2

REVIEW OF LITERATURE

The vast literature available pertained to the present study entitled **"Structure Based Virtual Screening and Validation of Potential Quorum Sensing Inhibitors Against LasR in *Pseudomonas aeruginosa*"** has been reviewed and presented under the following headings.

2.1. Biofilms

One of the preferred growth states for bacteria is a biofilm which exists in more than 90% of bacteria (Li and Lee, 2017). Naturally, microorganisms exist either as free-floating cells or enclosed within an architectural structure known as biofilms (Harmsen *et al.*, 2010). Biofilms may be regarded as "Microbial communities consisting of

various bacterial cells living in close association by encasing itself in an extracellular matrix made up of polymeric substances, adhered to a substratum or each other and exhibit an altered phenotype" (Wojtyczka *et al.,* 2014). Biofilms are heterogenous, with 15% of cells usually in microcolonies and 85% comprising of polymeric extracellular substances. The composition of the biofilm matrix varies among different species but generally contains proteins, polysaccharides, and nucleic acids (Flemming and Wingender, 2010).

Biofilms are polymicrobial with a tremendous rivalry for nutrients and space. The cohabitation of numerous microbes on a surface promotes cooperative behaviours such as metabolic cooperation, horizontal gene transfer, and other synergies leading to an increased potential for microorganisms to survive and exhibit resistance to antimicrobial agents (Wolcott *et al.,* 2013). In such an environmental niche, the bacterial communities are regulated by various biological processes and use advanced genotypic events to promote different molecular mechanisms and phenotypes necessary for survival in the new environment during pathogenesis and antibiotic treatment (Jamal *et al.,* 2018).

Biofilms form on many surfaces, including living tissues, hotels, industrial places, labs, wastewater channels, bathrooms, and indwelling medical devices. They are frequently found on hard surfaces immersed in or exposed to an aqueous solution. Nearly 99.9% of all microbes can develop biofilms on biotic and abiotic surfaces (Vetrivel *et al.,* 2021). The presence of biofilms on artificial surfaces imparts its significance in pathogenicity as they show resistance to cellular immunity in the host, antimicrobial, and biocide treatments (Harmsen *et al.,* 2010). Anton Van Leeuwenhoek first discovered this biofilm concept in 1684 when he observed a tooth's surface using a primitive microscope (Donlan and Costerton, 2002).

Adopting a standard clinical procedure could not easily remove an established biofilm. It could be detached only by eliminating the infected implant, which increases the trauma rate of the patients and treatment costs (Römling and Balsalobre, 2012). Biofilms cause infections by colonizing on inert surfaces, on dead tissues, on living tissues, and more commonly by dwelling on implanted devices like contact lenses, orthopaedic implants, urinary catheters, peritoneal dialysis catheters, central venous catheters, prosthetic joints, pacemakers, mechanical heart valves, voice prostheses, implantable electronic devices, and other orthopaedic and dental implants made up of composites and ceramics (Khatoon *et al.,* 2018). Other non-device-related infections are chronic infections,

periodontitis, and osteomyelitis. Microbial adhesion in implanted medical devices may be made of single or multiple types depending on the device and its duration of action. These adhesions are more severe and can cause life-threatening complications (Srivastava and Bhargava, 2016).

A report by the European Center for Disease Prevention and Control (2008) stated that annually around 4,100,000 patients acquire healthcare-associated infections (HAIs) in European hospitals, and the number of deaths due to these infections is estimated to be about 37,000. Mermel *et al.* (2001) stated that there are 200,000 cases of bloodstream infections in the United States annually due to implants of central venous catheters. According to reports, it is estimated that 449,334 patients are affected annually in US hospitals due to catheter-associated urinary tract infections (UTIs) (Klevens *et al.,* 2007).

Among the various biofilm-associated infections, UTIs are the most common bacterial infections affecting humans and serve as a public health issue (Flores-Mireles *et al.,* 2015). Nevertheless, in 2017, bloodstream infections associated with biofilms were ranked as the 12th leading cause of death, with an overall mortality rate of 15-30% (Pinto *et al.,* 2021). Wenzel and Edmond (2001) reported that approximately 80% of all microbial infections are of biofilm origin, of which 60-70% are nosocomial infections caused by biofilms on implanted medical devices. In a clinical environment, nosocomial infections account for merely 65% of hospital-acquired infections (Bryers, 2008).

According to the National Institute of Health (NIH), it is identified that biofilms formed by bacteria account for around 65% of infectious diseases caused by microbes and chronic infections for about 80% (Jamal *et al.,* 2018). The most common biofilm-associated infections caused in humans are chronic sinusitis, wound infection, osteomyelitis, prosthetic joint infection, prosthetic valve endocarditis, infections of cystic fibrosis patients, ventilator-related pneumonia, intravascular catheter, and breast implant infections (Reynold and Kollef, 2021). Thus, even after more than 80 years since the first report on biofilms (Zobell, 1943), there is still a need in various areas such as clinical, biomedical, industrial and environmental fields related to the problems encountered by biofilms.

2.2. Formation of biofilm

The growth of bacteria within biofilms is a naturally occurring phenomenon in which the whole microbe could be lively attached to an infection site. The ability of the

bacteria to colonize the environment and to mature as a biofilm on a surface is considered one of the survival strategies for biofilm-forming microorganisms (Lebeaux and Ghigo, 2012). In contrast to free-swimming planktonic cells, microorganisms are found as organized groups in biofilms that grow on diverse surfaces to constitute a distinct growth phase (Lerch *et al.*, 2017). The formation of biofilm is a complex and cyclic phenomenon involving transportation, diffusion, chemical reaction, and ecological evolution. It is controlled by regulatory mechanisms, namely bulk transport, adhesion, quorum sensing, detachment, death of cells, and dispersal (McCarty *et al.*, 2014).

Bacterial biofilms could be well established in a few hours. The formation of biofilm comprises four main stages: (a) Initial attachment of planktonic bacteria to a surface through physical forces and interaction occurs between bacteria and surface of attachment; (b) Adherent cells get attached to the surface irreversibly and encase themselves in extracellular polymeric substances matrix resulting in aggregation of cells; (c) Maturation of biofilm by microcolony formation to form a three-dimensional architecture of completely matured biofilm; (d) Release of microcolonies of cells from the matured biofilm to colonize new attachment site for spreading its infection (Jamal *et al.*, 2018). The steps in the biofilm formation cycle are depicted in Figure 1.

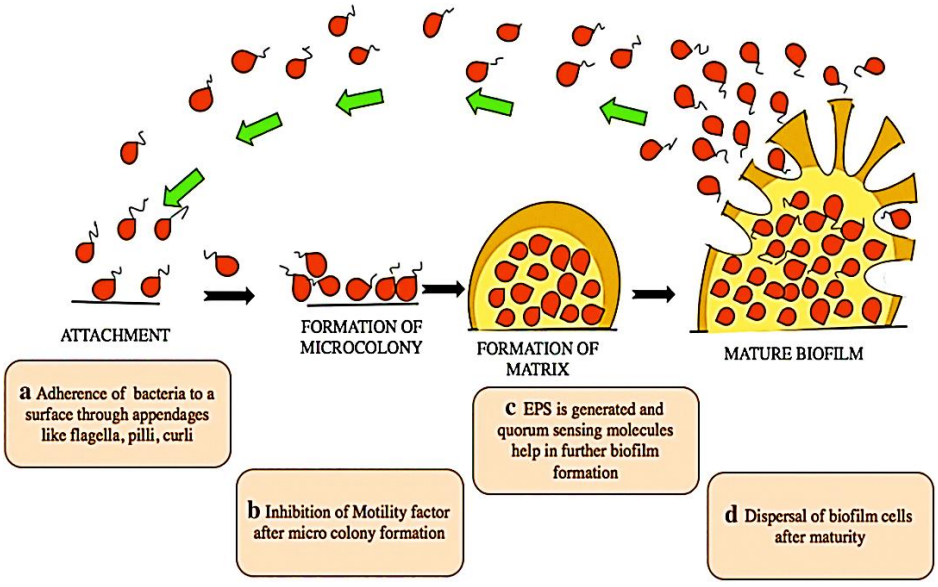

Figure 1

Steps involved in the biofilm formation cycle (Banerjee *et al.*, 2020)

The characteristic features of a matured biofilm, in general, are adherence to each other, adherence to either solid/liquid, solid/air, liquid/liquid, or liquid/air interfaces, attachment to surfaces, decreased antimicrobial susceptibility, decreased host defense

systems, the existence of one or more microbial species and three-dimensional structure (McCarty *et al.,* 2014). Synthesis of exopolysaccharides (EPS) matrix holds a crucial role in the biofilm development as it incorporates all the elements that make up 90% of the total organic matter found in the matrix material, the most important structural feature of bacterial biofilms (Limoli *et al.,* 2015).

The extracellular matrix renders the structural support and protection for bacteria in biofilms. The matrix is also involved in various other processes, such as adherence to surfaces, cell-to-cell communication, quorum sensing (QS), tolerance and deceiving nutrients (Harmsen *et al.,* 2010). The EPS of the matrix material holds all the biofilm cells in the near vicinity to enable intercellular interactions and facilitate the genetic material exchange by gene transfer method. It is reported that extracellular DNA (eDNA), another important constituent of the matrix, is necessary for pathogens to adhere and for its cell-to-cell coherence at the early stage of biofilm development (Strempel *et al.,* 2013).

The development of biofilm matrix and bacterial growth depend on factors, namely the availability of nutrients and hydrodynamic conditions. The cooperative interactions among species lead to various development states, structures, and functions of biofilm organization. Thus, biofilms are regarded as structural, architectural organizations of microorganisms that constantly evolve to adapt to their surroundings (Olivares *et al.,* 2020; Lee *et al.,* 2014). The matured biofilms can be detected by several biofilm detection methods that can be categorized into four divisions as physical, chemical, microscopical, and biological techniques (Azeredo *et al.,* 2017).

Typical types of potent biofilm-causing infectious microorganisms are *Pseudomonas aeruginosa, Burkholderia cepacia, Pseudomonas pseudomallei, Haemophilus influenza, Escherichia coli, Candida albicans, Streptococcus pyogenes, Streptococcus pneumonia,* other *Streptococcus species, Staphylococcus epidermidis,* and *Staphylococcus aureus* (Khatoon *et al.,* 2018). The various bacterial species involved in biofilm-associated infections and their adherent surfaces are listed in Table 1.

Among these, *Pseudomonas aeruginosa* is one of the most virulent opportunistic pathogens, which leads to a variety of acute infections and continues to possess a high rate of mortality and antibiotic failure (Jakobsen *et al.,* 2013). *Pseudomonas aeruginosa* was the fourth most frequently found pathogen, contributing to around 8% of chronic wound

infections, and the seventh leading pathogen, contributing to around 2% of bloodstream infections.

