In Silico Analysis, Molecular Modeling and Docking of *Pseudomonas Aeruginosa* Putative Choline Kinase

Abdonasr Mohamed Ali Almakfoukh

UNIVERSITI SAINS MALAYSIA

2021

In Silico Analysis, Molecular Modeling and Docking of *Pseudomonas Aeruginosa* Putative Choline Kinase

by

Abdonasr Mohamed Ali Almakfoukh

Dissertation submitted in partial fulfillment of the requirements of the degree of Master of Science (Biomedicine) Mixed Mode

October 2021

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my panel of supervisors; **Assoc. Prof. Dr. See Too Wei Cun**, the main supervisor, for his full support and guidance during my work on this project. He kept his eyes on the progress of my work and he was available for help whenever I needed it during the course of the study. He was always opened to listen and solve all the obstacles that I have met during the COVID-19 pandemic, thank you very much Dr. See Too… very much appreciated. Appreciation is also extended to my co- supervisor, **Assoc**. **Prof. Dr. Few Ling Ling**, for his valuable comments and critical advice during the writing of my thesis, and for his immense help in planning and executing the work especially with the econometric aspects of this thesis on time, thank you very much Dr. Few…very much appreciated. I also would like to express my greatest appreciation to **Dr. Shuhaila Mat Sharani** for her comments, suggestions and toning up the overall layout of my thesis, thank you very much Dr. Shuhaila..very much appreciated. I would like also to acknowledge all the staff members on top of them the dean of the faculty, secretary and also the postgraduate students especially Moad Alarabi who were helpful during my entire work on this project, thank you Moad for your help… very much appreciated. Not to forget my tutor **Dr. Abduladim Hmmier**, from the Biotechnology research centre- Libya, department of clinical proteomics for his unlimited support and for his encouraging me to step further and expand this piece of work for transfer as PhD project, thank you very much Dr. Abduladim…very much appreciated. I also would like to thank the Ministry of Higher Education and Scientific Research of Libya, for granting me a scholarship covering the tuition and bench fees, and all living expenses including medical insurance for my entire family as well as covering the expenses of my kids' school expenses. I also would like to thank the

cultural attaché and his team at the Libyan embassy- Malaysia for their remote assistance and communication with the university to ease and remove all the obstacles in front of the students. I would like also to thank all my friends and colleagues who were supporting me directly and indirectly, they were very patient in listening to my various arguments and provided valuable suggestions and support, thank you very much to all… very much appreciated. Finally, I would like to thank my wife for her patience and looking after our children during the uncomfortable circumstances in Libya which was exacerbated by the COVID-19 pandemic, thank you very much my wife, thank you very much my children for your patience, thank you for all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
CDC	Centers for Disease Control
WHO	The World Health Organization
ChoKIs	Choline kinase inhibitors
HC-3	Hemocholinium-3
PaChoK	*P. aeruginosa* choline kinase
CF	Cystic Fibrosis
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
TCA	Tricarboxylic Acid
MDR	Multidrug resistance
MBLs	Metallo-lactamases
Mex	Membrane Efflux
USM	Universiti Sains Malaysia
Cho	Phosphorylate choline
ChoP	Phosphorylcholine
Chok	Choline kinase
Cho	Choline
CCT	Phosphorylcholine Cytidylyl Transferase
CDP Colin	Cytidine diphosphate choline
DAG	Diacylglycerol
PC	Phosphatidylcholine
GRAVY	Grand Average of Hydropathy
NCBI	National Centre for Biotechnology Information
TCA	Tricarboxylic acid
MBLs	Metallo-lactamases
PI	Isoelectric point
M	Methionine
C	Carbon
H	Hydrogen

N	Nitrogen
O	Oxygen
S	Sulphur
QMEAN	Qualitative Model Energy Analysis
ORF	Open reading frame
MCS	Multiple cloning site

LIST OF APPENDICES

ANALISIS *IN SILICO*, PEMODELAN DAN PELABUHAN MOLEKUL KOLINA KINASE PUTATIF *PSEUDOMONAS AERUGINOSA*

ABSTRAK

Pseudomonas aeruginosa adalah organisma utama dalam jangkitan bakteria. Rawatan jangkitan *P. aeruginosa* telah menjadi suatu cabaran disebabkan kerintangan bakteria ini terhadap beberapa ubatan yang digunakan pada masa kini. Untuk menyelesaikan isu kerintangan antimikrob (AMR), pencarian antimikrobik baharu telah menjadi fokus utama dalam komuniti penyelidikan yang berkaitan. Fenomena AMR ini telah berlaku dengan cepat dan tidak dapat ditandingi oleh penemuan ubat-ubatan baharu. Oleh itu, agen antimikrob inovatif sangat diperlukan dengan segera. Penggunaan ubat-ubatan semasa untuk tujuan baharu memungkinkan penghasilan agen antimikrob yang baharu. Perencat kolina kinase (ChoKI) berkemungkinan menjadi salah satu agen antimikrob canggih yang menangani AMR dengan berkesan. ChoKI yang kebiasaannya digunakan sebagai ubat antikanser pada manusia mempunyai potensi untuk merencat kolina kinase *P. aeruginosa*. Perencatan ini mungkin mengganggu sintesis molekul lipopolisakarida / asid lipoteikoik yang diperlukan untuk integriti membran sel bakteria. Kajian ini bertujuan untuk melakukan pencirian asas PaChoK dengan kaedah *in silico* dan menjana struktur model untuk enzim ini bagi pelabuhan molekul dengan ChoKI untuk menilai potensi penggunaan ChoKIs sebagai agen antimikrob untuk menentang *P. aeruginosa*. Analisis asas bioinformatik menunjukkan PaChoK mempunyai kandungan asid amino alanina dan asid amino bercas negatif yang tinggi. PaChoK juga diramal mempunyai kelarutan yang rendah jika diekspresikan dalam sel *Escherichia coli*. Penjajaran jujukan amino asid dan superimposisi struktur protein menunjukkan homologi antara kolina kinase

manusia dan *P. aeruginosa*, ini menunjukkan ChoKI yang digunakan untuk merencat kolina kinase manusia juga berpotensi sebagai anti-PaChoK. Struktur model PaChoK telah dijana dan digunakan untuk pelabuhan dengan hemikolinium-3 (HC-3), suatu perencat kolina kinase manusia yang popular. Keputusan pelabuhan menunjukkan interaksi HC-3 boleh berlaku pada tujuh asid amino di dalam poket pengikat kolina pada PaChoK. Bukti ini jelas menyokong kesesuaian ChoKI seperti HC-3 sebagai molekul anti-PaChoK yang boleh terus dibangunkan menjadi agen antimikrob. Walau bagaimanapun, pengesahan eksperimen untuk interaksi dan perencatan PaChoK oleh ChoKI masih diperlukan bagi mencari perencat yang paling berkesan untuk mengakhiri AMR yang ditunjukkan oleh *P. aeruginosa*.

