose to Lipid A. Minimal amounts of divalent cations lead to polycationic antimicrobial resistance. This mechanism involves the introduction of the *arn* operon and the activation of PhoPQ and PmrAB regulatory systems. The function of the *arn* operon is reducing cation amounts upon stimulation in biofilms (Poole, 2011). PhoPQ and PmrAB control the lipolysaccharide modification *arnBCADTEFpmrE* operon in order to achieve resistance against cationic

antimicrobial peptides and polymyxin B under conditions of depleted cation concentration (Balasubramanian et al., 2012).

Another important mechanism of adaptive resistance involves the production of AmpC β-lactamases. β-lactamases inactivate β-lactam drugs. Upon the introduction of β-lactamases, cefotaxime, ceftazidime and penicillins can longer be used to effectively treat *P. aeruginosa* associated infections, as their main mode of action relies on *ampC* gene upregulation (Fernandez & Hancock, 2012).

The excessive expression of efflux pump genes is another means of adaptive resistance which is induced due to antibiotic sub-inhibitory dosages. MexXY efflux pump genes are overexpressed by the introduction of aminoglycosides. This results in adaptive resistance due to the quick outward flow of the aminoglycoside conferring multi-drug restistance (Fernandez & Hancock, 2012). This is observed in bacterial colonies growing in biofilms, where resistance is achieved through several mechanisms (Hoiby et al., 2010). One way includes mutation in the quorum sensing regulatory network resulting in the increased expression of genes that encode efflux pumps and enzymes. Another mode of resistance involves varying amounts of nutrient uptake causing diversity in the metabolic activities of different isolates (Breidenstein et al., 2011). Bacterial cells found in the interior of the growing biofilm are more likely to be metabolically inactive, while the bacterial cells found on the surface of the biofilm grow much faster, hence different antibiotics target different biofilm areas. Polymyxins target the interior cells, while β-lactams and aminoglycosides only affect the external layer. Biofilms, in addition, are described as being rich in persister cells with a lower percentage of planktonic cells. Persister cells are capable of surviving the harsh conditions of antibiotic pressure due to their slow growing nature, with little being known about the mechanism (Poole, 2011).

A particular type of movement carried out by *P. aeruginosa* is called swarming, and is unrelated to flagella-mediated swimming and pilus-mediated twitching. Dysregulation in more than 400 genes involved in adaptation, metabolic pathways, motility, regulation and secretion causes pseudomonal swarming in the lungs owing to its viscous nature and poor nitrogen availability. *lasB* and *pvdQ* are two virulence genes that are most needed for swarming (Overhage et al., 2008). Isolates that move by swarming are usually more resistant to a variety of antimicrobial drugs. They

also carry numerous virulence factors, genes and toxins. Adaptive resistance occurs in swarming cells by the dysregulation of a sensor kinase, PhoQ, which is mediated by two swarming regulators, Lon and CbrA (Poole, 2011; Sampedro et al., 2014).

Tokajian et al. (2012) studied the resistance pattern of 100 *P. aeruginosa* isolates collected mainly from tracheal aspirates, urine, sputum, pus and wounds. The study revealed that 25% of the isolates were resistant to impenem and pipercillin-tozabactam, while 29% were resistant to ceftazidime. Resistance was measured against the antibiotics mostly used in Lebanon to treat pseudomonal infections (Tokajian et al., 2012). On the other hand, Salma et al. (2012) reported that resistance to fluoroquinolones was due to one or more mutations in DNA gyrases and topoisomerase IV specifically in the *gyrA* and *parC* genes. Resistance to carbapenems was also detected in the Mediterranean region and was mainly linked to the production of carbapenemases (VIM-2 and IMP-15) and mutations in the *oprD* gene (Al Bayssari et al., 2014). Carbapenem resistant strains harboring the VIM-2 carbapenemase have disseminated into livestock animals in Lebanon posing a threat on humans (AL Bayssari et al., 2015).

2.3. Virulence Regulatory Systems of *P. aeruginosa*

2.3.1. Quorum Sensing

Quorum sensing (QS) is a mechanism used by bacteria to control the expression of different genes, during which bacterial cells synthesize small molecules called autoinducers. These molecules allow the continued existence of the bacterial cells upon diffusion when a certain concentration is met. The main reason behind utilizing the QS mechanism is to control the synthesis of virulence contributing factors including production of biofilms, expression of efflux pumps and motility. *P. aeruginosa* uses the QS system by means of two autoinducers: AHLs (*N*-acyl-homoserine lactones) and AQs (2-alkyl-4 quinolones) (Lee & Zhang, 2014).

2.3.1.1. AHL-mediated QS

The *las* and *rhl* systems are two AHL-mediated QS systems. *lasI* and *rhlI* lead to the activation of a family of regulators, LuxR, LasR or RhlR, by binding to them, resulting in multimer interactions due to their AHL occurrence (Balasubramanian et al., 2012).

2.3.1.2. AQ-mediated QS

PQS (2-heptyl-3-hydroxy-4-quinolone) and HHQ (2-heptyl-4-quinolone) are two AQ-mediated QS signals. Both of them allow the binding of the *pqsABCDE* operon promoter to LysR transcription regulator, MvfR. Hence, both PQS and HHQ act as effectors of MvfR. Because it is capable of sequestering iron molecules, PQS can influence the Fur regulon expression and can synthesize vesicles through membrane conformational changes. However, PmpR is a negative regulator of MvfR and is a YebC component (Gellatly & Hancock, 2013).

2.3.1.3. QS Regulation

QS genes are adjusted by global regulators such as the RpoS (Lee & Zhang, 2014). *lasR* and *rhlR* are modulated by RpoS. Another main global regulator is RsaL which plays a pivotal role in *las* signaling, regulates the expression of approximately 130 genes, specifically hydrogen cyanide and pyocyanin genes which are involved in AHL-related virulence repression, and is also implicated in the conversion from a planktonic status to a sessile status. CzcR is another regulator that binds to the promoter of *lasI* allowing *lasI*, *rhlI* and *rhlR* expression resulting in decreased susceptibility to carbapenems and heavy metals (Balasubramanian et al., 2012).

VqsR is another major regulator inhibiting QscR, the LuxR regulator, which in turn controls QS. QscR binds to LasR and RhlR and is required for the homeostasis of LasI (Liang et al., 2012). At the post-transcriptional level, LasR and RhlR are destabilized by the action of QteE. However, the QS threshold concentration, which is necessary for specific QS gene activation, drops to zero when *qteE* is absent. AHL amounts also drop upon subsequent activity of RsmA which is known for its negative effect on *rhl* and *las* pathways. Conversely, a positive regulator of *rhlI* is the RNA chaperone Hfq (Balasubramanian et al., 2012).

The most recent global regulator is the AmpR β-lactamase regulator, which positively regulates the QS genes (Balasubramanian et al., 2012). *P. aeruginosa* strains that do not harbor AmpR produce a very minute quantity of QS related virulence determinants, mainly LasA and LasB proteases and pycocanin. AmpR is

involved in preserving *P. aeruginosa* infections at the acute level and controlling non-β-lactam resistance (quinolones and aminoglycosides) (Kumari et al., 2014) by repressing efflux pump MexEF-OprN activity, production of biofilms, AlgT/U alignate regulator, expression of QS genes and production of pycocanin (Balasubramanian et al., 2012).

2.3.2. Two-Component Regulatory Systems

TCS are two-component regulatory systems implicated in cellular signaling. TCS consists of two constituents: a cytoplasmic response regulator (RR) and an integral membrane sensory histidine kinase (HK). A conserved acceptor domain and a variable donor domain compromise the RR (Balasubramanian et al., 2012). The HK C-terminal domain consists of a catalytic domain, a dimerization domain and a conserved histidine molecule. The HK utilizes its N-terminal domain in order to sense particular stimuli. The signaling process involves the dimerization of two HK molecules followed by phosphorelation at the histidine molecule. This occurs when the HK residues receive a signal. The receiver domain of RR accepts the phosphate and aids in its catalysis. The phosphate is added to its aspartate residue. This causes the donor domain, which often binds to DNA, to undergo a conformational change affecting the level of gene expression (Bentzmann & Plesait, 2011).

P. aeruginosa is characterized by having a great number of TCS proteins. The ability of *P. aeruginosa* to control its antibiotic resistance, metabolic activities and virulence is contributed to the vast amount of TCS proteins available. GacSA is the most important TCS protein found in *P. aerurginosa*, which is responsible for converting an acute infection into a chronic infection.
It also has a major role in the formation of biofilms, expression of virulence factors, metabolites and QS. Antibacterial drug resistance to ciprofloxacin, tetracycline and tobramycin is achieved by GacSA through the action of RsmA/rgRsmZ (Balasubramanian et al., 2013). The binary interaction of two additional TCSs in *P. aeruginosa*, PhoPQ and PmrAB, has a considerable effect on its virulence.

2.3.3. Biofilm Formation

P. aeruginosa isolates grow rapidly in biofilms, which generally occurs in patients with cystic fibrosis. Its increased resistance to a variety of antimicrobial drugs and to the immune system defense mechanisms results from its ability to form biofilms. Its

expanded resistance is mainly due to the presence of biofilm persister cells, which are produced by *algR* and *pilH genes* (Mulcahy et al., 2010; Poole, 2011). The process of biofilm cell lysis is achieved by the QS system, producing extracellular DNA required for bacterial biofilm formation. Rhamnolipid production is also controlled by the QS system, which generates the cap part of the biofilm mushroom. Rhamnolipid also preserves the channels of the mature biofilms and assists in biofilm movement (Harmsen et al., 2010).

2.3.4. Alginate Production

When switching from an acute infection to a chronic infection, the phenotype of *P. aeruginosa* isolates found in a patient suffering from cystic fibrosis changes from non-mucoid to mucoid cells with overproduction of alginate (Gellatly & Hancock, 2013). The lung foci are the restricted areas invaded by the chronic infection, and lung tissue are seriously damaged as a result of these foci disrupting regular lung activity. The microorganism is shielded from the action of antimicrobial drugs and phagocytosis owing to the synthesis of alignate (Bjarnsholt et al., 2010). However, this mucoid phenotype appears after antibiotics, hydrogen peroxide and leucocytes are given to eliminate the biofilm (Balasubramanian et al., 2012).

The chief factor in alginate production is the σ factor AlgT/U. MucA and MucB are two anti-σ factors that inhibit σ factor AlgT/U at the post-transcription level. When MucA and MucB lose their activity via mutations, mucoid cells appear. This takes place when a coordinated proteolytic pathway leads to the detachment of MucA from σ factor AlgT/U (Damron & Goldberg, 2012). The connection between alginate synthesis and antimicrobial drug resistance is AmpR. AmpR also negatively controls the expression of algT/U by interacting with QS. However, since it is a complex process, AlgT/U needs to regulate the action of several transcription regulators simultaneously (Balasubramanian et al., 2011).

2.3.5. Iron Uptake Regulation

The presence of iron is very vital, where toxic oxygen reactive species are created in response to a surplus of iron molecules. In order to find iron and bind to it, bacteria develop siderophores such as: pyochelin and pyoverdine. *P. aeruginosa* also utilizes a ferric uptake regulator called Fur, with its main function being to bind to the Fur

box to regulate the iron regulon and control other regulator expressions (Cornelis, 2010).

The expression of *P. aeruginosa* virulence factors are affected by the iron concentration. The correlation between the amount of iron and the expression of virulence factors is related to PvdS. PvdS regulates pyoverdine and pyoverdine receptor synthesis and PrpL and exotoxin A virulence factors, hence linking iron to virulence. In addition, lactoferrin prevents biofilm formation showing that iron plays a major role in the process. However, formation of biofilms is determined by the concentration of iron in the cell, but formation of biofilms is inhibited with superior iron amounts (Jimenez et al., 2012).

Minute concentrations of iron improve the functions of the QS systems. What the QS system regulators do is that they activate transcription by controlling an iron responsive gene, MvfR, when iron concentrations are minimal. Furthermore, the synthesis of PQS is positively regulated by small RNA molecules which are negatively regulated by Fur. Pyochelin and pyoverdine can more easily search for iron molecules in the cell due to PQS chelating iron (Balasubramanian et al., 2012).

2.3.6. Toxins and Exoproteins

Toxins and exoproteins strongly contribute to the virulence of *P. aeruginosa*. It contains a total of five secretion systems out of seven with numerous toxins. Of utmost importance is the type III secretion system. The type III secretion system injects ExoS, ExoT, ExoU and ExoY bacterial effector proteins directly into the host cell cytoplasm. Strains that contain ExoS and ExoU are more likely to be multi-drug resistant. Specifically, isolates that are ExoS positive can result in reduced DNA production, while isolates that are ExoU positive have the ability to combat the eukaryotic immune system. Besides, severe lung infections are associated with the presence of ExoS, which can lead to chronic disruption of the respiratory tract (Kipnis et al., 2012). Strains that harbor toxin genes are characterized by increasing the severity of the disease and symptoms, and are treated with anti-pseudomonal drugs resulting in drug resistance (Wong-Beringer et al., 2008).

2.3.7. Regulatory RNAs

In *P. aeruginosa*, regulatory RNAs (rgRNAs) are a necessary factor for the expression of virulence genes. rgRsmY and rgRsmZ are two important rgRNAs, which are required for the alteration from an acute infection into a chronic one. These two rgRNAs are positively regulated by GacA TCS, which proceeds by sequestering RsmA (RNA-binding protein) resulting in virulence factor expression dysregulation (Bentzamann & Plesait, 2011).

Additionally, translation can be inhibited by the action of antisense sRNAs (asRNAs) in *P. aeruginosa* strains by interacting with mRNAs. The expression of pyocyanin and PQS are influenced by asPhrS, an example of an asRNA. As mentioned previously, iron uptake is regulated by small RNA molecules, specifically, asRNAs which include asPrrF and asPrrF2. At conditions of high iron concentrations, Fur inhibits the two asRNAs. However, at conditions of iron-depletion, Fur activates the two asRNAs, which, interact with the mRNA of target genes such as superoxide dismutase *sodB* gene. Therefore, iron uptake, carbon metabolism and virulence work in synchrony due to regulation by asPrrF1 and asPrrF2. asPrrH is an additional asRNA regulating virulence mechanisms in *P. aeruginosa* which is activated at low iron concentrations (Balasubramanian et al., 2012).

2.4. Multi-drug Resistant *P. aeruginosa* Treatment

The antibiotic treatment needed for full eradication of this deadly pathogen is challenging due to the multiple antibiotic resistance mechanisms that it possesses. The most effective treatment determined is the use of an anti-pseudomonal β-lactam drug along with an aminoglycoside, or the use of an anti-pseudomonal β-lactam drug along with an anti-pseudomonal quinolone. However, it was suggested that β-lactams should be utilized more frequently than quinolones since β-lactam antibiotics were more effective in combating this microorganism. Developing resistance to quinolones is a much faster process. The optimum treatment involves one dosage of aminoglycoside antibiotic per day over a period of a maximum of five days. In some situations in intensive care units, polymyxins such as colistin are used as a last choice (Sun, 2011).

Antibiotic treatment can be replaced by other therapeutics such as bacteriophage treatment. In general, bacteriophages increase in number at the site of bacterial

infection, act on the bacterium of choice regardless of the normal flora and can interact with the antibiotic resistant bacteria. These are the three key differences between bacteriophage treatment and antibiotic treatment. Upon administration of bacteriophage treatment into the respiratory tract by nebulization, bacteriophages can stimulate host phagocytes to kill the pathogenic bacteria limiting their ability to initiate an infection in the lungs in cases of cystic fibrosis (Debarbieux et al., 2010).

2.5. High-throughput Whole Genome Sequencing

Before the discovery of the whole genome sequencing (WGS) approach, information on bacterial phylogeny was limited, and relationships among different pathogens were lacking. The emergence of high-throughput WGS along with bioinformatics techniques such as multi-locus sequence typing (MLST) allows the construction of phylogenetic trees and identification of the genetic structures of bacterial species (Klemm & Dougan, 2016). High-throughput WGS also drove the development of SNP-based phylogenetic analysis which proved to be useful in determining the relationship between genetic diversity and different niches for pathogenic bacteria, hence uncovering the epidemiology of outbreaks in hospital settings. The feasibility of these technological advances has facilitated the development of transmission mapping while identifying pathogen diversity within the host. This means that WGS has allowed microbiologists to monitor bacterial evolution and investigate the spread of bacterial pathogens at different scales (globally, locally and within a single patient). It can also allow the identification of previously unknown microorganisms, virulence factors and antibiotic resistance determinants (Robinson et al., 2013). High-throughput WGS has also proved to be useful in the development of antimicrobial drugs and vaccines. By combining WGS with SNP-based analysis, novel antibiotic targets can be determined by comparing the SNPS in the genomes of a sensitive bacterial parent strain and a resistant bacterial daughter strain (Loman & Pallen, 2015). Thus, high-throughput sequencing promises to be an effective approach for identifying, screening and preventing future threats to public health (Koser et al., 2014).

Chapter Three

MATERIALS AND METHODS

3.1. Clinical Isolates

Eighteen *P. aeurginosa* clinical isolates were collected from the American University Medical Center and were used for genome analysis. The isolates were recovered from bile, blood, catheter tip, sputum, tracheal-aspirate and urine, collected from nine females and nine males including two juniors (1-17 years), six adults (18-64 years) and ten seniors (>64 years). Isolates were designated as: PA3, PA5, PA9, PA15, PA16, PA28, PA 43, PA45, PA53, PA59, PA120, PA132, PA138, PA141, PA147, PA152, PA155 and PA177. All were cultured on Tryptic Soy Agar (TSA) (Bio-Rad, U.S.A) media at 37^{o}C for 24-48 h.

3.2. DNA Extraction

Bacterial DNA was extracted using the NucleoSpin kit (MACHEREY-NAGEL, Germany) and following the manufacturer's instructions.

3.3. Genome Sequencing

The Qubit 3.0 fluorometer (Life technologies, Carlsbad, CA) was utilized for accurately quantifying the DNA concentrations. Genomic DNA was used as input for library preparation using the Illumina Nextera XT library preparation kit (Illumina, Inc., San Diego, CA). The Agencourt AMPure XP PCR purification beads (Agencourt, Brea, CA) were used to allow effective clean up after library preparation. With the use of the Kapa library quantification kit (Kapa Biosystems, Woburn, MA), the CFX96-PCR system (Bio-Rad, U.S.A) was utilized to quantify the DNA libraries. The Illumina MiSeq Desktop Sequencer was finally used to multiplex, cluster and sequence the pooled libraries with a minimum of 250 bp read length.