Table 1

Bacterial species causing biofilm-associated infections and their adherent surfaces

S. No	Bacterial species	Infection/Diseases	Surface	References
1	*Streptococcus mutans*	Dental caries, Endocarditis	Tooth surface, Vascular grafts	Metwalli *et al.*, 2013; Abranches *et al.*, 2011
2	*Enterococcus faecalis*	Endocarditis, Root canal infection	Heart valves, Urinary catheters, Teeth, Central venous catheters	Minardi *et al.*, 2012
3	*Klebsiella pneumonia*	Pneumonia, Respiratory tract infection, Urinary tract infection, Pyogenic liver abscess	Lungs, Liver	Chung, 2016
4	*Pseudomonas aeruginosa*	Nosocomial infection, Otitis media, Cystic fibrosis	Central venous catheters, Middle ear, Prostheses, Lungs, Contact lenses	Huse *et al.*, 2013; Wiley *et al.*, 2012
5	*Staphylococcus sp. (Staphylococcus aureus; Staphylococcus epidermidis)*	Nosocomial infections, Chronic wounds, Endocarditis, Mucoloskeletal infections, Otitis media	Sutures, Central venous catheters, Arteriovenous shunts, Prostheses, Surfaces/deep skin, Heart valves, Bones, Middle ear	Arciola *et al.*, 2012; Qu *et al.*, 2010
6	*Escherichia coli*	Bacterial prostatitis, Urinary tract infection, Otitis media	Prostheses, Urinary tract, Urinary catheters, Middle ear	Jackson *et al.*, 2002
7	*Haemophilus influenza*	Otitis media	Middle ear	Takei *et al.*, 2013; Diaz *et al.*, 2011
8	*Burkholderia cepacian*	Cystic fibrosis	Lungs	Zlosnik *et al.*, 2011
9	*Mycobacterium tuberculosis*	Tuberculosis	Lungs	Qvist *et al.*, 2014

Epidemiological studies have proved that infections of *Pseudomonas aeruginosa* could significantly increase the rate of mortality, morbidity, need for surgical intervention, chronic care, and overall cost of treatment (Rahim *et al.,* 2017). Hence, focusing on the powerful treatment strategies to prevent *Pseudomonas aeruginosa*-mediated biofilm infections is an area of concern.

2.3. Characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a virulent rod-shaped, Gram-negative bacterium belonging to the group Pseudomonadaceae and found extensively inhabiting the water, plants, soil, and animals. It hardly causes infections in healthy individuals but can easily cause infections in immune-compromised individuals (Balasubramanian *et al.,* 2013). For over a decade, *Pseudomonas aeruginosa* has been among the 'top 10' common hospital 'superbugs' because of its widespread antimicrobial-resistant strains that cause life-threatening complications (Streeter and Katouli, 2016). It is the most commonly isolated species from chronic wounds and is considered a potent biofilm producer since they act as a barrier in wound healing and exhibits high resistance to antimicrobial therapy (Mantero *et al.,* 2017; Davis and Brown, 2016).

According to US National Healthcare Safety (2007), *Pseudomonas aeruginosa* was ranked to be the sixth most commonly occurring organism responsible for nosocomial infections, second most common pathogen responsible for ventilator-related pneumonia, and seventh major causative pathogen of catheter-linked bloodstream infections accounting for high death rate in individuals with AIDS, cystic fibrosis, and burn wounds (Bail *et al.,* 2021; Valderrey *et al.,* 2010). Infections caused by *Pseudomonas aeruginosa* can be life-threatening when inadequate therapy is employed, mainly when multidrug-resistant (MDR) strains are involved, which has threatened human and animal health for the last 30 years (Ito *et al.,* 2021).

Furthermore, *Pseudomonas aeruginosa* is one of the most prevalent pathogens in hospital environments, causing more than 50% of healthcare-acquired infections (Sarabhai *et al.,* 2013). Even though new antimicrobial drugs have been developed, the mortality rate due to *Pseudomonas aeruginosa* infections remains high, in the range of 20-60% (Tuon *et al.,* 2020). The significant risk factors for *Pseudomonas aeruginosa* infections are structural lung diseases, haematological neoplasms, transplantation, skin burns, recent use of antibiotics, presence of implants, prolonged hospitalization, and

mechanical ventilation (Reynolds and Kollef, 2021). A diagrammatic representation of the major infections caused by *Pseudomonas aeruginosa* is provided in Figure 2.

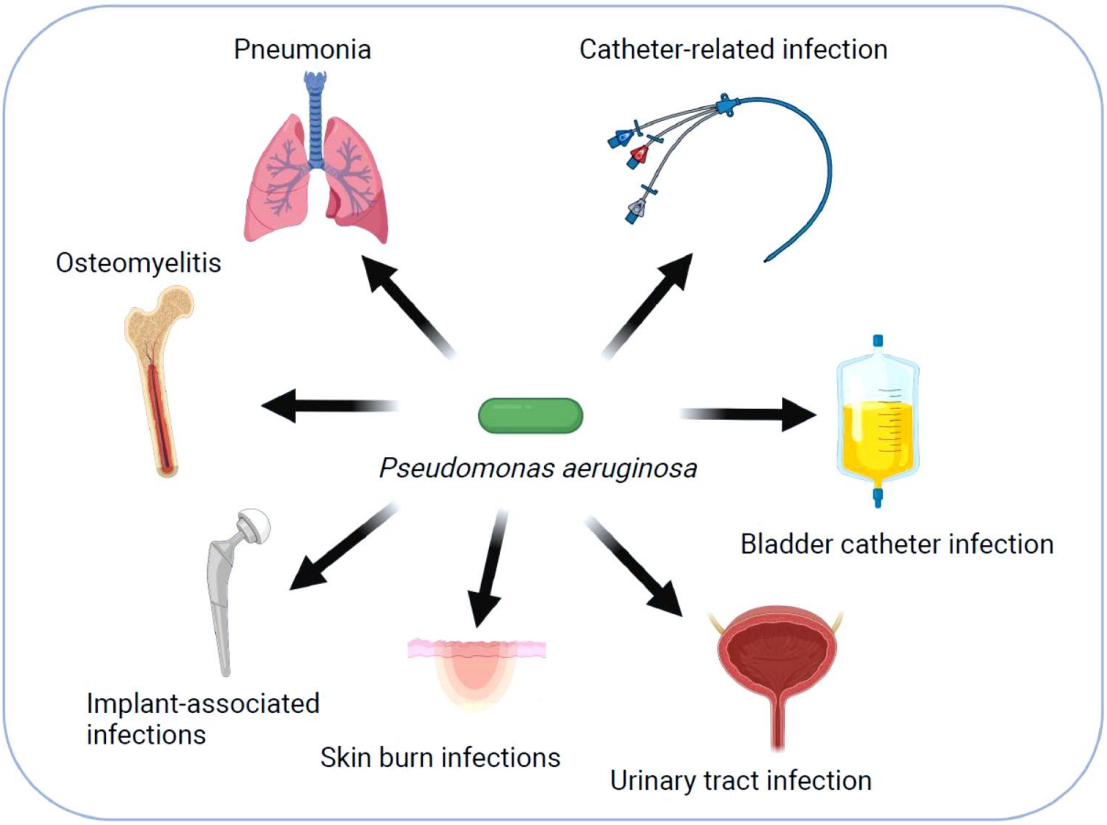

Figure 2

Schematic representation of major infections caused by *Pseudomonas aeruginosa* (Tuon *et al.*, 2022)

Pseudomonas aeruginosa causes infections with the aid of several cell-based virulence factors such as pili, lectins, alginate, lipopolysaccharide, and secreted virulence factors, namely, pyocyanin, cytotoxin, proteases, haemolysins, siderophores, exotoxin A, exoenzyme U, exoenzyme S, etc. (Strateva and Mitov, 2011). Regarding the pathogenesis of *Pseudomonas aeruginosa* infections, biofilm generation is the most critical virulence determinant, making it a suitable *in vitro* model for studying biofilm formation (Chang, 2017). Hence, *Pseudomonas aeruginosa* is identified as a pathogenic bacteria which colonizes several different surfaces, including medical materials and food industry equipment, and forms biofilms, leading to chronic infections and increased resistance to antibiotics (Tuon *et al.*, 2022).

2.4. *Pseudomonas aeruginosa* biofilm structure

The exopolysaccharides, namely alginate, Pel, and Psl, are the major constituents of the *Pseudomonas aeruginosa* biofilm matrix involved in surface adhesion, and together with eDNA, they determine the biofilm architecture. These EPS play an essential role in resistance to immune responses and antibiotic treatments (Gellatly and Hancock, 2013; Strempel *et al.,* 2013). The differential role of each EPS has been analysed at each stage of biofilm development (Ghafoor *et al.,* 2011). Various EPS, which exhibit different physiochemical properties, confers a survival strategy for increasing the flexibility and stability of biofilms under multiple conditions (Jennings *et al.,* 2015).

The Psl polysaccharide is a crucial element at the early stage of biofilm formation when cells explore surfaces for adhesion. It is anchored around cells in a helical arrangement initiating biofilm formation by enhancing cell migration, cell-cell interaction and cell-surface adhesion. In contrast, in mature biofilms it is located at the periphery of mushroom-shaped microcolonies (Zhao *et al.,* 2013). Psl can exist as a fibre-like matrix requiring type 4 pili-mediated migration of cells (Wang *et al.,* 2013). It protects cells against phagocytosis and oxidative stress during infection (Mishra *et al.,* 2012). Recent studies suggested that Psl can provide an instant protective role against antibiofilm agents and a broad spectrum of antibiotics, particularly at the early stage of biofilm development (Asma *et al.,* 2022; Billings *et al.,* 2013; Zegans *et al.,* 2012). Therefore, Psl provides a survival advantage during pathogenesis.

Similar to Psl, Pel is important for initiating and maintaining cell-cell interaction in biofilms (Colvin *et al.,* 2011). Pel and/or Psl are the primary matrix structural polysaccharides in non-mucoid *Pseudomonas aeruginosa* strains. However, the contribution of Psl and Pel to the structure of mature biofilms is strain-dependent, while both unique and functionally redundant roles have been reported for these exopolysaccharides (Colvin *et al.,* 2012). Recent studies elucidating Pel's chemical structure and biological function demonstrated that it is a major structural component of the biofilm stalk where it cross-links eDNA and structurally compensates for the absence of Psl in the periphery of mature biofilm (Le Mauff *et al.,* 2022; Jennings *et al.,* 2015). Furthermore, Pel was shown to protect bacteria against certain aminoglycoside antibiotics (Colvin *et al.,* 2011).

Alginate is predominately produced in the biofilm of mucoid *Pseudomonas* strains due to a mutation in the mucA22 allele. The mucoid phenotypes are found primarily on cystic fibrosis isolates, signifying the conversion from acute to chronic infection (Ciofu *et al.,* 2015; Folkesson *et al.,* 2012). Alginate is a negatively charged acetylated polymer consisting of mannuronic acid and guluronic acid residues (Ma *et al.,* 2012). A wide range of important functions of alginate, including biofilm maturation, protection from phagocytosis and opsonization, and decreased diffusion of antibiotics through the biofilm, has been well-documented (Hay *et al.,* 2013; Tseng *et al.,* 2013). The ratios between mannuronic acid and guluronic acid influence the viscoelastic properties of biofilms which lead to impairment of cough clearance in the lungs of cystic fibrosis patients infected with *Pseudomonas aeruginosa* (Gloag *et al.,* 2018).

eDNA is another important structural component for biofilm development, and along with the Pel polysaccharide, it can be detected within the stalks of mushroom-shaped microcolonies. However, eDNA has multifaceted roles in biofilm formation, such as contribution to the formation of cation gradients in the matrix via the chelating interaction of highly anionic DNA with cations such as Mg2+, Ca2+, Mn2+, and Zn2+, as a nutrient source during starvation, facilitating twitching motility and coordinating cell movements and conferring antibiotic resistance (Gloag *et al.,* 2013).