IN SILICO ANALYSIS, MOLECULAR MODELING AND DOCKING OF *PSEUDOMONAS AERUGINOSA* PUTATIVE CHOLINE KINASE

ABSTRACT

Pseudomonas aeruginosa is a major player in the bacterial invasion. Treating *P. aeruginosa* infections has become a serious challenge due to the bacterium's ability to survive several of the currently available medicines. To put an end to the antimicrobial resistance (AMR) issue, the quest for novel antimicrobics has been a key focus in the relevant research community. The AMR phenomenon has been evolving at a breakneck pace that has not been matched by the development of new drugs. As a result, innovative antimicrobials are urgently needed. Repurposing current medications allows for the development of novel antimicrobials. Choline kinase inhibitors (ChoKIs) could be one of these cutting-edge antimicrobials that puts an end to AMR once and for all. ChoKIs, which are used as anticancer drugs in human, have the potential to inhibit *P. aeruginosa* choline kinase (PaChoK). Inhibition of choline kinase would disrupt the synthesis of lipopolysaccharide/lipoteichoic acid molecules that are required for the bacterial cell membrane integrity. This study aims to characterize the basic properties of PaChoK by *in silico* approaches and generate the model structure of this enzyme for further molecular docking with ChoKI to assess the potential of utilizing ChoKIs as antimicrobial agent against *P. aeruginosa*. Basic bioinformatic analysis shows that PaChoK is abundant with alanine amino acids, has copious negatively charged residues and low solubility if expressed in *Escherichia coli*. Multiple amino acid sequence alignment and protein structure superimposition revealed homology between human and *P. aeruginosa* choline kinases, indicating the

ChoKIs previously used to inhibit human choline kinase as potential anti-PaChoK. The model structure of PaChoK was generated and used for docking with hemicholinium-3 (HC-3), a well known human ChoK inhibitor. The docking results showed feasible HC-3 interaction inside the choline-binding pocket of PaChoK, which interacts with seven pocket residues. The evidence clearly supports the ChoKIs such as Hemicholinium-3 appropriateness as anti-PaChoK that can be further develop into antimicrobials. Yet, experimental validation of ChoKIs interaction with and inhibition of PaChoK is still required to search for the most potent ChoKIs that can end AMR of *P. aeruginosa*.

Chapter I

Introduction

CHAPTER 1

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most serious public health issues of the twenty-first century, posing a challenge to the successful prevention and treatment of an ever-widening spectrum of infections caused by bacteria, parasites, viruses, and fungi that are no longer susceptible to common antibiotics. Antibiotic resistance in bacteria makes the issue of AMR much more urgent. Bacteria that cause common or serious infections have established resistance to each new antibiotic that comes to market over several decades, to varying degrees. Faced with this fact, immediate action is needed to avert a looming global healthcare crisis (Prestinaci, Pezzotti & Pantosti, 2015). Even more so when the world health organization has stated that ten million deaths every year by 2050 (Boerma & Mathers, 2015).

When microorganisms are exposed to antibiotic drugs, antibiotic resistance develops naturally. Susceptible bacteria are destroyed or inhibited by antibiotics, while bacteria that are naturally (or intrinsically) immune or have developed antibiotic-resistant traits have a better chance of surviving and multiplying. Antibiotic resistance is exacerbated not only by overuse, but also by improper use such as inappropriate choices, insufficient dosing, and poor adherence to treatment guidelines. The factors that contribute to the emergence and spread of antibiotic resistance are depicted in (figure 1.1). Human medicine in the community and hospital settings, animal production, and agriculture, and the environmental compartment were listed as the four key sectors involved in the development of antibiotic resistance (Prestinaci, Pezzotti & Pantosti, 2015).

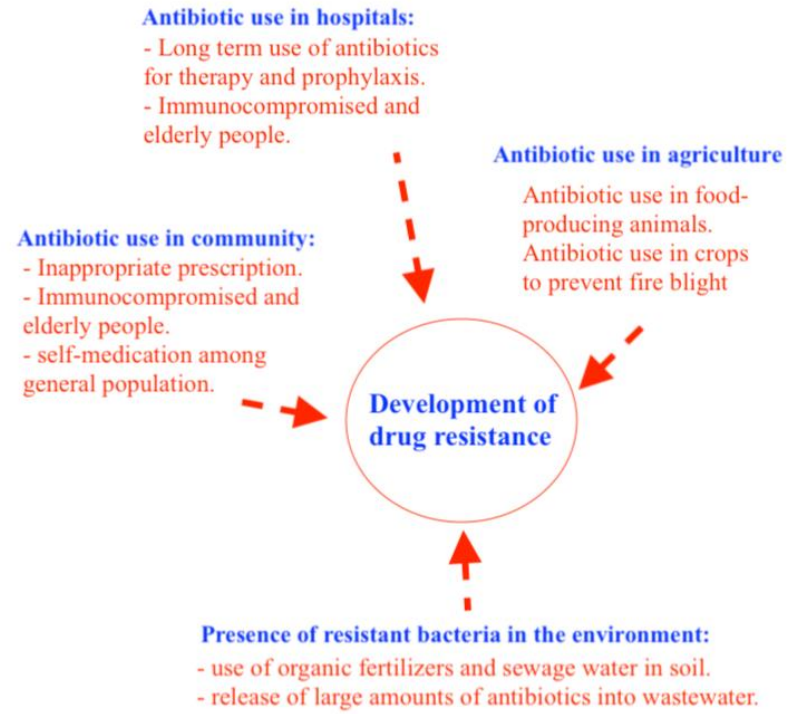

Figure1.1 Development of drug resistance. Antibiotic resistance is transmitted by factors in the following sectors: community and hospital medicine, animal development and agriculture, and the environment. These industries are also interconnected: the abuse of antibiotics in humans, livestock, and agriculture is the primary cause of resistant bacteria in the world. Modified from (Prestinaci, Pezzotti & Pantosti, 2015)