3.4. PCR Assays

3.4.1. Detection of Outer Membrane Protein Encoding Genes

PCR assays were performed using the following primers: oprI F (5'-ATGAACAACGTTCTGAAATTCTCTGCT-3') and oprI R (5'-CTTGCGGCTGGCTTTTTCCAG-3') amplifying a 249 bp fragment of the *oprI* gene, oprL F (5'-ATGGAAATGCTGAAATTCGGC-3') and oprL R (5'-CTTCTTCAGCTCGACGCGACG) amplifying a 504 bp fragment of the *oprL* gene, oprD F (5'- CGCCGACAAGAAGAACTAGC-3') and oprD R (5'-GTCGATTACAGGATCGACAG-3') amplifying a 1412 bp of the *oprD* gene (Adabi et al., 2015; Poonsuk et al., 2014). PCR mixture contained 2 µl of template DNA, 20 µM of each pair of primers, 25 mM $MgCl_2$, 2 mM dNTPs and PCR-grade sterile water achieving a final volume of 20 µL. The cycling conditions were: 1 cycle was set at 95˚C for 12 min followed by 30 cycles at 94˚C for 30 sec for denaturation, 1 cycle was set at 63˚C for 30 sec for annealing and finally, 1 cycle was set at 72˚C for 1 min followed by 1 cycle at 72˚C for 10 min for elongation. The PCRs were performed on a thermal cycler (Bio-Rad).

3.4.2. Detection of Efflux Pump Encoding Genes

PCR assays were performed using the following primers: mexA F (5'-CGACCAGGCCGTGAGCAAGCAGC-3') and mexA R (5'-GGAGACCTTCGCCGCGTTGTCGC-3') amplifying a 316 bp fragment of the *mexA* gene, mexC F (5'- GTACCGGCGTCATGCAGGGTTC-3') and mexC R (5'-TTACTGTTGCGGCGCAGGTGACT-3') amplifying a 164 bp fragment of the *mexC* gene and mexE F (5'- CCAGGACCAGCACGAACTTCTTGC-3') and mexE R (5'- CGACAACGCCAAGGGCGAGTTCACC-3') amplifying a 114 bp fragment of *mexE* gene (Adabi et al., 2015; Llanes et al., 2004). PCR mixture contained 2 µl of template DNA, 20 µM of each pair of primers, 25 mM $MgCl_2$, 2 mM dNTPs and PCR-grade sterile water achieving a final volume of 20 µL. The cycling conditions were: 1 cycle was set at 95˚C for 12 min followed by 30 cycles at 94˚C for 30 sec for denaturation, 1 cycle was set at 63˚C for 30 sec for annealing and finally, 1 cycle was set at 72˚C for 1 min followed by 1 cycle at 72˚C for 10 min for elongation. The PCRs were performed on a thermal cycler (Bio-Rad).

3.5. Antibiotic Susceptibility Test

Antibiotic susceptibility for the 18 pseudomonal strains was determined by the Kirby-Bauer disk agar diffusion method against 12 different antibiotics including: amikacin (AK), aztreonam (ATM), carbencilin (CAR), ceftazidime (CAZ), ceftriaxone (TX), ciproflaxin (CI), colistin (CST), gentamicin (GM), imipenem (IPM), piperacillin/tazobactam (TZP), tetracycline (TE) and tobramycin (TM). The bacterial-saline suspensions were adjusted to 0.5 Mcfarland standard. The test was performed on Mueller-Hinton agar followed by incubation at 37^{o}C for 24 h. The Clinical and Laboratory Standards Institute (CLSI) guidelines were used to determine the antibiotic resistance/susceptibility profiles for each respective antimicrobial drug (CLSI, 2013).

3.6. Analysis of Sequencing Results

3.6.1. Genome Assembly

De novo assembly was performed for the sequenced genomes by using the A5-miseq assembly pipeline (Tritt et al, 2012; Coil et al, 2015). The assembly was performed simultaneously with error correction, cleaning of data, control of quality and scaffolding (Tritt et al., 2012). .

3.6.2. Genome Annotation Using RAST

The *de novo* genome assemblies were annotated using RAST (http://rast.nmpdr.org) (Aziz et al, 2012). Genes encoding rRNA, tRNA and proteins were determined. The specific functions of each gene and the interaction systems of each genome were also identified (Aziz et al., 2008; Overbeek et al., 2014).

3.6.3. Determination of Resistance Genes

The bacterial genomes were analyzed using The Center for Genomic Epidemiology (CGE) website (www.genomicepidemiology.org) along with the SEED viewer service. Genes encoding antibiotic resistance were identified by the ResFinder 2.1 web server (Zankari et al., 2012).

3.6.4. Multi-Locus Sequence Typing (MLST) and Plasmid Detection

The CGE website includes a MLST 1.7 server which was used to determine the MLST of each sequenced isolate (Larsen et al, 2012). The CGE website also has a PlasmidFinder 1.2 web service that was used for detecting plasmids (Carattoli et al, 2014).

3.7. Phage Detection

The Phage Search Tool (PHAST) was used to detect the presence of phage sequences. RAST was used subsequently to annotate the phage genes (Zhou et al., 2011).

3.8. Phylogenetic Tree Construction

Phylogenetic tree construction was generated using Phylosift. The maximum likelihood tree was then inferred using FigTree v1.4.3. Six reference genomes DK2, LESB58, M18, NCGM2.S1, PAO1 and UCBPP-PA14 were downloaded from NCBI.

3.9. Circular Genomes

Informative figures of the bacterial circular genomes were obtained using the DNAPlotter release 1.11 (Carver et al., 2009), while a comparative figure was generated using the CGView server (Grant & Stothard, 2008).

Chapter Four

RESULTS

4.1. Sequencing Results

4.1.1. Genome Assembly Data

Whole genome sequencing was performed on 12 out of the 18 clinical isolates. The average genome size ranged between 6,579,978-7,009,409 bp, G+C% content between 65.90-66.30%, number of contigs 116-396, subsystems 566-583, coding sequences 6082-6559 and RNAs 61-69 (Table 1).

Table 1: Genomic features of *P. aeruginosa*

Isolate	Total length (bp)	G+C%	No. of contigs	No. of subsystems	No. of coding sequence	No. of RNAs
PA9	6,848,493	66.11	247	579	6380	66
PA15	7,005,560	66.00	116	583	6559	62
PA16	7,007,890	66.00	125	583	6557	61
PA28	7,007,723	66.00	118	583	6566	61
PA43	6,647,372	66.30	189	574	6178	65
PA45	7,009,409	66.00	120	582	6559	61
PA59	6,756,856	66.14	171	573	6292	64
PA120	6,624,004	66.10	122	566	6089	65
PA132	6,984,014	65.90	139	576	6485	67
PA141	6,757,082	66.08	178	573	6328	66
PA152	7,001,596	65.90	396	580	6506	69
PA155	6,579,978	66.11	204	569	6082	63

Individual MLST types and profiles of the 12 isolates were also obtained, and the results showed that the sequenced isolates belonged to different sequence types with the most common being ST-233 (Table 2).

Table 2: MLST types and profiles of the 12 sequenced *P. aeruginosa* isolates along with their site of isolation/infection

Clinical Isolate	Site of Isolation	MLST Type	MLST Profile
PA9	Catheter tip	ST-233	16,5,30,11,4,31,41
PA15	Tracheal aspirate	ST-233	16,5,30,11,4,31,41
PA16	Tracheal aspirate	ST-233	16,5,30,11,4,31,41
PA28	Tracheal aspirate	ST-233	16,5,30,11,4,31,41
PA43	Tracheal aspirate	ST-308	13,4,5,5,12,7,15
PA45	Tracheal aspirate	ST-233	16,5,30,11,4,31,41
PA59	Sputum	ST-235	38,11,3,13,1,2,4
PA120	Tracheal aspirate	ST-1182	5,1,109,54,1,1,47
PA132	Blood	ST-235	38,11,3,13,1,2,4
PA 141	Sputum	ST-296	44,8,5,3,15,6,26
PA 152	Urine	ST-654	17,5,26,3,4,4,26
PA 155	Tracheal aspirate	ST-1233	17,5,11,3,4,6,7

MLST, multilocus sequence typing; ST: sequence type. MLST genes are: *acs*, *aro*, *gua*, *mut*, *nuo*, *pps* and *trp*.

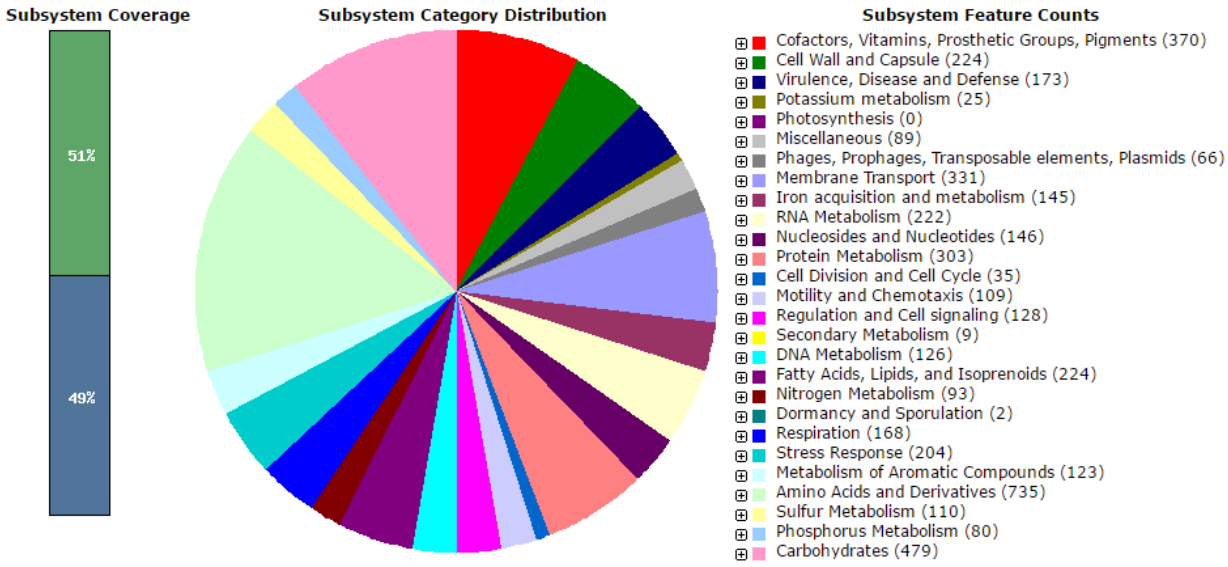


Figure 1. Subsystem category distribution of PA59 based on SEED databases

A total of 4719 subsystems were detected using RAST within the sequenced genomes, with the biggest subsystem being the genes encoding nutrient metabolism,

followed by those linked to stress response, virulence factors, regulation and cell signaling and phages (Figure 1).

4.1.2. Virulence Determinants

Virulence determinants were detected using the CGE VirulenceFinder 1.2 tool (Table 3). *lasI* and *lasR* genes, which are very important components of the quorum sensing network controlling virulence factors such as cell survival, biofilm formation and efflux pumps leading to enhanced pathogenesis (Balasubramanian et al., 2012), were detected in 11 out of the 12 sequenced isolates (PA28 and PA132 negative for *lasI* and *lasR*, respectively). *exoU* or *exoS*, T3SS effectors, were detected in nine isolates, with all being additionally XDR. Isolates PA9, PA45, PA59, PA120, PA132 and PA141 were positive for the *exoU* gene, a potent cytotoxin required for full pseudomonal virulence, PA9, PA15, PA16, PA45, PA152 and PA155 harbored the *exoS* and PA45 *exoS* and *exoU*. Finally, *gacA* and *gacS* were detected in all the sequenced genomes (Table 3).

Table 3: Virulence genes of *P. aeruginosa* strains

Strains	Category	Function	Name
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Quorum sensing	Transcriptional activators and regulators of the QS network. (Balasubramanian et al., 2012; Jimenez et al., 2012)	*rhlR*, *luxR*
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA141, PA152, PA155	Quorum sensing	Transcriptional activators and regulators of the QS network (Balasubramanian et al., 2012; Jimenez et al., 2012)	*lasR*
PA9, PA15, PA16, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Quorum sensing	Transcriptional regulators of the QS network that control the production of multiple virulence factors (Balasubramanian et al., 2012; Jimenez et al.,	*lasI*

		2012)	
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Regulation	Most important TCS protein in *P. aeruginosa* that is responsible for converting an acute infection into a chronic infection (Coggan & Wolfgang, 2012)	*gacA*, *gacS*
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Regulation	Result in resistance to cationic antimicrobial peptides and polymyxin B (Balasubramanian et al., 2012)	*phoP*, *phoQ*
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Adherence	Involved in antimicrobial drug resistance due to the production of biofilm persister cells (Mulcahy et al., 2010)	*pilH*
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Cell division	Results in intrinsic resistance to ciproflaxins (Breidenstein et al., 2011)	*ftsK*
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Transmembrane protein transporter	Results in intrinsic resistance to β-lactams (Breidenstein et al., 2011)	*ampG*
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155	Regulation	Suppresses regulation of the QS virulence genes and results in overproduction of β-lactamases and alginate (Balasubramanian et al., 2011)	ampR β-lactamase regulator
PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141,	Efflux pump regulation	Required for acquired resistance through mutational resistance causing efflux pumps	*mexR*, *nfxB*

PA152, PA155		MexAB-OprM and MexCD-OprJ derepression (Breidenstein et al., 2011)	
PA43, PA59, PA120, PA 132, PA141	Secretion system	Type III secretion system cytotoxin required for full pseudomonal virulence and increased pathology in the lungs (Hauser et al., 1999)	*exoU*
PA9, PA155	Secretion system	Type III secretion system toxin required for colonization and dissemination during infection (Lee et al., 2005)	*exoS*

4.2. Antimicrobial Susceptibility Test

Antibiotic susceptibility for the 18 studied isolates was determined by the Kirby-Bauer disk agar diffusion method against 12 different antibiotics (Table 4).

Table 4: Antimicrobial activity of *P. aeruginosa* tested against antimicrobial agents by disk diffusion method (R: Resistant; I: Intermediary Resistant; S: Sensitive)

	AK	**ATM**	**CAR**	**CI**	**TE**	**GM**
PA3	S	I	I	R	I	R
PA5	R	R	R	R	R	R
PA9	R	I	R	R	I	I
PA15	R	I	R	R	R	R
PA16	R	R	R	R	R	R
PA28	R	I	R	R	R	R

PA43	S	S	I	S	R	S
PA45	R	R	R	R	R	R
PA53	R	S	R	R	R	R
PA59	R	R	R	R	R	R
PA120	R	I	R	R	R	R
PA132	R	R	R	R	R	R
PA138	I	R	R	R	R	R
PA141	R	R	R	R	R	R
PA147	R	I	R	R	R	R
PA152	S	R	R	R	R	R
PA155	R	R	R	R	R	R
PA177	I	R	R	R	S	R

	IPM	**CAZ**	**TZP**	**TX**	**TM**	**COL**
PA3	S	S	S	I	R	S
PA5	R	R	S	R	R	S
PA9	I	S	R	R	S	S
PA15	R	R	R	R	R	S
PA16	R	R	R	R	R	S
PA28	R	R	R	R	R	S
PA43	S	S	S	R	S	S
PA45	R	R	R	R	R	S

PA53	R	R	R	R	R	S
PA59	R	R	R	R	R	R
PA120	R	R	R	R	R	S
PA132	R	R	R	R	R	S
PA138	S	R	R	R	R	S
PA141	R	R	R	R	R	S
PA147	R	R	R	R	R	S
PA152	R	R	R	R	R	S
PA155	R	R	R	R	R	S
PA177	S	R	R	R	R	S

Amikacin (AK), aztreonam (ATM), carbencilin (CAR), ceftazidime (CAZ), ceftriaxone (TX), ciproflaxin (CI), colistin (CST), gentamicin (GM), imipenem (IPM), piperacillin/tazobactam (TZP), tetracycline (TE) and tobramycin (TM)

4.2.1. Antibiotic Resistance

The disk agar diffusion method revealed that 94% of the isolates were resistant to ciproflaxin and ceftriaxone, with only 6% being resistant to colistin. Colistin was the most effective antimicrobial drug, followed by aztreonam. The results also showed that 33% of the isolates are intermediary resistant to amikacin, and 6% to each of ceftriaxone, gentamicin and imipenem (Figure 2).

Fluoroquinolone resistance (ciprofloxacin) was detected in only one isolate, PA152, mediated by the presence of the *aac(6')Ib-cr* gene (Table 5). The disc diffusion test confirmed that PA152 was resistant to fluoroquinolones (ciprofloxacin). The whole genome sequencing results (Table 5) were in agreement with the disc diffusion results (Table 4). The results of the PCR assays showed that the fluoroquinolone

resistant isolates harbored at least two of the *mexA*, *mexC* and *mexE* genes, which encode the efflux pumps MexAB-OprM, MexCD-OprJ and MexEF-OprN conferring resistance to fluoroquinolone (Table 7). This indicates genotype-phenotype matching except for isolate PA43, which was found to be sensitive to ciprofloxacin by the disk agar diffusion method while harboring all three *mex* efflux genes simultaneously (Tables 4 and 7).

The resistant isolates were additionally tested for other antimicrobial agents by the disk agar diffusion method, whole-genome sequencing and PCR assays. There was a high correlation between the detected phenotypes and genotypes (Tables 4, 5 and 7). All isolates (except PA43) were extensively drug-resistant (XDR), being resistant to almost all antimicrobial classes (Magiarakos et al., 2011). Isolates PA9, PA15, PA16, PA28 and PA45 belonged to the most prevalent sequence type (ST-233) and were XDR (Magiarakos et al., 2011), including resistance to aminoglycosides (amikacin, gentamycin and tobramycin), fluoroquinolones (ciproflocaxin), β-lactams (aztreonam) but susceptible to polymyxin E (colistin). Isolates PA59 and PA132 belonged to ST-235 and were XDR to the tested aminoglycosides (amikacin, gentamycin and tobramycin), monobactams (aztreonam), carbapenems (imipenem), penicillins (carbencillin and piperacillin/tazobactam), cephalosporins (ceftazidime and ceftriaxone), fluoroquinolones (ciprofloxacin) and tetracyclines, with it being additionally resistant to polymyxin E (colistin).

Among the detected genes were bla_{PAO} and $bla_{\text{OXA-50}}$ genes, which confers additional β-lactam/carbapenem resistance. PA152 also harbored $bla_{\text{IMP-15}}$ and $bla_{\text{GES-1}}$ genes, involved in hydrolyzing carbapenems. On the other hand, isolates PA9, PA15, PA16, PA28 and PA45 carried $bla_{\text{OXA-4}}$ and $bla_{\text{VIM-2}}$, which are linked to carbapenem resistance. (Table 5).

PCR assays targeting *mexA* and *oprD* showed that PA15, PA16 and PA28 carried the *mexA* gene (imipenem resistance), while PA141, PA147 and PA155 carried the *oprD* gene (imipenem resistance). PA45, PA120 and PA132 harbored both *mexA* and *oprD* (Table 7). However, the remaining imipenem resistant isolates PA5, PA9, PA53 and PA152 were negative to all the former genes, with resistance to carbapenems, specifically imipenem, being confirmed through the disk agar diffusion assay.