Among the proteinaceous biofilm constituents, both flagella and the type 4 pili are important during the maturation of the biofilm, but however, these cell appendages are not commonly considered classical matrix components of biofilms. Type 4 pili are important for adhesion and promote the initial attachment of cells to surfaces at the early stage of biofilm formation. Together with eDNA, flagella and the type 4 pili mediate migration required for the formation of the stalk and the cap in the mushroom-shaped microcolonies of the mature biofilm (Mann and Wozniak, 2012). The important polymeric substances contributing to the development of biofilm formation in *Pseudomonas aeruginosa* are provided in Table 2.

2.5. Multispecies biofilm and central network governing biofilm formation in *Pseudomonas aeruginosa*

Biofilms are polymicrobial structures, meaning that different microbial species can interact and coexist within the same biofilm community. In diseased conditions, microbial

interactions can affect a patient's prognosis. These synergic interactions of organisms are more significant than that produced by individual bacteria and can worsen the patient's outcome (Murray *et al.*, 2014). In this context, *Pseudomonas aeruginosa* could also grow and coexist with various microorganisms, including bacteria, fungi and viruses. Some of the clinically relevant microbial biofilm interactions of *Pseudomonas aeruginosa* are shown in Figure 3.

Table 2

Key polymeric substances in *Pseudomonas aeruginosa* biofilm formation and development

Name	Identity/Chemistry	Precursor(s)	Biosynthesis	References
Psl	Exopolysaccharide/Repeating pentasaccharide containing D-mannose, D-glucose and L-rhamnose	GDP-D-mannose, UDP-D-glucose and dTDP-L-rhamnose	The pslA-O operon	Colvin *et al.*, 2012
Pel	Exopolysaccharide/Partially acetylated (1→4) glycosidic linkages of N-acetylgalactosamine and N-acetylglucosamine	UDP-sugar nucleotide/ uncharacterized	The pelA-G operon	Jennings *et al.*, 2015
Alginate	Exopolysaccharide/O-acetylated (1→4) linked D-mannuronic acid and variable proportions of its 5-epimer L-glucuronic acid	GDP-mannuronic acid	The alginate operon (algD, alg8, alg44, algK, algE, algG, algX, algL, algI, algJ, algF, algA, and algC)	Hay *et al.*, 2010
eDNA	Nucleic acid	-	Cell lysis	Ma *et al.*, 2009
Type 4* pili	Multiprotein complex/Type 4a pili	-	The pilM/N/O/P/Q and the fimU-pilVWXY1Y2E operons	Burrows, 2012
Flagella *	Multiprotein complex	-	Atleast 41 genes clustered in three regions of the genome encode flagellin structural and regulatory components	Jyot and Ramphal, 2008

**Are not commonly considered classical matrix molecules of biofilm but are important for biofilm maturation.*

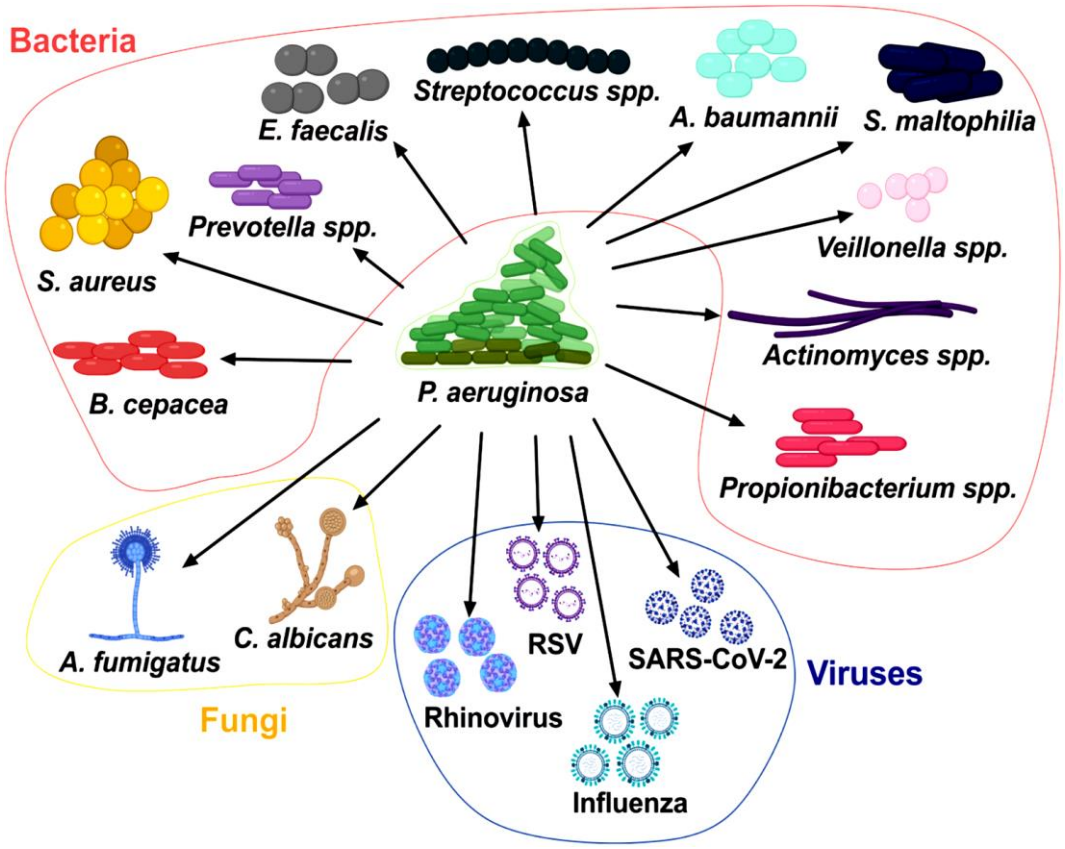

Figure 3

Scheme of the clinically relevant microbial biofilm interactions of *Pseudomonas aeruginosa* (Cendra and Torrents, 2021)

The transition from motility to sessility requires dynamic regulatory networks at transcriptional, post-transcriptional and Spost-translational levels resulting in the coordinated timely expression of hundreds of genes (Cendra and Torrents, 2021). During acute infection, the relationship between pathogen and host is reciprocally devastating as a variety of cytotoxic molecules produced by bacteria impair the host cellular processes while bacteria, on the other hand, encounter immune system responses such as the production of antimicrobial compounds and reactive oxygen species as well as enhanced phagocytosis.

In this case, motile *Pseudomonas aeruginosa* displays a more virulent phenotype. Various modes of *Pseudomonas aeruginosa* motility, such as swimming and swarming involving flagella and twitching using type 4 pili, are associated with virulent traits (Winstanley *et al.*, 2016). A motile cell is readily detectable by the host immune system via

flagellar and/or other motility components mediating recognition and induction of signaling pathways that trigger inflammatory responses and phagocytosis by murine or human macrophages (Amiel *et al.*, 2010).

Switching to a sessile lifestyle along with lower virulency is a survival advantage by which many pathogenic bacteria, such as *Pseudomonas aeruginosa*, evade stresses and adverse conditions. They lose motility and attach to surfaces and form cellular aggregations or microcolonies, which are embedded in EPS to protect bacteria from the surrounding environment. These structures are so-called biofilms confers an extreme capacity for persistence against phagocytosis, oxidative stresses, nutrient/oxygen restriction, metabolic waste accumulation, interspecies competitions, and conventional antimicrobial agents (Olsen, 2015). The central regulatory network governing the motility-sessility switch in *Pseudomonas aeruginosa* is shown in Figure 4.

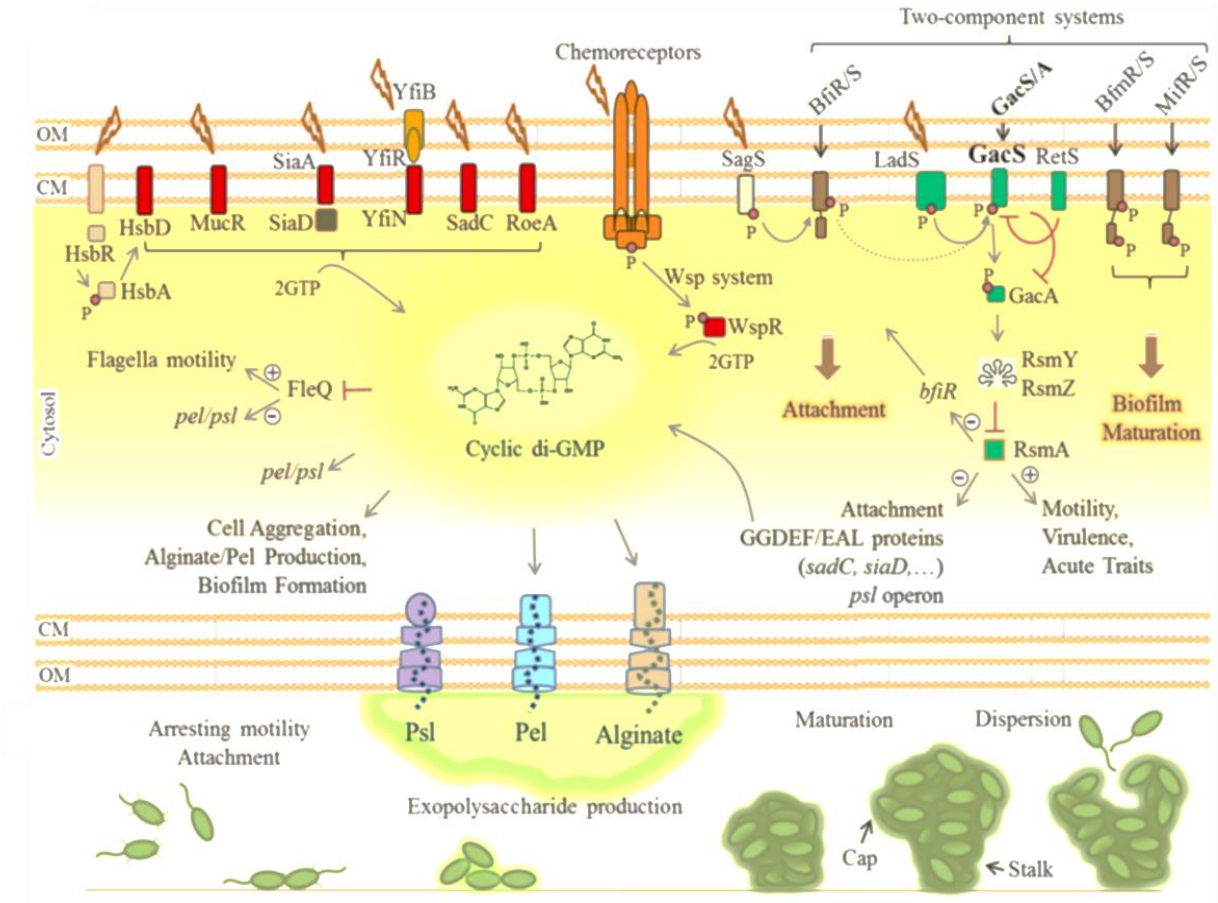

Figure 4

Regulatory networks underlying biofilm formation in *Pseudomonas aeruginosa* (Moradali *et al.*, 2017)

2.6. Role of quorum sensing in biofilm formation

An intercellular signaling system known as quorum sensing (QS) imparts a major part in the formation of biofilms by regulating gene expression using small molecules called autoinducers (AIs). The development and structural integrity of the biofilm is merely dependent upon QS. QS is a cell-to-cell interconnection mechanism that prevents cell density from reaching a threshold level to control its population density. A level at which the autoinducers reach a threshold concentration at a specific cell density is referred to as a "quorum level". At this level, autoinducers bind to their respective receptors to increase or decrease the activity of several genes responsible for maintaining biofilm size and coordinating phenotypic virulence. Thus, the viability of the biofilm community is always dependent upon quorum sensing or quorum diffusions (Dickschat, 2010).