Chemotherapy for cancer treatment, organ transplantation, hip replacement surgery, intensive care for pre-term newborn, and many other operations would be impossible to conduct without appropriate antibiotics. In reality, multidrug-resistant bacterial infections are one of the leading causes of morbidity and mortality in patients undergoing these procedures. Infections in cancer patients with chemotherapy-related neutropenia had high antibiotic resistance rates, according to a 2014 study from the University of Texas (Nesher & Rolston, 2014). Infections following orthotopic liver transplantation were found to have a high proportion of antibiotic-resistant bacteria, according to a new report from the Medical University of Warsaw (Kawecki *et al.*, 2014).

Antibiotic resistance's effect on mortality and public health costs is difficult to quantify, and few studies have been conducted on the subject. Antibiotic-resistant infections affect more than two million people in the United States per year, according to the US Centers for Disease Control and Prevention (CDC), with at least 23 000 people dying as a result of the infection (McGuire, 2014). Therefore, a novel antimicrobic is in urgent need.

1.1 Antibiotic resistance in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a prominent contributor to the bacterial onslaught. Owing to the bacterium's ability to withstand many of the currently available antibiotics, treating *P. aeruginosa* infections has become a major challenge. The World Health Organization (WHO) recently identified carbapenem-resistant *P. aeruginosa* as one of three bacterial species for which new antibiotics are urgently needed to treat infections. Furthermore, overuse of antibiotics during treatment hastens the emergence of multidrug-resistant *P. aeruginosa* strains, rendering scientific antibiotic therapy

ineffective against this microbe. Especially that there are multiple pathways of *P. aeruginosa* used to counter antibiotic attack can be divided into intrinsic, acquired, and adaptive resistance to a range of antibiotics, including aminoglycosides, quinolones, and β-lactams. Low outer membrane permeability, expression of efflux pumps that remove antibiotics from the cell, and development of antibiotic-inactivating enzymes are all part of the *P. aeruginosa* intrinsic resistance. *P. aeruginosa* acquired resistance can be accomplished by horizontal resistance gene transfer or mutational changes (Pang *et al.*, 2019). The development of resistant strains has become a threat in Malaysia to the point of urgency for novel solutions (Fazlul *et al.*, 2011; Fazlul, 2018).

1.2 Choline kinase inhibitors

Choline kinase inhibitors (ChoKIs) are known anti-cancer agents with promising prospects to be applied elsewhere. The chief ChoKI, Hemocholinium-3 (HC-3) is a well-characterized human choline kinase (ChoK) inhibitor with known inhibitor activity on both cancerous and parasitic cells. HC-3 directly competes and displaces the choline substrate from the active site of the human ChoK. HC-3 has been shown to inhibit cell division, minimize the development of lipoteichoic acid (LTA), deform the cell wall, and block cell division of *Streptococcus pneumoniae* ChoK activity. However, since HC-3 has a high MIC and IC_{50} (5400 M and >2700 M, respectively), it was essential to find stronger *S. pneumoniae* ChoK inhibitors. MN58b and RSM-932A were found to be more potent via a colorimetric system developed for quantifying ChoK enzymatic activity in cell extracts. Both drugs were found to be several orders of magnitude more powerful than HC-3, emulating eukaryotic system effects (Zimmerman *et al.*, 2020).

1.3 General objective

To perform *in silico* analysis and molecular modelling of *P. aeruginosa* putative choline kinase protein as well as molecular docking of potential inhibitors with the modelled structure.

1.4 Specific objectives

- To analyze the amino acid sequence of PaChoK to determine basic physicochemical parameters such as molecular weight, isoelectric point, aliphatic index, hydropathicity (GRAVY), estimated half-life, instability index, and the extinction coefficient.

- To compare the amino acid sequence of *P. aeruginosa* and align it with choline kinases from other organisms especially from bacteria (including human choline kinase).

- To construct a model of PaChoK.

- To determine the docking site of the choline kinase inhibitor hemicolinium-3 (HC-3) with the model structure of PaChoK

1.5 Rationale of the Study

With the antimicrobic resistance (AMR) running unchecked including the AMR developed by *Pseudomonas aeruginosa*, novel antimicrobics are in desperate demand. To establish the first step towards drug discovery, we need to bioinformatically discover brand new compounds or repurpose existing ones such as the choline kinase inhibitors (ChoKIs). Therefore, an *in-silico* approach to search for potential PaChoK was carried out. The potential inhibitors might be able to cause growth retardation of sturdy bacteria such as *P. aeruginosa*.

Chapter II

Literature review

CHAPTER 2

LITERATURE REVIEW

2.1 Pseudomonas aeruginosa bacterium

P. aeruginosa is the most well-known and studied member of the *Pseudomonas* genus, whose members are known for their metabolic versatility. *Pseudomonas syringe* pathovars are essential to plant pathogens, while *P. aeruginosa* infects immunocompromised people and people with cystic fibrosis (CF). *P. aeruginosa* is a Gram-negative bacterium with asymmetric phospholipid bilayer in the cytoplasm and an asymmetric outer membrane with a phospholipid inner face and a lipopolysaccharide outer layer that creates a permeability barrier. *P. aeruginosa* outer membrane comprises a variety of proteins, including lipoproteins and channels. β-barrel proteins, which produce water-filled diffusion channels and give these membranes a molecular sieve-like appearance, orchestrate nutrient exchange across the outer membrane. Because of their pore shape and function, these channels were first called porins and were studied using electron microscopy and conductance measurements in planar lipid membranes (Chevalier *et al.,* 2017).

Many of the outer membrane proteins of *P. aeruginosa* are highly conserved, according to studies of isolated outer membranes. Many of the outer membrane proteins from these strains are antigenically cross-reactive, even though there are various serologic forms (based on antigen evaluations of O-specific antigens) (Bitton *et al.,* 2015).