The *aph(3')-IIb gene*, an aminoglycoside-modifying enzyme, was detected in all the 12 isolates conferring resistance to aminoglycosides (Table 5). The disc diffusion assay (Table 4) showed that all the isolates were resistant to at least one of the following aminoglycosides: amikacin, gentamicin and tobramycin. Six isolates (PA9, PA15, PA16, PA28, PA45 and PA152) carried tetracycline resistant determinants *tet(A)* and *tet(G)*, and phenotypically showed tetracycline resistance (Tables 4 and 5). PCR assays revealed that PA43, PA120 and PA132 harbored *mexA*, which could be involved in mediating tetracycline resistance (Table 7).

Cephalosporin (ceftazidime), extended-spectrum cephalosporins (ceftriaxone), penicillin (piperacillin/tazobactam) and polymyxin (colistin) resistance were also determined (Table 4).

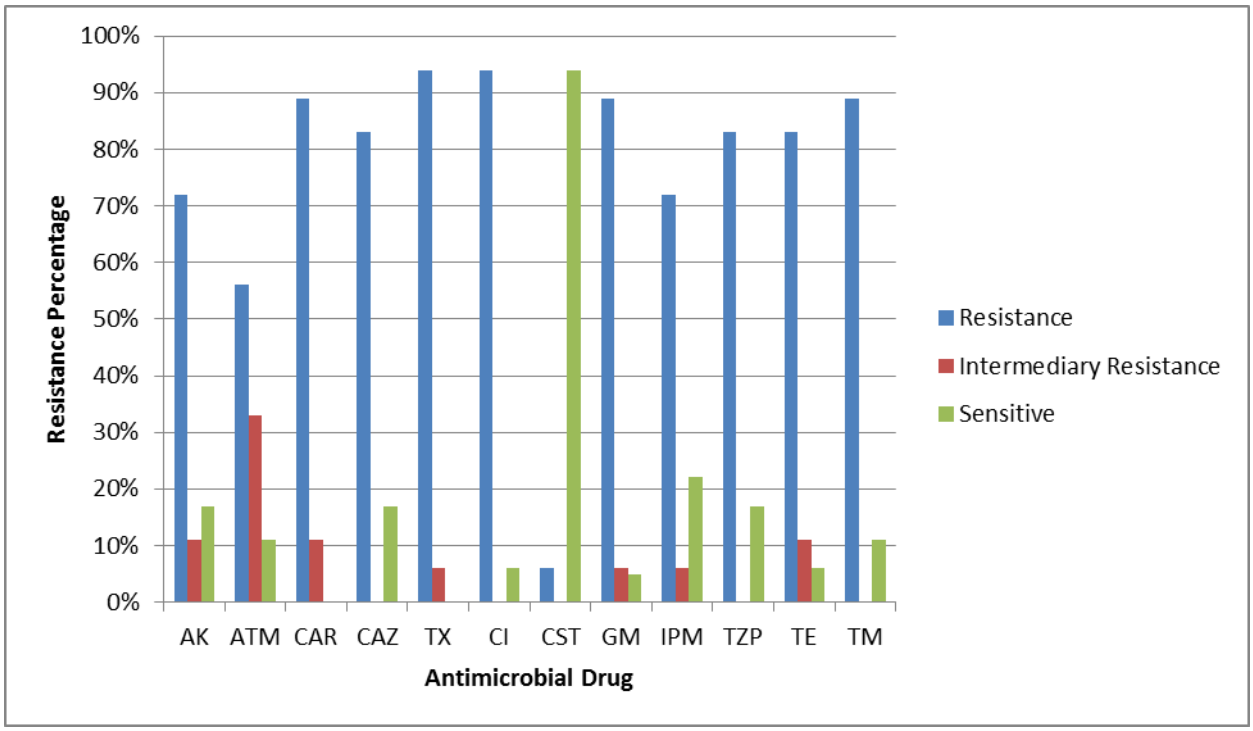


Figure 2. Histogram showing the percentage of resistant, intermediary resistant and sensitive isolates to each tested antimicrobial agents. Amikacin (AK), aztreonam (ATM), carbencilin (CAR), ceftazidime (CAZ), ceftriaxone (TX), ciproflaxin (CI), colistin (CST), gentamicin (GM), imipenem (IPM), piperacillin/tazobactam (TZP), tetracycline (TE) and tobramycin (TM)

Table 5: Antimicrobial resistance determinants of the 12 sequenced *P. aeruginosa* isolates

Antibiotic Resistance Determinant	Isolates
Aminoglycoside	
aph(3')-Via	PA9
aph(3')-IIb	PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155
aadA2	PA9, PA15, PA16, PA28, PA45
aadA6	PA59, PA132
aadA13	PA152
aac(6')Ib-cr	PA152
aadB	PA152

strA	PA15, PA16, PA28, PA43, PA152
strB	PA15, PA16, PA28, PA43, PA152
aac(3)-Id	PA15, PA16, PA28, PA45
β-lactam	
Bla_{PAO}	PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155
Bla_{OXA-50}	PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155
Bla_{OXA-4}	PA9, PA15, PA16, PA28, PA45
Bla_{IMP-15}	PA152
Bla_{GES-1}	PA152
Bla_{VIM-2}	PA15, PA16, PA28, PA45
Fosfomycin	
fosA	PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155
Phenicol	
catB7	PA9, PA15, PA16, PA28, PA43, PA45, PA59, PA120, PA132, PA141, PA152, PA155
cmlA1	PA9, PA15, PA16, PA28, PA45
Sulphonamide	
sul1	PA9, PA15, PA16, PA28, PA 45, PA59, PA132, PA152
Tetracycline	
tet(A)	PA9, PA152
tet(G)	PA9, PA15, PA16, PA28, PA45
Fluroquinolone	
aac(6')Ib-cr	PA152
Trimethoprim	
dfrB5	PA15, PA16, PA28, PA45

4.2.2. Extensive Drug Resistance (XDR) Patterns

The *P. aeruginosa* clinical isolates were analyzed for resistance patterns. A total of 11 patterns were detected among the 18 clinical isolates (Table 6). The most common XDR pattern was pattern A (aimkacin, aztreonam, carbencillin, ceftazidime, ceftriaxone, cirprofloxacin, gentamicin, imipenem, piperacillin/tazobactam, tetracycline and tobramycin), which was detected in five of the isolates: PA16, PA45, PA132, PA141 and PA155. It was followed by pattern B (amikacin, aztreonam (I), carbencilin, ceftazidime, ceftriaxone, cirproflaxin, gentamicin, imipenem, piperacillin/tazobactam, tetracycline and tobramycin) detected in: PA15, PA28, PA120 and PA147. All isolates, except PA43, showed resistance against 9-12 of the tested antibiotics.

Table 6. Extensive drug resistance patterns of the *P. aeruginosa* clinical isolates against antimicrobial agents

Pattern	XDR Isolate	No. of Antimicrobial Drugs	Intermediately Resistant to Antimicrobial Drugs	Resistant to Antimicrobial Drugs
Pattern A	PA16, PA45, PA132, PA141, PA155	11 (R)	-	AK, ATM, CAR, CI, TE, GM, IPM, CAZ, TZP, TX, TM
Pattern B	PA15, PA28, PA120, PA147	10 (R) 1 (I)	ATM	AK, CAR, CI, TE, GM, IPM, CAZ, TZP, TX, TM
Pattern C	PA59	12 (R)	-	AK, ATM, CAR, CI, TE, GM, IPM, CAZ, TZP, TX, TM, COL
Pattern D	PA3	3 (R) 4 (I)	ATM, CAR, TE,TX	CI, GM, TM

Pattern E	PA5	10 (R)	-	AK, ATM, CAR, CI, TE, GM, IPM, CAZ, TX, TM
Pattern F	PA9	5 (R) 4 (I)	ATM, TE, GM, IPM	AK, CAR, CI, TZP, TX
Pattern G	PA43	2 (R) 1 (I)	CAR	TE, TX
Pattern H	PA53	10 (R)	-	AK, CAR, CI, TE, GM, IPM, CAZ, TZP,TX, TM
Pattern I	PA138	9 (R) 1 (I)	AK	ATM, CAR, CI, TE, GM, IPM, CAZ, TZP, TX, TM
Pattern J	PA152	10 (R)	-	ATM, CAR, CI, TE, GM, IPM, CAZ, T
Pattern K	PA177	8 (R) 1 (I)	AK	ATM, CAR, CI, GM, CAZ, TZP, TX, TM

Amikacin (AK), aztreonam (ATM), carbencilin (CAR), ceftazidime (CAZ), ceftriaxone (TX), ciproflaxin (CI), colistin (CST), gentamicin (GM), imipenem (IPM), piperacillin/tazobactam (TZP), tetracycline (TE) and tobramycin (TM).

4.3. Prevalence of Porins, Efflux Pumps and Exotoxin Encoding Genes

PCR assays were performed to detect the presence of three porin encoding genes: *oprI*, *oprL* and *oprD* and 3 efflux pumps: *mexA*, *mexC* and *mexE* (amplifying the expected 249 bp fragment of the *oprI* gene, 504 bp fragment of the *oprL* gene, 1412

bp of the *oprD* gene, 316 bp fragment of *mexA* gene, 164 bp fragment of the *mexC* gene and 114 bp fragment of *mexE* gene). The PCR results are shown below (Table 7).

PA9, PA15, PA16, PA28 and PA45 belonging to ST-233, were positive for *oprI* and *oprL* genes, with PA45 being additionally positive for *oprD*. These isolates also harbored the *mexA*, *mexC* and *mexE* genes expect for PA9, which was negative for *mexA*. PA9, PA15 and PA16 were positive for *exoS*, PA45 carried both *exoS* and *exoU*, and PA28 was negative for both.

PA59 and PA132 typed as ST-235 carried the *oprI* and *oprL* genes, with PA132 being also positive for *oprD*. PA59 and PA132, which were positive for *exoU*, harbored the *mexC* and *mexE* genes, and PA132 also carried the *mexA* gene.

Table 7: PCR products detecting the presence (+) or absence (-) of *oprI*, *oprL*, *oprD*, *mexA*, *mexC*, *mexE*, *exoS* and *exoU* genes among the 18 *P. aeruginosa* clinical isolates

Clinical Isolates	***Porin Encoding Genes***			***Efflux Pump Encoding Genes***			***Exotoxin Encoding Genes***	
	oprI	***oprL***	***oprD***	***mexA***	***mexC***	***mexE***	***exoS***	***exoU***
PA3	+	+	+	_	+	+	+	_
PA5	+	+	_	_	+	+	+	_
PA9	+	+	_	_	+	+	+	_
PA15	+	+	_	+	+	+	+	_
PA16	+	+	_	+	+	+	+	_
PA28	+	+	_	+	+	+	_	_
PA43	+	+	_	+	+	+	+	+
PA45	+	+	+	+	+	+	_	_
PA53	+	+	_	_	+	+	+	_
PA59	+	+	_	_	+	+	_	+
PA120	+	+	+	+	+	+	_	+
PA132	+	+	+	+	+	+	_	+
PA138	+	+	+	_	+	+	+	_
PA141	+	+	+	_	+	+	_	+

PA147	+	+	+	_	+	+	_	+
PA152	+	+	_	_	+	+	+	_
PA155	+	+	+	_	+	+	+	_
PA177	+	+	+	+	+	+	_	+

4.4. Phages and Mobile Elements

Several phages were identified with the majority of them being classified under the phiCTX family of pseudomonal phages (Table 8). Other non-pseudomonal phages were also detected such as *Escherichia* and *Stenotrophomonas* phages. All phages had an average of 42 coding sequences, GC content of 63.52 %, and were 35.8 Kbp in length.

Table 8: Phages detected in the sequenced genomes

Isolate	Phage Name	Position	No. of CDS	Size (Kbp)	GC %
PA9	phiCTX	743077-779746	47	36.6	64.04%
	YMC11/02/R656	1150747-1182116	34	31.3	64.19%
PA15	YMC11/02/R656	632693-661109	35	28.4	65.12%
	phiCTX	5126788-5150819	28	24	60.47%
	phiCTX	5387820-5399371	16	11.5	65.55%
	F10	6490102-6565302	102	75.2	60.55%
PA16	YMC11/02/R656	1496853-1526834	34	29.9	65.80%
	phiCTX	4920620-4936555	21	15.9	65.78%
	D3	6397289-6429632	44	32.3	58.30%
	phiCTX	6590318-6606199	20	15.8	62.35%
	phiCTX	6808632-6850889	46	42.2	65.48%
PA28	YMC11/02/R656	1364979-1392112	32	27.1	65.40%
	phiCTX	5007127-5023794	22	16.6	65.83%
	F10	6389980-6464497	95	74.5	60.60%

	phiCTX	6819795-6862052	46	42.2	65.48%
PA43	YMC11/02/R656	2118560-2148833	35	30.2	64.48%
	phiCTX	4341803-4394545	52	52.7	64.43%
PA45	YMC11/02/R656	989751-1016691	33	26.9	65.44%
	phiCTX	2381297-2392694	17	11.3	65.71%
	phiCTX	5556401-5570151	17	13.7	65.69%
	PMG1	6513133-6547988	46	34.8	58.53%
	phiCTX	6798804-6839112	41	40.3	64.71%
PA59	phiCTX	577360-597499	25	20.1	65.25%
	D3	3192482-3251790	63	59.3	59.88%
	phiCTX	4139903-4165279	29	25.3	64.70%
	F10	4885051-4928085	52	43	61.15%
PA120	YMC11/02/R656	4119126-4149390	34	30.2	64.29%
	phi297	5648972-5709609	76	60.6	58.55%
PA132	phiCTX	1815802-1835941	25	20.1	65.25%
	D3	2451647-2505188	64	53.5	58.95%
	Bacter_Lily	6646296-6658579	17	12.2	64.43%
PA141	F10	840623-884601	59	43.9	60.83%
PA152	phiCTX	119784-163368	51	43.5	63.64%
	Escher_vB_EcoM_ECO1230_10	923732-945394	28	21.6	64.06%
	phiCTX	1388499-1408769	27	20.2	65.22%
	PMG1	3782350-3820977	48	38.6	59.71%
	D3	5283785-5418271	109	134.4	67.38%
	YMC11/02/R656	5610674-5632005	22	21.3	65.47%
PA155	Stenot_S1	2935327-2971800	20	36.4	64.53%