Generally, QS networks in Gram-negative bacteria and Gram-positive bacteria are modulated by signaling molecules such as N-acyl homoserine lactones and oligopeptides, respectively. Another signaling molecule, namely, autoinducer-2 (AI-2), regulates QS in both types of bacteria. Gram-negative bacteria, *Pseudomonas aeruginosa,* access acyl homoserine lactones (AHLs) as their signaling molecule for regulating their QS networks. AHL molecules consist of a fatty acyl chain connected by an amide bond to lactonized homoserine. Different AHL molecules are synthesized by various bacterial species, or the same bacterial species may synthesize different AHLs. Variations in the composition of acyl chains contribute to various physiological and biochemical functions of the bacterial species (Drees and Fetzner, 2015).

A wide range of regulatory proteins involved in the QS mechanism of Gram-negative bacteria have been identified; among them, LuxR-type protein is the widely studied model since most of the members of this protein are AHL-responsive transcriptional activators. LuxR-type proteins have two domains, namely, an N-terminus acyl-HSL-binding site and a C-terminus DNA binding site. The binding of acyl-HSL to the N-terminus domain promotes configurational changes that enable multimerization and DNA binding for transcriptional activation of the associated promoters (Boursier *et al.,* 2018). The mechanism of quorum sensing is illustrated in Figure 5.

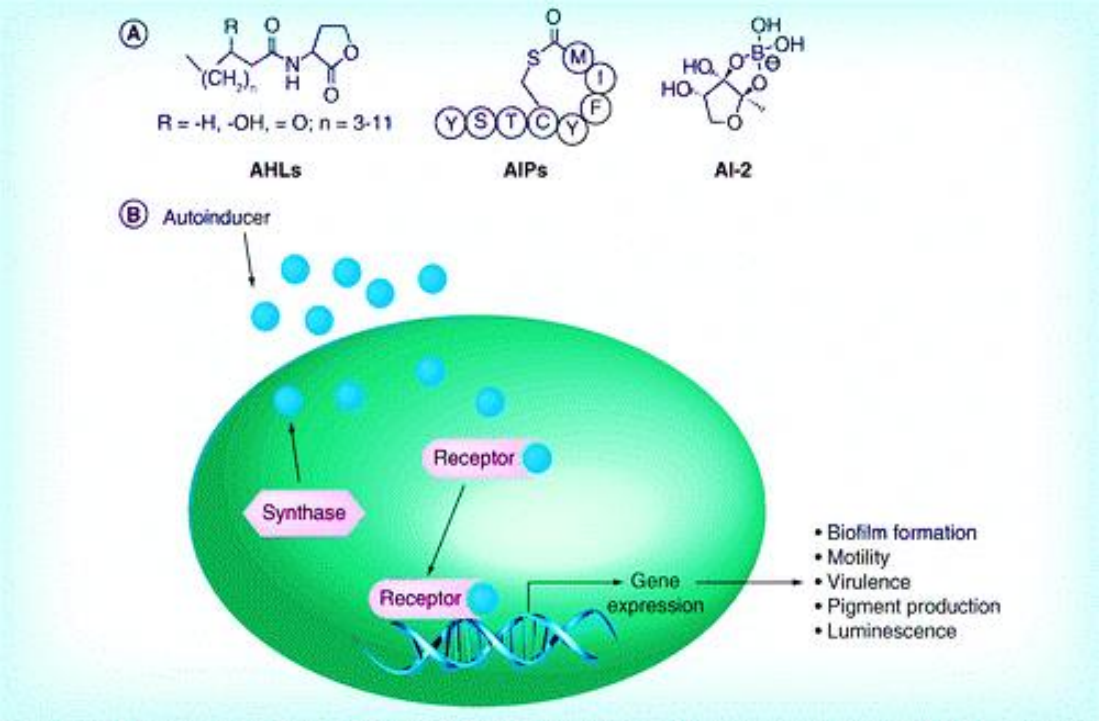

Figure 5

Quorum sensing mechanism (Rabin *et al.*, 2015)

The advantage of QS lies not only in controlling population density but also in spreading beneficial mutations to colonies of biofilms, which induce accessibility to nutrients and tolerance to antibiotics (Hannan *et al.*, 2010). Inadequacy in the regulation of QS networks alters the structure and architecture of biofilms (Rasamiravaka *et al.*, 2015a). As QS poses a trivial impact on most of the regulatory processes, interrupting this mechanism might serve as a critical approach and target of interest to control biofilm-forming pathogens (Storz *et al.*, 2012). Many approaches have been reported to hinder QS, such as blocking signal receptors and transduction, enzymatic degradation of signaling molecules, preventing autoinducers synthesis, etc., which destroy biofilms completely by the host immune system (Abraham, 2016; Scutera *et al.*, 2014).

2.7. *Pseudomonas aeruginosa* quorum sensing system

Among the various QS systems studied in different species, the *Pseudomonas aeruginosa* QS system is important because of its severe pathogenicity. *Pseudomonas aeruginosa* QS system is controlled by various pathways and exhibits interrelated effects. The importance of the QS system in *Pseudomonas aeruginosa* biofilms was first reported

in 1998 by Davis and his group (Davies *et al.,* 1998). Four types of QS systems have been so far studied in *Pseudomonas aeruginosa.* They are *las*, *rhl*, *pqs*, and *integrated QS (iqs). iqs* was added to the *Pseudomonas aeruginosa* QS system recently, and hence its mechanism is not much exploited (Lee and Yoon, 2017).

The *las* system comprises LasI synthase that induces the synthesis of signaling molecule N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), recognized by its LuxR-type receptor protein LasR to activate transcription of target genes. In the *rhl* system, the signaling molecule N-butyryl-L-homoserine lactone (C4-HSL) synthesized by RhlI synthase is recognized by its signal receptor RhlR to induce regulation of target gene expression. Among these two LuxR-type receptor proteins, LasR is activated the earliest and regulates the expression of RhlR (Ni *et al.,* 2009). Both *las* and *rhl* systems are not only involved in biofilm formation but also regulate various gene expressions necessary for virulence factors production (Lee and Yoon, 2017).

Pseudomonas aeruginosa QS system possesses hierarchical relationships among them, with the *las* system in the top position since it regulates other QS systems and *rhl* at the lowest position as it is regulated by other QS systems to activate QS-related virulence factors. *pqs* activates *rhl* and is regulated by *las* and *iqs. iqs* controls *pqs* and *rhl* systems and is activated by *las* (Lee and Zhang, 2015). Though the *Pseudomonas aeruginosa* QS system is an interlinked network, each system can be controlled by several environmental factors, including phosphate stress, starvation (Schafhauser *et al.,* 2014), low oxygen, low iron (Oglesby *et al.,* 2008), and host-derived factors (Lee and Zhang, 2015). The interconnected quorum sensing network of *Pseudomonas aeruginosa* is depicted in Figure 6.

LasR protein comprises two independently folded domains, an N-terminus ligand-binding region and a C-terminus DNA binding region (Bottomley *et al.,* 2007). By comparing the wild-type biofilm strain with that of mutant LasI strains, Davies *et al.* (1998) reported the importance of the *las* system in the development and maturation of biofilms. The mechanism of LasR is that LasR stabilizes itself and undergoes dimerization on binding to a signaling molecule. The resulting LasR homodimer complex regulates the transcriptional activation of target genes. It was reported by Gilbert *et al.* (2009) that LasR binds to the psl operon in the promoter region and regulates psl expression.

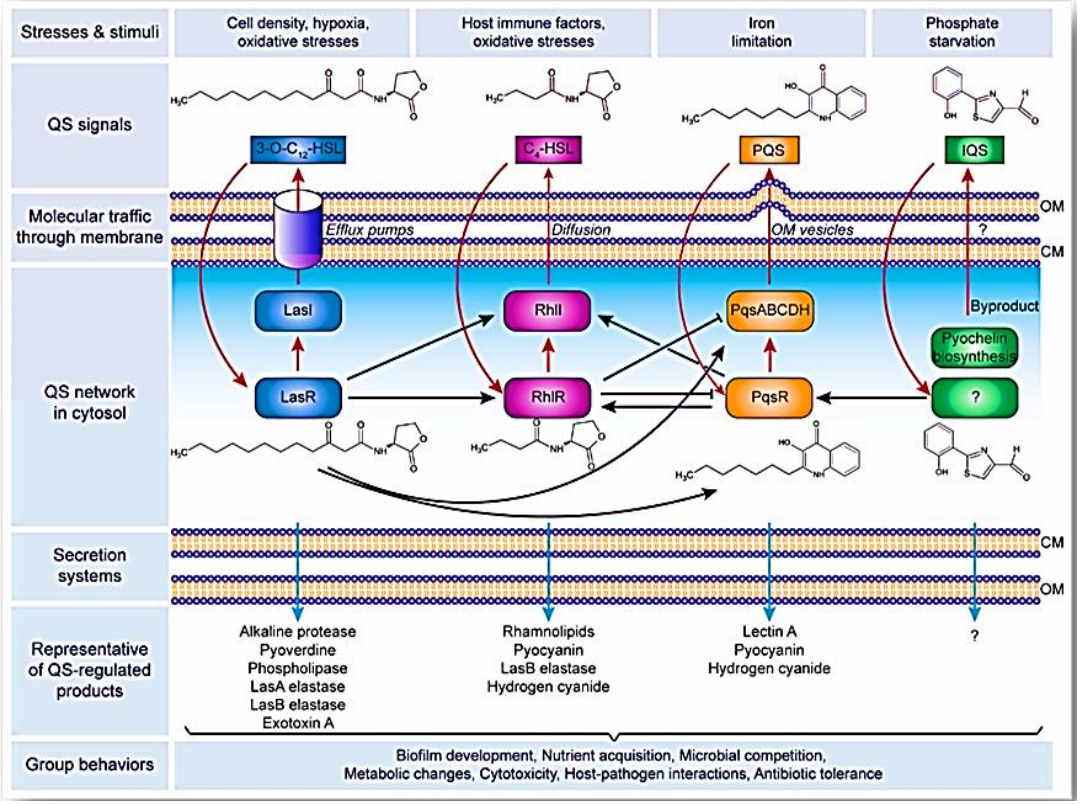

Figure 6

Hierarchical quorum sensing system of *Pseudomonas aeruginosa*

(Thi *et al.*, 2020)

On the other hand, RhlR binds to C4-HSL or an alternative signaling molecule synthesized by PqsE, a thioesterase intricated in alkyl quinolone synthesis to activate genes necessary for the production of virulence-associated QS factors and formation of biofilms (Drees and Fetzner, 2015). C4-HSL does not stabilize RhlR (O'Loughlin *et al.*, 2013) like LasR is stabilized since RhlR does not bind C4-HSL tightly, as evidenced by Boursier *et al.* (2018). The *rhl* system of *Pseudomonas aeruginosa* is involved in biofilm formation by modulating the synthesis of Pel polysaccharides (Rasamiravaka *et al.*, 2015a).