Porins are classified into three families: structural porins from the OmpA family, small porins from the OmpW family (8 β-sheets), and larger diffusion porins with 18 β-sheets. Larger TonB-dependent receptors for the absorption of siderophores, heme, and organic sulfur molecules are still present in the outer membrane, with 22 anti-parallel

β-sheets. The TonB inner membrane protein energizes them, relaying the proton motive force and opening the gate to allow large molecules to pass through. Other specialized channels engage in the efflux of harmful molecules such as antibiotics or secretion systems (Chevalier *et al.*, 2017).

P. aeruginosa is a Gram-negative rod that measures 0.5 to 0.8 m by 1.5 to 3.0 m. Almost all strains are motile thanks to a single polar flagellum, though some have two or three (Figure 2.1). Heat-labile antigens are produced by flagella (H antigen). Apart from its utility in serologic classification, the importance of antibodies directed against these antigens is unknown. Clinical isolates frequently have pili, which are antiphagocytic and likely help in bacterial attachment and colonization. *P. aeruginosa* cell envelope, which is identical to that of other Gram-negative bacteria, is made up of three layers: the peptidoglycan plate, the inner or cytoplasmic membrane, and the outer membrane. Phospholipid, protein, and lipopolysaccharide make up the outer membrane (LPS). *P. aeruginosa* LPS is less toxic than that of other Gram-negative bacteria. In addition to sidechain and core polysaccharides, most strains of *P. aeruginosa* LPS include heptose, 2-keto-3-deoxyoctonic acid, and hydroxy fatty acids. Recent evidence indicates that the LPS of a significant percentage of cystic fibrosis strains contains little to no polysaccharide side chain (O antigen) and that this result is linked to the strains' poly-agglutin-ability with typing sera (Bitton *et al.*, 2015).

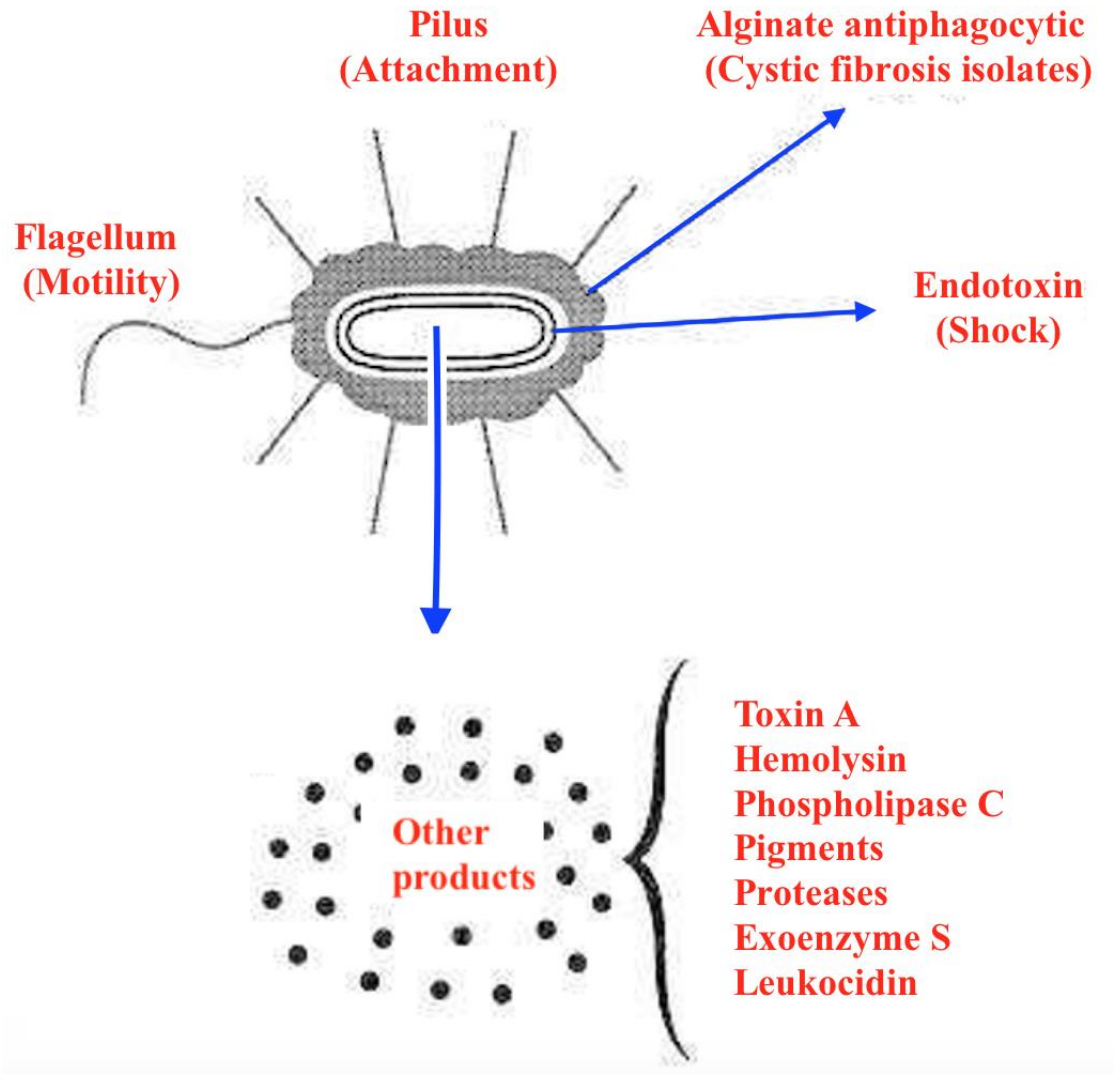

Figure 2.1 *P. aeruginosa* cell structure and pathogenic mechanisms. Modified from (Bitton *et al.*, 2015) .