4.5. Phylogenetic Analysis

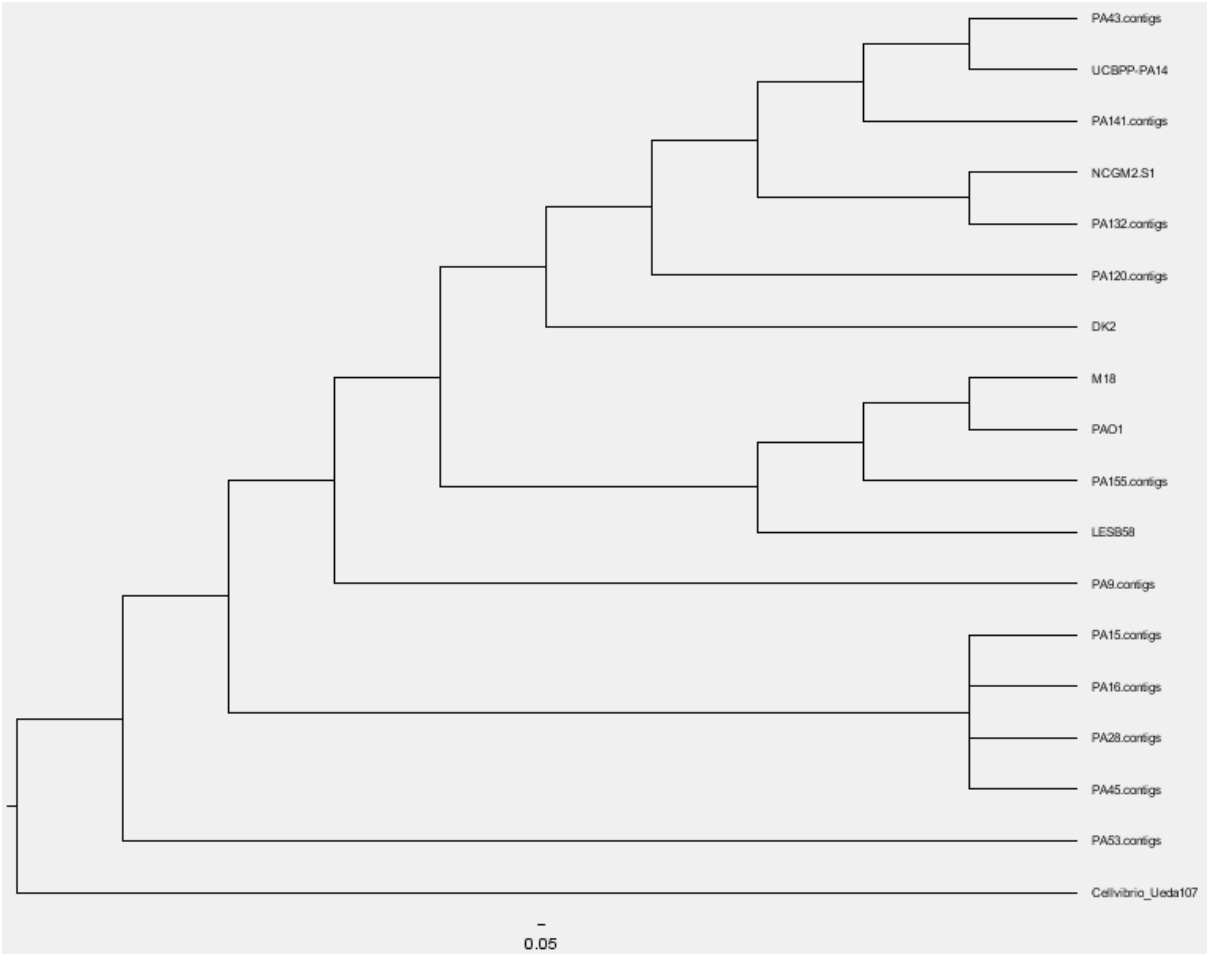


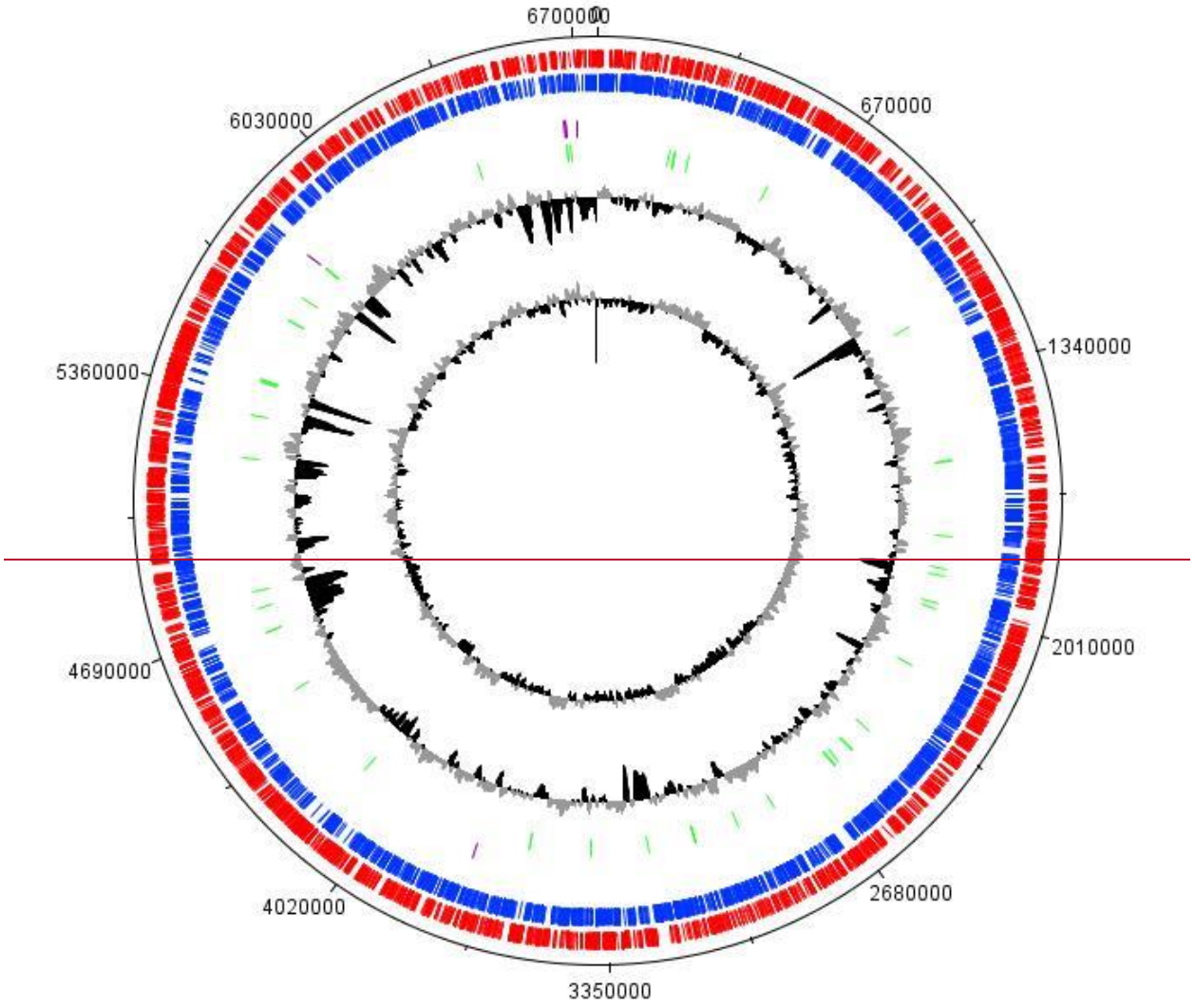
Figure 3. Maximum likelihood tree generated using PhyloSift. NCBI was used to download the genomes. The maximum likelihood tree was then inferred using FigTree including the 12 isolates along with six reference strains: DK2, LESB58, M18, NCGM2.S1, PAO1 and UCBPP-PA14. *Cellvibrio japonicus* Ueda107was excluded as an outlier.

A concatenated maximum likelihood tree was generated in order to determine epidemiological links between the sequenced isolates. DK2, LESB58, M18, NCGM2.S1, PAO1 and UCBPP-PA14 were used as reference genomes. Phylogenetic analysis showed the grouping of PA15, PA16, PA28 and PA45 into the same clade and in close association with PA9. PA43 was clustered with UCBPP-PA14 and in close association with PA141, while PA132 was clustered with NCGM2.S1 and in close association with PA120. PA155 was in close association with PAO1 and LESB58.

4.6. Comparative Circular Genomes

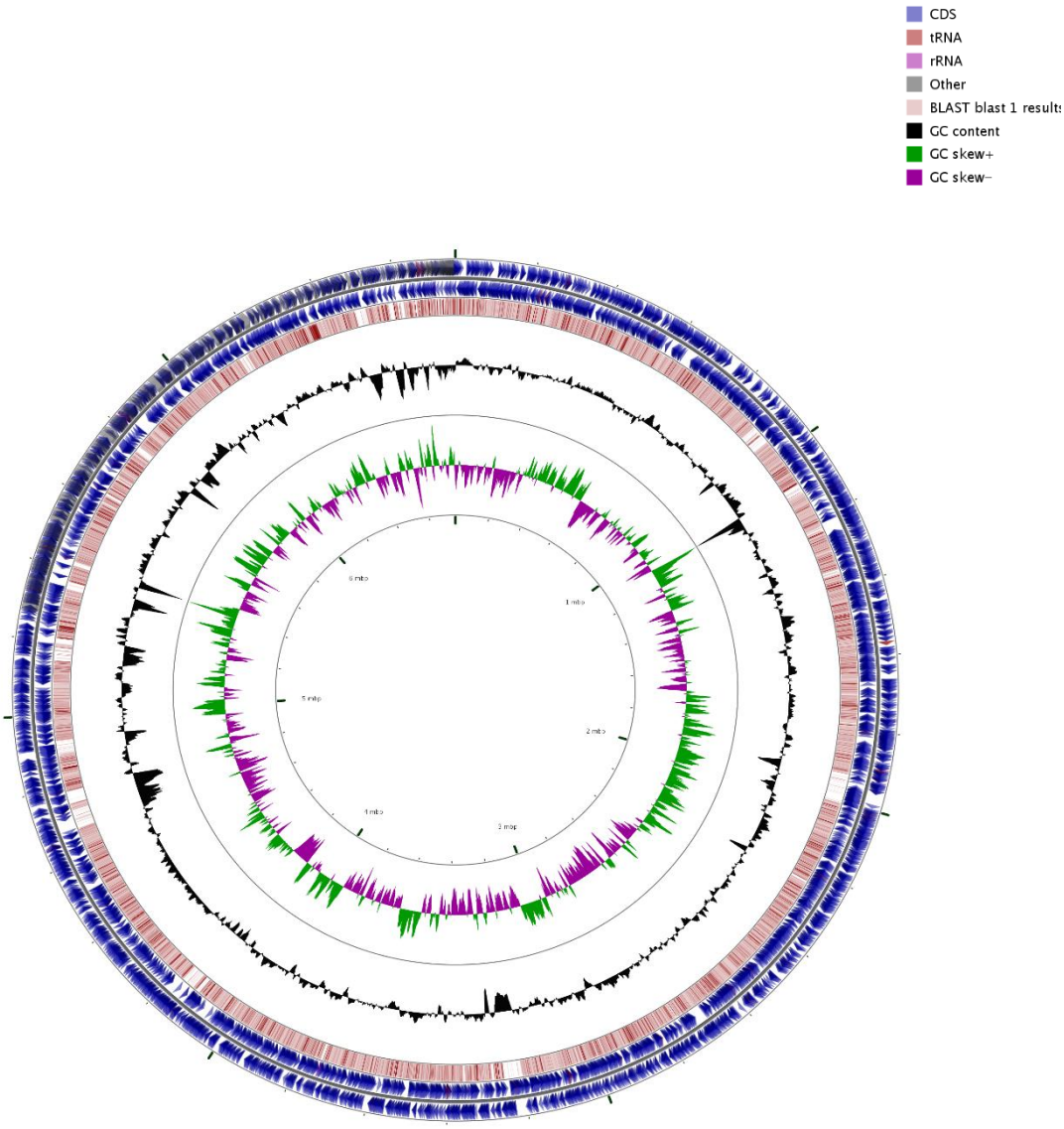
The genome of PA59 was 6,756,856 bp in length, which assembled as a single circular chromosome (ANNEX I: circular genomes of all sequenced isolates). The sequenced genomes were compared with the reference strain PAO1.

The results for PA59 additionally revealed 1887 ORFs including: systems for the synthesis of amino acids, carbohydrates and cofactors and those required for protein metabolism allowing it to grow on minimal media and survive in diverse environments. These genes made up 30% of the total ORFs found in *P. aeruginosa*. 331 ORFs (5.3%) were shown to be involved in membrane transport, both in terms of nutrient uptake through the di- and tri-carboxylates transporters and protein secretion through type I-VIII secretion systems. 459 ORFSs (7.3%) were found to be involved in regulation and cell signaling, which was significantly higher than what was reported in other sequenced bacterial genomes. PA59 also contained 173 ORFs (2.7%) encoding members of the RND family of efflux pumps and the MexA-MexB, MexC-MexD and MexE-MexF multidrug efflux pumps resulting in increased intrinsic resistance to antibiotics.



Pseudomonas aeruginosa PA59

Figure 4. Circular map of *P. aerugiosa* PA59 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (magenta), tRNAs (brown), GC plot and GC skew with positive value (green) and negative value (purple).



Pseudomonas aeruginosa PA59

(6,756,856 bp)

Figure 5. Comparative circular map of *P. aeruginosa* PA59 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color

indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.

Chapter Five

DISCUSSION

P. aeruginosa is a ubiquitous Gram-negative opportunistic pathogen capable of causing a wide range of diseases. In addition to its high rate of prevalence in the cystic fibrosis (CF) lung, *P. aeruginosa* is a frequent cause of acute infections such as burns, lung, urinary tract and wound infections (Mulcahy et al., 2014, Wong et al., 2012). It is involved in the healthcare setting where it poses serious threats to immunocompromised patients (Marvig et al., 2013). The increasing frequency of extensively drug resistant *P. aeruginosa* strains has made efficacious antibiotic treatment very limited and challenging (Morello et al., 2011). These extensively drug resistant strains are resistant to almost all approved antimicrobial agents mainly due to overexpression of multiple efflux pumps, production of β-lactamases and loss of outer membrane permeability compounded by their ability to form biofilms and release toxins (Magiorakos et al., 2011; Castanheira et al., 2014). The increasing prevalence of drug resistant and highly pathogenic strains of *P. aeruginosa* is of great concern in Lebanon and various neighbouring countries in the Middle East. Accordingly, the genomic features of 12 XDR *P. aeruginosa* recovered from clinical specimens in Lebanon were characterized in this study.

5.1. Multi Locus Sequence Typing (MLST)

MLST is a nucleotide sequence-based method used in epidemiological investigations targeting antibiotic drug resistant bacteria to understand and elucidate the genetic diversity among bacteria (Nunney et al., 2012). This scheme discriminates *P. aeruginosa* isolates by differences in the sequences of seven housekeeping genes: *acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE*. Due to recombination events in the non-clonal structure of this species, evolution of *P. aeruginosa* has occurred leading to a diversity of serotypes. The analysis of the seven loci in this study, demonstrated that the prevalent STs were: ST-233, ST-235, ST-296, ST-654, ST-1182 and ST-1233, with the most common ST being ST-233

(serotype O6; complex D; *bla*$_{VIM-2}$). PA15, PA16, PA28 and PA45 belonged to ST-233, harboured the *bla*$_{VIM-2}$ gene (Table 5) and had the same antibiotic resistance profile with the exception of aztreonam (PA16 and PA45 were resistant to ATM, while PA15 and PA28 were intermediary resistant). PA9 belonged to ST-233 and carried another carbapenemase encoding gene, *bla*$_{OXA-50}$. This strain belonged to the same ST but presented differences in its antibiotic resistance profile. ST-233 was first detected in an outbreak in Japan (2006-2009) (Tsutsui et al., 2011). ST-233 VIM-2 producing *P. aeruginosa* was detected in one isolate imported from Ghana and was imported to Scandinavia (Samuelsen et al., 2009; Zafer et al., 2015). These VIM-2 metallo-β-lactamase producing isolates (MBL) were also reported in several European countries including Croatia, Greece, Italy, the Netherlands and Turkey and in northern Africa including: Algeria and Tunisia and South Africa (Zafer et al., 2015). The *bla*$_{VIM-2}$ gene was more frequent in our study than other MBL encoding genes. This was in harmony with the findings of Walsh et al. where the *bla*$_{VIM-2}$ gene was the most dominant MBL linked to imipenem resistance (Walsh et al., 2008).

PA59 and PA132 were of sequence type ST-235 (serotype O11; complex B), in which aminoglycoside, β-lactam and quinolone resistance was detected with P59 being additionally colistin resistant (Table 4). ST-235 (predicted founder BG 11) was previously detected in different countries (Austria, Belgium, France, Greece, Hungary, Italy, Poland, Russia, Serbia, Singapore, Sweden and Turkey), carrying different β-lactamases such as: VIM- (Verona-integron-encoded MBL), BEL- (Belgium ESBL), IMP- (imipenem), OXA- (active on oxacillin), PAO- (*P. aeruginosa* strain PAO1), PER- (*Pseudomonas* extended resistance), PSE- (Pseudomonas-specific enzyme) and SPM- (São Paulo MBL) (Empel et al., 2007; Shevchenko & Edelstein, 2007; Edalucci et al., 2008; Lepsanovic et al., 2008; Libisch et al., 2008; Duljasz et al., 2009; Viedma et al., 2009; Glupczynski et al., 2010). Additionally, both isolates harbored *bla*$_{OXA-50}$ and *bla*$_{PAO}$ genes, coding for carbapenemases.

ST-654 (serotype O11; complex C) is another MDR isolate that was detected in this study (PA 152), and characterized by harboring *bla*$_{PAO}$, *bla*$_{OXA-50}$, *bla*$_{GES-1}$ and *bla* $_{IMP-15}$ genes. ST-654 is an international high-risk clone that was imported from Tunisia (Wright et al., 2015; Woodford et al., 2011), and detected in Poland, Sweden (carrying *bla*$_{VIM-2}$ gene) and the United Kingdom (carrying *bla*$_{VIM-2}$ and *bla*$_{IMP-15}$)

(Mataseje et al., 2016). Pasteran et al. revealed that strains belonging to ST-654 are of major importance due to their role in the worldwide dissemination of carbapenemases and metallo-β-lactamases (Pasteran et al., 2009).

5.2. Virulence

The increased pathogenesis of *P. aeruginosa* and the high mortality and morbidity rates associated with pseudomonal infections are linked to the presence of a combination of virulence determinants (Le Berre et al., 2009). The three most potent being quorum sensing (QS) components, type III secretion system toxins and two-component regulatory systems (Hauser, 2011).

The T3SS is the most significant in *P. aeruginosa* linked to reduced patient health outcome (Berube et al., 2016). Two T3SS effector proteins implicated in virulence, ExoS and ExoU, were investigated in this study. Our results revealed that 56% of the isolates harbored *exoS* gene and 39% the *exoU*, with one isolate having both (PA43) and two having none (PA28 and PA45). On the other hand, 50% of the isolates carrying *exoS* were recovered from tracheal aspirate. The fact that all the strains that were XDR were either positive for *exoU* or *exoS* is very alarming. PA43, however, was positive for both exotoxin genes and was resistant to only three out of 12 tested antimicrobial agents. However, and contrary to what has been reported previously (Adabi et al., 2015; Koutsogiannou et al., 2012; Mitov et al., 2010; El-Solh et al., 2012; Sawa et al., 2014), PA43 is a unique strain since it carries both exotoxin genes and is not XDR. ExoS is capable of disrupting the host, actin cytoskeleton and vesicular trafficking, causing host cell death and inhibiting endocytosis (Barbieri et al., 2001; Fraylick et al., 2001; Pederson et al., 1998; Rocha et al., 2003). Shaver et al. (2004) suggested that clinical strains harboring ExoS show reduced survival and increased bacterial spread from the lungs of patients with acute pneumonia. Strains carrying ExoU have increased virulence activity and lead to prompt necrotic cell death due to its phospholipase ability to damage membranes (Gendrin et al., 2011). The results of this study also revealed that 50% of the strains were characterized as being $exoS^{+}/exoU^{-}$ and 33% being $exoS^{-}/exoU^{+}$. Fleiszig et al. showed that $exoS^{+}$ strains are more invasive and can decrease DNA synthesis, while $exoU^{+}$ strains are more cytotoxic and are capable of evading the host immune response. In harmony with our results, strains carrying these virulent determinants are of great concern

because of their increased potential of causing disease and are consequently treated with antimicrobial agents resulting in the emergence of resistance.

Quorum sensing network systems, *las* and *rhl*, are very vital in regulating virulence determinants in *P. aeruginosa* (Moker et al., 2009). Eleven out of the 12 sequenced isolates carry the *lasI* and *lasR* genes (isolate PA28 lacks *lasI* and isolate PA132 lacks *lasR)* required for virulence. The *las* system is made up of LasR and LasI (the transcriptional activator and auto-inducer synthesis regulator, respectively), which induce the expression of a number of target genes and is essential for biofilm development. The *rhl* system is made up of RhlR and RhlI (transcriptional activator protein and auto-inducer synthesis regulator, respectively), regulating *rhlI* and *rhlAB* expression encoding a rhamnosyltransferase needed for rhamnolipids production (Jimenez et al., 2012). Rhamnolipids are required for the initial stage of infection providing the bacteria with an immune shield, protecting the bacterium from the antimicrobial activity of host polymorphonuclear neutrophilic leukocytes (PMNs) (Bjarnsholt et al., 2010). The AmpR β-lactamase regulator is another virulence factor that has been demonstrated to activate the expression of QS genes (Balasubramanian et al., 2012). All sequenced strains carried the AmpR- β-lactamase regulator (Table 3) and thus, are involved in increased *P. aeruginosa* virulence and probable production of QS linked virulence factors, where AmpR-deficient strains showed attenuated virulence (Balasubramanian et al., 2012; Kong et al., 2005).

In addition to the *las* QS system, a two-component signal transduction system, GacSA, controls biofilm formation and synthesis of several virulence determinants (Jimenez et al., 2012). Most importantly, the GacSA system is responsible for converting an acute infection into a chronic infection. Upon activation of the GacSA system, multiple genes involved in biofilm formation are activated, and many genes involved in acute stages of virulence are repressed (Coggan & Wolfgang, 2012). *gacA* and *gacS* genes were detected in all the sequenced genomes. GacSA system is also implicated in antibiotic resistance against ciprofloxacin, tetracyclines and tobramycin by controlling rgRsmZ and rgRsmY, two small regulatory RNAs (Linares et al., 2009). Our results showed that 10 out of the 12 sequenced isolates were resistant to the three antimicrobial classes at the same time and carried both *gacA* and *gacS* genes. Thus, both the QS and GacSA TCS systems act synergistically to enhance the virulence of *P. aeruginosa* during chronic infections in CF lungs and

biofilm formation. The GacSA TCS system also controls T3SS activity. Upon phosphorelation of GacA, rgRsmZ and rgRsmY block the negative regulator RNA-binding protein RsmA, which positively regulates genes of the T3SS. This interaction is highly involved in lung injury, to fully augment pseudomonal virulence and adaptation (Hauser, 2009).