The third QS system *pqs* synthesizes signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) that recognizes its cognate receptor PqsR for regulating eDNA release during biofilm formation (Yang *et al.*, 2009). Apart from this, the *pqs* system also regulates other metabolic processes in *Pseudomonas aeruginosa,* such as the secretion of elastase, rhamnolipid, the formation of membrane, and so on. Many researchers suggest *pqs* is important for virulence, and it is increasingly seen in patients

with cystic fibrosis affected by *Pseudomonas aeruginosa* infections. Recently discovered QS system *iqs* produces autoinducer molecule 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde, sensed by IqsR and modulated by *las* and PhoB, a phosphate stress response regulator. *iqs* controls the production of PQS, C4-HSL, and virulence factors, namely, elastase, rhamnolipids, and pyocyanin (Lee *et al.,* 2013).

More recently, it was discovered that PQS has been implicated in the first stages of biofilm development. It is an outer membrane vesicle (OMV) inducer that contributes at multiple stages. It has been demonstrated that PQS and OMVs are differentially produced during *Pseudomonas aeruginosa* biofilm development, providing evidence that effective biofilm dispersion depends on PQS-induced OMVs (Cooke *et al.,* 2020). It is interesting that these genes involved in QS constitute nearly 10% of the *Pseudomonas aeruginosa* genome and therefore account for a majority of the physiological processes and virulence phenotypes (Schuster and Greenberg, 2006).

In addition to the above, sigma factors like RpoS and RpoN, global regulators of transcription such as AlgQ, MvaT, DksA, and VfR, and two important homologs of LuxR, namely, QscR and VqsR, are also involved in the regulation of the QS signaling circuit (Schuster and Greenberg, 2006). Among these, the LuxR homolog QscR is an orphan QS control repressor receptor protein that utilizes LasR's signaling molecule since it does not have a synthase enzyme. QscR serves as a negative regulator of the QS system by repressing both *las* and *rhl* systems, thus holding a pivotal position in the *Pseudomonas aeruginosa* QS system. A two-component system, GacS/GacA system is considered a super-regulator of the QS network and regulates virulence-associated factors production and formation of biofilms (Mattmann *et al.,* 2011).

The stationary-phase sigma factor RpoS controls biofilm growth in *Pseudomonas aeruginosa*. Approximately 700 genes, most of which are related to QS, are under the direct or indirect control of RpoS (Schuster *et al.,* 2004). Bouillet *et al.* (2019) demonstrated that the post-translational regulation of RpoS depends on the HsbR-HsbA partner switch. This system is connected to the FlgM-HsbA partner switch, triggering the release or sequestration of RpoS. This mechanism is likely the most efficient system this bacterium possesses to decide whether to swim or to form and disperse its biofilm.

2.8. QS-controlled virulence factors and virulence mechanisms of *Pseudomonas aeruginosa*

Production of virulence factors is a survival strategy for pathogens to evade the host immune defense resulting in the progression of pathogenesis, particularly at the early stage of colonization and acute infection. A large number of virulence factors, including cell-associated or secreted compounds, both low and high molecular weight compounds, have been reported as important in the colonization and establishment of infections by *Pseudomonas aeruginosa*. Although they play a critical role in promoting bacterial growth and survival, they can cause devastating injuries to the host tissues and impair the immune responses.

QS-deficient mutants cause considerably less tissue damage and pathological changes during infections due to a significant decrease in virulence and cytotoxicity (Feng *et al.,* 2016). Production of many virulence factors is metabolically costly and requires community involvement. Hence, they are mainly under the regulatory control of the QS systems. The key QS-dependent virulence factors produced by *Pseudomonas aeruginosa* are enlisted in Table 3.

Pseudomonas aeruginosa encodes the above mentioned virulence factors to increase its fitness and chances of survival within the hosts. These virulence factors promote bacterial growth and survival, maneuvering the host cellular machinery by causing devastating injuries, tissue necrosis, evasion, and immune system impairment (Mues and Chu, 2020). The several virulence mechanisms adopted by *Pseudomonas aeruginosa* during its infections are described in Table 4.

Analysis of bronchial secretions of CF patients during different stages of pulmonary exacerbations showed that QS upregulates the expression of genes involved in the production of destructive virulence factors such as proteases (elastase, alkaline protease), phenazines (pyocyanin), toxins (exotoxin A), rhamnolipids and hydrogen cyanide (Lee and Zhang, 2015). Production of these toxic compounds is destructive to the host cells/tissues by impairing the permeability barrier and inhibiting protein production, promoting cell death.

Table 3

Key QS-dependent virulence factors produced by *Pseudomonas aeruginosa*

Virulence factor	Class/ Chemistry	Synthesis	Secretion	Property	References
Pyocyanin	Secondary metabolite/ tricyclic phenazine	*phzA1-G1* & *phzA2-G2* operons	T2SS	Redox-active, zwitterion	Hall *et al.*, 2016; van't Wout *et al.*, 2015
Pyoverdine	Pyoverdines/ dihydroquinoline-type chromophore linked to a peptide	Large multi-modular enzymes/ non-ribosomal peptide synthetases (NRPSs)	PvdRT-opmQ & MexAB-OprM efflux pumps	High affinity to Fe (III)/iron acquisition/ fluorescent	Schalk and Guillon, 2013; Hannauer *et al.*, 2012
LasA Elastase	β-lytic zinc metallo-endopeptidase (staphylolytic) /serine protease	*lasA*	The Sec pathway & T2SS	Protease and elastolytic activity/ cleaving a wide range of glycine-containing proteins	Hoge *et al.*, 2010
LasB Elastase	M4 thermolysin peptidase family/ zinc metallo-protease	*lasB*	The Sec pathway & T2SS	Protease and elastolytic activity	Hoge *et al.*, 2010
Alkaline protease	M10 peptidase family/ zinc metallo-endopeptidase	*aprA*	T1SS	Wide protease activity	Laarman *et al.*, 2012; Hoge *et al.*, 2010
Lectin A	Tetrameric protein	*lecA* (or *pa1L*)	Intracellular; only a small fraction present on the cell surface	Galactophilic/ adhesive	Chemani *et al.*, 2009; Diggle *et al.*, 2006
PlcB	Phospholipases C	*plcB*	The Sec pathway & T2SS	Hydrolysing phosphatidyl-choline & phosphatidyl-ethanolamine	Barker *et al.*, 2004

Virulence factor	Class/ Chemistry	Synthesis	Secretion	Property	References
Rhamnolipids	Rhamnose-containing glycolipidic compounds	*rhlAB* operon & *rhlC*	-	Biosurfactant/ detergent-like structure/ hemolytic activity	Wittgens *et al.*, 2011; Alhede *et al.*, 2009
Exotoxin A (ToxA)	PE belongs to the two-component AB toxin family/ NAD^+-diphthamide-ADP-ribosyl-transferase	*toxA*	T2SS	Modifying the elongation factor-2 in eukaryotic cells	Michalska and Wolf, 2015; Daddaoua *et al.*, 2012
Hydrogen cyanide (HCN)	Secondary metabolite	*hcnABC* operon	Diffusible	Highly toxic/potent inhibitor of cytochrome c oxidase and other metallo-enzymes	García-García *et al.*, 2016; Ghafoor *et al.*, 2011

Table 4

Virulence mechanisms employed during *Pseudomonas aeruginosa* infections

Virulence mechanism	Causative agent
Antibiotic resistance	Efflux pumps, modifying enzymes
Motility	Flagella, type IV pili
Biofilm structure & dynamics	Rhamnolipids, alginate
Iron scavenging	Proteases, siderophores (pyochelin and pyoverdine)
Cytotoxicity	Pyocyanin, T3SS, endotoxin A, HCN
Immune evasion	Elastase, alkaline protease

Recent findings suggested a correlation between systemic concentrations of QS signaling molecules with the clinical status of pulmonary exacerbation and found that at least some QS signaling molecules were elevated at the start of either pulmonary exacerbation or antibiotic treatment when assessed in different biofluids (Dellol and Hamzah, 2021; Barr *et al.*, 2015). Thus, virulence factors assist bacteria in colonization and survival aligned with the worsened clinical course of infections.

2.9. *Pseudomonas aeruginosa* biofilm challenge to antimicrobial agents

Planktonic cells are at greater risk to the effect of antibiotics and are sufficiently sensitive to antimicrobial agents, whereas bacteria within a biofilm structure are not susceptible to the host immune systems and antimicrobials as they exhibit a high tolerance and resistance to antimicrobial agents. Most of the resistance mechanisms of microorganisms are transferable and devoid of the target's interaction with antibiotics (Olivares *et al.,* 2020). The biofilm structure of *Pseudomonas aeruginosa* exhibits a greater extent of antibiotic resistance due to various reasons, such as moderate or deficient penetration of antibiotics, the altered chemical environment within the biofilm, and cell differentiation in a biofilm. All these mechanisms occur due to the multicellular nature of biofilms, thereby leading to antibiotic resistance of biofilm structure and failure in treatment strategies (Kragh *et al.,* 2016; Sugimoto *et al.,* 2016).

It has become a great challenge to treat patients with infections of *Pseudomonas aeruginosa* as they exhibit high-level of resistance to most of the available antibiotics in use (Lister *et al.,* 2009). Recently, World Health Organization (WHO) has mentioned *Pseudomonas aeruginosa* as a life-threatening species for which new antibiotics must be developed to prevent its infections (Tacconelli *et al.,* 2018). To date, empirical antibiotic therapy is used to treat cases of *Pseudomonas aeruginosa* infections, but more use of antibiotics for therapy may develop multidrug-resistant strains of *Pseudomonas aeruginosa* and can cause the failure of empirical antibiotic therapy against this microbe (Park *et al.,* 2012; Hirsch and Tam, 2010). The various antibiotic resistance mechanisms adopted by *Pseudomonas aeruginosa* are illustrated in Figure 7.

In addition to the above resistance mechanisms, multidrug-tolerant persister cells can form in the biofilms that withstand antibiotic attacks and cause prolonged periodic infections in individuals with cystic fibrosis (Mulcahy *et al.,* 2010). Persisters are bacterial subpopulation with a multidrug tolerance phenotype rather than genetic variations (Ayrapetyan *et al.,* 2015; Helaine and Kugelberg, 2014). Mulcahy *et al.* (2010) have evidenced high levels of persister cells in cystic fibrosis patients compared to wild-type strains of *Pseudomonas aeruginosa* suggesting them as highly antibiotic-resistant and can change as multidrug-tolerant.

Most biofilm cells enter the stationary phase with time, and persister cells are high in number at this phase. The main reason for decreased susceptibility of persister cells to antibiotics as 1% of the population in the stationary phase becomes tolerant (Amato *et al.*, 2014; Keren *et al.*, 2011). This potential remodels the regulatory networks of *Pseudomonas aeruginosa* through a process referred to as adaptive radiation, where intense genetic mutations lead to diverse genotypes and phenotypes within bacterial populations followed by the selection of colonizers leading to a transition from acute to chronic infections (Figure 8).

The ability of microbial biofilms to resist exposure to a high concentration of antimicrobials and components of the host immune system makes *Pseudomonas* biofilms incredibly challenging to eradicate and a public health concern (Høiby *et al.*, 2015). This type of antibiotic-resistant state of biofilm cells becomes a causative agent for the majority of human infections and pose a serious threat to humans due to their pathogenicity and contribute to various pathogenic infections (Kaur *et al.*, 2017; Flemming *et al.*, 2016). The most common chronic infections caused by *Pseudomonas aeruginosa* biofilms are shown in Figure 9.