P. aeruginosa is a non-fermentative bacterium that gets its energy from oxidation rather than carbohydrate fermentation. It can grow on media containing only acetate for carbon and ammonium sulphate for nitrogen, even though it can use more than 75 different organic compounds. It can also expand anaerobically, using nitrate as an electron acceptor, despite being aerobe. This organism grows well at temperatures ranging from 25 to 37 degrees Celsius, but it can also grow slowly or live at higher and lower temperatures. Its ability to grow at 42°C sets it apart from many other *Pseudomonas* species. Therefore, this bacterium has adapted to living in harsh environment. *P. aeruginosa* is resistant to high concentrations of salt, dyes, poor antiseptics, and many widely used antibiotics, in addition to its nutritional versatility. These characteristics clarify its widespread distribution and contribute to its dominance as a source of nosocomial infections (Bitton *et al.*, 2015).

2.2 Genome structure

The genomes of *P. aeruginosa* strains are wide (5–7 Mbp). Their metabolic potential is vast, as shown by their ability to generate a wide range of secondary metabolites and polymers, as well as their ability to use a wide range of carbon sources and electron acceptors. The large number of regulatory genes and networks found in *P. aeruginosa* genomes indicate the highest proportion of regulatory genes and networks found in known bacterial genomes, which is essential for the bacteria to react and adapt to a variety of environments (Moradali, Ghods & Rehm, 2017).

The versatility and adaptability encoded in the *P. aeruginosa* genome contribute significantly to its effectiveness as an opportunistic pathogen. According to the NCBI Entrez database, 36 strains of *P. aeruginosa* from clinical and environmental sources had been entirely or partially sequenced as of September 2012. *Pseudomonas* has a relatively broad genome, ranging from 6.22 to 6.91 Mb when compared to most other

bacteria that cause disease. The genome is organized as an array of conserved regions interspersed by regions of genomic plasticity that contain genes unique to each strain, according to the sequencing of multiple strains. As a result, *P. aeruginosa* has been classified as having a ‘core' genome, which contains a conserved collection of genes common to the genus and accounts for up to 90% of genomic material, and an ‘accessory’ genome, which contains genes contained in only a few strains. The large number of paralogous genes that have developed from genetic replication, since they evolved independently to produce families of gene products that overlap functionally but have distinct properties or are regulated differently, is a key feature of the P. aeruginosa genome. When combined with *P. aeruginosa* increased metabolic and functional diversity, it appears that the *P. aeruginosa* genome evolved as a result of selective pressure for environmental adaptability (Silby *et al.*, 2011).

P. aeruginosa is known for its metabolic versatility and has been isolated from a variety of nutrient-poor environments, including medical facility surfaces. Any real or imagined hydrocarbon can be catabolized by a *Pseudomonas* species given oxygen or nitrite and enough time, according to a popular anecdote among *Pseudomonas* scientists. In the laboratory, *Pseudomonas* prefers to develop on tricarboxylic acid (TCA) intermediates over sugars (mediated by CbrAB/Crc/CrcZ), and the sequencing of strain PAO1 (the first strain to be sequenced) revealed 300 cytoplasmic transport systems and a large number of genes encoding enzymes predicted to be involved in -oxidation of various carotenoids (Stover *et al.*, 2000).

2.3 Pathogenicity of Pseudomonas aeruginosa

Many factors produced by *P. aeruginosa* may contribute to its virulence. On blood agar plates, almost all strains of *P. aeruginosa* are hemolytic, and much hemolysis

have been identified. Purification of a heat-stable hemolytic glycolipid containing two molecules of L-rhamnose and one molecule of 1-β-hydroxydecenoic acid While this hemolytic glycolipid is not particularly toxic to animals (5 mg intraperitoneally is enough to destroy a mouse), it is toxic to alveolar macrophages. Furthermore, *P. aeruginosa* strains isolated from respiratory tract infections produce more haemolysing than environmental strains, implying that this glycolipid haemolysing is involved in *P. aeruginosa* pulmonary infections (Gregson, Thomas & Elphick, 2021).

Several important virulence factors are known or suspected to play a role in the pathogenesis of respiratory infections (Figure 2.2). However, since clinical evidence is inherently correlative and can be skewed by multiple mutations in a single isolate or the existence of multiple isolates with different genotypes and phenotypes, the contribution of particular virulence factors to human disease is rarely established. Flagella and type 4 pili are some of these key virulence factors. A single polar flagellum and several much shorter type 4 pili are found at the cell pole of each *P. aeruginosa* cell. These proteinaceous appendages serve as adhesins as well as significant motility mechanisms. Inflammatory responses may also be triggered by flagella and pili (Gellatly & Hancock, 2013).

In an aqueous environment, the whip-like flagellum provides swimming motility through a rotating corkscrew motion and is an important component of bacterial chemotaxis. Tumbles, in which flagella rotation is temporarily reversed and motility is halted to allow the bacterium to reorient itself, are interspersed with bursts of straight-line swimming. During an infection, the bacterium will cling to host epithelial cells by binding its flagellum to the asialyated glycolipid asialoGM1, as well as elicit a strong NFκB-mediated inflammatory response through TLR5 signalling and a caspase-1-mediated response via the Nod-like receptor (Miao *et al.*, 2007).