Whole-genome sequencing also revealed the presence of *phoP* and *phoQ* genes in all the 12 sequenced isolates. The *phoP-phoQ* two-component regulatory system is required for resistance to cationic antimicrobial peptides and polymyxin B (Balasubramanian et al., 2012) by responding to Mg^{2+} depleted conditions leading to *arnB* operon overexpression (Lee et al., 2014). The *phoP-phoQ* two-component regulatory system is involved in adaptation to epithelial surfaces, which is very important for the pathogenesis of Gram-negative pathogens (Gellatly et al., 2012). Although all the sequenced isolates carried *phoP* and *phoQ* genes, PA59 was the only strain found to be resistant to colistin. Mutations that result in changes in this phoP-phoQ two-component regulatory system lead to increased polymyxin E (colistin) resistance in *P. aeruginosa* clinical strains (Lee et al., 2014). Many studies have previously reported that colistin-resistant *P. aeruginosa* strains divert from causing an acute to one causing a chronic infection as a result of mutations in the *phoQ* gene (Barrow et al., 2009; Miller et al., 2011; Schurek et al., 2009; Sriramulu et al., 2005). According to Gellatlly et al., these mutations along with the added pressures of epithelial adherence and polymxin resistance have resulted in the increased emergence of MDR and XDR strains.

5.3. Antibiotic resistance

A worrying problem is the dissemination of resistant *P. aeruginosa* strains in Lebanon (Al Bayssari et al., 2014; Al Bayssari et al., 2015). The results of our study also showed the presence of XDR *P. aeruginosa* in Lebanese patients upon testing against 12 antimicrobial agents. Resistance is most common in the isolates recovered from tracheal aspirates All the clinical isolates (except PA43) showed resistance to at least nine to 12 of the tested antibiotics, with only one, PA59, being resistant to colistin. MDR Gram-negative bacteria have been rapidly spreading in many countries worldwide, including Lebanon (Hamouche et al., 2012).

One of the most effective treatments against *P. aeruginosa* infections is the carbapenems, followed by fluoroquinolones and aminoglycosides (Morita et al., 2015). Eight of the 12 sequenced *P. aeruginosa* isolates were negative for the *oprD* gene as confirmed by the PCR assay (Table 7), which caused the loss of the OprD protein and hence explains their resistance to imipenem (Annex I). All 12 isolates harbored the *ampG* gene linked to intrinsic resistance to β-lactams (Breidenstein et al., 2011), out of which four (PA15, PA16, PA28 and PA43) carried the *mexA* gene involved in carbapenem resistance. Each of the sequenced isolates also carried at least one of the following β-lactamases: bla_{PAO}, $bla_{\text{OXA-50}}$, $bla_{\text{OXA-4}}$, $bla_{\text{IMP-15}}$, $bla_{\text{GES-1}}$ and/or $bla_{\text{VIM-2}}$. *P. aeruginosa* has acquired several resistance mechanisms against a wide array of antibiotics. *P. aeruginosa* become resistant to carbapenems through: outer membrane porin protein OprD loss or alteration, metallo-β-lactamases (IMP and VIM) production and MexA-MexB multidrug efflux pump overexpression (Al Bayssari et al., 2014). Carbapenems enter into the periplasmic space of *P. aeruginosa* through the OprD outer membrane porin (Horii et al., 2003; Carmeli et al., 1999). Diminished expression or loss of the OprD porin is rather frequent during imipenem treatment conferring imipenem resistance, which was similar to what was detected in this study. The tripartite system on the other hand, MexA-MexB efflux pump, acts synergistically with low outer membrane permeability through loss of oprD porin conferring intrinsic multi-drug resistance to *P. aeruginosa* (PA15, PA16, PA28 and PA43) (Poole et al., 2001).

All types of carbapenemases, except SIM-114 (Seoul imipenemase), have been detected in *P. aeruginosa* isolates around the world. Among them, the MBLs are considered as the most clinically important for *P. aeruginosa* (Walsh et al., 2005). MDR and XDR *P. aeruginosa* are the outcome of a combination of multiple mechanisms in a single isolate or the action of a single potent resistance mechanism (Tavajjohi et al., 2011).

The ability of MBL-producing *P. aeruginosa* to reach high level endemicity can outpace the loss of oprD and up-regulated efflux pumps as leading factors causing reduced carbapenem susceptibilitble1y unfolding their potential for causing outbreaks (Diab et al., 2013). Our results showed that VIM-2 is the most dominant MBL implicated in imipenem resistant *P. aeruginosa* as supported by results of other studies and confers the greatest clinical threat (Walsh et al., 2008, Elias et al.,

2010). Worldwide, VIM-2 is the dominant MBL gene associated with nosocomial outbreaks due to MBL-producing *P. aeruginosa*. Since MBL-producing isolates can cause serious infections that are difficult to treat, their presence in various hospitals in Lebanon is of nationwide concern (Al Bayssari et al., 2014). Thus, the combined effect of the multiple resistance mechanisms may be the cause of increased carbapenem resistance in Lebanon (Al Bayssari et al., 2014, Halat et al., 2017).

Senda et al. (1996) reported an increasing frequency of MBLs and concluded that bacteria producing these enzymes have been responsible for persistent nosocomial outbreaks that were accompanied by severe infections (Senda et al., 1996).

The two main mechanisms of fluoroquinolone resistance in *P. aeruginosa* include: drug target modifications (*gyrA, gyrB, parC* and *parE*) and/or multidrug efflux pumps (MexA-MexB, MexC-MexD and/or MexE-MexF) overexpression (Llanes et al., 2011). Carbapenem-resistant *P. aeruginosa* infections are best treated with fluoroquinolone. This however, led to an increase in the emergence of fluoroquinolone resistant strains especially in South Korea resulting in treatment failure (Lee et al., 2003). Two isolates, PA59 and PA152 (ST-235; serotype O11; complex B), were fluoroquinolone resistant. Our results also showed that all 12 sequenced ones harbored the *ftsK* gene associated with intrinsic resistance to ciprofloxacins (Breidenstein et al., 2011), and *mexR* and *nfxB* genes linked to acquired ciprofloxacin resistance through mutational resistance causing the derepression of efflux pumps: MexAB-OprM and MexCD-OprJ (Breidenstein et al., 2011). In addition, all the 18 isolates carried *mexC* and *mexE* (Table 7) and were phenotypically resistant to ciprofloxacin (Annex I). Interestingly, PA43 was the only strain that was susceptible to ciprofloxacin, while harboring all three *mex* efflux genes simultaneously (Table 7). This could be due to mutations producing non-functional proteins and the lack of promoters upstream of the site of insertion (Poole et al., 2000). Increased resistance to fluoroquinolones is characterized by the simultaneous presence of *mexA, mexC* and *mexE* genes (seen in eight out of the 18 tested isolates) (Adabi et al., 2015). On another note, an interesting characteristic of strains expressing efflux-mediated quinolone resistance is their cross-resistance to several unrelated antimicrobial agents such as: aminoglycosides, carbapenems, penicillins, as a results of the broad substrate specificity of the FQ efflux systems

accommodating a variety of clinically relevant antimicrobial agents in addition to FQs (Poole, 2000).

The most widely recognized aminoglycoside resistance mechanism in *P. aeruginosa* is enzymatic alterations of the aminoglycosides via aminoglycoside-acetyltransferases (AAC), aminoglycoside-adenyltransferases (AAD), and aminoglycoside-phosphotransferases (APH). These modifying enzymes spread by mobile genetic elements (Teixeira et al., 2016). Two isolates, PA59 and PA132, which were typed as ST-235 (serotype O11; complex B), were aminoglycoside resistant. This sequence type is associated with aminoglycoside, β-lactam and quinolone resistance (Zafer et al., 2015). The most common aminoglycoside-modifying enzyme detected in this study was the *aph(3')-IIb gene*, while *aac(6')Ib-cr* gene was only positive in PA152 (Table 5). Such differences however, could be attributed to country-based differences in aminoglycoside usage, with *aac(6')Ib-cr* gene being either the most prevalent or absent (Kim et al., 2008; Gad et al., 2011; Vaziri et al. 2011). The fact that the isolates tested in this study were positive for multiple aminoglycoside resistance genes was inconsistent with previous studies reporting that the majority of resistant isolates harbor only a single aminoglycoside-modifying gene (Miller et al., 1997). The synthesis of more than one enzyme simultaneously can lead to pan-aminoglycoside resistance (Gad et al., 2011), as seen among the isolates in this study with 17 out of the 18 being phenotypically resistant to at least one of the following aminoglycosides: amikacin (AK), gentamicin (GM) and tobramycin (TM).

5.4. Phages

Eight phages were identified in 12 of the sequenced isolates: phiCTX, YMC11/02/R656, F10, D3, PMG1, phi297, Bacter_Lily, Escher_vB_EcoM_EC01230_10 and Stenot_S1. phiCTX was the most common phage detected in this study. phiCTX is dsDNA cytotoxin-converting phage from the Myoviridae family that carries the *ctx* gene and increases virulence potential (Kung et al., 2010; Kwan et al., 2006). Battle et al. (2008) showed that *P. aeruginosa* strains harbor the phiCTX phage but lack the *ctx* gene, due to several deletion and recombination events, show attenuated virulence. Four out of 12 of the sequenced bacterial genomes (PA16, PA59, PA132 and PA152) carried the D3 bacteriophage,

which is known to harbor serotype-converting genes. Thus, these strains are more capable of attaching to epithelial cells and evading the human immune response due to seroconversion, and thus, more pathogenic (Kung et al., 2010). The F10 bacteriophage was also detected in four out of the 12 sequenced genomes (PA15, PA28, PA59 and PA141). Strains that harbor the F10 bacteriophage have been shown to be involved in increased virulence in a rat lung chronic infection model (Kwan et al., 2006; Wistanley et al., 2009).

5.5. Phylogenetic Analysis

Phylogenetic analysis revealed that the isolates were mainly grouped with the reference strains that had a similar sequence type and resistance profiles. PA9, PA15, PA16, PA28 and PA45 were grouped into the same clade and in close association with PA9 and clustered separately from all the 12 isolates and reference strains used in our study. The five isolates were of sequence type ST-233 and were recovered from tracheal aspirates or catheter tip (PA9). PA132 and NCGM2.S1 clustered together, and had the same ST/clonal complex (ST235 and CC235). PA43 clustered with reference strain UCBPP-PA14 but belonged to different STs, and PA132 clustered with reference strain NCGM2.S1 but also belonged to different STs.

Overall, our study presented a deep understanding on the multiple resistance mechanisms and virulence pathways required for the full potential of *P. aeruginosa* to initiate severe infections. Since novel antimicrobial agents that are FDA approved are limited, it is very important to find new approaches to hamper the threat of antimicrobial resistance. Such strategies include: targeting regulation of resistance mechanisms to enhance antimicrobial drug efficiency and gene expression and developing ways to overcome resistance mechanisms. We are aware that a lot is to be further studied and discovered regarding how *P. aeruginosa* regulates its dynamic and intricate resistance mechanisms to resist antimicrobial activity.

5.6. Future Works

The potential of XDR and cytotoxic *P. aeruginosa* strains in Lebanon is a great threat to public health, especially for immuno-compromised patients. Thus, the genotype and phenotype of this important human pathogen must be rapidly determined. Drug susceptibility testing and surveillance programs are required to

monitor changes in drug resistance patterns and determine the incidence of XDR and virulent isolates to limit and prevent outbreaks of pathogenic *P. aeruginosa*. Once the virulent and antibiotic-resistant determinants that cause injury, sepsis and mortality are identified, opportunities are available to enhance the clinical outcome of the infected patients. Thus, further clinical investigations and suitable protocols must be implemented for the proper use of antimicrobial drugs not only to combat infections, but also to prevent the spread of *P. aeruginosa* resistance genes.

Chapter Six

CONCLUSION

- This study showed the emergence of XDR in *P. aeruginosa* (except isolate PA43) against the antimicrobial drugs being routinely used for treatment.
- The average genome size ranged between 6,579,978-7,009,409 bp, G+C% content between 65.90-66.30% and number of contigs 116-396.
- The isolates belonged to the following sequence types: ST-233, ST-235, ST-296, ST-654, ST-1182 and ST-1233.
- Out of the 12 sequenced isolates, 56% of the isolates harboured *exoS* toxin gene and 39% the *exoU*, with one isolate having both (PA45) and two having none (PA28 and PA43). The fact that all the strains that were XDR were either positive for *exoU* or *exoS* is very alarming.
- PA45 is a unique strain in this study since it lacks both exotoxin genes but is XDR at the same time.
- Other virulence determinants present included: *lasI*, *lasR*, ampR β-lactamase regulator, *gacS*, *gacA*, *phoP* and *phoQ*.
- Although all the sequenced isolates carried *phoP* and *phoQ* genes (which confer resistance to polymyxin B, PA59 was the only strain found to be resistant to colistin which may be due to haboring a mutated *phoQ* gene.
- Out of the 12 sequenced isolates, 67% were negative for the oprD gene, 100% were positive for the ampG gene, 33% were positive for the mexA gene all of which confer carbapenem resistance.
- A large variety of the β-lactamase genes (bla_{PAO}, $bla_{\text{OXA-50}}$, $bla_{\text{OXA-4}}$, $bla_{\text{IMP-15}}$, $bla_{\text{GES-1}}$ and/or $bla_{\text{VIM-2}}$ genes) were detected in all the sequenced strains conferring carbapenem resistance.
- Our results showed that VIM-2 is the most dominant MBL implicated in imipenem resistant *P. aeruginosa* as supported by results of other studies and confers the greatest clinical threat.

- All sequenced isolates isolates harbored the *ftsK* gene, *mexR* and *nfxB* genes which are linked to fluoroquinolone resistance. In addition, all the 18 isolates carried *mexC* and *mexE* and were phenotypically resistant to ciprofloxacin.
- PA43 was the only strain that was susceptible to ciprofloxacin while harboring all three mex efflux genes simultaneously. This could be due to mutations producing non-functional proteins and the lack of promoters upstream of the site of insertion.
- In this study, *P. aeruginosa* isolates contained multiple aminoglycoside resistance genes, which is inconsistent with previous studies reporting that the majority of resistant isolates harbor only a single aminoglycoside-modifying gene
- The most frequent aminoglycoside-modifying enzyme detected in this study was the *aph(3')-IIb gene* conferring aminoglycoside resistance.
- PA43 carried three aminoglycoside resistance encoding genes but was still susceptible to the aminoglycosides tested. The exact reason for the sensitive phenotype of this strain is unknown.
- Eight plasmids were identified: phiCTX, YMC11/02/R656, F10, D3, PMG1, phi297, Bacter_Lily, Escher_vB_EcoM_EC01230_10 and Stenot_S1. phiCTX was the most common detected plasmid in this study, followed by YMC11/02/R656.
- Drug susceptibility testing and surveillance programs are required to monitor changes in drug resistance patterns and determine the incidence of XDR and virulent isolates to limit and prevent outbreaks of pathogenic *P. aeruginosa*.
- Further clinical investigations and suitable protocols must be implemented for the proper use of antimicrobial drugs not only to combat infections, but also to prevent the spread of *P. aeruginosa* resistance genes.

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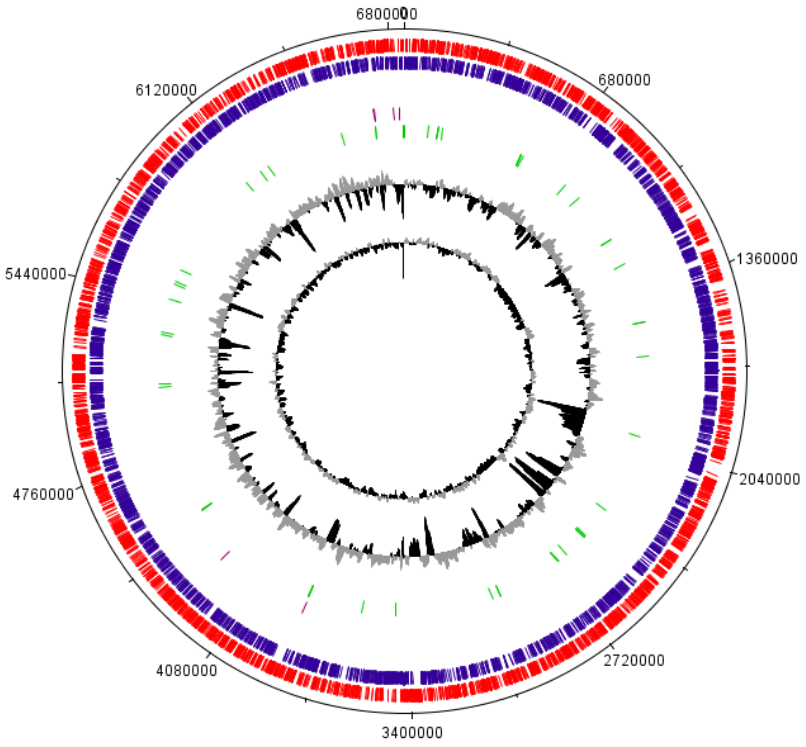
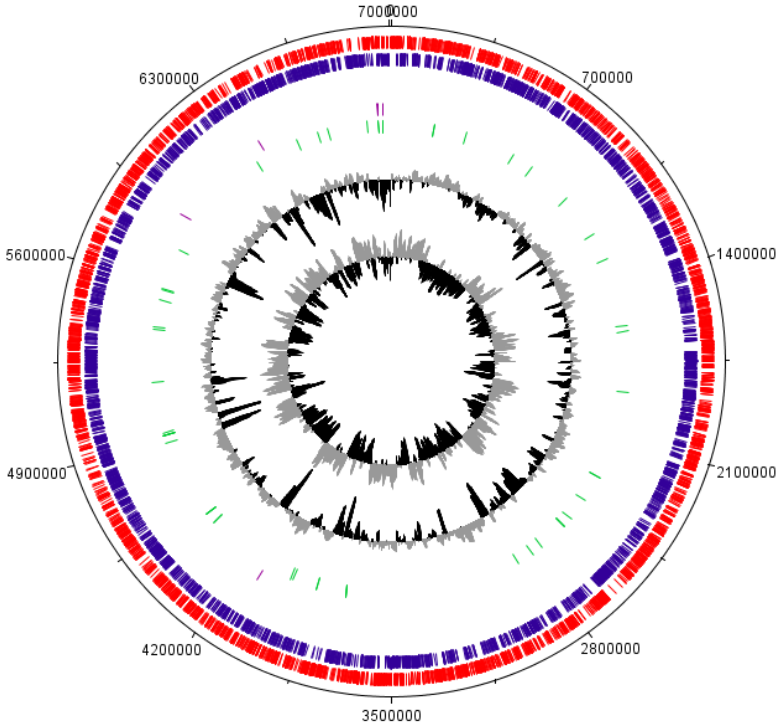
ANNEX I