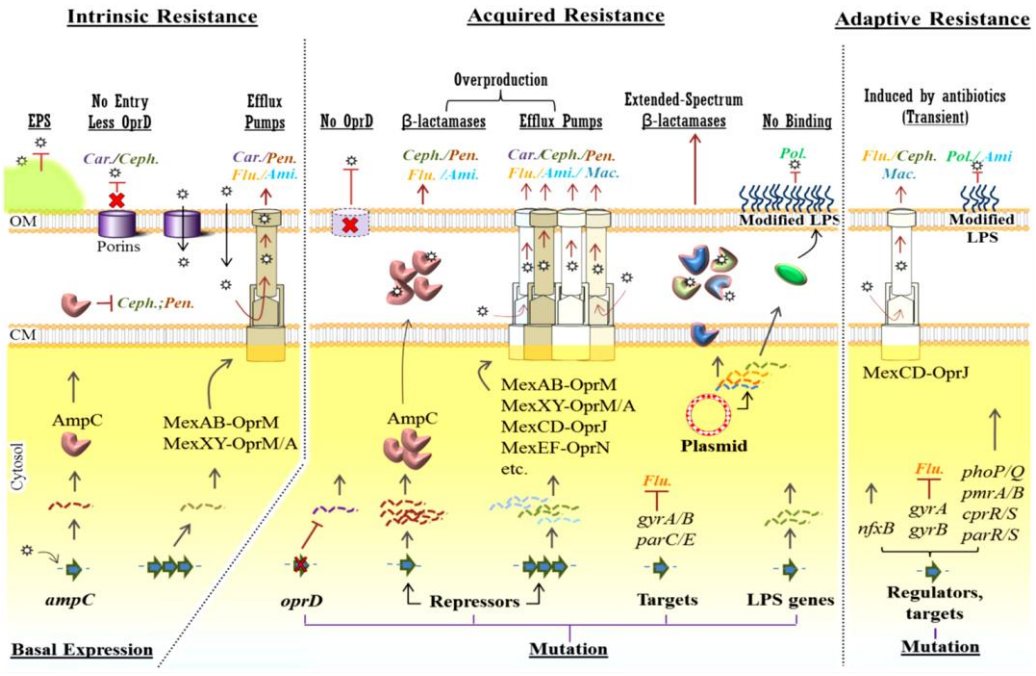

Figure 7

Resistance mechanisms conferred against antibiotics in *Pseudomonas aeruginosa* (Moradali *et al.*, 2017)

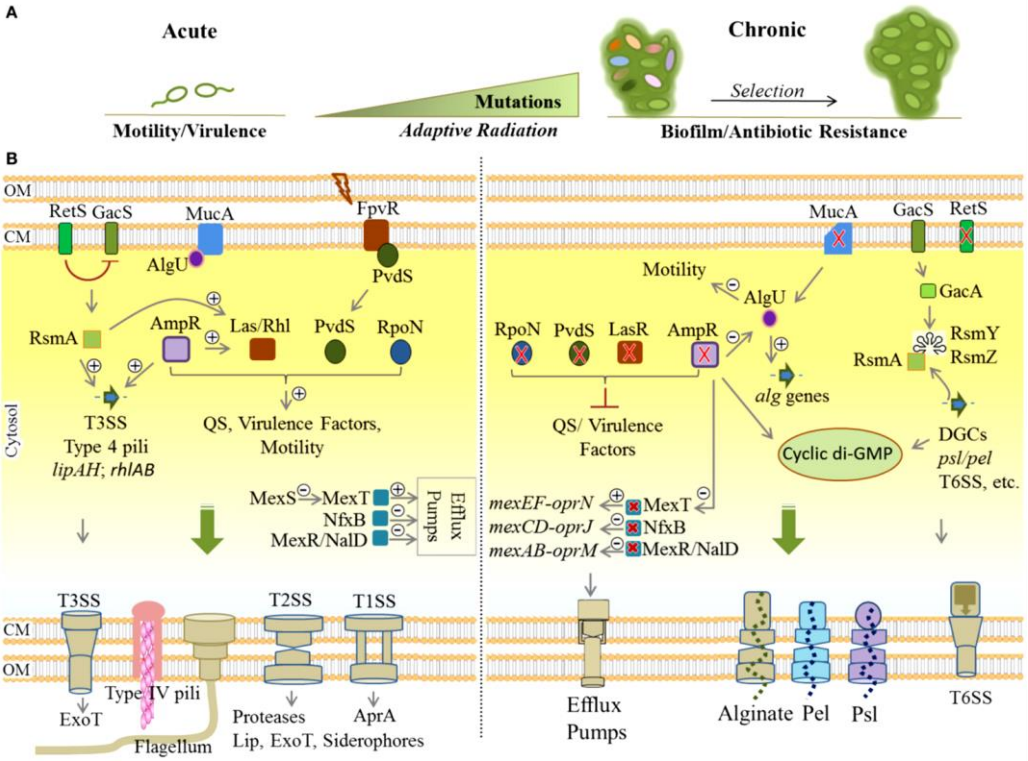

Figure 8

Remodelling of regulatory network and transition from acute to chronic infections in *Pseudomonas aeruginosa* (Moradali *et al.*, 2017)

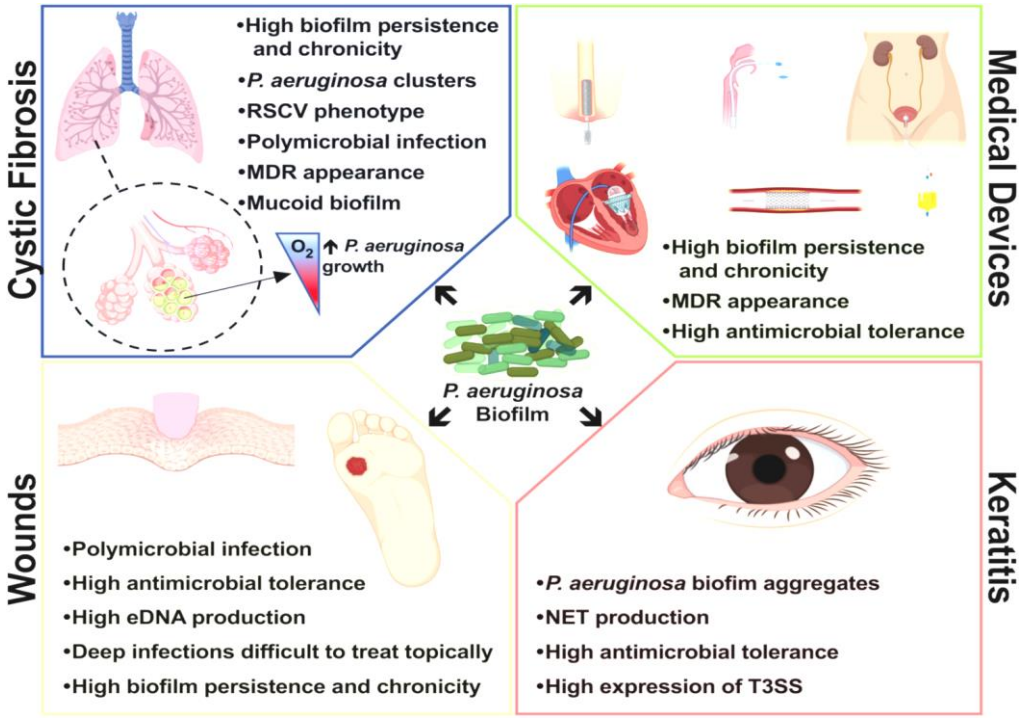

Figure 9

Consequences of progression of *Pseudomonas aeruginosa* biofilm chronic infections (Cendra and Torrents, 2021)

2.10. Strategies to control *Pseudomonas aeruginosa* biofilm-associated infections

Increased rates of mortality and morbidity are seen in healthcare facilities among patients affected by *Pseudomonas aeruginosa* infections due to failure in developing new antibiotics and its widespread resistance (Algburi *et al.,* 2017). Successful treatment of biofilm-associated infections is troubled due to its high antibiotic-resistant nature. Classical antibiotics chemotherapy cannot completely eradicate bacterial cells, which are situated in the central region of the biofilm and lead to the emergence of a worsening situation globally (Sharma *et al.,* 2019a).

The development of new antibiofilm strategies could effectively treat biofilm-related infections, thereby reducing their complications (Bernardes *et al.,* 2015). Therefore, to overcome the drug resistance of bacterial biofilm communities; alternative strategies and novel antibiofilm agents have been studied earlier (Misba and Khan, 2018; Misba *et al.,* 2017; Zaidi *et al.,* 2017; Zuberi *et al.,* 2017; Misba *et al.,* 2016; Muñoz-Egea *et al.,* 2016; Kulshrestha *et al.,* 2014; Sun *et al.,* 2013; Kolodkin-Gal *et al.,* 2012; Baek and An, 2011; Hochbaum *et al.,* 2011; Iannitelli *et al.,* 2011; Kolodkin-Gal *et al.,* 2010). A summary of current therapeutic strategies targeting *Pseudomonas aeruginosa* biofilms, including the advantages and limitations of individual approaches, is provided in Table 5.

2.10.1. Plants as a natural source of antibiofilm agents

For the past two decades, novel approaches in preventing QS and biofilm formation have been employed by natural products from plants that demonstrate chemo-protective and antimicrobial properties. It is already well known that natural products and herbal remedies have been used in practice by different human cultures for many years for therapy and to prevent the spread of infectious diseases (Lu *et al.,* 2019). The following table (Table 6) presents the various natural plant-based products screened for antibiofilm properties to treat *Pseudomonas aeruginosa* biofilm-associated infections.

2.10.2. Enzymes against *Pseudomonas aeruginosa* biofilms

Another possible approach to control biofilms is the incorporation of enzymes to destroy polymers of the extracellular matrix and enable the disruption of biofilms

(Bernardes *et al.*, 2015). Since biofilm matrix is a complex architecture, multi-enzymatic formulations are needed to control biofilms effectively. Researchers have reported a variety of enzymes that can fight against *Pseudomonas aeruginosa* biofilms (Torres *et al.*, 2011).