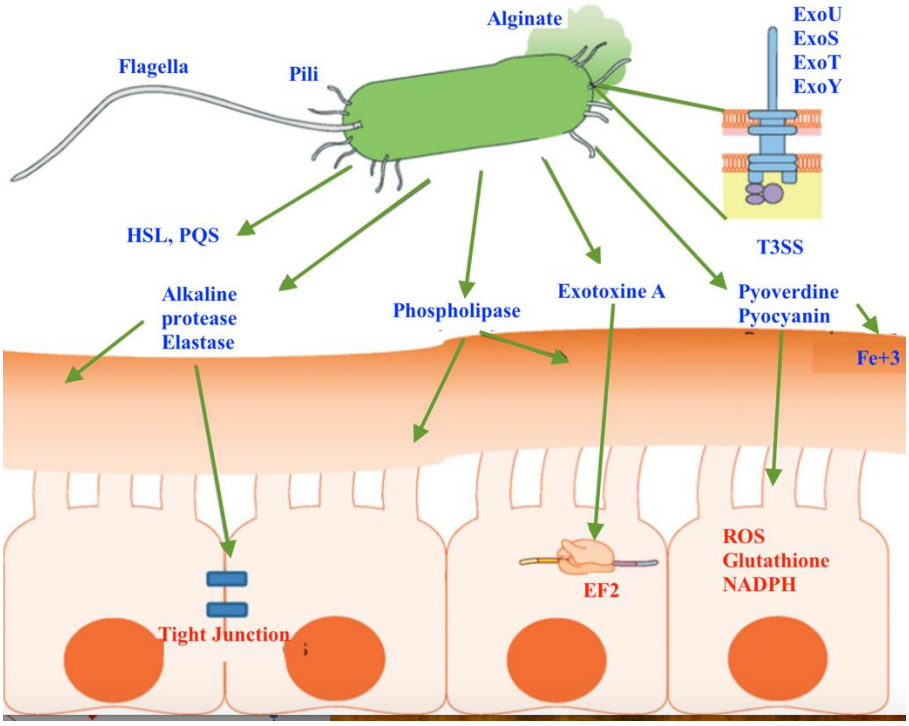

Figure 2.2 *P. aeruginosa* virulence factors. The major adhesins are flagella and type 4 pili, which can bind to the host epithelial gangliosides asialoGM1 and asialoGM2. These surface appendages, like lipopolysaccharide, are highly inflammatory. After contacting the host epithelia, the T3SS is triggered, allowing it to inject cytotoxins directly into the host cell. P. aeruginosa secretes some virulence factors, each of which has a different effect on the host. Several proteases are made, which can degrade host complement factors, mucins, and disrupt epithelial tight junctions, allowing bacteria to spread. Lipases and phospholipases can attack both surfactant lipids and host cell membrane lipids. Pyocyanin, a blue-green pigment, can disrupt electron transport and redox cycling in the host cell. Pyoverdine binds to Fe 3+ to give it a competitive advantage in a world where free iron is scarce. Obtained from (Gellatly & Hancock, 2013).

Type 4 Pilli are the most essential adhesins of *P. aeruginosa*, and they are also involved in twitching motility and biofilm formation. Type 4 pili extend and retract like grappling hooks to pull the cell along solid surfaces through a process known as "twitching motility". Swarming motility, a highly organized type of motility on semi-solid surfaces, is facilitated by pili in conjunction with flagella. Pili may also cause aggregation, which causes bacteria to form microcolonies on target tissues, essentially trapping the bacteria in one place and potentially protecting them from the host immune system and antibiotics. Microcolonies of *P. aeruginosa* have been found in the sputum of chronically infected CF patients, and they mimic mucoid colonies grown in the lab (Gellatly & Hancock, 2013).

Another key virulence factor is the type 3 secretion system (T3SS). Many pathogenic Gram-negative bacteria use T3SS as a way of directly injecting toxins into host cells. As a result, the T3SS of *P. aeruginosa* is a key determinant of virulence, and its expression is often related to acute invasive infections and increased mortality in infected patients. The T3SS's needle-like appendage, which evolved from flagella, allows effector proteins from the bacterium to enter the host cell through a pore created in the host cell membrane. Only four effectors have been identified – ExoY, ExoS, ExoT, and ExoU – Compared to many other well-studied T3SS, there are even fewer (e.g. Salmonella enterica SPI-1 has 13, Shigella sp. have 25) (Hauser, 2009).

2.4 Diagnosis and clinical importance of *Pseudomonas aeruginosa*

The isolation and laboratory identification of *P. aeruginosa* are needed for diagnosis. It can be isolated on blood agar plates or eosin-methylthionine blue agar, and it grows well on most laboratory media. It is recognized by its Gram morphology, inability to ferment lactose, positive oxidase reaction, fruity odour, and ability to grow at 42°C. Fluorescence under ultraviolet radiation aids in the early detection of *P.*

aeruginosa colonies and can also be used to indicate the presence of the bacteria in wounds. Specific laboratory methods are used to identify other pseudomonads (Gregson, Thomas & Elphick, 2021).

P. aeruginosa is an opportunistic human pathogen that can cause a wide range of life-threatening acute and chronic infections, particularly in patients with weakened immune systems. It is especially important because it is the leading cause of morbidity and mortality in cystic fibrosis (CF) patients, as well as one of the most common nosocomial pathogens affecting hospitalized patients, and it's intrinsically resistant to a broad variety of antibiotics. *P. aeruginosa* is also linked to several hospital-acquired infections (Table 2.1), such as ventilator-associated pneumonia, central line-associated bloodstream infection, urinary catheter-related infection, and surgical/transplantation infections. *P. aeruginosa* nosocomial infections have become a worldwide healthcare concern, according to the International Nosocomial Infection Control Consortium (Moradali, Ghods & Rehm, 2017).

Patients' poor health, the high carriage rate of sometimes multidrug-resistant strains in hospital wards, and previous use of broad-spectrum antibiotics all lead to the high incidence of *P. aeruginosa* in healthcare facilities. Acute lung infections can also affect people who are unable to mount a proper immune response. Immune defects such as old age, neutropenia from cancer chemotherapy, or immunosuppression from an organ transplant can all predispose to *Pseudomonas* infection. As a result, community-acquired pneumonia is more common in these patients than in otherwise healthy patients. Since immune-compromised patients are often hospitalized and thus exposed to *Pseudomonas* reservoirs in the healthcare environment, nosocomial infections are also common (Gellatly & Hancock, 2013).

Table 2.1 Common pseudomonal infections and risk factors. Adapted from (Gellatly & Hancock, 2013).

Infection	Major risk factors
Soft tissue	Burns, open wounds, postsurgery
Urinary tract	Use of urinary catheter
Bacteraemia	Immunocompromised
`	Diabetes, impaired microvascular circulation
Respiratory/pneumonia	Old age, chronic obstructive pulmonary disease, cystic fibrosis, mechanical ventilation
Otitis externa (swimmer's ear)	Tissue injury, water blockage in the ear canal
Keratitis (corneal infection)	Extended contact lens wear, contaminated contact lens solution
Otitis media folliculitis (hot tub rash)	Improperly cleaned hot tubs

2.5 Treatment of Pseudomonas aeruginosa

Antibiotics that are commonly used to treat *P. aeruginosa* infections include aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase (colistin, polymyxin B) (Bassetti *et al.*, 2018).