6.1. Circular Images



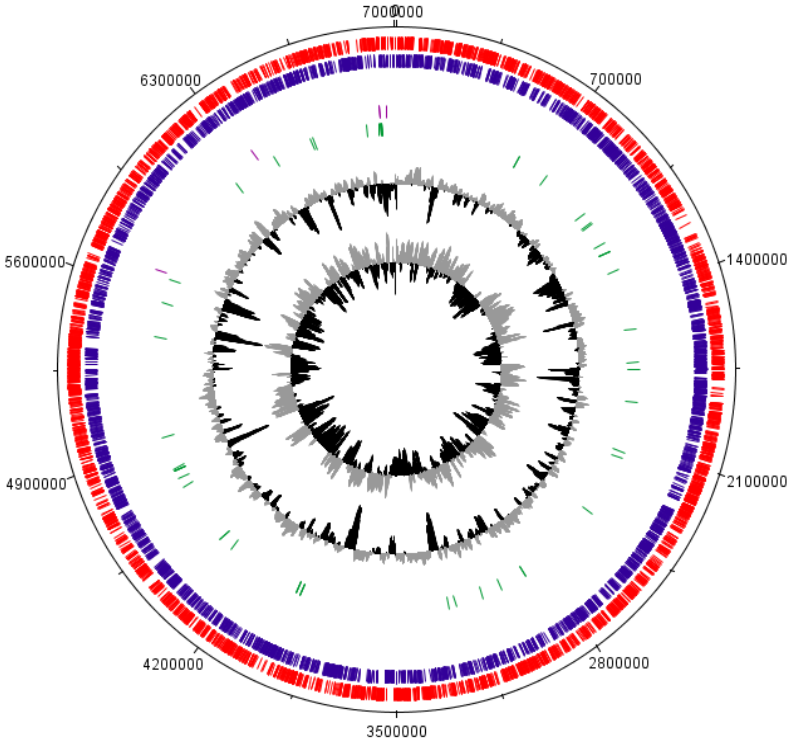
Pseudomonas aeruginosa PA9

Figure 1. Circular map of *P. aeruginosa* PA9 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



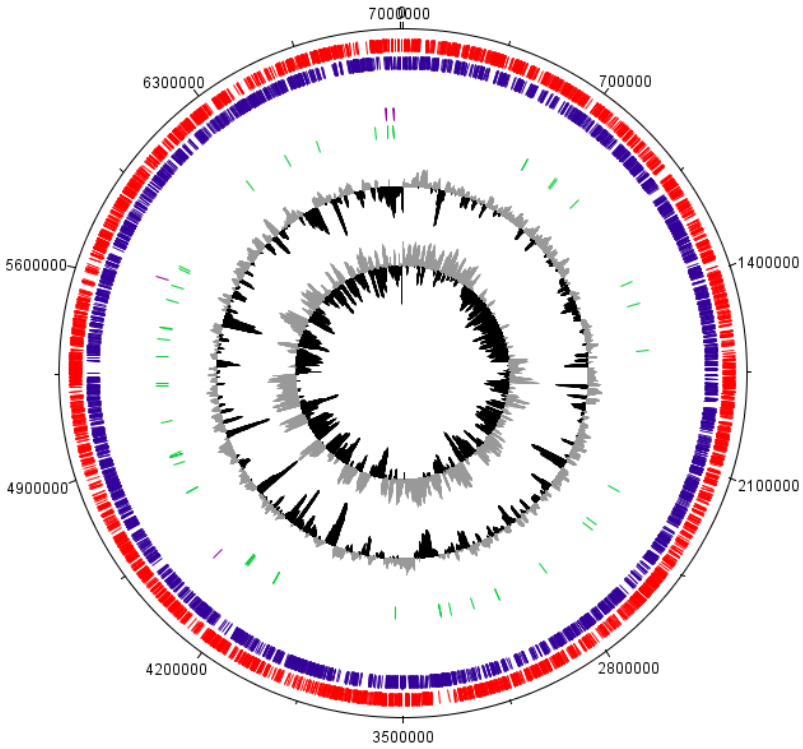
Pseudomonas aeruginosa PA15

Figure 2. Circular map of *P. aeruginosa* PA15 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



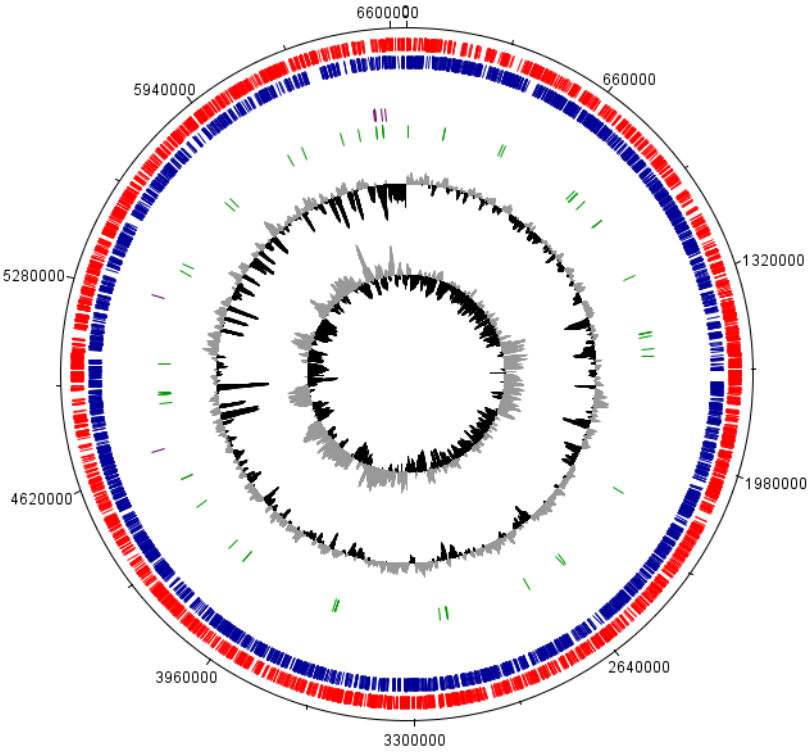
Pseudomonas aeruginosa PA16

Figure 3. Circular map of *P. aeruginosa* PA16 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



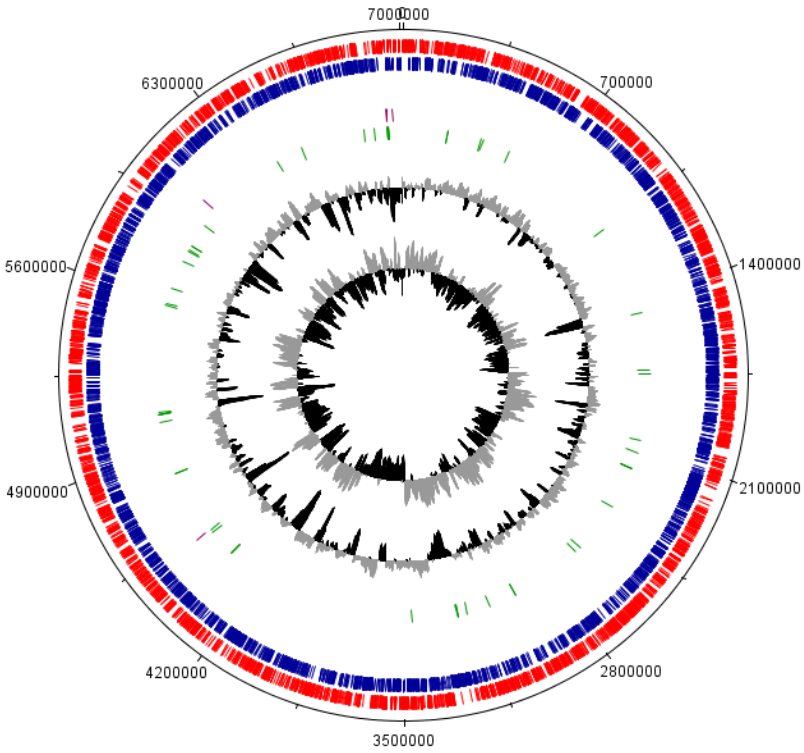
Pseudomonas aeruginosa PA28

Figure 4. Circular map of *P. aeruginosa* PA28 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



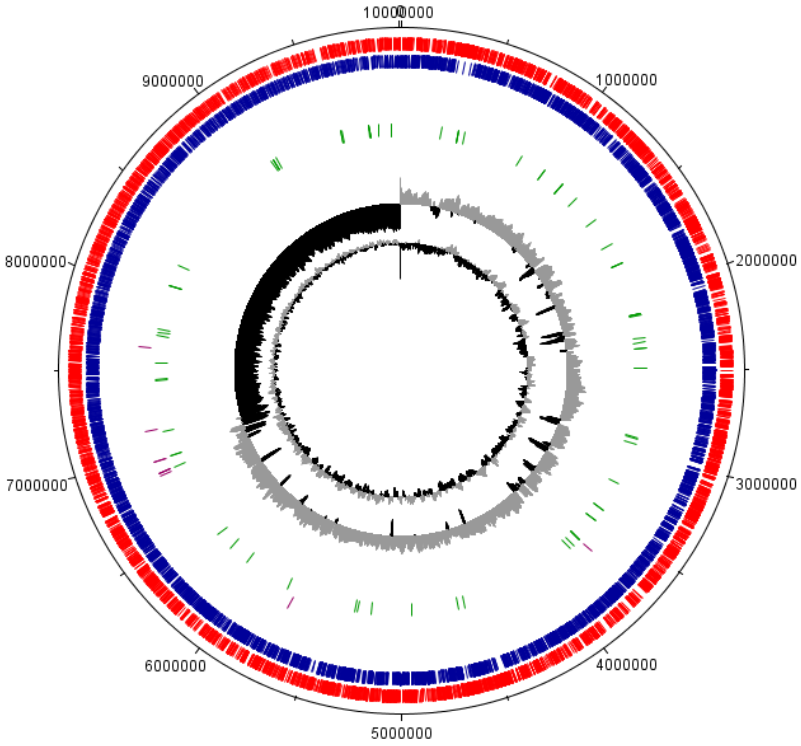
Pseudomonas aeruginosa PA43

Figure 5. Circular map of *P. aeruginosa* PA43 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



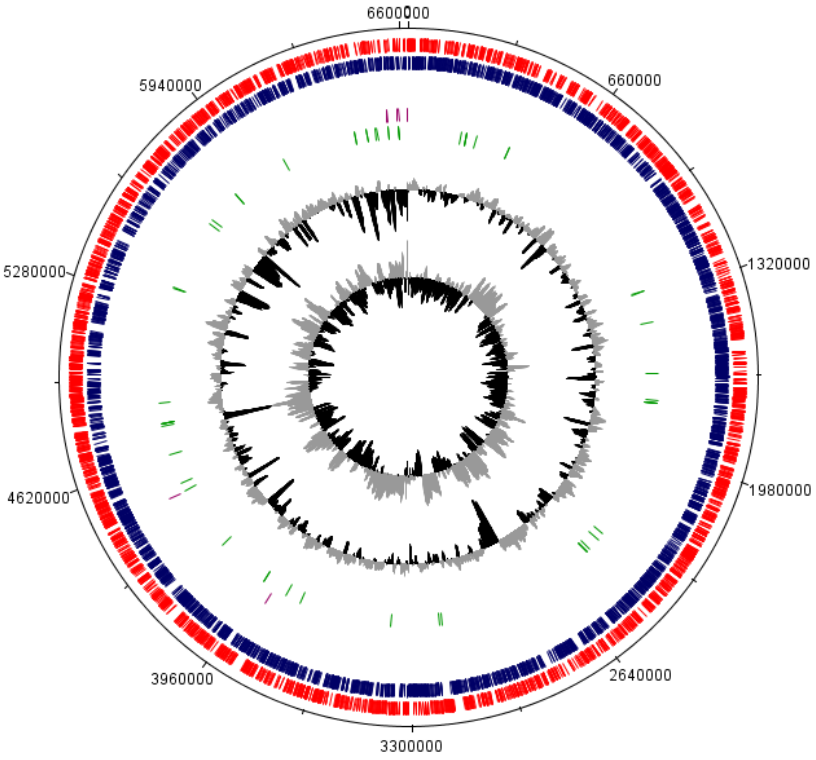
Pseudomonas aeruginosa PA45

Figure 6. Circular map of *P. aeruginosa* PA45 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



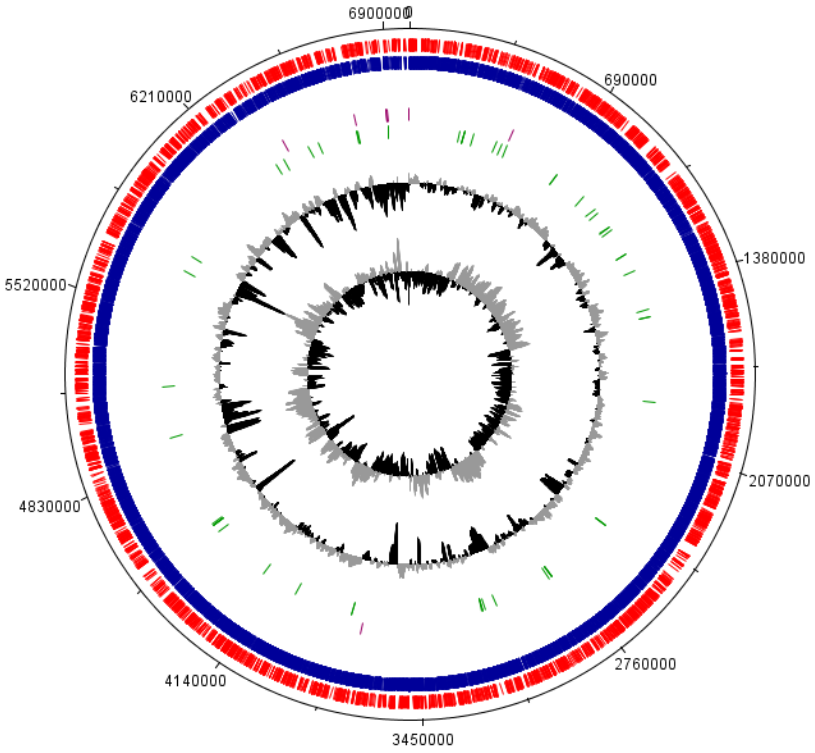
Pseudomonas aeruginosa PA53

Figure 7. Circular map of *P. aeruginosa* PA53 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



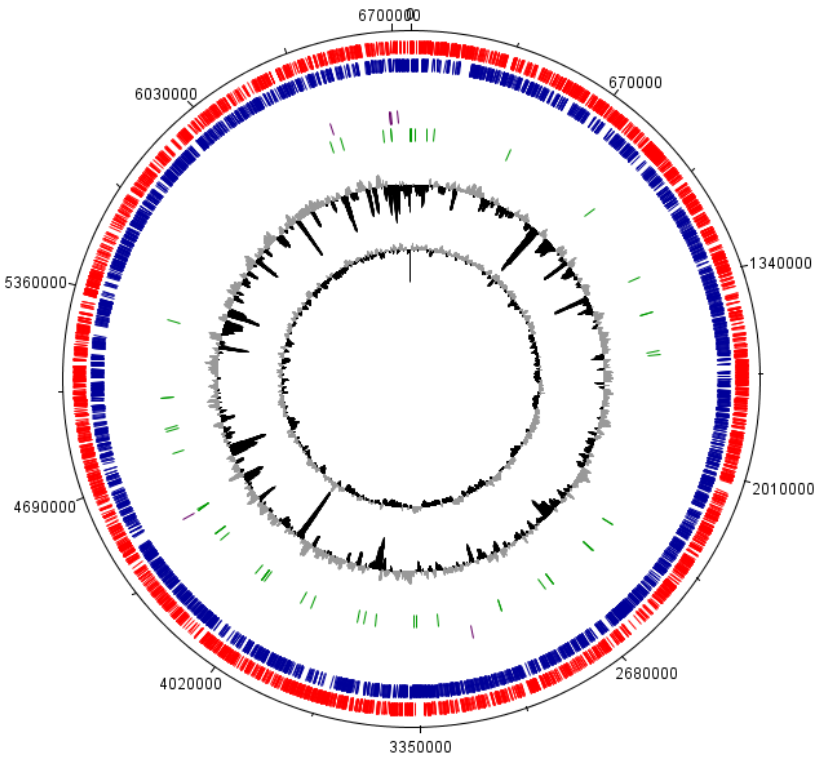
Pseudomonas aeruginosa PA120

Figure 8. Circular map of *P. aeruginosa* PA120 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



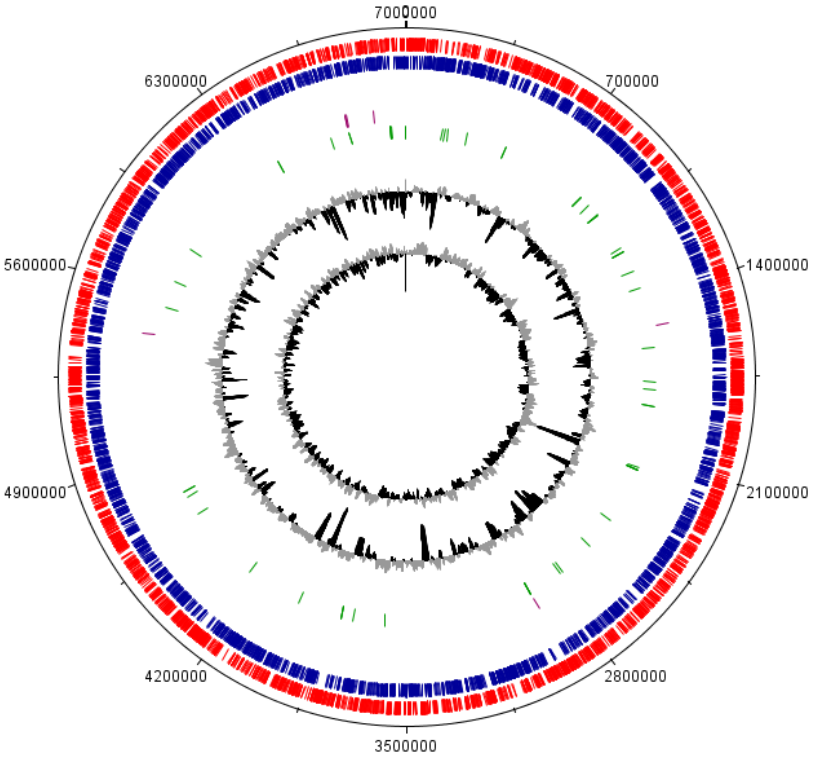
Pseudomonas aeruginosa PA132

Figure 9. Circular map of *P. aeruginosa* PA132 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (prurple), tRNAs (green), GC plot and GC skew with above mean value (grey) and below mean value (black).