Table 5

Summary of therapeutic strategies against *Pseudomonas aeruginosa* infections

Therapeutic approach	Activity	Advantages	Limitation	References
Antimicrobial peptides	Antibacterial, Antibiofilm, Immunological modulator	Low cytotoxicity, Combined treatment possibility	Sensitive to salt, serum and pH, Susceptible to host proteolysis	Shin *et al.*, 2017; Wnorowska *et al.*, 2015
Antibiotics	Antibacterial	Inhaled antibiotic class, Broad-spectrum, Safety, Improvement of lung function in CF patients	Resistance development	Flume *et al.*, 2016
Lectin inhibitor	Antibiofilm	High stability, Low resistance	No *in vivo* data, Toxicity, Narrow spectrum	Wagner *et al.*, 2017; Krachler and Orth, 2013
Bacteriophages	Antibacterial	Delivery at the infection site, High specificity, Fewer side effects, Easy administration	Poor stability, Undesired cytotoxicity, Insufficient pharmacokinetics and pharmacodynamics data	Hesse and Adhya, 2019; Pires *et al.*, 2015
Natural products	Antibacterial, Antibiofilm, Quorum sensing modulator	Broad-spectrum, Multiple mechanisms of action	Cytotoxicity, Resistance development, Limited penetration into biofilm, Availability	Melander *et al.*, 2020
Iron chelators	Interference with iron metabolism	Bactericidal activity, Biofilm prevention, Low risk of resistance development	Cytotoxicity	Moreau-Marquis *et al.*, 2009

Therapeutic approach	Activity	Advantages	Limitation	References
Nanoparticles	Antibacterial, Antibiofilm	Broad-spectrum, Small size, thus direct delivery to targets	Cytotoxicity, Host metabolism of nanoparticles	Chen *et al.*, 2019; Salomoni *et al.*, 2017
Nanocarriers (Liposomes, solid lipid and polymeric)	Drug delivery	Protection of therapeutic agents from inactivation and degradation by bacterial and host system, Enhancement of efficacy	Cytotoxicity, Host metabolism of nanoparticles	Türeli *et al.*, 2017
EPS inhibitors	Anti-EPS	Biofilm matrix degradation, Limited/no effect on bacterial viability, Low risks of resistance development	Incomplete biofilm matrix disruption, Cytotoxicity	Powell *et al.*, 2018
Biofilm dispersers	Dispersal induction	Augumentation of antibiotic efficacy to clear the infection, Low risk of resistance development	Release of harmful dispersed cells for re-colonization and lethal septic event, Cytotoxicity	Zhu *et al.*, 2017
QS inhibitors	Biofilm prevention, Biofilm inhibition	Reduction of virulence factors, No effect on bacterial viability	Narrow spectrum, Unwanted effect on bacteria	Birmes *et al.*, 2019; Kiymaci *et al.*, 2018
Photodynamic therapy	Antibacterial, Antibiofilm	No resistance development, Improved sensitivity, No photocytotoxicity	Potential side effects	Vassena *et al.*, 2014
Photothermal therapy	Antibacterial, Antibiofilm	No resistance development, Improved sensitivity, Negligible cytotoxicity	Photothermal ablation of host tissues	Bilici *et al.*, 2020; Peng *et al.*, 2020

Table 6

Plant species screened to treat *Pseudomonas aeruginosa* biofilm infections

S. No	Plant species	Part of the plant used	Extract	References
1	*Allium cepa*	Outer scales	Methanol	Al-Haidari *et al*.,2016
2	*Allium sativa*	Bulbs	Methanol	Al-Haidari *et al*.,2016
3	*Ananas comosus*	Fruit	Aqueous	Musthafa *et al.*, 2010
4	*Centella asiatica*	Leaves	Ethanol	Vasavi *et al.*, 2016
5	*Citrus sinensis*	Seeds	Methanol	Al-Haidari *et al*.,2016
6	*Coriandrum sativum*	Fruit	Methanol	Al-Haidari *et al*.,2016
7	*Couroupita guianensis*	Fruit	Chloroform	Al-Dhabi *et al.*, 2012
8	*Elettaria cardamomum*	Seeds	Methanol	Al-Haidari *et al*.,2016
9	*Euphorbia hirta* L.	Aerial parts	Methanol	Perumal and Mahmud, 2013
10	Garlic	Bulbs	Toluene	Bjarnsholt *et al.*, 2005
11	*Hemidesmus indicus* (L.)	Root	Ethanol	Zahin *et al.*, 2010
12	*Holarrhena antidysenterica* (Roth.)	Bark	Ethanol	Zahin *et al.*, 2010
13	*Laurus nobilis*	Leaves	Methanol	Al-Haidari *et al*., 2016
14	*Mangifera indica* L.	Seed	Ethanol	Zahin *et al.*, 2010
15	*Manilkara zapota*	Fruit	Aqueous	Musthafa *et al.*, 2010
16	*Mentha longifolia*	Aerial part	Methanol	Al-Haidari *et al.*, 2016
17	*Musa paradiciaca*	Stem	Aqueous	Musthafa *et al.*, 2010
18	*Ocimum sanctum*	Leaves	Aqueous	Musthafa *et al.*, 2010
19	*Panax notoginseng*	Roots	Aqueous	Song *et al.*, 2010
20	*Psidium guajava*	Leaves	Methanol	Al-Haidari *et al*., 2016
21	*Psoralea corylifolia* L.	Seeds	Ethanol	Zahin *et al.*, 2010
22	*Senecio brasiliensis*	Stem bark	Aqueous	Silva *et al.*, 2012
23	*Syzygium aromaticum*	Bud	Methanol	Krishnan *et al.*, 2012
24	*Terminalia catappa*	Leaves	Methanol	Taganna *et al.*, 2011
25	*Amphypterygium adstringens*	Stem bark	Hexane	Juarez *et al.*, 2013
26	*Sclerocarya birrea*	Stem bark	Methanol	Sarkar *et al.*, 2014
27	*Ocimum basilica*	Whole plant	Aqueous	Vattem *et al.*, 2007
28	*Brassica oleracea*	Whole plant	Aqueous	Vattem *et al.*, 2007
29	*Zingiber officinale*	Whole plant	Aqueous	Vattem *et al.*, 2007
30	*Myristica cinnamomea*	Bark	Methanol	Chong *et al.*, 2011
31	*Melicope lunu-ankenda*	Leaves	Hexane	Tan *et al.*, 2012
32	*Psidium guajava*	Leaves	Methanol	Al-Haidari *et al.*, 2016
33	*Phyllanthus amarus*	Whole plant	Methanol	Priya *et al.*, 2013
34	*Capparis spinosa*	Dried fruit	Methanol	Abraham *et al.*, 2011
35	*Thymus sp.*	Whole plant	Aqueous	Vattem *et al.*, 2007
36	*Nymphaea tetragona*	Whole plant	Aqueous	Hossain *et al.*, 2015
37	*Terminalia bellirica*	Fruits	Methanol	Shukla and Bhathena, 2016

S. No	Plant species	Part of the plant used	Extract	References
38	*Terminalia chebula*	Fruits	Methanol	Shukla and Bhathena, 2016
39	*Syzygium cumini*	Seeds	Methanol	Shukla and Bhathena, 2016
40	*Punica granatum* L.	Pericarp	Ethanol	Zahin *et al.*, 2010
41	*Sclerocarya birrea*	Bark	Methanol	Sarkar *et al.*, 2014
42	*Triumfetta welwitschii*	Leaves	Methanol	Mombeshora *et al.*, 2021
43	*Corchorus olitorius*	Stem	Ethanol	Al-Youef *et al.*, 2021
44	*Phrynium capitatum*	Leaves	Ethanol	Jalli *et al.*, 2019
45	*Dryptes indica*	Leaves	Ethanol	Jalli *et al.*, 2019
46	*Plantain herb*	Whole plant	Ethanol	Li *et al.*, 2019
47	*Cinnamomum camphora*	Bark	Distilled water	Topa *et al.*, 2020
48	*Centella asiatica*	Leaves	Ethanol	Vasavi *et al.*, 2016
49	*Anogeissus acuminata*	Whole plant	Methanol	Hnamte *et al.*, 2019
50	*Mallotus roxburghianus Muell*	Whole plant	Ethanol	Hnamte *et al.*, 2019
51	*Camellia kissi wall*	Leaves	Methanol	Jalli *et al.*, 2020
52	*Plectranthus tenuiflorus*	Leaves	Methanol	Hnamte *et al.*, 2018
53	*Persicaria maculosa*	Aerial plants	Ethanol	Jovanović *et al.*, 2020
54	*Bistorta officinalis*	Rhizome	Ethanol	Javanović *et al.*, 2020
55	*Syzygium legatii*	Leaves	Acetone	Famuyide *et al.*, 2019
56	*Syzygium masukuense*	Leaves	Acetone	Famuyide *et al.*, 2019
57	*Syzygium species A*	Leaves	Acetone	Famuyide *et al.*, 2019
58	*Berginia ciliate*	Rhizome with skin	Methanol	Alam *et al.*, 2020
59	*Lavandula coronopifolia*	Aerial parts	Methanol: water	Emam *et al.*, 2021
60	*Centella asiatica*	Leaves	Methanol	Jahan *et al.*, 2018
61	*Mentha spicata*	Leaves	Methanol	Jahan *et al.*, 2018
62	*Azadirachta indica*	Leaves	Methanol	Jahan *et al.*, 2018
63	*Psidium guajava*	Leaves	Methanol	Jahan *et al.*, 2018
64	*Syzygium aromaticum*	Whole plant	Ethyl acetate	Jahan *et al.*, 2018
65	*Cinnamomum zeylanicum*	Whole plant	Ethyl acetate	Jahan *et al.*, 2018

A study by Kovach *et al.* (2020) reported that the effect of EPS-specific enzymes, namely, alginate lyase and DNase is greater on *Pseudomonas aeruginosa* biofilms, whereas non-specific enzymes such as glycoside hydrolases, cellulases, and α-amylases did not significantly alter the biofilm mechanics *in vitro*. However, the mechanism of how these enzymes hinder the biofilm mechanism remains still unknown. In the case of *in vivo* studies on a mouse model of wound infections, glycoside hydrolases were more productive than

other specific enzymes since there might be a difference in the formation of biofilms developed *in vivo* and *in vitro* by genetically similar strains of bacteria.

Quorum quenching was initially described in 2000 with the invention of a quorum quenching enzyme from *Erwinia carotovora* that degrade AHL signals. Most of the identified quorum quenching enzymes, namely phosphotriesterase-like lactonases (PLLs), lactonases, acylases, and oxidoreductases, target AHLs (Weiland-Bräuer *et al.,* 2016; Fetzner, 2015). Example of enzymes that can act as anti-biofilm agents for grafting wounds and removes barriers that weaken wound healing, such as weakened tissues, bacterial biofilms, and scars, includes bromelain-derived debridase, collagenase, trypsin, fibrinolysin, lysozyme, streptokinase, and dispersin B (Gawande *et al.,* 2014; Demidova-Rice *et al.,* 2012). Different classes of the enzyme known to control biofilms are shown in Table 7.

Table 7

Classes of enzymes used in controlling *Pseudomonas aeruginosa* biofilms

Class of enzyme	Example	Target	References
Oxidoreductases	Glucose oxidase, Curvularia haloperoxidase	Directly or indirectly retarding bacterial growth by production of H_2O_2	Ge *et al.,* 2012; Hansen *et al.,* 2003
Transferases	Transaminase	EPS matrix	Barton *et al.,* 2004
Hydrolases	AiiA, α-amylase, Proteinase K	QS molecules, Exopolysaccharides, Exoproteins	Fagerlund *et al.,* 2016; Kalpana *et al.,* 2012; Dong *et al.,* 2000;
Lyases	Alginate lyase	Exopolysaccharides	Lamppa and Griswold, 2013

Acylase reduces *Pseudomonas aeruginosa* ATCC 10145 and *PAO1* growth to 60% by disrupting QS signaling (Grover *et al.,* 2016; Ivanova *et al.,* 2015). A study by Vogel *et al.* (2020) stated that immobilizing quorum quenching enzyme with quorum quenching properties such as acylase PvdQ over the polydimethylsiloxane silicone (PDMS) surface in a biosensor setup exhibited a 6-fold decrease of the autoinducer OdDHL compared to untreated material. Researchers have stated that lysozyme and proteinase K could inhibit the biofilm formation of many species of bacteria (Hukić *et al.,* 2018; Sebaa *et al.,* 2017). In connection with this, Eladawy *et al.* (2020) reported lysozyme as a potent biofilm inhibitor as it reduces 19% biofilm formation at a physiological concentration of 30 μg/mL,

whereas proteinase K exhibited a biphasic effect on *Pseudomonas aeruginosa* biofilms at different concentrations. Another class of enzyme, BpiB09 oxidoreductases, was evidenced to reduce the motility, biofilm formation, production of pyocyanin, and prevent the induction of OdDHL in *Pseudomonas aeruginosa PAO1* strains (Bijtenhoorn *et al.,* 2011).