Proper isolation procedures, aseptic technique, and thorough cleaning and monitoring of respirators, catheters, and other instruments are the best ways to prevent *P. aeruginosa* from spreading. Topical antibacterial agents like mafenide or silver sulfadiazine applied to burn wounds, combined with surgical debridement, have greatly decreased the occurrence of *P. aeruginosa* sepsis in burn patients. Many widely used antibiotics are immune to *P. aeruginosa*. Even though several strains are susceptible to gentamicin, tobramycin, colistin, and amikacin, resistant strains have emerged, necessitating susceptibility testing. Treatment of severe *Pseudomonas* infections with gentamicin and carbenicillin is common, particularly in patients with leukopenia. Several vaccines are currently being tested, but none are ready for widespread use (Gregson, Thomas & Elphick, 2021).

2.6 Antimicrobial resistance mechanisms of *Pseudomonas aeruginosa*

Multidrug resistance (MDR) has skyrocketed in recent years, and it is now recognized as a major global challenge. Several studies have looked into the factors that contribute to the growth of MDR strains. In a case-control study in Brazil, 142 patients with Metallo-lactamases (MBLs) strains were compared to 26 patients with non-MBLs strains. ICU stay and urinary tract infection was found to be important factors in MBL infections in the multivariate analysis. MBLs strains were also linked to a quicker onset of infection and progression to death (Bassetti *et al.*, 2018).

P. aeruginosa infections are notoriously difficult to treat due to the bacteria's inherent ability to withstand certain antibiotic groups as well as its ability to develop resistance. This bacterium will exhibit all established mechanisms of antibiotic resistance (intrinsic, acquired, and adaptive), often all within the same isolate (Tables 2.2 and 2.3). Despite the use of combination drug treatments, resistance is on the rise (Moore & Flaws, 2011).

Intrinsic resistance is encoded in the chromosome of the microorganism. The poor permeability of *P. aeruginosa* outer membrane, the constitutive expression of membrane efflux (Mex) pumps, and the normal occurrence of an inducible chromosomal -lactamase, AmpC, all contribute to intrinsic resistance. Acquired resistance can be the result of mutations in targets or genes, including regulators, that stabilize or enhance intrinsic resistance mechanisms, or it can be the result of the genetic transfer and subsequent expression of a resistance cassette taken up by the bacterium. Plasmids and transposons are DNA elements that can be transferred between bacteria through conjugation, transformation, or transduction and can confer antibiotic resistance to an otherwise susceptible recipient. These elements can also improve *P. aeruginosa* natural resistance. Adaptive resistance occurs when environmental stresses, such as subinhibitory antibiotic concentrations, or growth states, such as biofilm formation, swarming or surfing motility, or contact with epithelial surfaces, cause an increase in resistance. These circumstances result in a shift in gene expression, which leads to the upregulation of genes that impart resistance (Gellatly & Hancock, 2013).

Table 2.2. *P. aeruginosa* resistance mechanisms examples. Adapted from (Gellatly & Hancock, 2013).

Mechanism	Resistance class	Example(s)
Efflux pumps	Intrinsic	MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM (cephalosporins, carbapenems, aminoglycosides, quinolones, ureidopenicillins)
Outer membrane impermeability	Intrinsic	OprF, OprD, OprB (carbapenems, aminoglycosides, quinolones)
β-lactamases	Intrinsic	AmpC (penicillins)
Targeted mutation	Acquired	DNA gyrase, DNA topoisomerase (quinolones)
MexZ (quinolones, cefapimes, aminoglycosides)		
Horizontal transfer	Acquired	Metallo-β-lactamases extended-spectrum β-lactamases (penicillins, cephalosporins, carbapenems)
Membrane changes	Adaptive	Lipid A modification (aminoglycosides, polymyxins)

Table 2.3. *P. aeruginosa* resistance mechanisms that are chromosomally encoded or imported. Adapted from (Bassetti *et al.*, 2018).

Location	Resistance mechanisms	Targeted antibiotics	Type of resistance
Intrinsic (chromosomal)	AmpC–type cephalosporinase	β-lactams	Antibiotic inactivation
	Class D oxacillinase OXA-50	β-lactams	Antibiotic inactivation
	Aminoglycosides inactivating enzymes	Aminoglycosides	Antibiotic inactivation
	Efflux systems (overexpression)	Multiple antibiotic classes	Efflux systems
	Decreased membrane permeability	Multiple antibiotic classes	Membrane impermeability and purines
	DNA gyrase and topoisomerase IV	Fluoroquinolones	Target modification
	LPS modification	Colistin	Target modification
Imported (Mobile genetic elements)	Class A serine β-lactamases (PSE, CARB, TEM)	β-lactams	Antibiotic inactivation
	Class B Metallo-β-lactamase (IMP, VIM, SPM, GIM)	Carbapenems	Antibiotic inactivation
	Class A serine carbapenemase (KPC)	Carbapenems	Antibiotic inactivation
	Class D carbapenemase (OXA-types: OXA-40)	Carbapenems	Antibiotic inactivation
	Aminoglycosides inactivating enzymes	Aminoglycosides	Antibiotic inactivation
	Ribosomal methyltransferase enzymes	Aminoglycosides	Target modification

2.7 Choline kinase

ChoK is expressed by bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Clostridium perfringens*, and *Clostridium botulinum*. Thousands of bacterial species are known to carry a putative ChoK gene, suggesting that ChoK inhibitors could be used to control the development of some of them (Zimmerman, Lacal & Ibrahim, 2019).

ChoK is considered a promising drug target for Gram-positive bacteria as well as Gram-negative bacteria (Zimmerman, Lacal & Ibrahim, 2019), especially after the successful inhibition of the *S. pneumoniae* ChoK. The anti-cancer agents, ChoKIs have been tested with promising results on *S. pneumoniae* ChoK such as Hemicholinium-3 (Zimmerman & Ibrahim, 2017) and the more potent RSM-932A and MN58b (Zimmerman *et al.*, 2020) would be the ideal candidate to target the ChoK of other bacteria such as *P. aeruginosa*.

P. aeruginosa cell envelope contains lipopolysaccharide (LPS), which occupies the outer leaflet of the outer membrane in this Gram-negative opportunistic pathogen. It is a significant virulence factor for this organism and is essential for bacterium–host interactions. *P. aeruginosa* LPS is made up of three different domains: lipid A, core oligosaccharide, and distal O antigen (O-Ag) (Lam *et al.*, 2011).