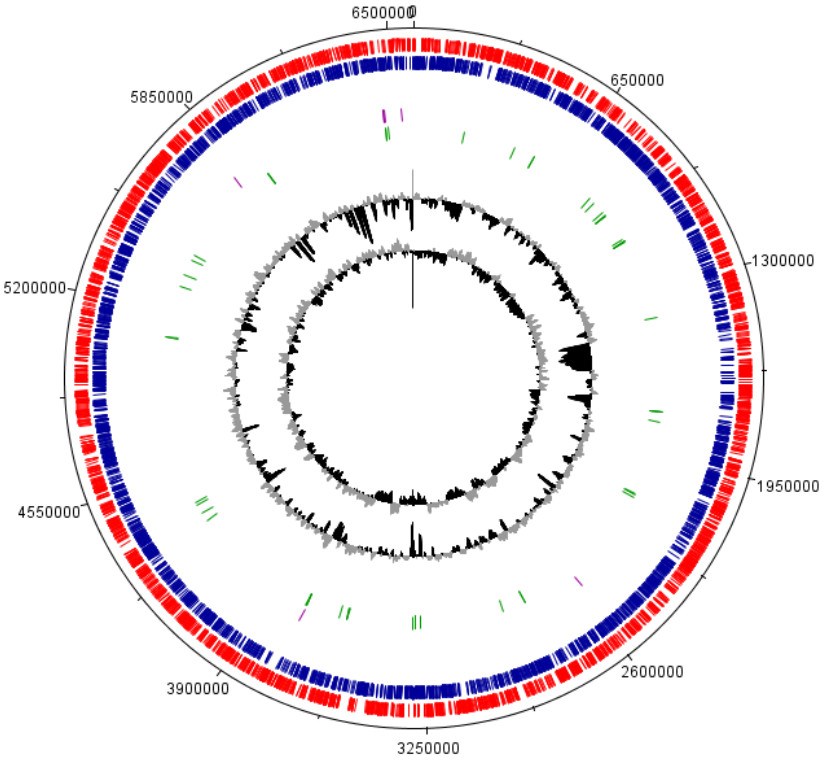
Pseudomonas aeruginosa PA141

Figure 10. Circular map of *P. aeruginosa* PA141 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (magenta), tRNAs (brown), GC plot and GC skew with positive value (green) and negative value (purple).



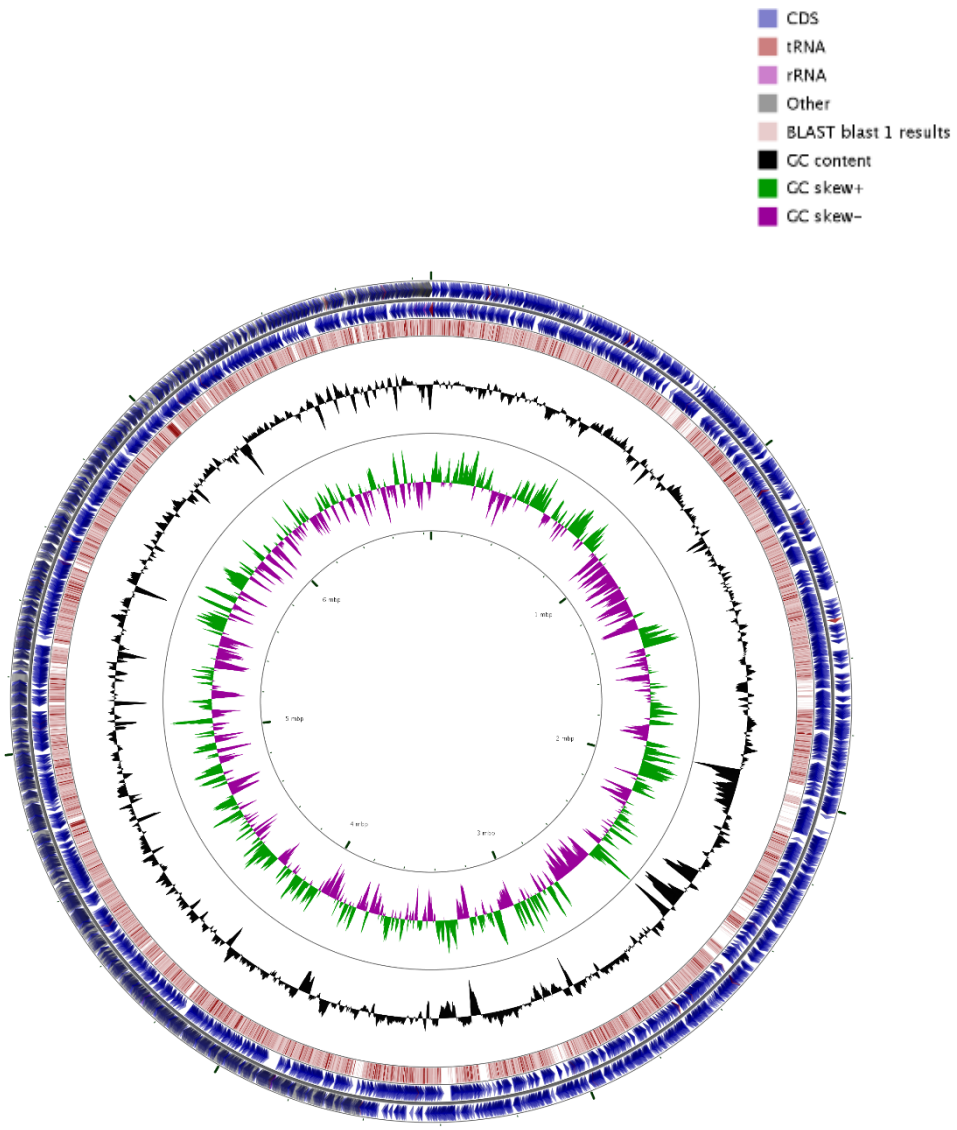
Pseudomonas aeruginosa PA59

Figure 11. Circular map of *P. aeruginosa* PA152 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (magenta), tRNAs (brown), GC plot and GC skew with positive value (green) and negative value (purple).



Pseudomonas aeruginosa PA155

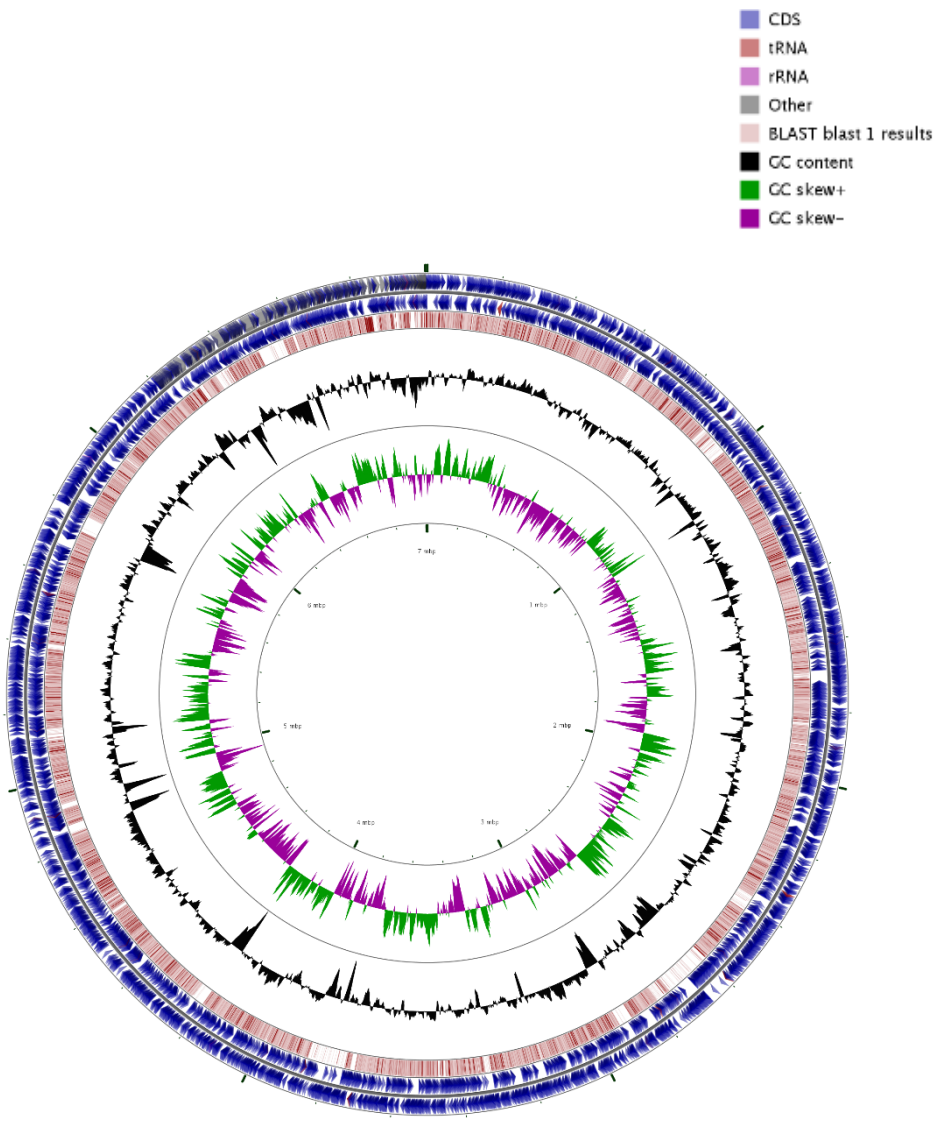
Figure 12. Circular map of *P. aeruginosa* PA155 genome. Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: CDSs of PA59 on the forward stand (red), CDSs of PA59 on the reverse stand (blue), rRNAs (magenta), tRNAs (brown), GC plot and GC skew with positive value (green) and negative value (purple).



Pseudomonas aeruginosa PA9

Figure 13. Comparative circular map of *P. aeruginosa* PA9 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs.

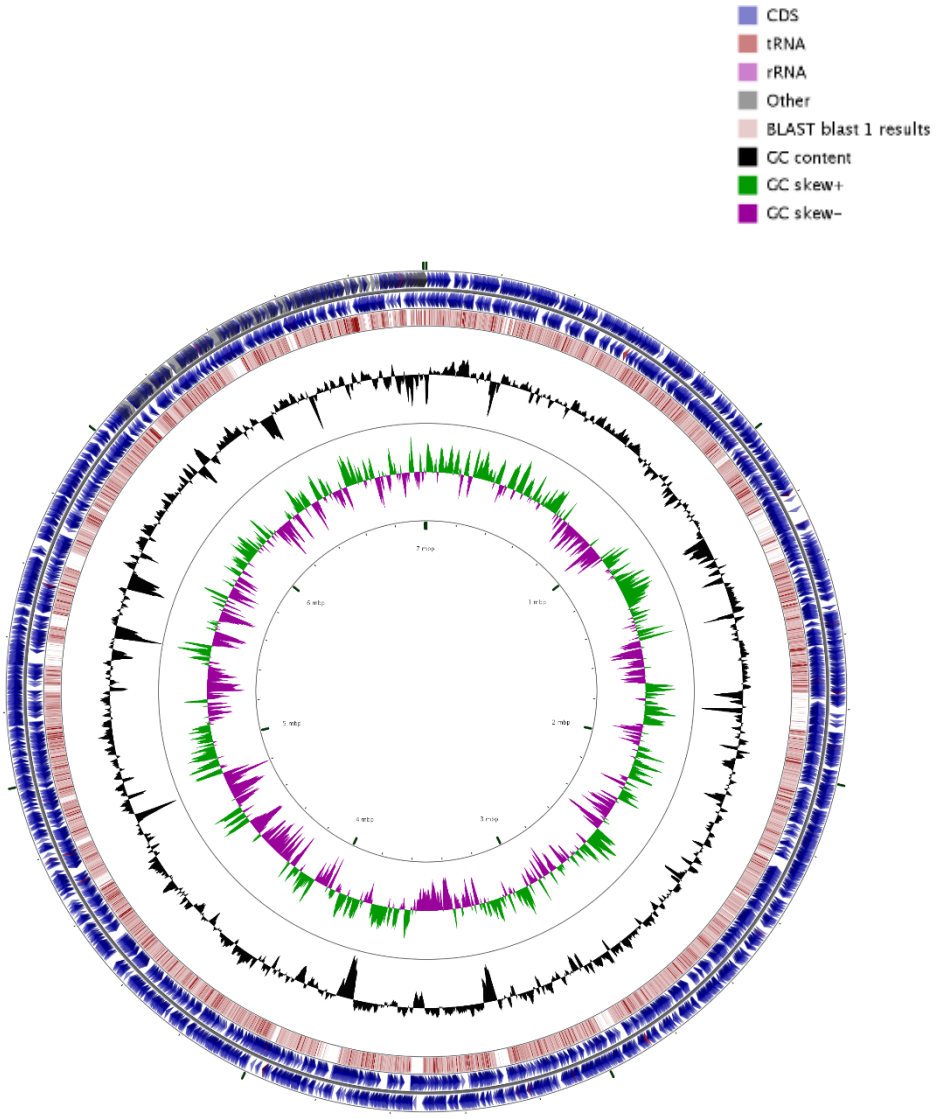
Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA15

Figure 14. Comparative circular map of *P. aeruginosa* PA15 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts

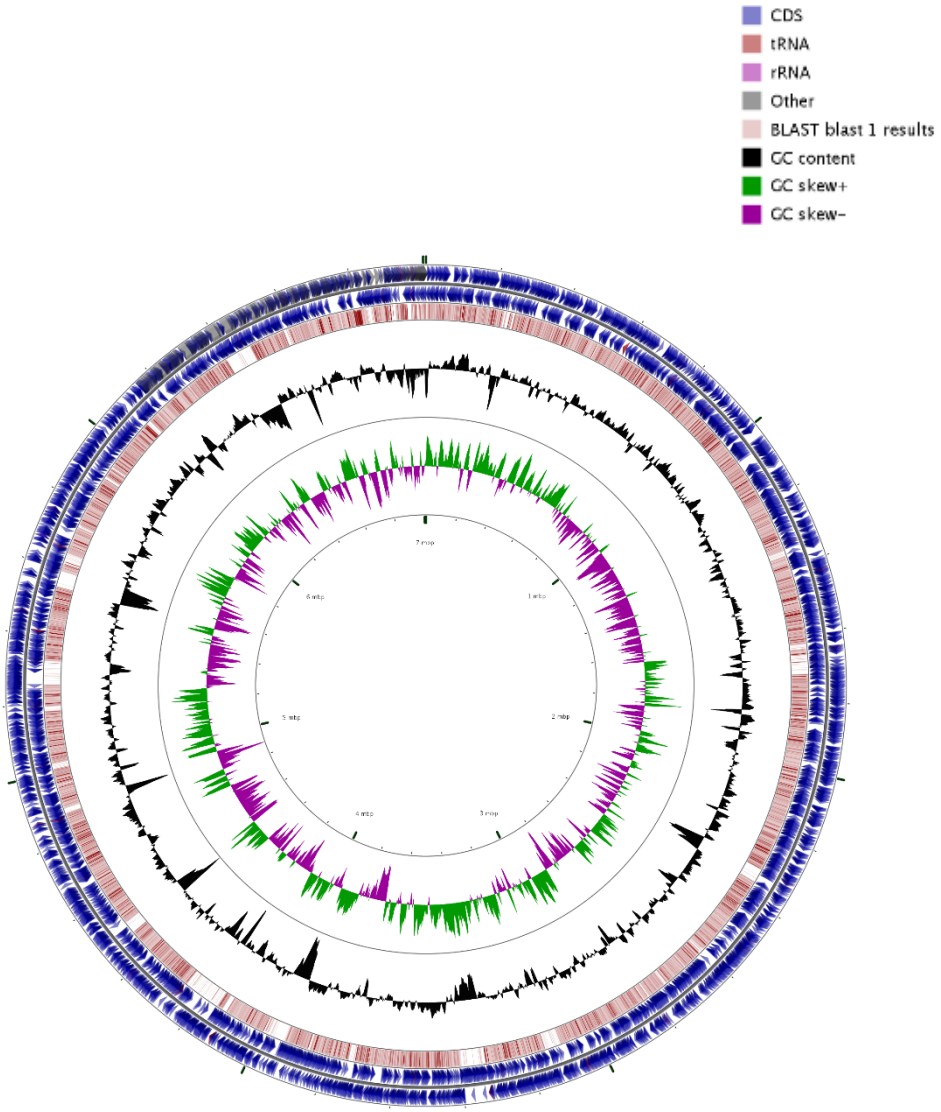
of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA16

Figure 15. Comparative circular map of *P. aeruginosa* PA16 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN

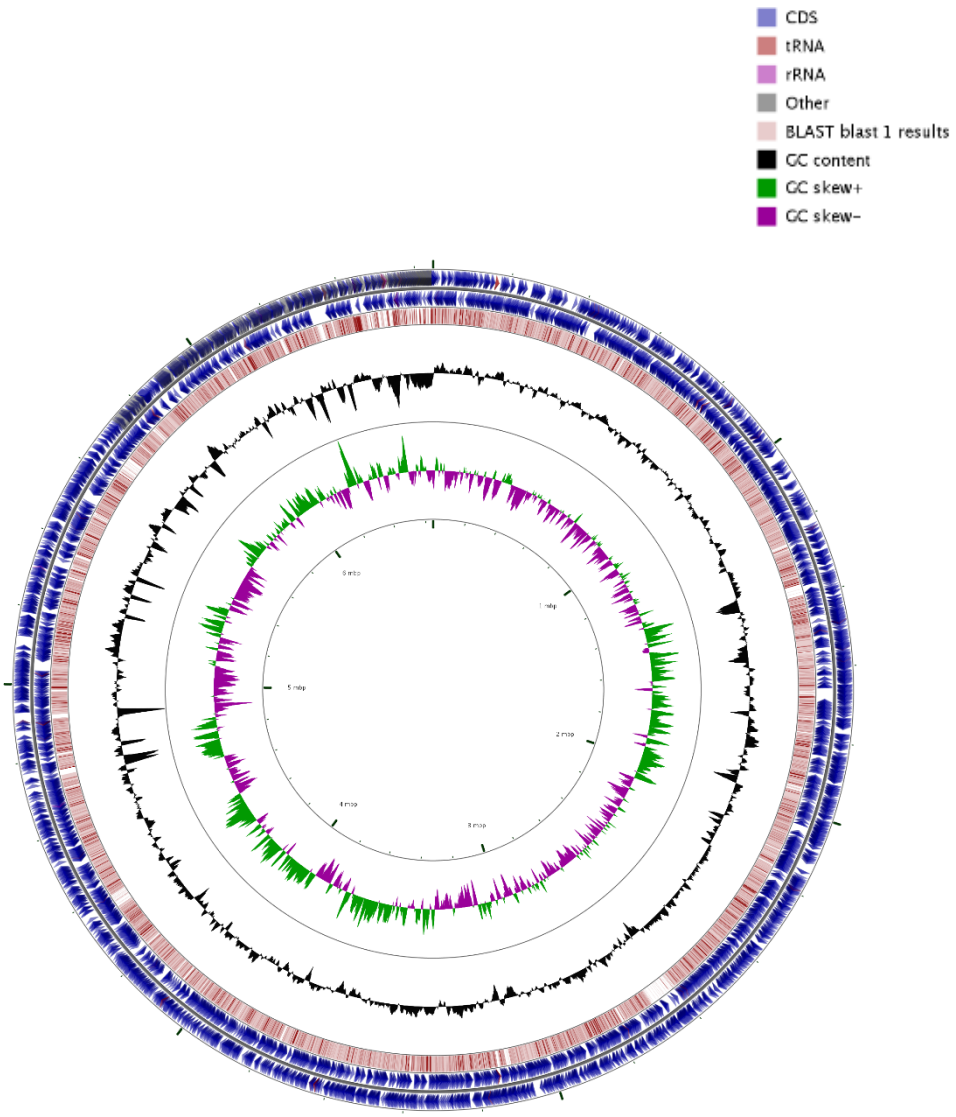
alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA28

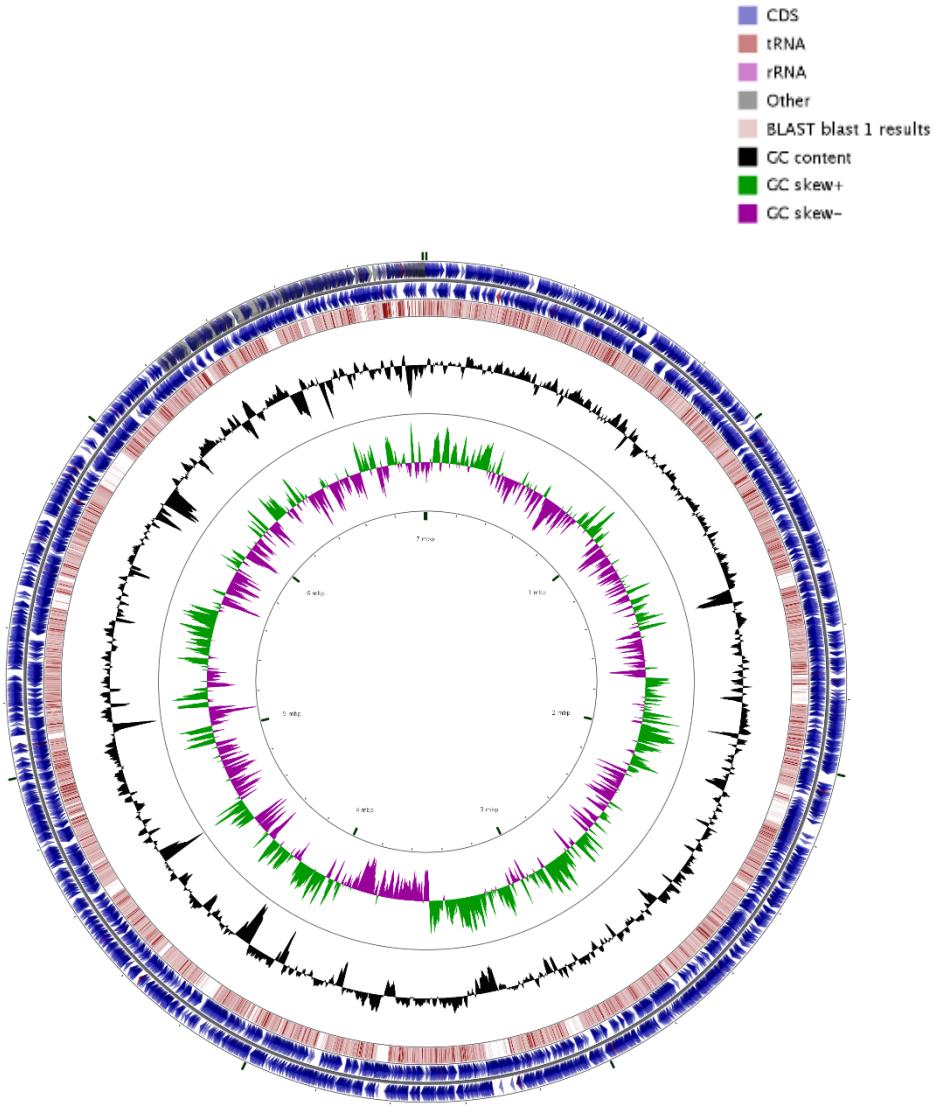
Figure 16. Comparative circular map of *P. aeruginosa* PA28 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008).

Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA43

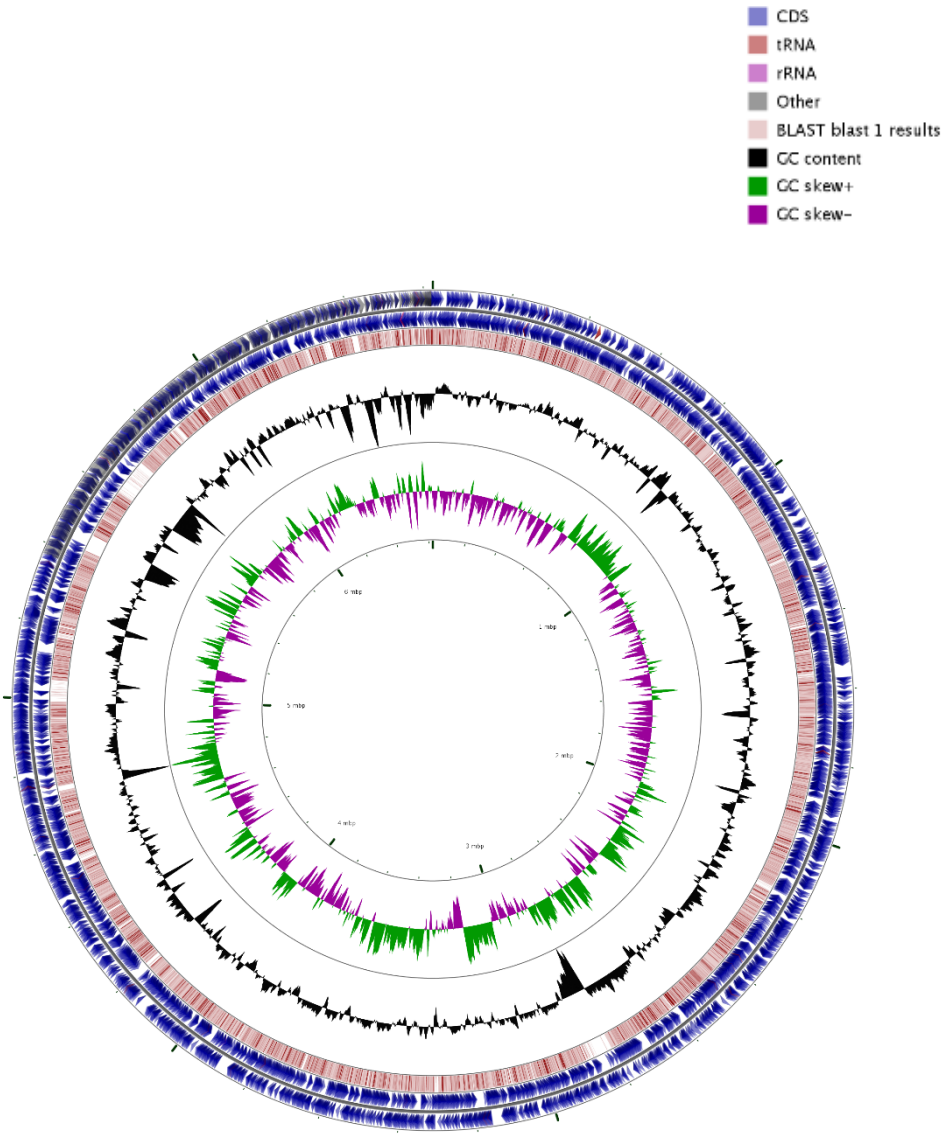
Figure 17. Comparative circular map of *P. aeruginosa* PA43 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA45

Figure 18. Comparative circular map of *P. aeruginosa* PA45 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs.

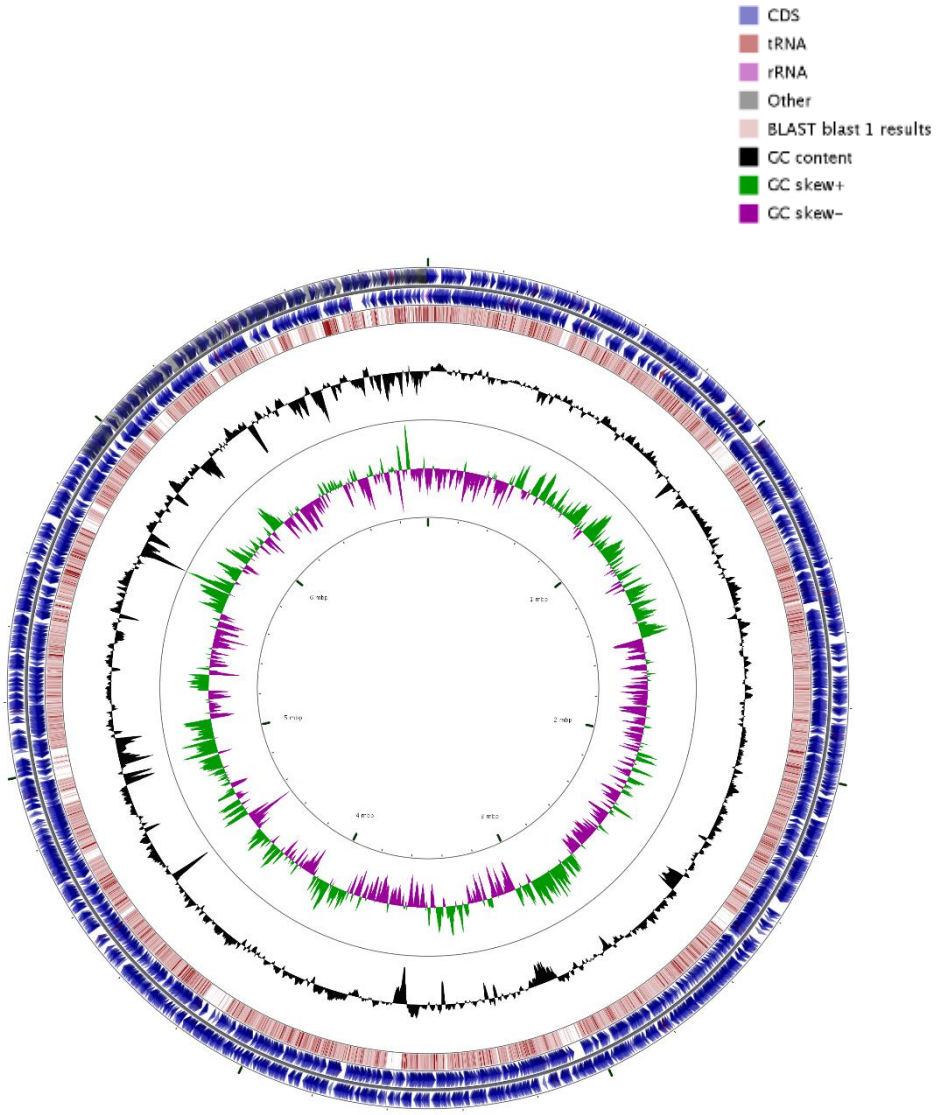
Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA120

Figure 19. Comparative circular map of *P. aeruginosa* PA120 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence

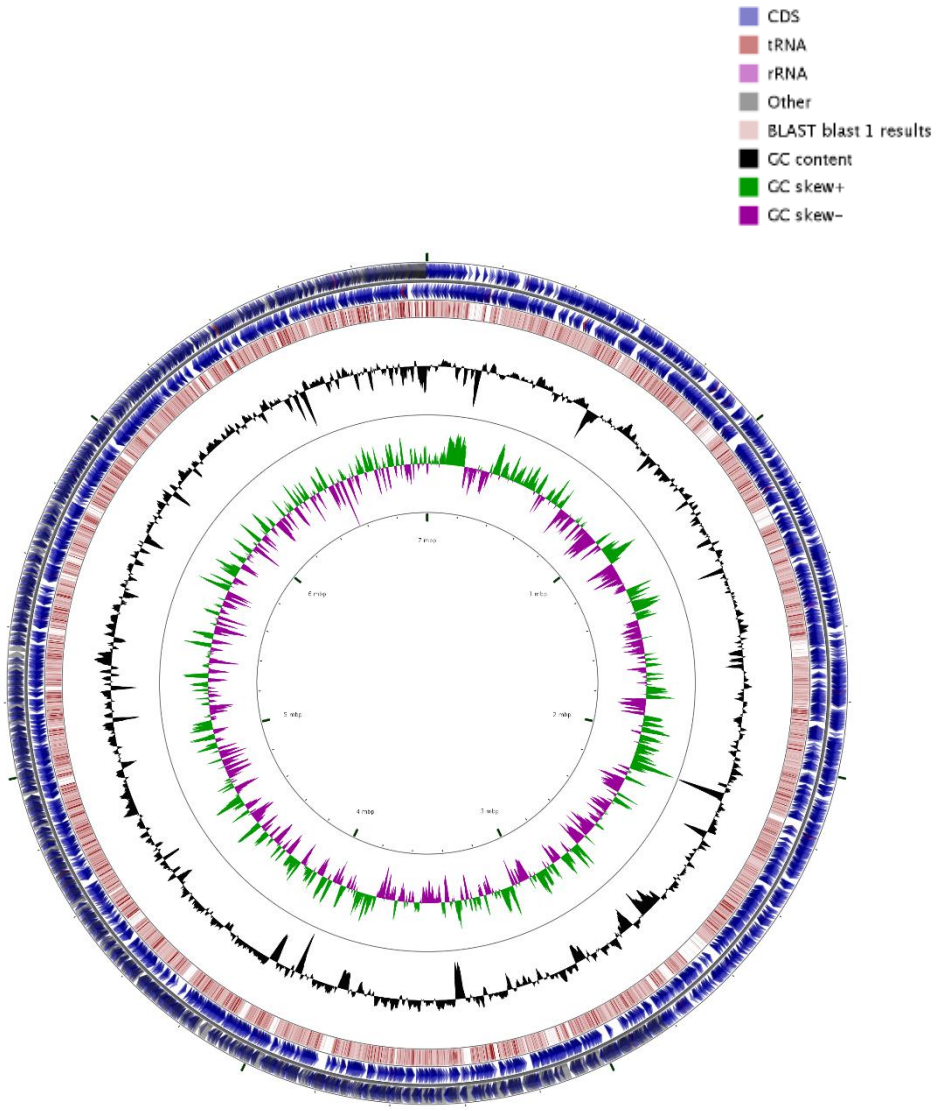
that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA132

Figure 20. Comparative circular map of *P. aeruginosa* PA132 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN

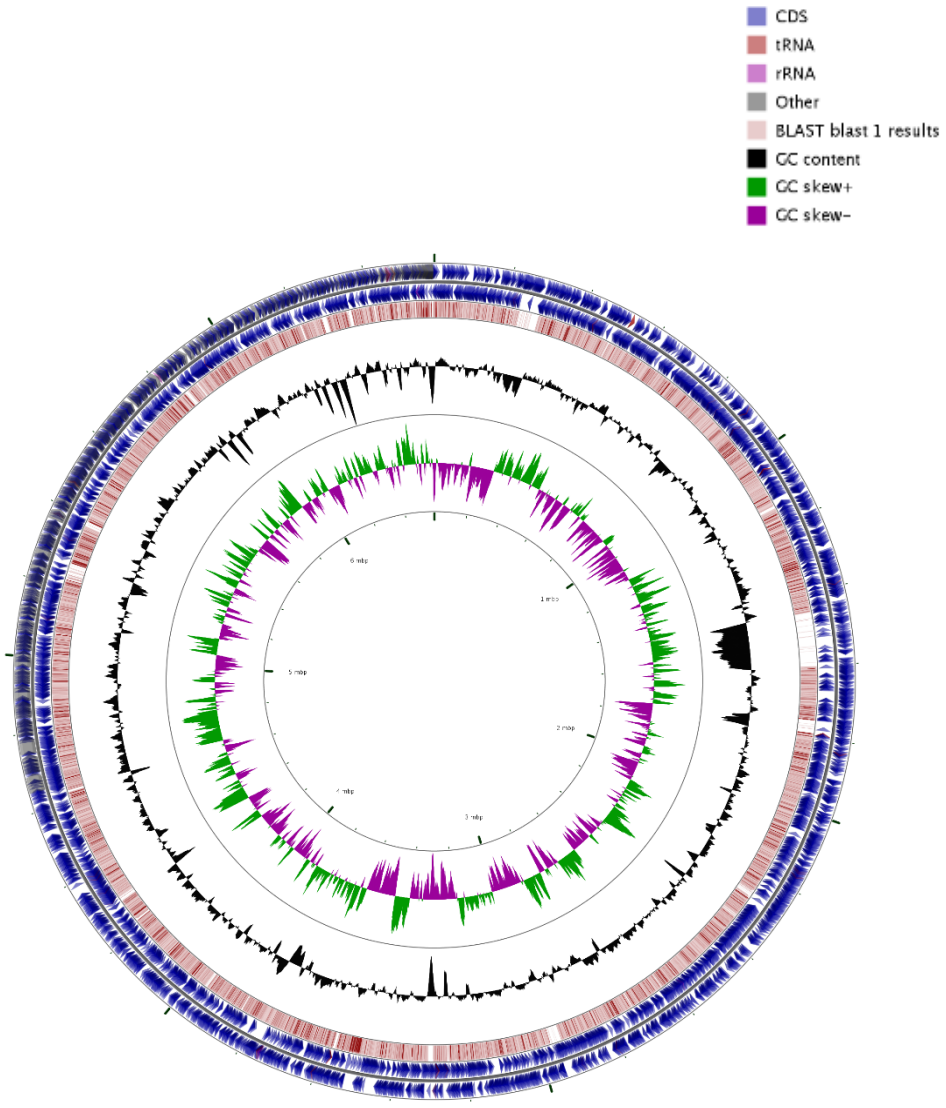
alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA152

Figure 21. Comparative circular map of *P. aeruginosa* PA152 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008). Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P.*

aeruginosa PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.



Pseudomonas aeruginosa PA155

Figure 22. Comparative circular map of *P. aeruginosa* PA155 genome BLASTed against the genome of reference strain PAO1. This graphical representation of the genome was generated using CGview server V 1.0 (Grant & Stothard, 2008).

Circular representation of the PA59 genome; from outside to inside, the tracks display the following information: clockwise CDSs, anticlockwise CDSs, *P. aeruginosa* PAO1 BLAST comparison, GC content and GC skew. The BLASTN alignment tracks displays white regions which indicate parts of the input sequence that did not yield a BLAST hit. Regions with a little pink/green color represents parts of the input sequence that yield one blast hit. Regions with a dark pink/green color indicate numerous BLAST hits which are commonly attributed to rRNAs or tRNAs. Then, GC plot and GC skew with positive value (green) and negative value (purple) are shown.