Dioxygenases destroy 2-heptyl-3-hydroxy-4 (1H) quinolone-based signaling molecules and have been shown to inhibit the quinolone signals in the *Pseudomonas aeruginosa* QS system (Witzgall *et al.,* 2018). Banar *et al.* (2019) studied the mechanism of β-glucosidase and lyticase enzymes on biofilms formed by various strains of *Pseudomonas aeruginosa* and found that both enzymes degraded and altered the biofilm states. These enzymes also significantly reduced the colony-forming units and revealed no cytotoxicity when treated against cell lines of A-549 human lung carcinoma and A-431 human epidermoid carcinoma.

Another study by Daboor *et al.* (2019) revealed that alginate lyase (AlyP1400), a class of alginolytic enzyme purified from marine bacteria *Pseudoalteromonas sp.,* reduced *Pseudomonas aeruginosa* biofilms after 24 h of incubation by 69% and could serve as a specific combinational therapeutic strategy when used along with conventional antibiotics. A lactonase group of quorum quenching enzyme, SsoPox-W263I reduced protease, elastase, and pyocyanin production among bacteriophage-resistant strains and degraded acyl-homoserine lactones (Mion *et al.,* 2019).

Snarr *et al.* (2017) reported that microbial glycoside hydrolases developed by recombinant technology may serve as a potent therapeutic agent with promising antibiofilm potential by destroying biofilms and inhibiting virulence. An enzyme-based endoscope cleaner was invented for clearing biofilms in medical devices by Stiefel *et al.* (2016) using an optimized base formulation of enzyme mixture and discovered that it removed about 90% of biofilms formed by *Pseudomonas aeruginosa* in 96-well plate with >99% decrease of colony forming unit (CFU) and >90% decrease in extracellular polymeric substances.

A study on trypsin, α-mannosidase, and β-mannosidase enzymes effect on *Pseudomonas aeruginosa* biofilm from wound infections caused due to burns by Banar *et al.* (2016) proved that only enzyme trypsin had no cytotoxic effect on cell lines of A-431 human epidermoid carcinoma and possessed antibiofilm features better than other enzymes. The only enzyme that is found to disrupt *Pseudomonas aeruginosa* biofilms in clinical fields is Dornasealfa (deoxyribonuclease I), which promotes destruction by eDNA

hydrolyzation within the extracellular matrix (Hymes *et al.,* 2013). However, immature *Pseudomonas aeruginosa* biofilms were more prone and sensitive when treated with deoxyribonuclease I than mature biofilms (Parks *et al.,* 2009). Another therapeutic enzyme, glycoside hydrolase DspB (Dispersin B), hydrolyzes the poly-b-1,6-N-acetyl-D-glucosamine (PNAG/PIA) exopolysaccharide found in biofilms (Gawande *et al.,* 2014) but this PNAG is not found in *Pseudomonas aeruginosa* biofilms (Messiaen *et al.,* 2014). Baker *et al.* (2016) identified naturally derived glycoside hydrolases, namely, PelAh and PslGh, that specifically target psl and pel polysaccharides, and can clear *in vitro* biofilms of clinical, environmental, and laboratory isolates at nanomolar concentrations.

A study by Kiran *et al.* (2011) stated that lactonase treatment on *Pseudomonas aeruginosa* biofilms significantly quenches all the major lactones synthesized by *Pseudomonas aeruginosa* strains, namely OdDHL and C4-HSL that regulate virulence factor expression. In addition, extracellular hydrolases secreted by mucoid *Pseudomonas aeruginosa* strains during biofilm growth EstA, LasB, and LipC can cause changes in the composition of EPS and alter the motility of cells as reported by Tielen *et al.* (2010).

One of the main drawbacks of enzyme-mediated antibiofilm therapy is that it should be carried out in combination with antibiotic agents as it may become a preventive measure rather than a way of treatment. Another concern is their typical higher cost when compared to other costs of conventional chemical disinfectants and antimicrobial agents. But apart from this, many researchers have reported that biofilm matrix-degrading enzymes could be a potent antibiofilm agent to reduce the incidence of medical device infections (Kaplan, 2009).

2.10.3. *In silico* approach to control *Pseudomonas aeruginosa* biofilms

Many studies have reported various chemical tools to exploit new knowledge to inhibit bacterial virulence by hindering the QS systems as a novel means to reduce *Pseudomonas aeruginosa* infections efficiently, which makes it harder for the bacteria to develop drug resistance. Wang *et al.* (2020) identified cladodionen isolated from extracts of the marine fungal strain *Cladosporium sp.* Z148 as a novel QS inhibitor that showed effective binding conformation to LasR and PqsR compared to native ligands through a molecular docking approach. He also reported that the QS-related mRNA gene expressions were down-regulated by cladodionen. A recent study by Sadiq *et al.* (2020) reported sulfamerazine, a synthetic FDA-approved compound, as an inhibitor of LasR by

performing virtual screening and molecular docking by employing a pharmacophore hypothesis-based screening and elucidating the stability of their binding conformation by a simulation study. A study by Baldelli *et al.* (2020) suggested two antibiotic compounds, namely, nitrofurazone and erythromycin estolate, as PqsE inhibitors by screening a library of FDA-approved drugs and found that these compounds reduce the expression of PqsE-dependent virulence and formation of biofilm in *Pseudomonas aeruginosa PAO1* model strain.

Abelyan *et al.* (2020), through *in silico* virtual screening approach, reported benzamides, a synthetic derivative of flavones, could be promising LasR inhibitors as they exhibit higher binding affinity to the LasR ligand-binding domain compared to the DNA binding domain. Also, the selected compounds conformationally bind to the same amino acid residues of the ligand-binding region similar to the natural ligand, which indicates the competitive nature of the compounds. Mellini *et al.* (2019) attempted a virtual screening on in silico FDA-approved drugs library consisting of 1467 compounds through molecular docking and selected five top hit compounds that possessed a stable binding affinity for the QS receptor PqsR as novel antagonists of the *pqs* system, which imparts its effect on *Pseudomonas aeruginosa* PqsR associated expression of virulence factors.

Another report by Shah *et al.* (2019) found a synthetic compound potassium 2-methoxy-4-vinylphenolate to be the most powerful *Pseudomonas aeruginosa* quorum sensing inhibitor that targets LasIR/RhlIR circuits and inhibits the formation of biofilm, production of virulence-associated factors like LasA protease, pyocyanin, LasB elastase, and motilities in bacteria. Recently, a study by Nain *et al.* (2020) utilized energy-optimized pharmacophore coupled virtual screening to discover QS inhibitors for LasR of *Pseudomonas aeruginosa* based on hydrogen bond networking and further exploited the stability of the binding complexes through a dynamics simulation study. Singh and Bhatia (2018) performed a study on an FDA-approved clinical drug, Albendazole, by a structure-based molecular docking approach that exhibited putative interactions with LasB and CviR receptor protein of *Pseudomonas aeruginosa* and *Chromobacterium violaceum,* respectively.

Paczkowski *et al.* (2017) screened a highly diverse library of chemical compounds consisting of 60,000 molecules to identify putative flavone-based QS inhibitors. The structure-activity relationship analysis reported that flavone A ring backbone with two

hydroxyl moieties is necessary for LasR/RhlR inhibition and thus stated as flavonoids might function in a non-competitive way to hinder LasR/RhlR DNA-binding by altering the transcriptional regulation of quorum sensing mediated target promoters and thereby leading to a decrease in the production of virulence factors. An *in silico* approach with combined pharmacophore and molecular docking studies was carried out by Xu *et al.* (2017) for screening a library of 167,740 compounds derived from the Specs database for potent QscR agonists and LasR antagonists since QscR is an indirect suppressor and represses the las system by binding to the promoter region of LasI or forming an inactive hetero multimer with LasR. Concerning this mechanism, it was suggested that QscR agonists and LasR antagonists can have synergistic effects, which led to the identification of four potential compounds as biofilm inhibitors with novel scaffolds.

A report by Jha *et al.* (2014) stated a high throughput computational docking approach to identify an inhibitor for LasR of *Pseudomonas aeruginosa* and suggested [(4E)-1-hydroxy-3-methylpyridin-4(1H)-ylidene]azinic acid] as the best antagonist of LasR as it satisfied the ADMET profile for drug properties among the top five inhibitors obtained to post the screening process. Tan *et al.* (2013) discovered novel QSI candidates using 3040 structures of natural compounds and their derivatives by a structure-based virtual screening (SBVS) protocol and found five compounds to have the efficiency to block QS-associated expression of genes in *Pseudomonas aeruginosa* utilizing a live reporter gene assay.

Wei *et al.* (2016) designed a computational network to measure cell-cell interactions directly and biofilm dynamics at a fundamental level, which revealed the quorum sensing inhibitor-based therapies to decrease the spread of QSI resistance through simulations. Sharma *et al.* (2016) reported a web server, dPABBS, which could predict, and design anti-biofilm peptides based on the whole composition of amino acid residues, selected features of residue, and positional preference of amino acid residues to fight *Pseudomonas aeruginosa* biofilms. Another interesting piece of research by Kim *et al.* (2015) found 6-gingerol from fresh ginger, a pungent oil, antagonizes *Pseudomonas aeruginosa* QS receptors through hydrogen bonding and hydrophobic interactions with LasR as revealed by molecular docking analysis. Supporting the above information, transcriptomics analysis also proved that 6-gingerol repressed QS-induced genes, particularly virulence factor production.

Using a bio-sensor strain of *Pseudomonas aeruginosa*, Gopu *et al.* (2015) reported quercetin as a competitive inhibitor of LasR through *in silico* methods, namely molecular docking and simulation studies. A system-level approach was carried out to quantify the capacity of biofilm formation by mutants for identifying the target genes necessary for metabolism in the planktonic state of bacteria. It was seen that the essential gene mutation treatment made *Pseudomonas aeruginosa* survive by regulating the metabolism of acetate, arginine, and glutamate (Xu *et al.*, 2013).

Computational methods employ a crucial role in the current drug discovery process, starting from the designing and maintaining of small molecule libraries and improvisation of pharmacological properties of the lead compounds to the final stage in clinical development. Thus, *in silico* methods could be a productive approach and form alternative tools in all stages of drug development for efficacy, safety, the speed with more certainty, and lower cost (Sahner *et al.*, 2013). On the other side, though several QS inhibitors have been identified so far, many do not suit further drug development as lead compounds because of their undesirable pharmacological properties and cytotoxicity (Mellini *et al.*, 2019). Also, much of the literature on computational approaches has resulted in lead compounds but does not provide further experimental validation of their biological activity (Amin and Welsh, 2006).

In addition, molecules identified by the virtual screening approach might be false positives resulting from molecule aggregation and could be considered “promiscuous inhibitors” if they have not been properly investigated by *in vitro* experimental verifications. For many years, the concept of computational studies has come into play after the synthesis of molecules and a significant illustration of the data. However, now predictions are made for pharmacological activity without *in vitro* or *in vivo* validation. In such a case, it is important to understand that predictive models only have a limited prediction domain, and they are not very accurate and reliable (Ekins *et al.*, 2007). Hence, with this background information, the present investigation was carried out to screen for potential quorum sensing inhibitors against the principal protein LasR in *Pseudomonas aeruginosa.*