Bacterial ChoKs, like their eukaryotic relatives, phosphorylate choline (Cho) into phosphorylcholine (ChoP) (Zimmerman *et al.*, 2020). ChoP is a precursor molecule used to make LPS. The choline transporter is responsible for extracellular choline uptake (licB). ChoP is then activated in the cytoplasm by phosphorylcholine cytidylyl transferase (licC), which uses cytidine triphosphate to convert it to CDP-choline (CTP). Phosphorylcholine transferases, then transfer ChoP from CDP-choline to LPS (Figure 2.3) (Khalifa, Few & See Too, 2020). Therefore, inhibition of ChoK would be detrimental to the *P. aeruginosa* bacterium.

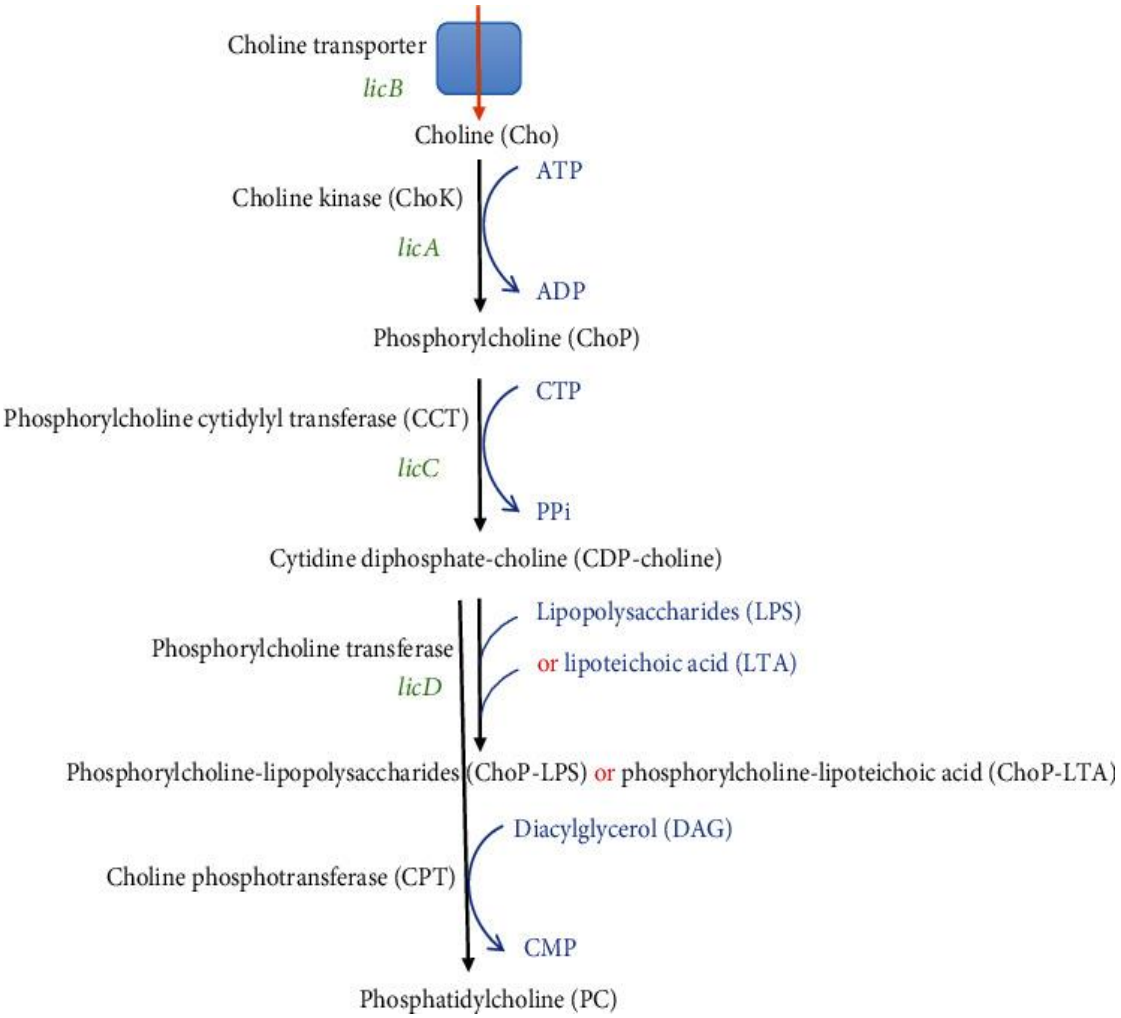

Figure 2.3. Bacterial ChoK phospholipid synthesis pathway. Obtained from (Khalifa, Few & See Too, 2020).

2.8 Molecular modeling and molecular docking

Molecular docking is a method which analyses the conformation and orientation (referred together as the “pose”) of molecules into the binding site of a macromolecular target. Searching algorithms generate possible poses, which are ranked by scoring functions (Torres *et al.*, 2019). Virtual screening is a cost-effective way to complete the screening of millions of chemicals in a matter of days (Wang & Zhu, 2016).

Finding protein targets for a query ligand is an essential part of drug development. In modern times, the drug design method is heavily reliant on Ehrlich's assumption that drugs function as "magic bullets," modulating a single target of particular interest to a disease. This basic assumption has had a lot of success, but it has also had some drawbacks in recent years. The most obvious drawback is the high attrition rate (roughly 90%) of new compounds in late-stage clinical trials due to efficacy and clinical safety issues. According to recent research, each current drug binds to an average of six target proteins rather than only one. If all interested ligand targets can be detected early in the development of a new drug, side effects, and toxicities that occur later in clinical trials can be effectively prevented. As a result, a prescreening procedure will greatly improve the success rate and lower the total production cost of a drug pipeline. This where molecular docking steps in for the prediction of a query ligand binding mode (such as a small-molecule drug) and affinity against a receptor (such as a target protein) as illustrated in figure 2.4 (Xu, Huang & Zou, 2018).

The most popular technique, which has been in use since the early 1980s, is molecular docking. The molecular docking method can be used to model the atomic level interaction between a small molecule and a protein, allowing us to characterize small-molecule activity in target protein binding sites as well as to elucidate fundamental biochemical processes. The docking process consists of two main steps: